SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
INVENTARIO

Industria y Comercio

SUPERINTENDENCIA

03-23841

Delegatura de propiedad Industrial

Organizacion CSA Ltda

División de Nuevas Creaciones

PCT

B.T.

SOLICITUD

PATENTE DE INVENCIÓN

03-23841

21. **EXPEDIENTE No.**

54. **TÍTULO** "Batería del acido lactico capaz de reducir la tendencia de un individuo a desarrollar reacciones alergicas"

51. **CLASIFICACIÓN INTERNACIONAL** C12N 9/52

71. **SOLICITANTE** Societe Des Produits Nestlé S.A.

DOMICILIO Vevey, Suiza

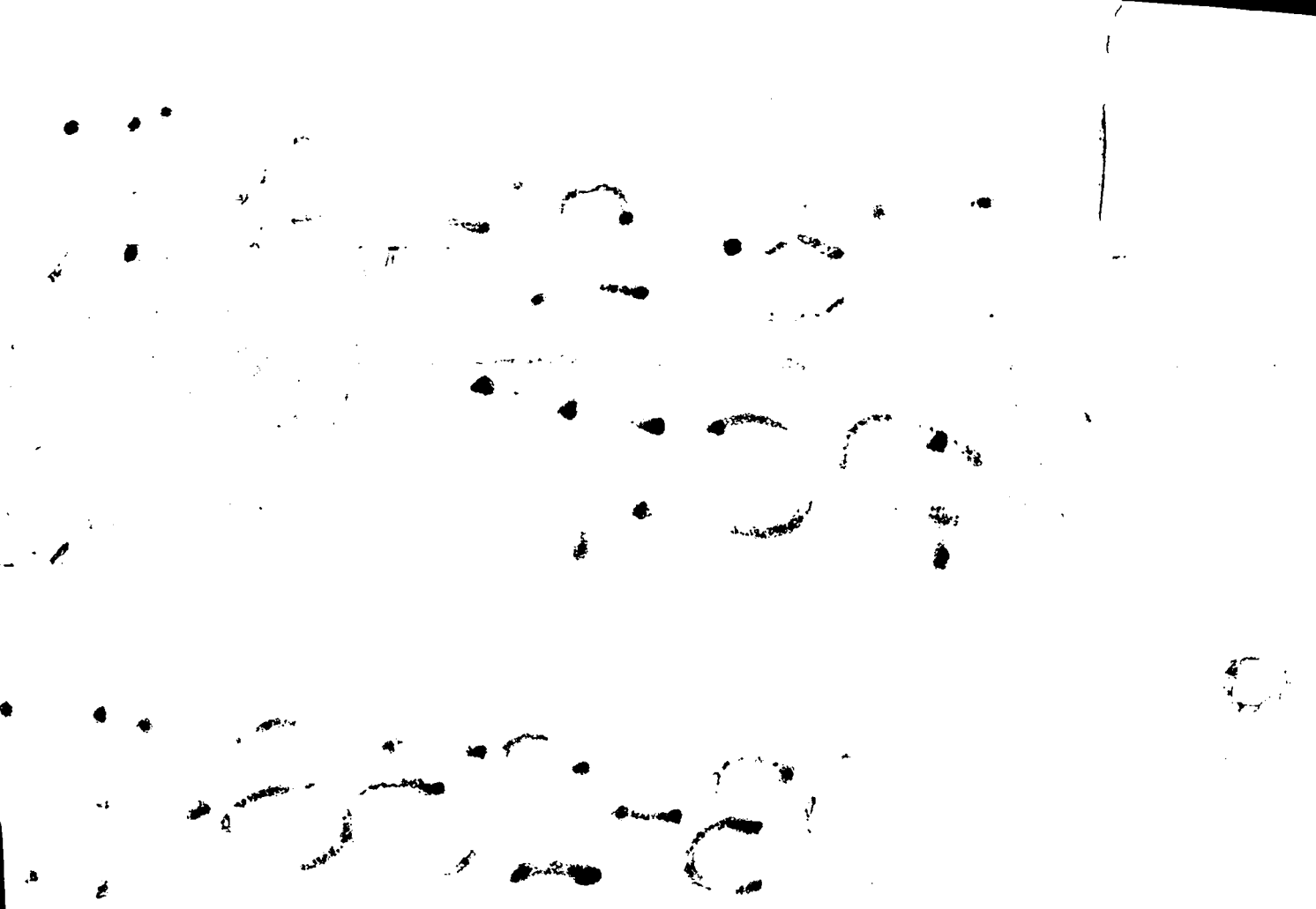
APODERADO Jose Antonio Uribe
T.P. 10.784 codigo 231

22. **BOGOTÁ, D. C.,** 20 Marzo 2003

202038

Q-F-89

(FORMA P-10)



No hay Ref 45

PETITORIO

Industria y Comerc
SUPERINTENDENC

03 023841 00

SUPERINDUSTRIA Y COMERCIO
Radicacion : 03023841 00000000 Folios: 347
Fecha (AMD): 2003-03-20 16:39:36
Tramite : 011 PCT-PATENT 379 FASE NACI 411 PRESENTAC
Dependencia: 2020 DIVISION DE NUEVAS CREACIONES

FORMULARIO UNICO DE SOLICITUD DE PATENTE
PCT
ENTRADA EN FASE NACIONAL

(1) SOLICITUD DE:

☒ Patente de Invención ☐ Patente de Modelo de Utilidad

☐ Capítulo I. ☒ Capítulo II.

(2) SOLICITANTE (71)	Nombre: **SOCIÉTÉ DES PRODUITS NESTLÉ S.A.** Dirección: P.O. Box 353, CH-1800 Vevey, Suiza. Nacionalidad o Domicilio: Vevey, Suiza. Lugar de Constitución: Suiza. Teléfono: 3264270 Fax: 6069701 E-mail: jllcco@lloredacamacho.com	**IDENTIFICACION** C.C. ☐ NIT ☐ C.E. ☐ Otro ☐ Cual Número
(3) REPRESENTANTE O APODERADO	Nombre: **JOSE ANTONIO LLOREDA** Dirección: Calle 72 No. 5-83 Piso 5º, Bogotá- Colombia Teléfono: 3264270 Fax: 6069701 E-mail: jllcco@lloredacamacho.com	C.C. ☒ NIT ☐ C.E. ☐ Otro ☐ Cual Número 17.159.681 TP 10.784
(4) INVENTOR(ES) (72)	Nombre: GERMOND, Jaques, Edouard; CORTHESY, Blaise; MOLLET, Beat y FRITSCHE, Rodolphe. Dirección: Ch. des Cèdres 18, CH-1023 Crissier; Chemin du Pony 34C, CH-1066 Epalinges; Avenue de l'Esplanade 9, CH-1012 Lausanne y Ch. de la Chaumény 48, CH-1814 La Tour-De-Peilz. Nacionalidad o Domicilio: Suiza.	

(5) PCT (86)

Solicitud Internacional No. PCT/EP01/10956 Fecha: 21/09/01

Publicación Internacional No. WO 02/24883 A2 Fecha: 28/03/02

(6) Título (54) "BACTERIA DEL ACIDO LACTICO CAPAZ DE REDUCIR LA TENDENCIA DE UN INDIVIDUO A DESARROLLAR REACCIONES ALERGICAS"

(7) Clasificación Internacional (51) C12N 9/52

(8) Prioridad SI ☒ NO ☐

(33) País de Origen	(32) Fecha	(31) Número de Solicitud
EP	25/09/00	00120867.7

(9) Comprobante de Pago No. 02-86,474
03-11,830

Fecha: Diciembre 24 de 2002
Febrero 17 de 2003

(10) **ANEXOS**

- [x] Comprobante de pago de la tasa de presentación de la solicitud.
- [x] Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica.
- [x] Documento de Representación Legal. Protocolizado bajo el número 11.145.
- [ ] Documento de cesión del inventor al solicitante o a su causante.
- [ ] Declaraciones.
- [ ] Copia de la solicitud internacional. (resumen, descripción, reivindicaciones, dibujos)
- [x] Traducción de la solicitud internacional al castellano. (resumen, descripción, reivindicaciones, dibujos, listado de secuencias)
- [x] Copia del examen preliminar internacional efectuado por la Autoridad Internacional de Examen Preliminar (IPEA).
- [x] Traducción del examen preliminar internacional efectuado por la IPEA.
- [ ] De ser el caso, copia del contrato de acceso.
- [ ] De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales las comunidades indígenas.
- [ ] Arte Final 12X12.
- [ ] De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionados parcial o totalmente con la invención de esta solicitud.
- [ ] De ser el caso, modificaciones efectuadas a la solicitud internacional.
- [x] Otros Reporte de Búsqueda Internacional con su traducción y Anexos, Publicación con su traducción, Opinión Escrita con su traducción.

(11) **FIGURA CARACTERISTICA:**

(12) Solicito la concesión de la patente,

NOMBRE: JOSE ANTONIO LLOREDA

FIRMA:

C.C. 17.159.681 Btá. T.P. 10.784

2

Industria y Comercio

SUPERINTENDENCIA

SUPERINDUSTRIA Y COMERCIO
Radicacion : 03023841 00000000 Folios: 347
Fecha (AMD): 2003-03-20 16:39:36
Tramite : 011 PCT-PATENT 379 FASE NACI 411 PRESENTAC
Dependencia: 2020 DIVISION DE NUEVAS CREACIONES

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 02 - 86,474
FECHA : DICIEMBRE 24 DE 2002

***** C O N S I G N A C I O N *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
LLOREDA Y CIA S. A	CONSIGNACION	BANCO POPULAR	050-00110-6	3139931	24/12/2002	149,623,700.00

***** C O N C E P T O *****

CANT.	RENTISTICO	CONCEPTO		TOTAL CONCEPTO
1	50005-01-01 SOLICITUDES	1	TRAMITES DE SOL. DE PATENTE DE IN	378,000.00
			TOTAL : $	378,000.00

SON: TRESCIENTOS SETENTA Y OCHO MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

Carrera 13 No. 27-00 Pisos 5, 7 y 10
Conmutador 382 0840
Fax 350 5220
E-mail: info@sic.gov.co
www.sic.gov.co
Bogotá, D.C., Colombia

NIT : 800176089-2

SUPERINDUSTRIA Y COMERCIO
Radicacion : 03023841 00000000 Folios: 347
Fecha (AMD): 2003-03-20 16:39:36
Tramite : 011 PCT-PATENT 379 FASE NACI 411 PRESENTAC
Dependencia: 2020 DIVISION DE NUEVAS CREACIONES

CERTIFICACION DE EXCEDENTE : 03 - 11,830
FECHA : FEBRERO 17 DE 2003

***** C O N S I G N A C I O N *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
LLOREDA Y CIA S. A	CONSIGNACION	BANCO POPULAR	050-00110-6	8284102	17/02/2003	898,000.00

***** C O N C E P T O *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-09 OTROS SERVICIOS DE PROPIEDAD I	99 OTROS SERVCIOS ADMINISTRATIVOS	22,000.00
		TOTAL : $	22,000.00

SON: VEINTIDOS MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

ESTA CERTIFICACION DE EXCEDENTE DE PAGO SOLO PODRA SER UTILIZADA COMO PARTE DE LA CANCELACION DE UNA TARIFA VIGENTE Y NO PODRA SER SUSTITUIDA POR NINGUN RECIBO.

Carrera 13 No. 27-00 Pisos 5, 7 y 10
Conmutador 382 0840
Fax 350 5220
E-mail: info@sic.gov.co
www.sic.gov.co
Bogotá, D.C., Colombia

ANEXO

El documento que acredita la calidad de REPRESENTANTES LEGALES en Bogotá de los doctores **JOSE ANTONIO LLOREDA** y/o **ALFONSO LLOREDA CAMACHO** para TODOS LOS ASUNTOS RELACIONADOS CON LA PROPIEDAD INDUSTRIAL de la sociedad peticionaria, que faculta a cada uno de dichos abogados para actuar individualmente, se encuentra protocolizado ante esa División bajo el No. 11.145. En consecuencia, ruego a usted reconocer la personería de cada uno de dichos Representantes Legales para actuar dentro del presente expediente, a fin de que cualquiera de ellos pueda intervenir en cualquier tiempo respecto de la solicitud en él contenida.

JOSE ANTONIO LLOREDA
T.P. No. 10.784
Código 231

TRATADO DE COOPERACION DE PATENTES
PCT
REPORTE DE BUSQUEDA INTERNACIONAL

(PCT Artículo 18 y Reglas 43 y 44)

Registro de referencia del solicitante o agente 80263 WO	**PARA FUTURAS ACCIONES** ver Notificación o Transmisión del Reporte de Búsqueda Internacional (Forma PCT/ISA/220) así como, donde aplique, el ítem 5 a continuación.	
Solicitud internacional No. PCT/EP 01/10956	Fecha de registro internacional *(dia/mes/año)* 21/09/2001	Fecha de Prioridad (Primera) *(dia/mes/año)* 25/09/2000
Solicitante **SOCIETE DES PRODUITS NESTLE S.A.**		

Este Reporte de Búsqueda Internacional ha sido preparado por esta Autoridad de Búsqueda Internacional y es transmitido al solicitante Según el Artículo 18. Una copia será transmitida a la Oficina Internacional.

Este Reporte de Búsqueda Internacional consta de un total de 4 hojas.

☒ También está acompañado de una copia de cada documento técnico anterior citado en este reporte.

1. **Bases del reporte**

a. Respecto al **lenguaje**, la búsqueda internacional se llevó a cabo según la solicitud internacional en el lenguaje en el cual se presentó, a menos que se indique de otro modo en este ítem.

☐ la búsqueda internacional se llevó a cabo según la traducción de la solicitud internacional proporcionada a esta Autoridad (Regla 23.1(b)).

b. Respecto a una secuencia de **ácido nucleótido y/o amino revelado** en la solicitud internacional, la búsqueda internacional se llevó a cabo según la lista de la secuencia:

☐ contenido en la solicitud internacional en forma escrita.

☐ presentada junto con la solicitud internacional en forma legible por computador.

☒ proporcionada subsiguientemente a esta Autoridad en forma escrita.

☒ proporcionada subsiguientemente a esta Autoridad en forma legible por computador.

☒ se ha proporcionado la afirmación de que el listado de secuencias escrito proporcionado subsiguientemente no va más allá de la revelación en la solicitud internacional según se presentó.

☒ se ha proporcionado la afirmación de que la información registrada en forma legible por computador es idéntica al listado de la secuencia escrita.

2. ☐ **Ciertas reivindicaciones no pudieron localizarse** (Ver Casilla I).

3. ☐ **Falta unidad de invención** (ver Casilla II).

4. Respecto al **título,**

☒ El texto es aprobado según lo presenta el solicitante.

☐ Esta Autoridad ha establecido que el texto se lea así:

5. Respecto al **resumen,**

☐ El texto es aprobado según lo presenta el solicitante.

☒ Esta Autoridad ha establecido el texto de acuerdo con la Norma 38.2(b), según aparece en el Recuadro III. El solicitante puede, un mes después de la fecha de haber enviado por correo este reporte de búsqueda internacional, presentar comentarios a esta Autoridad.

6. La figura de los **dibujos** que se publicará con el resumen es la Figura No. ——

☐ como lo sugirió el solicitante.

☒ porque el solicitante no sugirió una figura.

☐ porque esta figura caracteriza mejor a la invención.

☒ Ninguna de las figuras.

REPORTE DE BUSQUEDA INTERNACIONAL

Solicitud internacional No.
PCT/EP 01/10956

Casilla III TEXTO DEL RESUMEN (Continuación del ítem 5 de la primera hoja)

La presente invención tiene que ver con cepas novedosas de bacterias lácticas que son capaces de reducir la tendencia de un individuo a elevar reacciones alérgicas. En particular, la presente invención se refiere a la generación de cepas de bacterias lácticas que expresan proteínas que incluyen péptidos inductores de tolerancia y el uso de los mismos en la reducción de la tendencia de un individuo a desarrollar reacciones alérgicas. La invención también tiene que ver con composiciones alimenticias o farmacéuticas que contienen dichos microorganismos o fracciones activas de los mismos.

REPORTE DE BUSQUEDA INTERNACIONAL

Solicitud Internacional No.
PCT/EP 01/10956

A. CLASIFICACION DE MATERIA OBJETO

IPC 7 C12N9/52 C12N15/62 C12N1/21 C07K14/47 C07K16/18
A61K35/74 A61K38/17 A23J3/34 A23L1/305 A23C9/123
C07K7/06 C07K7/08 //(C12N1/21, C12R1:46)

Según la Clasificación de Patente Internacional (IPC) o según la clasificación nacional y la IPC

B. CAMPOS BUSCADOS

Documentación mínima buscada (sistema de clasificación seguido de símbolos de clasificación)

IPC 7 C12N C07K C12R A61K A23C A23J A23L

Documentación buscada fuera de documentación mínima hasta donde dichos documentos se incluyan en los campos buscados

Base de datos electrónica consultada durante la búsqueda internacional (nombre de la base de datos y, donde sea práctico, buscar términos usados)

EPO-Internacional, WPI Dato, PAJ, FSTA, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTOS CONSIDERADOS RELEVANTES

Categoría °	Citación de documento, con indicación, donde sea apropiado, de los textos relevantes	Relevante a la reivindicación No.
Y	WO 96 32486 A (TNO; LEER ROBERT JAN (NL); POUWELS PIETER HENDRIK (NL); BOERSMA WI) 17 de Octubre de 1996 (1996-10-17) página 4, línea 14-18 página 5, línea 5-16 ---	1-14
Y	WO 97 09437 A (REMAUT ERIK; STEIDLER LOTHAR (BE); WELLS JEREMY MARK (GB)) 13 de Marzo de 1997 (1997-03-13) página 4, línea 25-página 9, línea 8 reivindicaciones 18,19 --- -/--	1-14

☒ En el recuadro C se enumeran más documentos.

☒ En el anexo se enumeran miembros de la familia de patentes.

° Categorías especiales de documentos citados:

'A' documento que define el estado general de la técnica el cual no se considera de relevancia particular

'E' documento anterior pero publicado en o después de la fecha de presentación internacional

'L' documento que puede arrojar dudas sobre reivindicaciones de prioridad o que se cita para establecer la fecha de publicación de otra citación u otra razón especial (según se especifica)

'O' documento que se refiere a una revelación oral, uso, exhibición u otros medios

'P' documento publicado antes de la fecha de presentación internacional pero después de la fecha de prioridad reivindicada

'T' documento posterior publicado después de la fecha de presentación internacional o fecha de prioridad y que no está en conflicto con la solicitud pero citado para entender el principio o teoría que sustenta la invención

'X' documento de relevancia particular; la invención reivindicada no puede considerarse nueva o no puede considerarse que comprende un paso inventivo cuando el documento se toma solo

'Y' documento de relevancia particular; la invención reivindicada no puede considerarse que comprende un paso inventivo cuando el documento se combina con uno o más documentos, siendo dicha combinación obvia para una persona experta en la técnica

'&' documento miembro de la misma familia de patentes

Fecha de la terminación real de la búsqueda internacional	Fecha de envío por correo del reporte de búsqueda internacional
29 de Agosto de 2002	05/09/2002

Nombre y dirección de correo de ISA	Funcionario autorizado
European Patent Office, P. B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx 31 651 epo nl, Fax (+31-70) 340-3016	Van de Kamp, M

REPORTE DE BUSQUEDA INTERNACIONAL

Solicitud Internacional No.
PCT/EP 01/10956

C. (Continuación) DOCUMENTOS CONSIDERADOS RELEVANTES

Categoría °	Citación de documento, con indicación, donde sea apropiado, de los textos relevantes	Relevante a la reivindicación No.
Y	MAASSEN C B M ET AL.: "Instrumentos para estrategias orales de prevención de enfermedades: Lactobacillus casei recombinante que expresa el fragmento C de toxina tetanica para vacunación o proteínas de mielina para inducción de tolerancia por vía oral en esclerosis múltiple" VACCINE, vol. 17, no. 17, 23 de Abril de 1999 (1999-04-23), páginas 2117-2128, XP004164996 resumen página 2117, columna de la derecha, línea 1-página 2118, columna de la derecha, línea 24	1-14

Y	WO 97 00078 A (SYVAEOJA EEVA LIISA; ISOLAURI ERIKA (FI); KORHONEN HANNU (FI); SAL) 3 de Enero de 1997 (1997-01-03) página 3, línea 28-página 6, línea 26 ejemplo 2	1-14

Y	WO 00 42863 A (FRITSCHE RODOLPHE; MAYNARD FRANCOISE (CH); NESTLE SA (CH); PECQUET) 27de Julio de 2000 (2000-07-27) citado en la solicitud el documento completo	1-14

A	POUWELS P H ET AL.: "Bacteria de ácido láctico como vehículo de liberación para propósitos de inmunización por vía oral" INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, vol. 41, 1998, páginas 155-167, XP000921209 el documento completo	1-14

A	GILBERT C ET AL.: "Una nueva proteinasa de superficie de célula: secuenciación y análisis del gen prtB de Lactobacillus delbrueckii subsp. bulgaricus" JOURNAL OF BACTERIOLOGY, vol. 178, no. 11, 1 de Junio de 1996 (1996-06-01), páginas 3059-3065, XP 000608165 citado en la solicitud resumen	1,3

A	WO 95 21926 A (UNIV ROCHESTER) 17 de Agosto de 1995 (1995-08-17) página 2, línea 12-página 3, línea 30	1

REPORTE DE BUSQUEDA INTERNACIONAL

Solicitud Internacional No.
PCT/EP 01/10956

C. (Continuación) DOCUMENTOS CONSIDERADOS RELEVANTES

Categoría °	Citación de documento, con indicación, donde sea apropiado, de los textos relevantes	Relevante a la reivindicación No.
A	BOREL Y ET AL.: "Inducción de tolerancia sistémica por tolerógenos" NESTLÉ NUTRITION WORKSHOP SERIES. INTESTINAL IMMUNOLOGY AND FOOD ALLERGY. 34TH NESTLÉ NUTRITION WORKSHOP VERSAILLES, FRANCE, JUNE 21-24, 1993. NESTEC LTD. VEVEY, SWITZERLAND, vol. 34, 21 de Junio de 1993 (1993-06-21), páginas 179-189, XP000981593 página 180, línea 1-35 página 186, línea 29-página 187, línea 4 -----	1

REPORTE DE BUSQUEDA INTERNACIONAL

Informe sobre miembros de familia de patentes

Solicitud Internacional No.
PCT/EP 01/10956

Documento de patente citado en el reporte de búsqueda	Fecha de publicación	Miembro(s) de la familia de patentes			Fecha de publicación
WO 9632486 A	17-10-1996	WO	9632486	A1	17-10-1996
		AU	2149495	A	30-10-1996
		AU	5163996	A	30-10-1996
		WO	9632487	A1	17-10-1996
WO 9709437 A	13-03-1997	AU	729449	B2	01-02-2001
		AU	6884496	A	27-03-1997
		BR	9610133	A	21-12-1999
		CN	1201493	A	09-12-1998
		EP	0848756	A1	24-06-1998
		WO	9709437	A1	13-03-1997
		JP	11511983	T	19-10-1999
		NO	980976	A	06-05-1998
		NZ	316580	A	29-09-1999
		US	6190662	B1	20-02-2001
WO 9700078 A	03-01-1997	FI	952926	A	15-12-1996
		AU	710126	B2	16-09-1999
		AU	6127896	A	15-01-1997
		BG	63153	B1	31-05-2001
		BG	102144	A	30-12-1998
		BR	9607827	A	30-11-1999
		CA	2224320	A1	03-01-1997
		CZ	9703997	A3	17-06-1998
		EE	9700339	A	15-06-1998
		EP	0833649	A1	08-04-1998
		WO	9700078	A1	03-01-1997
		JP	11510791	T	21-09-1999
		NO	975832	A	11-12-1997
		NZ	310478	A	28-01-2000
		PL	324010	A1	27-04-1998
		SK	171597	A3	04-11-1998
		ZA	9605088	A	22-01-1997
WO 0042863 A	27-07-2000	AU	3421000	A	07-08-2000
		BR	0007597	A	16-10-2001
		WO	0042863	A1	27-07-2000
		EP	1146794	A1	24-10-2001
		US	2002037357	A1	28-03-2002
WO 9521926 A	17-08-1995	US	5817308	A	06-10-1998
		CA	2182977	A1	17-08-1995
		EP	0741788	A1	13-11-1996
		JP	9509316	T	22-09-1997
		WO	9521926	A1	17-08-1995
		US	2002048562	A1	25-04-2002

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
80263 WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 01/10956	21/09/2001	25/09/2000
Applicant		
SOCIETE DES PRODUITS NESTLE S.A.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[ ] the text is approved as submitted by the applicant.

[X] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[X] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 01/10956

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

The present invention pertains to strains of lactic acid bacteria that are capable to reduce an individual's tendency to mount allergic reactions. In particular, the present invention relates to the generation of lactic acid bacteria strains that express proteins including tolerogenic peptides and the use thereof in reducing an individual's tendency to mount allergic reactions. The invention also pertains to food or pharmaceutical compositions containing said micro-organisms or active fractions thereof.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/10956

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/52 C12N15/62 C12N1/21 C07K14/47 C07K16/18
A61K35/74 A61K38/17 A23J3/34 A23L1/305 A23C9/123
C07K7/06 C07K7/08 //(C12N1/21,C12R1:46)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K C12R A61K A23C A23J A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, FSTA, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 32486 A (TNO ;LEER ROBERT JAN (NL); POUWELS PIETER HENDRIK (NL); BOERSMA WI) 17 October 1996 (1996-10-17) page 4, line 14-18 page 5, line 5-16 ---	1-14
Y	WO 97 09437 A (REMAUT ERIK ;STEIDLER LOTHAR (BE); WELLS JEREMY MARK (GB)) 13 March 1997 (1997-03-13) page 4, line 25 -page 9, line 8 claims 18,19 --- -/--	1-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 2002	05/09/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van de Kamp, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/10956

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	MAASSEN C B M ET AL.: "Instruments for oral disease-intervention strategies: recombinant Lactobacillus casei expressing tetanus toxin fragment C for vaccination or myelin proteins for oral tolerance induction in multiple sclerosis" VACCINE, vol. 17, no. 17, 23 April 1999 (1999-04-23), pages 2117-2128, XP004164996 abstract page 2117, right-hand column, line 1 -page 2118, right-hand column, line 24 ---	1-14
Y	WO 97 00078 A (SYVAEOJA EEVA LIISA ;ISOLAURI ERIKA (FI); KORHONEN HANNU (FI); SAL) 3 January 1997 (1997-01-03) page 3, line 28 -page 6, line 26 example 2 ---	1-14
Y	WO 00 42863 A (FRITSCHE RODOLPHE ;MAYNARD FRANCOISE (CH); NESTLE SA (CH); PECQUET) 27 July 2000 (2000-07-27) cited in the application the whole document ---	1-14
A	POUWELS P H ET AL.: "Lactic acid bacteria as antigen delivery vehicles for oral imunization purposes" INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, vol. 41, 1998, pages 155-167, XP000921209 the whole document ---	1-14
A	GILBERT C ET AL.: "A new cell surface proteinase: sequencing and analysis of the prtB gene from Lactobacillus delbrueckii subsp. bulgaricus" JOURNAL OF BACTERIOLOGY, vol. 178, no. 11, 1 June 1996 (1996-06-01), pages 3059-3065, XP000608165 cited in the application abstract ---	1,3
A	WO 95 21926 A (UNIV ROCHESTER) 17 August 1995 (1995-08-17) page 2, line 12 -page 3, line 30 ---	1
	-/--	

2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/10956

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BOREL Y ET AL.: "Induction of systemic tolerance by tolerogens" NESTLÉ NUTRITION WORKSHOP SERIES. INTESTINAL IMMUNOLOGY AND FOOD ALLERGY. 34TH NESTLÉ NUTRITION WORKSHOP VERSAILLES, FRANCE, JUNE 21-24, 1993. NESTEC LTD. VEVEY, SWITZERLAND, vol. 34, 21 June 1993 (1993-06-21), pages 179-189, XP000981593 page 180, line 1-35 page 186, line 29 -page 187, line 4 -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 01/10956

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9632486 A	17-10-1996	WO 9632486 A1	17-10-1996
		AU 2149495 A	30-10-1996
		AU 5163996 A	30-10-1996
		WO 9632487 A1	17-10-1996
WO 9709437 A	13-03-1997	AU 729449 B2	01-02-2001
		AU 6884496 A	27-03-1997
		BR 9610133 A	21-12-1999
		CN 1201493 A	09-12-1998
		EP 0848756 A1	24-06-1998
		WO 9709437 A1	13-03-1997
		JP 11511983 T	19-10-1999
		NO 980976 A	06-05-1998
		NZ 316580 A	29-09-1999
		US 6190662 B1	20-02-2001
WO 9700078 A	03-01-1997	FI 952926 A	15-12-1996
		AU 710126 B2	16-09-1999
		AU 6127896 A	15-01-1997
		BG 63153 B1	31-05-2001
		BG 102144 A	30-12-1998
		BR 9607827 A	30-11-1999
		CA 2224320 A1	03-01-1997
		CZ 9703997 A3	17-06-1998
		EE 9700339 A	15-06-1998
		EP 0833649 A1	08-04-1998
		WO 9700078 A1	03-01-1997
		JP 11510791 T	21-09-1999
		NO 975832 A	11-12-1997
		NZ 310478 A	28-01-2000
		PL 324010 A1	27-04-1998
		SK 171597 A3	04-11-1998
		ZA 9605088 A	22-01-1997
WO 0042863 A	27-07-2000	AU 3421000 A	07-08-2000
		BR 0007597 A	16-10-2001
		WO 0042863 A1	27-07-2000
		EP 1146794 A1	24-10-2001
		US 2002037357 A1	28-03-2002
WO 9521926 A	17-08-1995	US 5817308 A	06-10-1998
		CA 2182977 A1	17-08-1995
		EP 0741788 A1	13-11-1996
		JP 9509316 T	22-09-1997
		WO 9521926 A1	17-08-1995
		US 2002048562 A1	25-04-2002

DOCUMENTOS ANEXOS AL REPORTE DE BÚSQUEDA INTERNACIONAL

1. WO 96 32486 A
2. WO 97 09437 A
3. MAASSEN C B M ET AL
4. WO 97 00078 A
5. WO 00 42863 A
6. POUWELS P H ET AL
7. GILBERT C ET AL
8. WO 95 21926 A
9. BOREL Y ET AL

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/74, 15/62, 15/46, 15/44, 15/70, 15/31, C07K 14/14, 14/11 // C12N 1/21, C12R 1:225

A1

(11) International Publication Number: WO 96/32486

(43) International Publication Date: 17 October 1996 (17.10.96)

(21) International Application Number: PCT/NL95/00135

(22) International Filing Date: 11 April 1995 (11.04.95)

(71) Applicant *(for all designated States except US)*: NEDERLANDSE ORGANISATIE VOOR TOEGEPAST-NATUURWETENSCHAPPELIJK ONDERZOEK TNO [NL/NL]; Schoemakerstraat 97, NL-2628 VK Delft (NL).

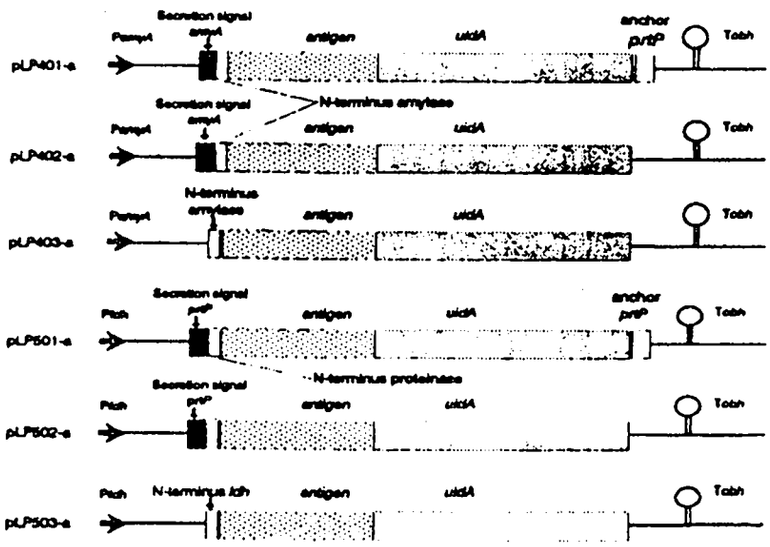
(72) Inventors; **and**
(75) Inventors/Applicants *(for US only)*: LEER, Robert, Jan [NL/NL]; Tulpentuin 36, NL-2272 XK Voorburg (NL). POUWELS, Pieter, Hendrik [NL/NL]; Delftweg 14, NL-2289 AJ Rijswijk (NL). BOERSMA, Wilhelmus, Johannes [NL/NL]; Burgemeester Merkusstraat 7, NL-2645 NJ Delfgauw (NL).

(74) Agent: DE BRUIJN, Leendert, C.; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).

(81) **Designated States:** AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD FOR THE CONSTRUCTION OF VECTORS FOR LACTIC ACID BACTERIA LIKE LACTOBACILLUS SUCH THAT THE BACTERIA CAN EFFICIENTLY EXPRESS, SECRETE AND DISPLAY PROTEINS AT THE SURFACE



(57) Abstract

The invention relates to a method to construct multi-purpose plasmid vectors that can be used for the introduction, stable maintenance and efficient expression and secretion of foreign genes or gene fragments in a variety of lactic acid strains such as *Lactobacillus* strains and *Bifidobacterium*, as well as to expression vectors to be used in this method. The invention also comprises a method of production of proteins and polypeptides heterologous and homologous to lactic acid bacteria at increased levels. Also comprised are lactic acid bacteria containing these expression vectors and producing these proteins or polypeptides.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia
AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

Method for the construction of vectors for lactic acid bacteria like Lactobacillus such that the bacteria can efficiently express, secrete and display proteins at the surface.

FIELD OF THE INVENTION

The present invention relates to a method to construct multi-purpose plasmid vectors that can be used for the introduction, stable maintenance and efficient expression and secretion of foreign genes in a variety of lactic acid strains such as *Lactobacillus* strains and *Bifidobacteria*, as well as to expression vectors to be used in this method. The invention also comprises a method of production of proteins and polypeptides heterologous to lactic acid bacteria at increased levels as well as some novel products.

BACKGROUND OF THE INVENTION

Introduction

Recombinant DNA technology has opened the way to an efficient production of proteins and enzymes from human, animal, plant or bacterial sources in a variety of organisms. During the past fifteen years it has become routine to (over)produce a protein of interest by cloning the corresponding gene onto a (multi-copy) plasmid vector, and expressing the gene under the control of strong expression signals, in a microorganism such as for example the bacterium *Escherichia coli*. Application of this methodology to other bacterial hosts, in particular industrially important bacteria like Lactic Acid Bacteria (LAB), more specifically *Lactobacilli*, requires that cloned DNA sequences can be stably maintained and expressed. Over the past decade systems have been developed with which homologous and heterologous genes can be introduced in *Lactobacilli* on (multi-copy) plasmid vectors. Such hybrid vectors are, in general, structurally stable and can be stably maintained under selective conditions. Systems for site-specific integration of cloned genes have also been described [Chopin et al, Appl. Environm. Microbiol. 55, 1769-1774 1989; Leenhout et al, Appl. Environm. Microbiol. 55, 394-400 1989; Leer et al, Mol. Gen. Genet. 239 269-272 1993]. Cloned genes that are integrated into the *Lactobacillus* chromosome are both structurally and segregationally stable, in the presence or absence of selective pressure.

BNSDOCID: <WO 9632486A1 I >

Heterologous gene expression in *Lactobacillus*

Rapid advances in applying gene technology for the development of strain improvement programmes for *Lactobacilli* are limited by i) structural instability of *Lactobacillus* DNA in working organisms like *Escherichia coli* and ii) inefficiency of expression and secretion of heterologous proteins in *Lactobacillus*.

Structural instability of *Lactobacillus* DNA sequences cloned in *Escherichia coli* was reported in a number of publications [Bates and Gilbert, Gene 85, 253-289 (1989); Taguchi and Ohta, J. Biol. Chem. 266, 12588-12594 (1991); Bernard et al, FEBS Lett. 290, 61-64 (1991); Kochhar et al, Biochem. Biophys. Res. Commun. 185, 705-712 (1992); Lee et al, J. Biol. Chem. 266, 13028-13034 (1989); Lerch et al, Gene 78, 47-57 (1989); Vanderslice et al, J. Biol. Chem. 261, 15186-15191 (1986); Posno et al, Appl. Environm. Microbiol. 57, 2764-2769 (1991); Branny et al, J. Bacteriol. 175, 1993]. In general, instability increases when cloned *Lactobacillus*-derived DNA fragments are expressed in *Escherichia coli*. For example when the *xylA* gene of *Lactobacillus pentosus*, encoding xylose isomerase, was introduced in *Escherichia coli* on a high copy vector (pUC19) in two parts (one of which also contained the promoter region), these recombinant vectors were structurally stable. However, reconstitution in *Escherichia coli* of the entire *xylA* gene from the two parts proved to be impossible [Posno et al, Appl. Environm. Microbiol. 57, 2764-2766 (1991)]. Similarly, reconstitution of the *Lactobacillus plantarum* D-lactate dehydrogenase gene by joining parts of the gene present on two separate clones could not be achieved in *Escherichia coli*. It was suggested that the product of the intact D-*ldh* gene was toxic for *Escherichia coli* [Taguchi et al, J. Biol. Chem. 266, 12588-12594 (1991)]. This type of instability can be explained in general by assuming that (over)production of a *Lactobacillus* gene product is toxic for *Escherichia coli*.

Structural instability of plasmid vectors with strong cloned promoters has been reported by different groups [e.g. Gibson et al, Gene 53, 275-281 1987] and has also been observed for the promoter of the *Lactobacillus casei ldh* gene by the present inventors. This type of instability is explained by assuming that transcription starting at the promoter interferes with efficient replication of the plasmid vector. The instability can, in general, be overcome by insertion of a transcription

terminator sequence between the promoter and the origin of replication.

Although the inconvenience of structural instability during construction of recombinant DNA molecules and performance of other necessary genetic manipulations might be overcome by using another host organism, e.g. *Lactobacillus* itself, the bacterium *Escherichia coli* is preferred as "working organism" because of the wealth of genetic and biochemical data that are available concerning this organism. In addition, genetic manipulation of lactic acid bacteria like *Lactobacilli* is characterized by intrinsic difficulties which make this host less suitable for standard recombinant DNA experiments than a standard working organism like *Escherichia coli*.

Preliminary studies which deal with expression of heterologous proteins in lactic acid bacteria like *Lactobacilli* have shown that expression of foreign proteins, in particular mammalian proteins, in *Lactobacillus* is rather inefficient. In none of the studies published sofar was expression of heterologous nucleic acid sufficiently efficient to obtain levels of production that surmount 1% of total soluble protein. The inventors have observed that the efficiency of expression in *Lactobacillus* of the *Escherichia coli* enzyme β-galactosidase was in fact reduced ten- to twenty-fold (final level 0.1-0.2% of total soluble protein) when β-galactosidase was fused at its N-terminal end with a twenty to thirty amino acid antigenic determinant from the Foot and Mouse Disease Virus capsid protein VP1 or the Rotavirus capsid protein VP7.

Object of the invention

The objective of the present invention is to overcome the above mentioned difficulties and to provide

i) a method for the construction of plasmid expression and expression-secretion vectors in a working organism other than the lactic acid bacterium to be transformed, for example in *Escherichia coli*,

ii) a series of expression and expression-secretion vectors for transforming lactic acid bacteria like *Lactobacillus* and Bifidobacteria, said vectors being characterized by at least a translation initiation region allowing efficient expression of heterologous nucleic acid encoding foreign proteins or polypeptides, and

iii) a series of vectors that efficiently express, secrete and display antigens from pathogens such as influenza virus or rotavirus at the

BNSDOCID: <WO 9632486A1_I_>

bacterial surface of their host, said vectors preferably being food grade. Thus providing a method for expressing and optionally secreting and displaying heterologous proteins or polypeptides in lactic acid bacteria strains. The products thus obtainable are also considered to fall within the scope of the invention.

SUMMARY OF THE INVENTION

The present invention provides a method to construct by recombination DNA technology a series of expression vectors and expression-secretion vectors in a working organism other than the lactic acid bacterium to be transformed, in particular a non *Lactobacillus* microorganism such as *Escherichia coli* that can be used to genetically modify a bacterium, in particular a lactic acid bacterium so as to efficiently produce foreign proteins or polypeptides. Especially interesting is the production of immunogenic proteins and peptides from pathogenic organisms and proteins e.g. mammalian proteins that can be used for the induction of immunological tolerance. Other interesting objectives are production of micronutrients, vitamins and prebiotic factors. In particular products that could now be obtained are human and pig rotavirus envelope proteins, influenza virus haemagglutinins, influenza virus nucleo proteins, heat labile toxin of *E. coli* and fusion proteins thereof, cholera toxin (ß-subunits) and fusion proteins thereof, urease A and/or B from *Helicobacter pylori*. The nucleic acid sequences encoding these proteins form part of the state of the art and recombinant lactic acid bacteria expression of such sequences will lead toproducts that are illustrative of the products that have now become available. In particular the fact that such products can be obtained as more than 1% of the total soluble protein content of a lactic acid bacterium like *Lactobacillus* in a simple manner using an expression vector according to the invention is particularly interesting. It now also becomes possible to provide oral vaccines and immunologically stimulating preparations. Use of a foodgrade host cell and food grade expression vector enables production of food grade proteins and polypeptides in particular of proteins and polypeptides that are heterologous to the host cell.

It was found that the level of expression of a heterologous protein or polypeptide in a lactic acid bacterium, whilst the corresponding nucleic acid is under control of a homologous promoter of the lactic acid bacterium, can be considerably increased by insertion of a nucleotide

BNSDOCID: <WO _9632486A1_I_>

sequence coding for the N-terminal sequence of a gene homologous to a lactic acid bacterium gene, immediately downstream of the translation start codon.

The present invention more specifically provides
i) a series of plasmid vectors with which foreign proteins, in particular immunogenic proteins from pathogenic organisms or mammalian proteins that can induce immunological tolerance, can be efficiently expressed in *Lactobacillus* and optionally accumulated or excreted and/or secreted either into the culture fluid or subsequently anchored to the membrane and displayed at the surface of bacteria, in particular of *Lactobacilli*,
ii) a method to construct such plasmid vectors in *Escherichia coli*, circumventing structural instability of the *Lactobacillus* vectors,
iii) *Lactobacillus* strains harbouring plasmid vectors that efficiently express antigenic determinants from e.g. influenza virus and rotavirus fused to an immunological carrier protein like β-glucuronidase.

DETAILED DESCRIPTION OF THE INVENTION

i) Facilitated construction in a non lactic acid bacterium for example in a working organism like *Escherichia coli*

The present invention is characterized by a novel method to construct the vectors in a non lactic acid bacterium for example in a working organism like *Escherichia coli* prior to their introduction in a lactic acid bacterium like *Lactobacillus*. Application of the invention thus in particular circumvents the structural instability of *Lactobacillus* vector material in *Escherichia coli* caused by cloning of strong *Lactobacillus* promoters and/or by cloning and expression in *Escherichia coli* of *Lactobacillus* genes. The construction route in a non lactic acid bacterium for example in a working organism like *Escherichia coli* involves the insertion of a nucleotide sequence containing a transcription terminator between the promoter controlling the expression of the heterologous nucleic acid and the transcription terminator for the heterologous gene located downstream from the promoter. The newly introduced terminator serves to prevent transcription in *Escherichia coli* of open reading frames (ORF) that are to be inserted downstream from the promoter sequence so as to prevent their expression in *Escherichia coli*. Preferably the newly introduced terminator sequence will be located such that no product controlled by the promoter is produced in the working organism. This can be achieved by locating the newly introduced

BNSDOCID: <WO _9612486A1_I_>

terminator prior to the first codon of the heterologous expression product. A person skilled in the art will realise that in some instances a short expression product, i.e. a partial amino acid sequence expressed from lactic acid bacterium nucleic acid will be tolerated by the working organism, so that location of the newly introduced terminator sequence within the heterologous sequence encoding heterologous protein or polypeptide could be acceptable to prevent the negative consequences that expression of the complete product would otherwise have on the working organism. The newly introduced terminator sequence is preferably a strong terminator sequence. The newly introduced terminator sequence must be recognised as such by the working organism in which the vector is to be constructed or manipulated prior to transformation of a lactic acid bacterium. A preferred embodiment comprises a newly introduced terminator sequence that is recognised by the working organism, a non lactic acid bacterium but is not recognised by the lactic acid bacterium in which expression of the heterologous protein or polypeptide is desired.

In particular when a newly introduced terminator sequence is employed that can also be recognised by a lactic acid bacterium in which the heterologous protein or polypeptide is to be expressed it is necessary for the transcription block to be lifted. In order to achieve this the newly introduced terminator sequence is flanked at both sides such that it is excisable from the vector in a manner known per se to a person skilled in the art. This can be the case for example when it is flanked by a nucleic acid sequence encoding one or more very infrequently occurring restriction enzyme sites (e.g. *Not*I) which do not occur elsewhere in the plasmid, allowing easy removal of the newly introduced terminator sequence from the vector prior to its introduction into the lactic acid bacterium for example a *Lactobacillus*. Alternatively the newly introduced terminator sequence may be flanked by direct repeats such that the terminator sequence is excisable through homologous recombination of the host. After removal of the newly introduced terminator sequence the cloned ORF with the heterologous protein or polypeptide will be transcribed from the promoter in the lactic acid bacterium.

A person skilled in the art will realise that the working organism need not necessarily be a non lactic acid bacterium; this is merely a preferred embodiment due to the inherent problems of working in a lactic

NSDOCID: <WO___9632486A1_I_>

acid bacterium as working organism. Furthermore a person skilled in the art will realise that the nucleic acid sequence encoding a protein or polypeptide i.e. the product to be expressed need not necessarily be heterologous to a lactic acid bacterium. The system according to the invention may also be employed for homologous nucleic acid sequences.

ii) Enhanced expression in *Lactobacillus*

The present invention also provides expression and expression-secretion vectors that allow efficient expression of cloned foreign genes. It was found that the level of expression of a heterologous protein or polypeptide like the *Escherichia coli* enzyme β-galactosidase in a lactic acid bacterium such as *Lactobacillus*, whilst the corresponding nucleic acid is under control of a homologous promoter of the lactic acid bacterium, for example a *Lactobacillus* promoter, can be considerably increased by insertion of a nucleotide sequence coding for the N-terminal sequence of a gene homologous to a lactic acid bacterium like a *Lactobacillus* immediately downstream of the translation start codon.

In particular it is postulated that the presence of A and T rich nucleic acid sequences at the beginning of a nucleic acid sequence to be expressed in a lactic acid bacterium is conducive to high expression being achieved from such sequences. As in particular lactic acid bacteria like *Lactobacillus* have A and T rich sequences in parts of their genes encoding the N terminal part of their proteins this could be the reason why the presence of such a sequence ensures good expression in a lactic acid bacterium. It is possible that a high number of G and C residues in this region would favour the formation of stable RNA duplexes, which may interfere with translation initiation.

In order to obtain good expression in a lactic acid bacterium and optionally surface display of a protein or polypeptide, said vector must comprise an expression promoter sequence and a nucleic acid sequence encoding said protein or polypeptide with expression of said nucleic acid sequence being controlled by said promoter sequence, characterised in that said encoding nucleic acid sequence is preceded by a 5' non-translated nucleic acid sequence comprising at least the minimal sequence required for ribosome recognition and RNA stabilisation followed by a translation initiation codon, said translation initiation codon being immediately followed by a fragment of at least 5 codons of the 5'

(5)

terminal part of the translated nucleic acid sequence of a gene of a lactic acid bacterium or a structural or functional equivalent of said fragment, said fragment also being controlled by said promoter, thus resulting in an expression product which is a fusion polypeptide.

A structural or functional equivalent of said fragment of at least 5 codons is understood to include fragments encoding the same amino acid sequence but using different codons, fragments derived from other organisms than lactic acid bacteria but encoding corresponding parts of functionally related polypeptides, and equivalents of said fragment having at least 60% or even 80% nucleotide homology and preferably containing at least all the A and T residues of said fragment.

The gene from which the 5' terminal part of the translated nucleic acid sequence is derived is preferably the same gene from which the 5' non-translated region located between the promoter which is used to control the expression of the heterologous nucleic acid is derived. The resulting expression product thus becomes a hybrid of homologous and heterologous amino acid sequences, whereby the presence of the N-terminal sequence of the lactic acid bacterium gene in the resulting expression product does not detract from the functionality of the heterologous product that needs to be expressed. It is not necessary for the homologous N-terminal sequence to be very long. A nucleic acid sequence encoding at least 5 amino acids is sufficient to obtain good expression results. The maximum length to be used will depend on the expression host and the nature of the expression product. The maximum length is not really critical. One embodiment can comprise as small a sequence as possible in order not to risk the loss of functionality of the resulting expression product due to tertiary folding like e.g. proteolytic instability sufficiently to disturb the activity of the heterologous protein or polypeptide. A sequence of ten amino acids has been found to form an embodiment that is capable of achieving the objective of the invention quite adequately. N-terminal sequences with lengths over 20 amino acids are acceptable embodiments as well. Alternatively longer sequences are contemplated embodiments forming e.g. complete genes, without the corresponding gene terminator. By way of example even polycistronic mRNA encoding at least two proteins can be envisaged.

The expression and expression-secretion vectors of the present invention

SNSDOCID: <WO_ 9632486A1_I_>

can comprise the entire non-translated 5' region including the Shine-Dalgarno sequence [Shine and Dalgarno, Proc. Natl. Acad. Sci. USA 71, 5463-5467 (1974)], translation initiation codon and the A/T-rich N-terminal sequences preferably of a lactic acid bacterium gene. The selection of the gene from which the N-terminal sequence and the promoter are to be used will be obvious to a person skilled in the art. The criteria that are relevant are the organism in which expression is desired and the degree of expression that is normal for the gene system of which some components are to be used in the vector when the gene system is functioning normally in its natural environment. For *Lactobacillus* for example a number of proteins are known or are expected to be expressed to a high degree and any of the relevant components from the corresponding gene systems of *Lactobacillus* are suitable for use in vectors according to the invention. A large deal of sequencing has already taken place and a person skilled in the art can quite readily ascertain suitable sequences that can be used. The lactate dehydrogenase encoding gene, *ldh*, the proteinase encoding gene, *prtP*, or the α-amylase encoding gene, *amy* the S layer protein encoding gene slpA (Boot et al. Journ. of Bacteriol. Oct. 93 p. 6089-6096) (Figure 12), the ribosomal RNA encoding gene rrn, the glyceraldehyde-3-phosphate dehydrogenase encoding gene gapdh are examples of genes that are efficiently expressed in *Lactobacillus*. Their promoters and N terminal sequences can quite suitably be employed in vectors according to the invention. The configuration around the translation initiation start site in the expression vectors is chosen so as to warrant translation of foreign proteins in a manner equally efficient to that of the authentic *Lactobacillus*. L-lactate dehydrogenase, proteinase, α-amylase, S layer protein, the ribosomal RNA and the glyceraldehyde-3-phosphate dehydrogenase are all proteins that are efficiently translated in *Lactobacillus*. L-Lactate dehydrogenase is a key enzyme in the glycolytic pathway of lactobacilli which mediates the conversion of pyruvate to lactate. The proteinase encoded by *prtP* is an enzyme secreted and anchored to the cell surface through a so-called anchor sequence by a number of *Lactococci* and *Lactobacilli* which enables the bacteria to proteolytically hydrolyse casein and therefore grow on milk. Finally, α-amylase is an enzyme secreted by *Lactobacillus amylovorus* that enables the bacterium to grow on starch as sole energy source.

Furthermore a person skilled in the art will realise that the nucleic

BNSDOCID: <WO___9632486A1_I_>

acid sequence encoding a protein or polypeptide i.e. the product to be expressed need not necessarily be heterologous to a lactic acid bacterium. Naturally the system according to the invention may also be employed for expression of homologous nucleic acid sequences.

iii) Preferred embodiments

-a- cassette structure

The present invention is further in preferred embodiments characterized by the use of fully sequenced, multi-copy plasmid expression and expression-secretion vectors carrying a replicon from the lactic acid bacterium in which the heterologous nucleic acid is to be expressed. Such an organism can be a *Lactobacillus*. The vectors preferably have a modular structure facilitating easy manipulation and exchange of cassettes from one vector to another. The cassettes can comprise in addition to the essential components described in the preceding description of embodiments of the invention and those components of neccesity present in a vector in order to be operable a number of additional components. The vector includes in the order 5' to 3' one or more additional components. the additional components follow the constitutive promoter or the strong regulatable promoter, which promoter could e.g. be from a lactic acid bacterium such as *Lactobacillus* and/or follow the transcription termination sequence for blocking transcription of the heterologous protein or polypeptide in the working host. The components can be

i) a DNA sequence encoding the signal sequence of a secreted protein recognisable by a lactic acid bacterium as such, this could for example be a signal sequence from a lactic acid bacterium,

ii) a reporter gene encoding an easily assayable product in the working organism like e.g. β-glucuronidase from *Escherichia coli*, said reporter gene can be located either in frame between the initiation codon and the nucleic acid sequence encoding a protein or a polypeptide or in frame after the nucleic acid sequence encoding a protein or a polypeptide under the control of the same promoter as the nucleic acid sequence encoding a protein or polypeptide,

iii) a DNA sequence encoding an anchor sequence from a gene of a lactic acid bacterium for example like *Lactobacillus*, said DNA sequence being located in frame downstream from the nucleic acid sequence encoding a protein or a polypeptide and

iv) a transcription termination sequence of a lactic acid bacterium gene like a *Lactobacillus* gene located such as to ensure only expression of

BNSDOCID: <WO___9632486A1_I_>

the desired heterologous protein or polypeptide and
v) A DNA sequence encoding a probiotic product.

The working host is preferably a non lactic acid bacterium, in particular not a *Lactobacillus* and preferably an E coli.

-b- selected cloning sites

Additional embodiments of the invention are further characterized by the presence in the vectors, between the translation initiation region and reporter gene, of a series of restriction enzyme sites (MCS) in three reading frames, allowing in-phase translation of any foreign DNA sequence, coding for e.g. an antigenic determinant. The advantages of MCS in vectors are well known to a person skilled in the art. In the distal part of the MCS the present restriction enzyme sites can be easily manipulated to allow in-phase translation of inserted ORF sequences with the downstream reporter gene; or when the reporter gene is omitted, in-phase fusion with an anchor sequence if immobilisation on the surface of the organism in which the sequence is expressed is required. Such an anchor sequence can be that of prtP of *Lactobacillus* if *Lactobacillus* is to be transformed. In phase fusion may also be desirable when the product to be expressed should not contain additional amino acids at the carboxyl end. This can be realised through fusion to the last amino acid codon of the *cbh* gene.

These restriction enzyme sites can be easily manipulated so as to allow in-phase translation of the inserted DNA sequence with a downstream reporter gene. A further embodiment of the invention is the presence in the vector of restriction enzyme sites that permit easy deletion of the reporter gene. Another option is the presence of sites such that substitution of the reporter gene by other genes coding for desired proteins, polypeptides or parts thereof can occur. Such proteins or polypeptide can serve the purpose of an immunogenic carrier. e.g. *Escherichia coli* β-galactosidase can be suitably used for this purpose.

-c- reporter gene

Further embodiments of the present invention are additionally characterized by the possibility to fuse the antigenic determinant of interest to a large protein moiety (e.g. β-glucuronidase) which serves two purposes. Because of its ability to hydrolyse the chromogenic

BNSDOCID: <WO__9632486A1_I_>

substrate X-gluc (5-bromo-4-chloro-3-indolyl-β-D glucuronic acid), β-glucuronidase can serve as reporter protein. Synthesis of antigenic determinants fused with β-glucuronidase can easily and semiquantitatively be monitored after growth of transformed bacteria on solid media, supplemented with X-gluc. Beta-glucuronidase activity can be quantitatively determined after growth of the bacteria in liquid media. Fusion of an antigenic determinant to a large immunogenic protein (e.g β-glucuronidase), enhances the immune response against the antigenic determinant, in particular if the determinant itself is small. The immunogenic protein serves as the carrier. The cassette structure allows deletion of the reporter gene at will, for example for expression of non-fused heterologous proteins.

Another option is to include nucleic acid encoding products with health stimulating properties to further enhance the value of a transformed lactic acid bacterium. Examples of such proteins are immunomodulators, e.g. cytokines, bacteriocines, glutathione-S-transferase, immuno-globulins and levanase. The sequences encoding such proteins are comprised in the state of the art or can be derived without undue burden in a number of cases from clones comprising the relevant sequences. The sequences of such products are hereby enclosed by reference.

-d- site of protein expression

Embodiments of the present invention can additionally be characterized by the possibility, in the case of the synthesis of proteins that are to be secreted and surface-bound e.g. via the membrane or the S layer protein. For binding to the membrane the length of the spacer region between the antigenic determinant and the anchor sequence can be varied, depending on the thickness of the peptidoglycan layer and the sensitivity of the fusion protein to proteolytic attack.

-e- food-grade expression vector

Embodiments of the present invention can further be characterized by the possibility to replace commonly used antibiotic resistance markers and *Escherichia coli* DNA sequences of the vectors by a food grade marker e.g. a nutritional selection marker from a food-grade organism, to obtain a food-grade vector comprising elements derived from food-grade organisms only. The food grade marker preferably originates from *Lactobacillus*, enabling the construction of vectors to be performed under conditions

3NSDOCID: <WO__9632486A1_I_>

that are exempt from regulations for recombinant DNA research of the EC.

Basic vector construction for a vector suitable for Lactobacillus

Firstly as starting material for the construction of the expression and expression-secretion vectors, pGEM-3 was used. The strategy for the construction of the expression vector is as follows. Expression from strong *Lactobacillus* promoters like the *Lactobacillus casei ldh* promoter in *E. coli* frequently leads to structural instability which can be overcome by inserting a terminator directly behind the promoter sequence. Consequently, the transcription terminator (e.g. T_{cbh}) was first inserted in pGEM-3 before a *Lactobacillus* promoter sequence was introduced. In addition, as expression of genes under the control of *Lactobacillus* promoters in *Escherichia coli* frequently results in structural instability, the transcription terminator sequence of the *Lactobacillus casei ldh* gene was inserted between the promoter and the *Lactobacillus plantarum cbh* terminator, prior to the introduction of the *Escherichia coli uidA* gene.

The nucleotide sequence of pGEM-3 (Promega), of the *Lactobacillus* plasmid vector pLPE323 [Leer et al, Mol. Gen. Genet. 234, 265-274 1992] and of all other elements used for the construction of the expression vectors, such as promoter sequences, terminator sequences etc, is fully known. All elements were provided with flanking nucleotide sequences harbouring restriction enzyme sites which do not occur elsewhere in the plasmid, permitting easy manipulation of the elements.

Multi-purpose cassette construction

The final set of expression cassettes was equipped with a number of unique restriction enzyme sites permitting further modification of the vectors, if wanted. To achieve this, a 260-bp *Hpa*I-*Hin*dIII fragment containing the last amino acid codon and transcription terminator sequence of the *cbh* gene of *Lactobacillus plantarum* 80 [Christiaens et al, Appl. Environm. Microbiol. 58, 3792-3798 1992] was ligated between the *Hin*cII and *Hin*dIII sites of pGEM-3. Subsequently, the *Hin*dIII site was converted into an *Eag*I site using a synthetic linker sequence. The remaining multiple cloning sequence was replaced by a chemically synthesized oligonucleotide (PRESS) containing the following restriction enzyme sites, *Eco*RI, *Bgl*II, *Spe*I, *Sph*I, *Bam*HI, *Bst*EII. The *Bst*EII site is adjacent to the *Hin*cII site used for cloning of the *cbh* fragment. The

BNSDOCID: <WO__9632486A1_I_>

resulting vector which was designated pGTC3 (Figure 1) was used for the insertion of three DNA fragments containing expression and expression-secretion sequences.

Secondly, promoter bearing DNA fragments were inserted into the new PRESS of pGTC3 (Figure 1).

i) The promoter, translation initiation region and first 8 specific codons of *Lactobacillus casei ldh*, which are present on a 513-bp *Sau*3AI fragment were cloned between the *Bgl*II and *Bam*HI sites of pGTC3, leaving the *Bam*HI site 3' from the promoter sequence intact. First, a DNA fragment containing the 5' part of the *Lactobacillus casei ldh* gene [Kim et al, Appl. Environm. Microbiol. 57, 2413-2417 1991] was synthesized by PCR and used as a probe to isolate a 3.4-kb *Pst*I-*Eco*RI fragment from a partial genomic library cloned into pUC19. This fragment was used to generate the 513-bp *Sau*3AI fragment (Figure 1). The resulting vector comprising pGTC3 and the expression sequence of the L-*ldh* gene of *Lactobacillus casei* ATCC 393 was called pGLC3 (Figure 2A-b).

ii) To obtain a P_{ldh}-directed expression system with which an expressed protein can also be secreted, the secretion signal sequences of the *Lactobacillus casei* ATCC 393 *prtP* gene were isolated by PCR. With primers based on the *ptrP* gene of *L. lactis* [Kok et al, Appl. Environm. Microbiol. 54, 231-238 1988] and *Lactobacillus paracasei* subsp. *paracasei* NCDO 151 [Holck and Naes, J. Gen. Microbiol.138, 1353-1364 1992] a PCR fragment from *Lactobacillus casei* ATCC 393 was amplified which contains the Shine-Dalgarno sequence, translation startcodon and the first 70 codons of the *prtP* gene. The identity of this fragment was confirmed by DNA sequence analysis of the cloned fragment. From this cloned PCR fragment a 239-bp *Sph*I-*Bam*HI fragment was isolated and used to replace the 46-bp *Sph*I-*Bam*HI fragment containing the translation initiation region and first 8 codons of the *ldh* gene. The resulting vector was named pGLC3-ss (Figure 2A-c).

iii) A *Bgl*II-*Sph*I fragment (864 bp) from pLPCR2-1 [Jore et al, 1995 submitted for publication] containing the transcription initiation sequences, translation initiation region and first 61 codons of the *Lactobacillus amylovorus* α-amylase gene, was inserted between the *Bgl*II-*Sph*I sites of pGTC3. The first 38 amino acids of the primary translation product (preproprotein) comprise the typical tripartite structure of a

BNSDOCID: <WO___9632486A1_I_>

Sec-dependent signal sequence. The vector constructed in this way was called pGAM3 (Figure 2B-b).

iv) To obtain a P_{amy}-directed expression system with its regulatable expression characteristics, but without secretion directing signals, the *Pst*I-*Sph*I sequence (236 bp) was replaced by a synthetic oligonucleotide with the same restriction enzyme termini. This oligonucleotide contained the entire sequence from *Pst*I to the translation start codon and the first 7 amino acid codons of the *amy* gene. The shortened sequence comprises the AT-rich 5' region but lacks the signal sequence peptidase site. The vector constructed in this way was called pGAMΔss (Figure 2B-c).

Thirdly, the terminator sequence of the *Lactobacillus casei ldh* gene was inserted in the vectors. Between the *Bam*HI and *Bst*EII sites of the three promoter-terminator (P_{ldh}, P_{ldh}-ss_{ariP}, P_{amy}-Δss and P_{amy}) expression vectors, a synthetic oligonucleotide was inserted encompassing the transcription terminator of the *Lactobacillus casei ldh* gene flanked by two *Not*I sites and followed by a nucleotide sequence containing *Bam*HI and *Nco*I sites. Insertion of this oligonucleotide destroyed the original *Bam*HI site but left the *Bst*EII site intact. In the three vectors obtained in this way the promoter sequences are separated from the terminator of the *cbh* gene by an additional terminator. ORF-encoding DNA fragments, to be expressed in *Lactobacillus* but not to be expressed in *Escherichia coli*, can be cloned in the region between the two terminator sequences, preventing transcription (Figure 3).

Fourth, the *E. coli uidA* gene coding for β-glucuronidase was isolated from pNOM2 [Roberts et al, Curr. Genet. 15, 177-180 1989] and cloned into a pUC19 derivative. The *uidA* gene was extended with two codons by insertion of a hexanucleotide comprising an *Xho*I site (Figure 4). A *Bst*EII site was introduced 6 nucleotides downstream from the translation stop codon. All changes, which were introduced by PCR, were confirmed by sequence analysis. An 1.8-kb *Nco*I-*Bst*EII fragment from this pUC-*uidA* plasmid with the modified *uidA* gene was cloned into the expression vector which was cut with *Nco*I and *Bst*EII, between the *ldh* and *cbh* terminators (Figure 5-b). The presence of unique *Xho*I and *Bst*EII sites facilitates insertion of additional sequences.

BNSDOCID: <WO__9632486A1_I_>

Fifth, the series of vectors was further extended by insertion of a PCR-generated *Xho*I-*Bst*EII fragment of the *prtP* gene from *Lactobacillus casei* ATCC 393 encoding 117 C-terminal amino acids (Figure 5-c). This C-terminal sequence is, in analogy with similar sequences from lactococcal proteases, thought to be responsible for anchorage to the bacterial cell surface. The vectors can be further adapted to accomodate differences in thickness of the peptidoglycan layers of different bacterial species by extension of the cell wall spanning domain. The cell wall spanning peptide can be easily extended by replacement of the present *Xho*I-*Bst*EII fragment by a larger *Xho*I-*Bst*EII fragment encoding additional aminoacid sequences, or insertion into the *Xho*I site of a sequence encoding additional amino acids of the cell wall spanning domain. Alternatively, by using a smaller *Xho*I-*Bst*EII fragment (less amino acids in cell wall spanning sequence), the anchored protein will be closer to or in direct contact with the cell surface.

Sixth, a synthetic oligonucleotide comprising restriction enzyme sites for the following enzymes, *Cla*I, *Bam*HI, *Mun*I, *Kpn*I, *Hin*dIII, *Sma*I, *Bbs*I, *Eco*RI, *Sal*I, *Apa*I, *Nco*I (MCS) was inserted between the *Bam*HI and *Nco*I sites of one of the expression cassetttes. The original *Bam*HI site was destroyed using this procedure, but a new *Bam*HI site was present within the newly inserted synthetic oligonucleotide (Figure 6-b). From these prototype cassettes the MCS sequences can easily be transferred to other cassettes as all cassettes contain unique *Bam*HI and *Nco*I sites. With these cassettes any open reading frame (ORF) can be aligned in-frame with that of the N-terminal sequence of the *amy*, *ldh* or *prtP* genes. ORF's that are cloned in one of the sites of the MCS or between two sites of the MCS, can be easily aligned with the *uidA* gene. Alignment of the reading frame of the new ORF's to sequences downstream from the MCS can be achieved by adjusting the sequence in the distal part of this MCS. For this purpose the *Bbs*I restriction enzyme recognition sequence was incorporated within the synthetic oligonucleotide (MCS). The enzyme *Bbs*I recognizes the non-palindromic sequence GAAGAC and cleaves the DNA strand 2 nucleotides downstream from this sequence. Cleavage in the anti-parallel strand occurs 6 nucleotides downstream from the recognition sequence (Figure 7).

By cutting the MCS (depicted in Figure 6-b,c,d) in one of the expression or expression-secretion cassettes with *Bbs*I, followed by filling in with

BNSDOCID: <WO__9632486A1_I_>

dNTP and T4 DNA polymerase and ligation, the MCS was enlarged by 4 nucleotides. The reading frame of the sequences upstream of and including the *Bbs*I site shows a +1 frameshift with respect to the methionine codon within the *Nco*I site (Figure 8-b). The thus obtained frameshifted MCS containing expression cassette can again undergo *Bbs*I cutting, filling in with dNTP and ligation. The resulting sequence shows again a +1 frameshift of the sequences upstream of and including the *Bbs*I site, relative to the *Nco*I site (Figure 8-c). The *Bbs*I recognition sequence is not affected by this procedure. The *Bbs*I recognition sequence in the MCS can also be used to clone *Eco*RI compatible sequences in the MCS. The nucleotides for the MCS directly following the *Bbs*I site were chosen to yield compatible termini after *Bbs*I and *Eco*RI cutting. The latter enzyme will not be used in practice since it is not unique in the expression vectors.

The presence of unique *Xho*I and *Bst*EII sites in the expression cassettes allows variation at will of the length of the anchor sequence, so as to adjust the peptidoglycan spanning region to the thickness of the peptidoglycan layer of the host bacterium.

Seventh, the six expression cassettes, digested with *Eco*RI were ligated to pLPE323 [Posno et al, Appl. Envionm. Microbiol. 57, 1822-1828 1991] which was also cut with *Eco*RI, resulting in the following *Escherichia coli-Lactobacillus* shuttle vectors, pLP401-T, pLP402-T, pLP403-T and pLP501-T, pLP502-T and pLP503-T.

Finally, the nucleotide sequence with the terminator, T_{ldh}, was removed from the pLP400-T and pLP500-T series of plasmids by digestion with *Not*I and religation. The religated plasmids were used to transform *Lactobacillus*, resulting in plasmids pLP401, pLP402, pLP403, and pLP501, pLP502 and pLP503 (Figure 9a and 9b).

Food-grade vectors

The applicability of the vectors can be further improved by removal of the *Escherichia coli* DNA sequences and replacement of the erythromycin-resistance gene by a food-grade selection marker, resulting in a food-grade vector. These alterations can be achieved by digestion of plasmids of the pLP400 and pLP500 series with *Bgl*II and *Nhe*I and insertion of a DNA fragment comprising the *xylR*, *xylA/B* genes and a replicon for

BNSDOCID: <WO__9632486A1_I_>

Lactobacillus (Figure 10). *Lactobacillus casei* or *Lactobacillus plantarum* transformed with vectors harbouring these *xyl* genes can be selected on plates with xylose as sole energy source.

To demonstrate the usefulness of the vectors for the efficient expression of foreign proteins, first the expression of β-glucuronidase was measured. Beta-glucuronidase activity of transformants expressing the *uiA* gene was determined semi-quantitatively in a plate assay using the chromogenic substrate X-gluc. In such assays colonies of *Lactobacillus* transformants that express *uidA* stain blue, whereas colonies of transformants that do not express the *uidA* gene are white. Beta-glucuronidase activity of transformants expressing the *uidA* gene can be quantitatively and reproducibly assayed enzymatically or immunologically using anti-β-glucuronidase antibodies.

The results, which are detailed below in the Examples, show that
i) expression of *uidA* under the control of the *ldh* promoter is constitutive, whereas expression under the control of the *amy* promoter is induced by cellobiose and repressed by glucose,
ii) efficient expression of β-glucuronidase takes place with vectors harbouring the *ldh* promoter or the *amy* promoter,
iii) β-glucuronidase is secreted when the promoter is followed by the α-amylase or proteinase secretion signal,
iv) β-glucuronidase is secreted and remains surface-bound when the *uidA* gene is fused to the anchor sequence of the *prtP* gene.

It is apparent from this and the following examples that expression of a nucleic acid sequence encoding a protein or polypeptide in a lactic acid bacterium using an expression vector according to the invention in any of the embodiments disclosed and claimed can be improved in comparison to existing systems. It has now become possible to express such a sequence in an amount exceeding 1% of the total amount of protein, preferably exceeding 2% and more preferably even exceeding 3% in an expression host. In particular this is quite spectacular for heterologous nucleic acid expression in a lactic acid bacterium. Most especially this is relevant for expression in *Lactobacillus* a microorganism renown for difficulties for expression of heterologous sequences. The subject invention now enables production of foodgrade lactic acid bacteria comprising foodgrade heterologous sequences. The possibilities for production of proteins and

BNSDOCID: <WO__9632486A1_I_>

polypeptides that are foodgrade using vectors and methods according to the invention are numerous.

A method for production comprises use of an expression vector and/or host cell comprising such a vector according to the invention in a manner known per se. This involves transformation of a lactic acid bacterium with an expression vector according to the invention comprising the nucleic acid sequence encoding the product of interest. A number of such nucleic acid sequences have been indicated elsewhere in the description. Following introduction of the expression vector in the lactic acid bacterium, the host cell must be cultured in a medium in a manner to be determined in a manner known per se by a person skilled in the art best suited to the kind of host cell, the promoter and/or nucleic acid sequence to be expressed such that expression occurs. Determination of the optimal conditions lies within reach of a person skilled in the art without requiring inventive step or undue experimentation.

The method for constructing an expression vector according to the invention also falls within the scope of protection. Such a method comprises introduction of a vector according to the invention for maintenance and manipulation in a working organism other than the strain of lactic acid bacterium in which expression is to occur, manipulating the vector in a manner known per se to obtain desired characteristics in said working organism and finally introducing the vector comprising a nucleic acid sequence encoding a protein or polypeptide in the strain of lactic acid bacterium in which expression is to occur, said sequence preferably being heterologous to the strain of lactic acid bacterium in which expression is to occur. In particular the method may be useful when the working organism is a non lactic acid bacterium. In such a method when the vector comprises the transcription terminator sequence located between the promoter and the first codon of the nucleic acid sequence encoding a protein or a polypeptide the vector can be treated in a manner known per se for excising nucleic acid sequences to excise the terminator sequence in order to allow transcription of the nucleic acid sequence encoding a protein or a polypeptide. This can occur e.g. by treatment of the vector with restriction enzyme for cutting the vector at the sites flanking the terminator sequence. Another possibility is that the vector for maintenance is introduced into the expression host prior to the excision and due to the presence of direct repeats flanking the

BNSDOCID: <WO___9632486A1_I_>

terminator sequence and treatment rendering homologous recombination possible subsequently the expression can be rendered possible due to excision of the terminator sequence in such a fashion.

A vector according to the invention for maintenance and manipulation in a working organism of a nucleic acid sequence encoding a polypeptide or protein, said vector comprising an expression promoter sequence and a cloning site for said nucleic acid sequence encoding a polypeptide or protein with the cloning site being located such that said nucleic acid sequence when present in the vector is or will be controlled by said promoter sequence and is or will be present in an open reading frame in phase with a translation initiation region, is characterised in that a terminator sequence recognisable as such by the working organism in which the vector is to be maintained prior to introduction into a lactic acid bacterium is located on the vector such that the complete polypeptide or protein encoded by the nucleic acid sequence cannot be expressed. Preferably the working organism is a non lactic acid bacterium. A multiple cloning site will be preferable as cloning site.

In another embodiment a vector according to the invention will comprise the nucleic acid sequence encoding the protein or polypeptide at the appropriate cloning site. In such a vector the terminator sequence is present in one particular embodiment between the promoter sequence and the first codon of the nucleic acid sequence encoding a protein or polypeptide. Specifically in a particular embodiment hereof the terminator sequence is present between the promoter sequence and the translation initiation region preceding the nucleic acid sequence. Preferably any of the preceding embodiments may further comprise the transcription terminator sequence such that it is excisable from the vector in a manner known per se to a person skilled in the art e.g when the transcription terminator sequence is flanked by restriction enzyme sites uniquely present in the vector at such flanking position or is flanked by direct repeats e.g. excisable by homologous recombination by the host.

All the components described for the expression vector according to the invention may naturally also be comprised in the maintenance and manipulation vector according to the invention. Thus such a vector may further comprise one or more of the components:

BNSDOCID: <WO___9632486A1_I_>

i) a replicon for a lactic acid bacterium

ii) a DNA sequence encoding the signal sequence of a secreted protein recognisable by a lactic acid bacterium as such this could e.g. be a signal sequence from a lactic acid bacterium, said signal sequence being located in frame between the initiation codon and the nucleic acid sequence encoding a protein or a polypeptide,

iii) a reporter gene encoding an easily assayable product, such a reporter gene e.g. being β-glucuronidase from *Escherichia coli*,

iv) a transcription terminator sequence recognisable by a lactic acid bacterium, said sequence being located downstream of the nucleic acid sequence encoding a protein or polypeptide,

v) a DNA sequence encoding an anchor sequence from a lactic acid bacterium gene and said DNA sequence being located in frame downstream from the nucleic acid sequence encoding a protein or a polypeptide and

vi) a DNA sequence encoding a product with health stimulating properties.

vii) solely foodgrade components.

Example 1

The expression and expression-secretion vectors pLP401.2.3-T and pLP501.-2.3-T, that were constructed in *Escherichia coli*, were digested with *Not*I and ligated, in order to obtain vectors in which the T_{ldh} sequence was omitted. *Lactobacillus casei* ATCC 393 was transformed with the ligation mixtures using electroporation [Posno et al, Appl. Envionm. Microbiol. 57, 1822-1828 (1991)]. Transformants were selected on selective media. Selective media contained 10 g of proteose pepton, 5 g meat extract, 5 g yeast extract, 1 ml Tween 80, 5 g Na-acetate.$3H_2O$, 0.2 g $MgSO_4.7H_2O$, 50 mg $MnSO_4.4H_2O$), 2 g diammoniumcitrate, 17 g agar per 800 ml of deionized water. After autoclaving (20 min at 120ºC) 200 ml 0.5 M potassium-phosphate buffer pH 7.1 was added. After cooling down to 60ºC, erythromycin and X-gluc (final concentrations 5 and 40µg/ml, respectively) and an energy source (glucose or cellobiose; 0.5-1.0%) were added. The media were poured into petri dishes (13 cm diameter; 25-30 ml medium), after solidification the agar containing plates were dried for 20 min at 60ºC and used immediately. *Lactobacillus* transformants containing vectors from the pLP400 or pLP500 series were streaked onto the surface of the agar plates. Colonies and their phenotypical characteristics (blue or white) could be seen within 24 hrs of incubation at 37ºC.

When transformants of the pLP400 series were plated on indicator plates with X-gluc containing cellobiose as energy source all colonies were light blue, but were white when glucose was used as energy source. When transformants of the pLP500 series were plated on indicator plates all colonies were dark blue, irrespective of whether cellobiose or glucose was used as energy source.

Lactobacillus transformants containing plasmids pLP401, pLP402, pLP403 and with pLP501, pLP502 and pLP503, showed the expected phenotype, whereas *Lactobacillus* transformants containing the pLP401,2,3-T or pLP501,2,3-T plasmids containing the T_{ldh} sequence displayed the white phenotype on selective medium. Reintroduction of pLP503 and pLP402 isolated from *Lactobacillus casei* transformants into *Escherichia coli* DH5α yielded transformants with blue (30%) or white (70%) phenotype on selective medium (L medium, 0.5% glucose, 1.5% agar, 50 µg/ml ampicillin, 40 µg/ml X-gluc). The white phenotype was correlated with structurally instable plasmids (size smaller than starting material). When *Escherichia coli* transformants with a blue phenotype were suspended in physiological salt solution and streaked onto agar plates with selective medium, most colonies showed a white phenotype, indicating plasmid instability.

Example 2

A DNA fragment encoding the SA epitope (Site A; Wiley et al, Nature 89, 373-378 (1981)) of influenza virus comprising 18 amino acids (VTAACS-HAGKSSFYRNLL) was inserted between the *Bam*HI and *Nco*I sites of plasmids pLP401,2,3-T and plasmids pLP501,2,3-T. No structural instability of these vectors was observed in *Escherichia coli*. After removal of the T_{ldh} terminator sequence by digestion with *Not*I and ligation, *Lactobacillus casei* ATCC 393 was transformed with the resulting plasmids pLP401-SA, pLP402-SA, pLP403-SA and with pLP501-SA, pLP502-SA and pLP503-SA. Transformants were selected on selective media as described in Example 1. When transformants of the pLP400-SA series were plated on indicator plates with X-gluc containing cellobiose as energy source all colonies were light blue, but were white when glucose was used as energy source. When transformants of the pLP500-SA series were plated on indicator plates all colonies were dark blue, irrespective of whether cellobiose or glucose was used as energy source.

Example 3

3NSDOCID: <WO__ 9632486A1_I_>

A DNA fragment encoding the SA epitope of influenza virus comprising 18 amino acids was inserted between the *Bam*HI and *Nco*I sites of derivatives of plasmids pLP401 and pLP501 that lack the *uidA* gene, resulting in plasmids pLP401-SA-1 and pLP501-SA-1. In plasmids pLP401-SA-1 and pLP501-SA-1 the SA encoding sequence is directly fused, in-frame, to the anchor region encoding sequence. *Lactobacillus casei* ATCC 393 was transformed with plasmids pLP401-SA-1 and pLP501-SA-1. Transformants were selected on selective media as described in Example 1. Western analysis shows that transformed bacteria synthesize a product of the expected size that specifically reacts with antibodies against the SA epitope.

Example 4

Amplification of antigenic determinant encoding the SA epitope of influenza virus. An oligonucleotide duplex with *Bam*HI and *Nco*I compatible termini encoding the SA epitope was synthesized (Figure 11) and inserted between the *Bam*HI and *Nco*I sites of plasmids pLP401 and pLP501, resulting in plasmids pLP401-T-SA-1 and pLP501-T-SA-1. No structural instability of these vectors was observed in *Escherichia coli*. Ligation of the *Bam*HI compatible terminus with the *Bam*HI site of plasmids pLP401-T and pLP501-T destroyed the *Bam*HI recognition sequence. Ligation of the *Nco*I compatible terminus with the *Nco*I site of these plasmids left the *Nco*I site intact. The sequence within the synthetic oligonucleotide duplex between the epitope encoding nucleotides and the *Nco*I recognition sequence contains a *Bbs*I site (see Figure 7) which gives rise to a *Bam*HI compatible terminus after cutting with *Bbs*I (Figure 11). Digestion of pLP401-T-SA-1 and pLP501-T-SA-1 with *Bbs*I and *Nco*I permits insertion of a second SA epitope encoding oligonucleotide (pLP401-T-SA-3 and pLP501-T-SA-3). By repeating *Bbs*I and *Nco*I digestion, followed by insertion of an SA epitope encoding oligonucleotide, expression-secretion vectors (pLP401-T-SA-3 and pLP501-T-SA-3) with 3 copies of the SA epitope sequence fused, in-frame, with the *uidA* gene sequence were obtained.

The procedure described in the previous paragraph allows to easily construct vectors with multiple copies of the same, or different antigenic determinants, provided that the sequence distal to the antigenic determinant encoding sequence contains *Bbs*I (as described in the preceding paragraph) and *Nco*I recognition sequences. Although the basic expression and expression-secretion vectors contain a PRESS with a *Bbs*I site (Figure 6-b,c,d), this *Bbs*I site is removed when antigenic determinant encoding

sequences are inserted between the *Bam*HI and *Nco*I sites.

After removal of the T_{ldh} terminator sequence by digestion with *Not*I and ligation, *Lactobacillus casei* ATCC 393 was transformed with the resulting plasmids pLP401-SA-1,2,3 and pLP501-SA-1,2,3. Transformants were selected on selective media as described in Example 1. When transformants of the pLP400-SA series were plated on indicator plates with X-gluc containing cellobiose as energy source all colonies were light blue, but were white when glucose was used as energy source. When transformants of the pLP500-SA series were plated on indicator plates all colonies were dark blue, irrespective of whether cellobiose or glucose was used as energy source. Western analysis shows that transformed bacteria synthesize a product of the expected size that specifically reacts with antibodies against the SA epitope and antibodies against β-glucuronidase.

Example 5

A DNA fragment encoding the Hacket epitope of influenza virus comprising 10 amino acids (FERFEIFPKE; Hacket et al, J. Immunol. 135, 1391-1395 (1985)) was inserted between the *Bam*HI and *Nco*I sites of plasmids pLP401-T, pLP402-T and pLP503-T, resulting in plasmids pLP401-T-HA, pLP402-T-HA and pLP503-T-HA. No structural instability of these vectors was observed in *Escherichia coli*. After removal of the T_{ldh} terminator sequence by digestion with *Not*I and ligation, *Lactobacillus casei* ATCC 393 was transformed with the resulting plasmids pLP401-HA, pLP402-HA and pLP503-HA and *Lactobacillus plantarum* strains ATCC 14917, ATCC 8014, NCIB 8826, 80, NCDO 1193 with pLP402-HA and pLP503-HA. Transformants were selected on selective media as described in Example 1. When transformants of the pLP400-HA series were plated on indicator plates with X-gluc containing cellobiose as energy source all colonies were light blue, but were white when glucose was used as energy source. When transformants of pLP503-HA were plated on indicator plates all colonies were dark blue, irrespective of whether cellobiose or glucose was used as energy source. Western analysis shows that transformed bacteria synthesize a product of the expected size that specifically reacts with antibodies against the HA epitope and antibodies against β-glucuronidase.

The amount of fusion protein synthesized by pLP503-HA was quantitatively determined by measuring the β-glucuronidase activity and by quantitative ElISA. The enzymatic assay and the ELISA determination indicate that the

3NSDOCID: <WO__9632486A1_I_>

fusion protein amounts to 1-3% of total cellular protein.

Example 6

A DNA fragment encoding pig rotavirus VP8 polypeptide comprising the first 248 amino acids of the VP4 protein (this part corresponds with the region of the VP4 protein that arises after trypsic cleavage [Lopez et al, 65, 3738-3745 (1991)]) was inserted between the *Bam*HI and *Nco*I sites of plasmids pLP401-T, pLP402-T, pLP501-T and pLP502-T, resulting in plasmids pLP401-T-R08, pLP402-T-R08, pLP501-T-R08 and pLP502-T-R08. No structural instability of these vectors was observed in *Escherichia coli*. After removal of the T_{ldh} terminator sequence by digestion with *Not*I and ligation, *Lactobacillus casei* ATCC 393 was transformed with plasmids pLP401-R08, pLP402-R08, pLP501-R08, and pLP502-R08. Transformants were selected on selective media as described in Example 1. When transformants of the pLP400-R08 series are plated on indicator plates with X-gluc containing cellobiose as energy source all colonies are light blue, but are white when glucose is used as energy source. When transformants of the pLP500-R08 series are plated on indicator plates all colonies are dark blue, irrespective of whether cellobiose or glucose is used as energy source.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1

Schematic representation of the construction route of some embodiments of the expression vectors.

a. Structure of plasmid pGTC3. T_{cbh}, transcription terminator of *Lactobacillus plantarum cbh* gene.

b. Nucleotide sequence comprising PRESS, poly restriction enzyme site sequence, and part of the sequence containing the *cbh* terminator.

Figure 2: Schematic representation of the construction route of the expression vectors.

Figure 3: Sequence of the synthetic oligonucleotide duplex encompassing the transcription terminator of the L-*ldh* gene of *Lactobacillus casei*. Inverted repeats capable of forming a stem loop structure are marked by horizontal arrows. The overhanging terminal sequences on the left and right side of the sequences are compatible to the *Bam*HI and *Bst*EII sites.

respectively, of the promoter-terminator cassette vectors.

Figure 4

Nucleotide sequence of the 3'end of the *uidA* gene before (a) and after (b) mutagenesis by PCR. An *Xho*I site was introduced immediately upstream of the translation stop codon to allow in-frame fusion of amino acid encoding sequences of the *uidA* gene with an ORF to be fused with the 3' end of this *uidA* gene.

Figure 5

a. Physical map of promoter-double-terminator cassette vector pPTT3. P stands for P_{ldh}, P_{ldh}-ss_{prtP}, P_{amy}-Δss, or P_{amy}, T_{ldh} and T_{cbh}: transcription terminators of the *Lactobacillus casei ldh* and *Lactobacillus plantarum cbh* gene, respectively.

b. Physical map of promoter-terminator cassette in which an adjusted *Escherichia coli uidA* gene was cloned.

c. As b. The reading frame of the *uidA* gene is fused to that of the 3' end of the *prtP* gene coding for the anchor sequence.

Figure 6

Nucleotide sequences of the region between the *ldh* gene transcription terminator sequence and the *uidA* gene of the expression cassette vectors.

a. Nucleotide sequence between the *Bam*HI and *Nco*I sites in pGTT3; this sequence is part of the synthetic oligonucleotide sequence encompassing the *Lactobacillus casei ldh* transcription terminator signal.

b. Nucleotide sequence of MCS inserted between *Bam*HI and *Nco*I sites of pPTUT. The original *Bam*HI was destroyed using this strategy but a new *Bam*HI site is present in the MCS. Starting from this MCS two new, slightly modified MCS were obtained in which the *Bam*HI site and following sites are frame-shifted.

c. A vector containing the MCS as depicted in b) was digested with *Cla*I and the resulting DNA termini were filled in with dNTP's using T4 DNA polymerase. After ligation the MCS contained a *Bam*HI site shifted two nucleotides with respect to *G*.

d. Nucleotide sequence of a MCS obtained by replacing the *Cla*I-*Bam*HI sequence in b) by a *Cla*I-*Bsp*EI-*Bam*HI sequence. The *Bam*HI site is shifted four nucleotides with respect to *G*.

DNA fragments can be cloned in MCS depicted in b, c or d depending on the

BNSDOCID: <WO___9632488A1_I_>

reading frame needed. To ensure alignment of the cloned ORF with the *uidA* gene, restriction enzyme sites just upstream of the *Nco*I site (containing translation startcodon ATG of the *uidA* gene) can be modified with other restriction enzymes (see elsewhere in the description).

Figure 7
Cleavage pattern of *Bbs*I. The bold hexanucleotide duplex represents the *Bbs*I recognition sequence, the arrows show the cleavage sites. N and N' stand for any nucleotide and its complementary counterpart.

Figure 8
a) Nucleotide sequence of MCS sequences as depicted in Figure 6-b,c,d.
b) Nucleotide sequence of MCS after filling up the *Bbs*I restriction enzyme generated termini of MCS depicted in a) with dNTP's using T4 polymerase and ligation.
c) Nucleotide sequence of MCS after filling up the *Bbs*I restriction enzyme generated termini of MCS depicted in b) with dNTP's using T4 polymerase and ligation. The line between G-G corresponds with the nucleotide sequences of Figure 6-b,c,d.

Figure 9
a. Schematic representation of expression vector.
b. Schematic structure of cassettes present in plasmids of the pLP400 and pLP500 series. P_{ldh} and P_{amy} represent the promoters of the *ldh* gene of *Lactobacillus casei* and *amy* promoter of the α-amylase gene of *Lactobacillus amylovorus*, respectively. *uidA*, β-glucuronidase gene of *Escherichia coli*. T_{ldh}, transcription terminator of the *ldh* gene of *Lactobacillus casei*. T_{cbh}, transcription terminator of the *cbh* gene of *Lactobacillus plantarum*. Anchor *prtP*, sequence from *Lactobacillus casei* encoding the membrane anchor sequence and peptidoglycan spanning region. The versatility of the vectors was further improved by introduction, between the *Bam*HI and *Nco*I sites, of a multicloning region (MCS) comprising the following restriction enzyme sites, *Cla*I, *Bam*HI, *Mun*I, *Kpn*I, *Hin*dIII, *Sma*I, *Bbs*I, *Eco*RI, *Sal*I, *Apa*I, *Nco*I.

In the promoter-terminator (P_{ldh}, P_{ldh}-ss_{prtP}, P_{amy}-Δss and P_{amy}) expression vectors, the *Bam*HI site, and all other sites of the MCS, are present in three reading frames, allowing in-frame translation of any DNA sequence fused to the N-terminal sequence of LDH, α-amylase or PrtP. The presence

of a unique cloning sites in the MCS permits easy alignment, if necessary, of the reading frames of the antigenic determinant encoding sequence and of *uidA*.

Figure 10
Schematic representation of the structure of part of the xylose cluster of genes of *Lactobacillus pentosus*. Replacement of the erythromycin-resistance marker by these genes allows a transformed *Lactobacillus* strain to grow on xylose as sole energy source.

Figure 11
Nucleotide sequence of the distal part of the MCS containing the *Nco*I and *Bbs*I recognition sites. The recognition sequences for *Bam*HI, *Nco*I and *Bbs*I are indicated with bold letters.

Figure 12
The nucleotide sequence of S layer protein in particular the promoter region of *L. acidophilus slpA*.

BNSDOCID: <WO___9632486A1_I_>

Claims

1. Expression vector for expression and optionally for excretion and optionally for surface display of a protein or polypeptide in a lactic acid bacterium, said vector comprising an expression promoter sequence and a nucleic acid sequence encoding said protein or polypeptide with expression of said nucleic acid sequence being controlled by said promoter sequence, characterised in that said encoding nucleic acid sequence is preceded by a 5' non-translated nucleic acid sequence comprising at least the minimal sequence required for ribosome recognition and RNA stabilisation, followed by a translation initiation codon, said translation initiation codon being immediately followed by a fragment of at least 5 codons of the 5' terminal part of the translated nucleic acid sequence of a gene of a lactic acid bacterium or a structural or functional equivalent of said fragment, said fragment also being controlled by said promoter.

2. Expression vector according to claim 1, wherein said gene is homologous to a lactic acid bacterium.

3. Expression vector according to claim 1 or 2 wherein said 5' terminal part of the translated nucleic acid sequence is richer in A-T than the rest of said gene such that a person skilled in the art will consider it an A-T rich sequence.

4. Expression vector according to any of the preceding claims, wherein the 5' non translated nucleic acid sequence is the complete 5' non translated region of the gene including the Shine Dalgarno sequence.

5. Expression vector according to any of the preceding claims, wherein the 5' non-translated nucleic acid sequence and/or the 5' terminal part of the translated nucleic acid sequence are derivable from the same gene as the promoter.

6. Expression vector according to any of the preceding claims, wherein the promoter is derived from a lactic acid bacterium.

7. Expression vector according to any of the preceding claims, wherein the lactic acid bacterium is a *Lactobacillus* or a *Bifidobacterium*.

8. Expression vector according to any of the preceding claims, wherein the nucleic acid sequence encoding the protein or polypeptide encodes an antigenic determinant.

9. Expression vector according to any of the preceding claims, wherein the lactic acid bacterium gene encodes a protein that is expressed to a degree at least equivalent to that of 1%, preferably more than 2% with more preference for more than 3% of total protein of the lactic acid bacterium and is for example selected from the following *Lactobacillus* genes: lactate dehydrogenase encoding gene, the proteinase encoding gene, the amylase encoding gene, the S layer protein encoding gene, the ribosomal RNA encoding gene, the glyceraldehyde 3 phosphate dehydrogenase encoding gene.

10. Expression vector according to any of the preceding claims, wherein the nucleic acid sequence encoding the protein or polypeptide to be expressed in a lactic acid bacterium is heterologous or homologous to the lactic acid bacterium.

11. Host cell comprising an expression vector according to any of the preceding claims, said host cell preferably being a lactic acid bacterium, most preferably being a *Lactobacillus* bacterium or a *Bifidobacterium*.

12. Host cell according to claim 11, wherein the host cell including the expression vector is food grade.

13. Vector for maintenance and manipulation in a working organism, preferably a non lactic acid bacterium of a nucleic acid sequence encoding a polypeptide or protein, said vector comprising an expression promoter sequence and a cloning site, preferably a multiple cloning site for said nucleic acid sequence encoding a polypeptide or protein with the cloning site being located such that said nucleic acid sequence when present in the vector is or will be controlled by said promoter sequence and is or will be present in an open reading frame in phase with a translation initiation region, said vector being characterised in that a terminator sequence recognisable as such by the working organism in which the vector is to be maintained prior to introduction into a lactic acid bacterium is located on the vector such that the complete polypeptide or

BNSDOCID: <WO__9632486A1_I_>

protein encoded by the nucleic acid sequence cannot be expressed.

14. Vector according to claim 13, wherein the nucleic acid sequence encoding the protein or polypeptide is present in the vector at the appropriate cloning site.

15. Vector according to claim 13 or 14, wherein the terminator sequence is present between the promoter sequence and the first codon of the nucleic acid sequence encoding a protein or polypeptide.

16. Vector according to any of claims 13-15, wherein the terminator sequence is present between the promoter sequence and the translation initiation region preceding the nucleic acid sequence.

17. Vector according to any of claims 13-16, wherein the transcription terminator sequence is present such that it is excisable from the vector in a manner known per se e.g. when the transcription terminator sequence is flanked by restriction enzyme sites uniquely present in the vector at such flanking position or is flanked by direct repeats e.g. excisable by homologous recombination by the host.

18. Vector according to any of claims 13-17, wherein the components of an expression vector according to any of claims 1-10 are present.

19. Vector according to any of the preceding claims further comprising one or more of the components:

i) a replicon for a lactic acid bacterium

ii) a DNA sequence encoding the signal sequence of a secreted protein recognisable by a lactic acid bacterium as such this could e.g. be a signal sequence from a lactic acid bacterium, said signal sequence being located in frame between the initiation codon and the nucleic acid sequence encoding a protein or a polypeptide,

iii) a reporter gene encoding an easily assayable product, such a reporter gene e.g. being β-glucuronidase from *Escherichia coli*,

iv) a transcription terminator sequence recognisable by a lactic acid bacterium, said sequence being located downstream of the nucleic acid sequence encoding a protein or polypeptide,

v) a DNA sequence encoding an anchor sequence from a lactic acid bacterium gene and said DNA sequence being located in frame downstream

from the nucleic acid sequence encoding a protein or a polypeptide and vi) a DNA sequence encoding a product with health stimulating properties.

20. Vector according to any of the preceding claims, wherein the vector is a foodgrade vector.

21 Method for obtaining improved stability and degree of expression of a nucleic acid sequence heterologous to a lactic acid bacterium, said heterologous sequence being present on a vector according to any of claims 1-10 as such or in combination with claims 19 or 20, said vector being introduced into a lactic acid bacterium in a manner known per se, said expression of heterologous nucleic acid sequence occurring in the lactic acid bacterium.

22 Method for constructing a vector according to any of claims 1-10 as such or in combination with claim 19 or 20, comprising introducing a vector according to any of claims 13-20 into a working organism other than the strain of lactic acid bacterium in which expression is to occur, manipulating the vector in a manner known per se to obtain desired characteristics in said working organism and finally introducing the vector comprising nucleic acid sequence encoding a protein or polypeptide in the strain of lactic acid bacterium in which expression is to occur, said sequence preferably being heterologous to the strain of lactic acid bacterium in which expression is to occur.

23. Method according to claim 22, wherein the working organism is a non lactic acid bacterium.

24. Method according to claim 22 or 23 wherein the transcription terminator sequence located between the promoter and the first codon of the nucleic acid sequence encoding a protein or a polypeptide is treated in a manner known per se for excising nucleic acid sequences to excise the terminator sequence in order to allow transcription of the nucleic acid sequence encoding a protein or a polypeptide e.g. by treatment of the vector with restriction enzyme for cutting the vector at the sites flanking the terminator sequence.

25. A method for producing a protein or polypeptide heterologous to a lactic acid bacterium in a lactic acid bacterium, preferably a

BNSDOCID: <WO___9632486A1_I_>

Lactobacillus or *Bifidobacterium* in an amount exceeding 1%, preferably exceeding 2% of the total amount of protein, said method comprising expression of nucleic acid encoding such a protein or polypeptide from an expression vector according to any of claims 1-10 as such or in combination with 19 or 20 or a host cell according to claim 11 or 12 in a manner known per se.

26. Protein or polypeptide obtainable by the method of claim 25.

27. Protein or polypeptide according to claim 26, wherein the protein or polypeptide is foodgrade.

BNSDOCID: <WO___9632486A1_I_>

Fig. 1a

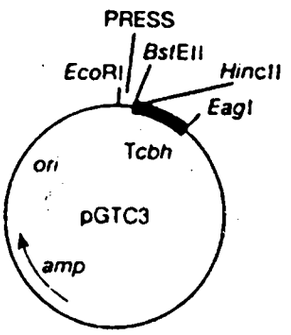


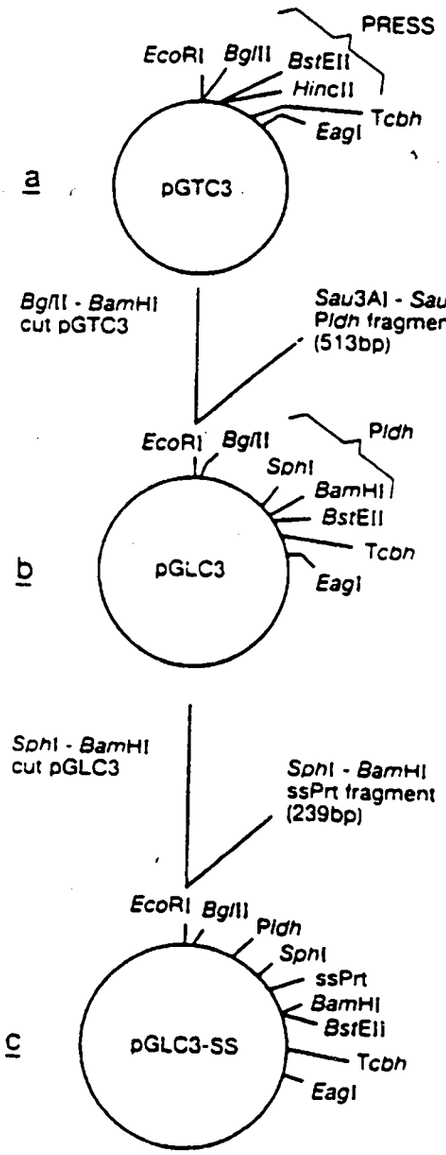
Fig. 1b.

EcoRI BglII SpeI XhoI SphI BamHI
GAATTC AGATCT ACTAGT CTCGAG GCATGC GGATCC ATTCAA

BstEII HincII
GGTCACC CCTAGA GTCAAC TAAAAGCCACTACTGT
N stop ---->
T_{cbh} sequences

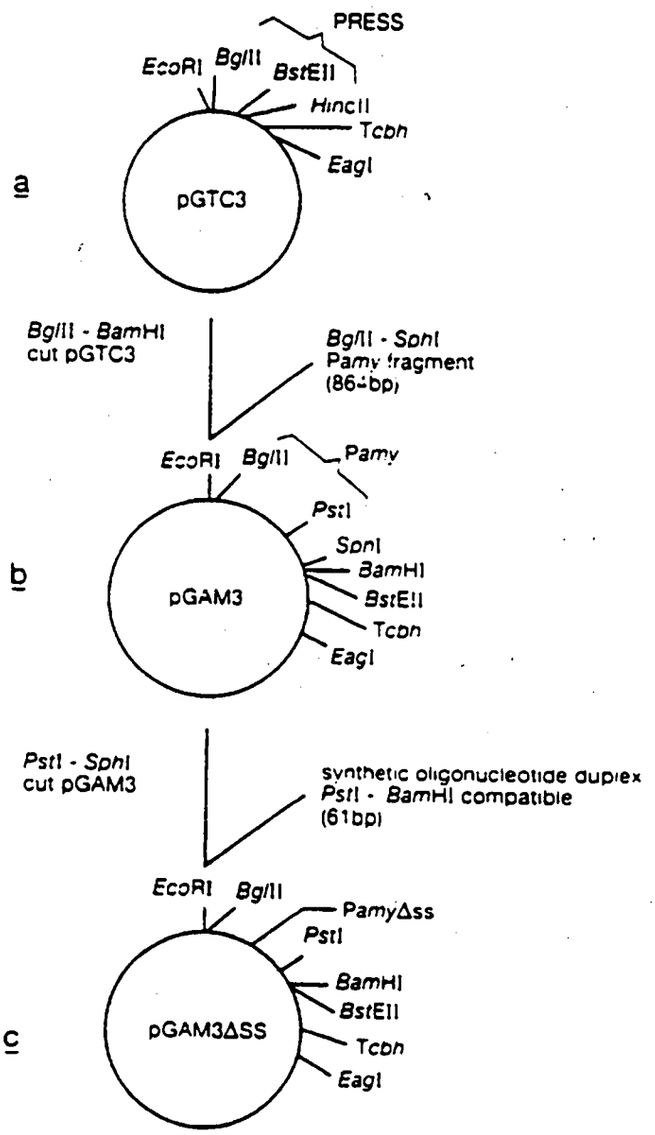
BNSDOCID: <WO__9632486A1_I_>

Fig.2A



BNSDOCID: <WO__9632486A1_I_>

Fig. 2B



BNSDOCID: <WO___9632486A1_1_>

SUBSTITUTE SHEET (RULE 26) <WO 9632486A1_I_>

Fig. 3

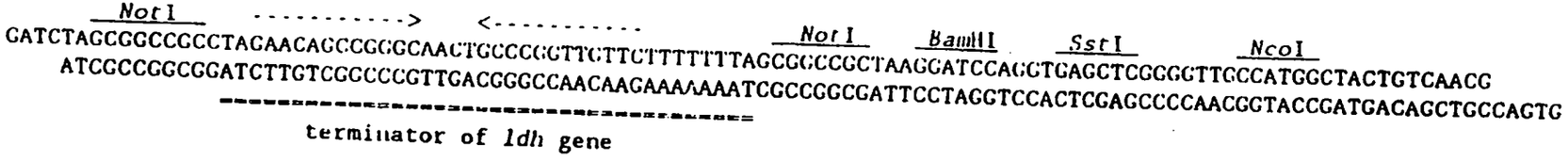


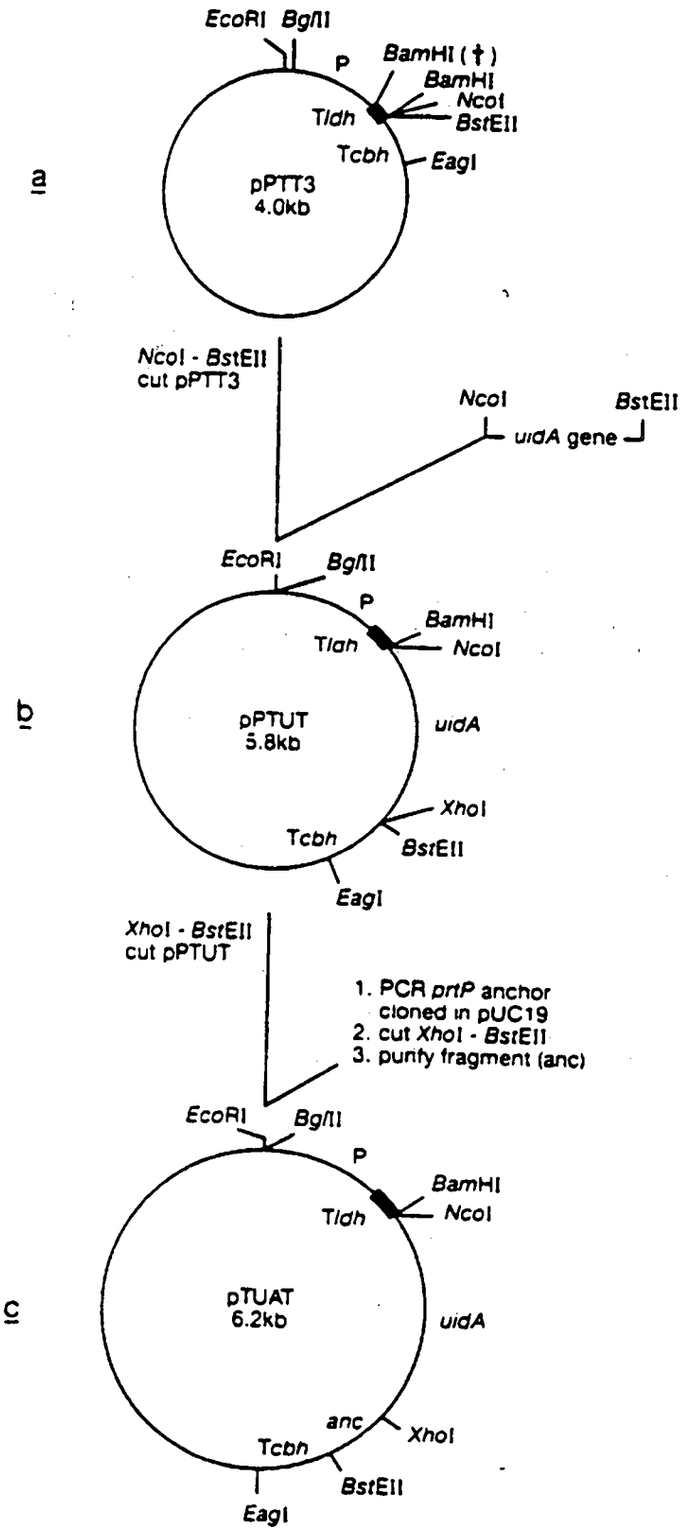
Fig. 4

Nucleotide sequence of the 3'end of the *uidA* gene

a. GGC.AAA.TGAATCACA
G K stop

b. XhoI BstEII HindIII
GGC.AAA.CTC.GAG.TAATGTAAGGTCACCTCGACAAGCTT
G K L E stop pUC cloning site

Fig. 5


a
EcoRI
BglII
P
BamHI (†)
BamHI
NcoI
BstEII
Tldh
Tcbh
EagI
pPTT3
4.0kb
NcoI - BstEII
cut pPTT3
NcoI
BstEII
uidA gene
b
EcoRI
BglII
P
BamHI
Tldh
NcoI
pPTUT
5.8kb
uidA
XhoI
Tcbh
BstEII
EagI
XhoI - BstEII
cut pPTUT
1. PCR prtP anchor
cloned in pUC19
2. cut XhoI - BstEII
3. purify fragment (anc)
c
EcoRI
BglII
P
BamHI
Tldh
NcoI
pTUAT
6.2kb
uidA
anc
XhoI
Tcbh
BstEII
EagI

BNSDOCID: <WO__9632486A1_1>

Fig. 6

a. BamHI SstI NcoI
G GAT CCA GGT GAG CTC GGG GTT GCC ATG G
D P G E L G V A M

b. ClaI BamHI MunI KpnI HindIII
G GAT CTT ATC GAT TTA CGG ATC CAA CAA TTG CAA GGT ACC GAT CAA GCT T
D L I D L R I Q Q

SmaI BbsI EcoRI SalI ApaI NcoI
ACC CGG GAA GAA GAC AGA ATT CAC AAG TCG ACC GGG CCC TTG CCA TGG

c. NruI BamHI MunI
G GAT CTT ATC GCG ATT TAC GGA TCC AAC AAT TG ---> sequence as in B
D L I A I Y G S N

d. ClaI BspEI BamHI MunI
G GAT CTT ATC GAT GTC CGG ATG GAT CCA ACA ATT G ---> sequence as in B
D L I D V R M D P

Fig. 7

↓
-----GAAGAC N N N N N N N N N------
-----CTTCTG N'N' N'N'N'N' N'N'N'-----
↑

Fig.8

a.
```
                BbsI  EcoRI          SalI   ApaI          NcoI
G-------GA AGA CAC AAT TCA CAA GTC GAC CGG GCC CTT GCC ATG G
        R  R  Q  N  S  Q  V  D  R  A  L  A  M
```

b.
```
                BbsI
G-------GAA GAC AGA ATT AAT TCA CAA GTC GAC CGG GCC CTT GCC ATG G
        E  E  D  R  I  N  S  Q  V  D  R  A  L  A  M
```

c.
```
                BbsI
G-------G AAG AAG ACA GAA TTA ATT AAT TCA CAA GTC GAC CGG GCC CTT GCC ATG G
        K  K  T  E  L  I  N  S  Q  V  D  R  A  L  A  M
```

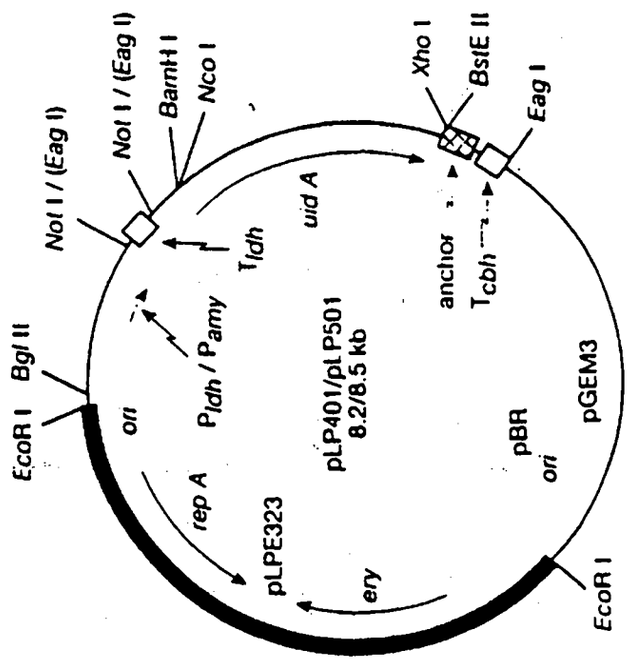


Fig.9a

BNSDOCID: <WO___9632486A1_I_>

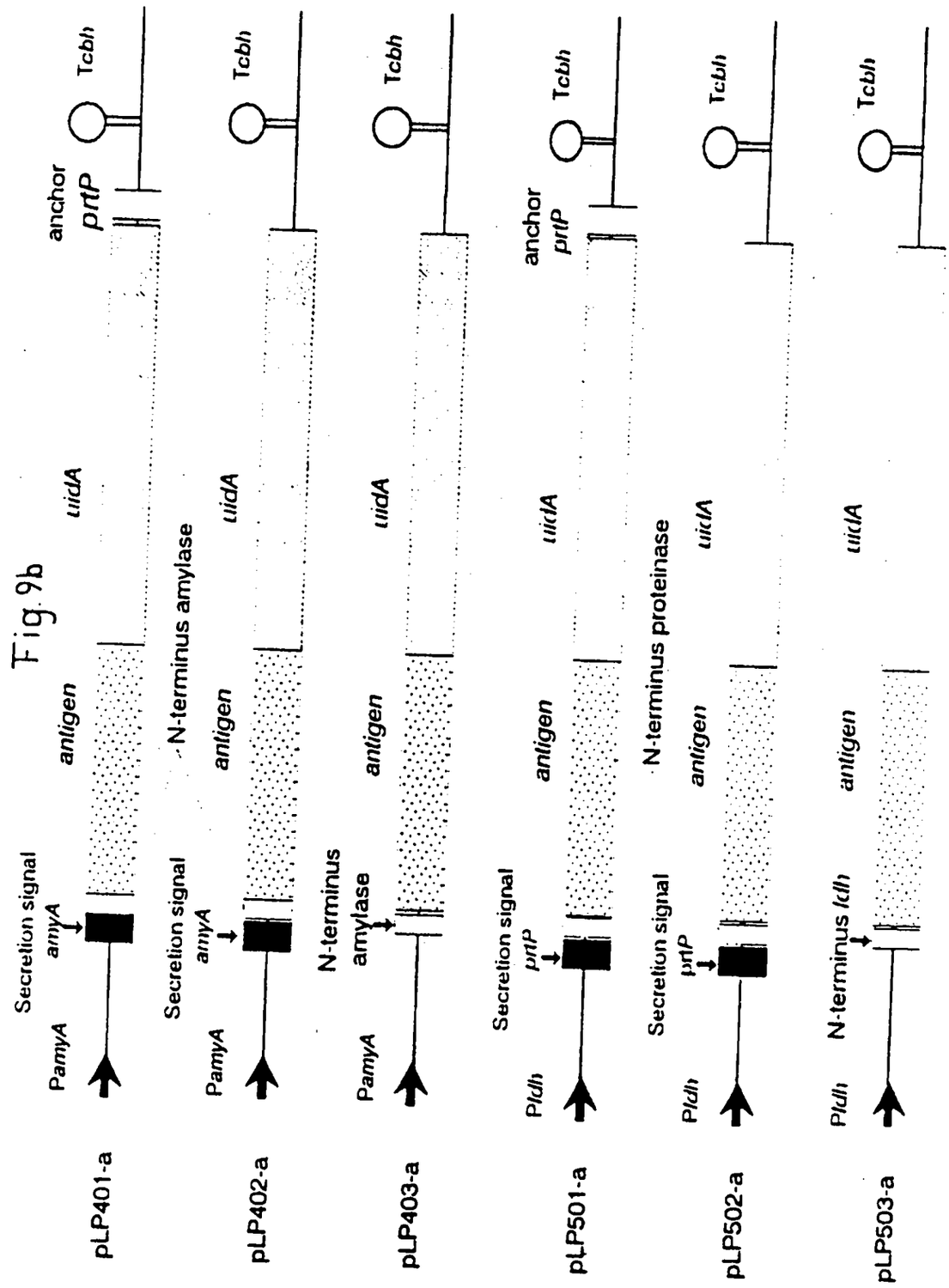

Fig 9b
pLP401-a
PamyA
Secretion signal amyA
antigen
uidA
anchor prtP
Tcbh
pLP402-a
N-terminus amylase
pLP403-a
N-terminus amylase
pLP501-a
Ptdh
Secretion signal prtP
pLP502-a
N-terminus proteinase
pLP503-a
N-terminus tdh

Fig.10

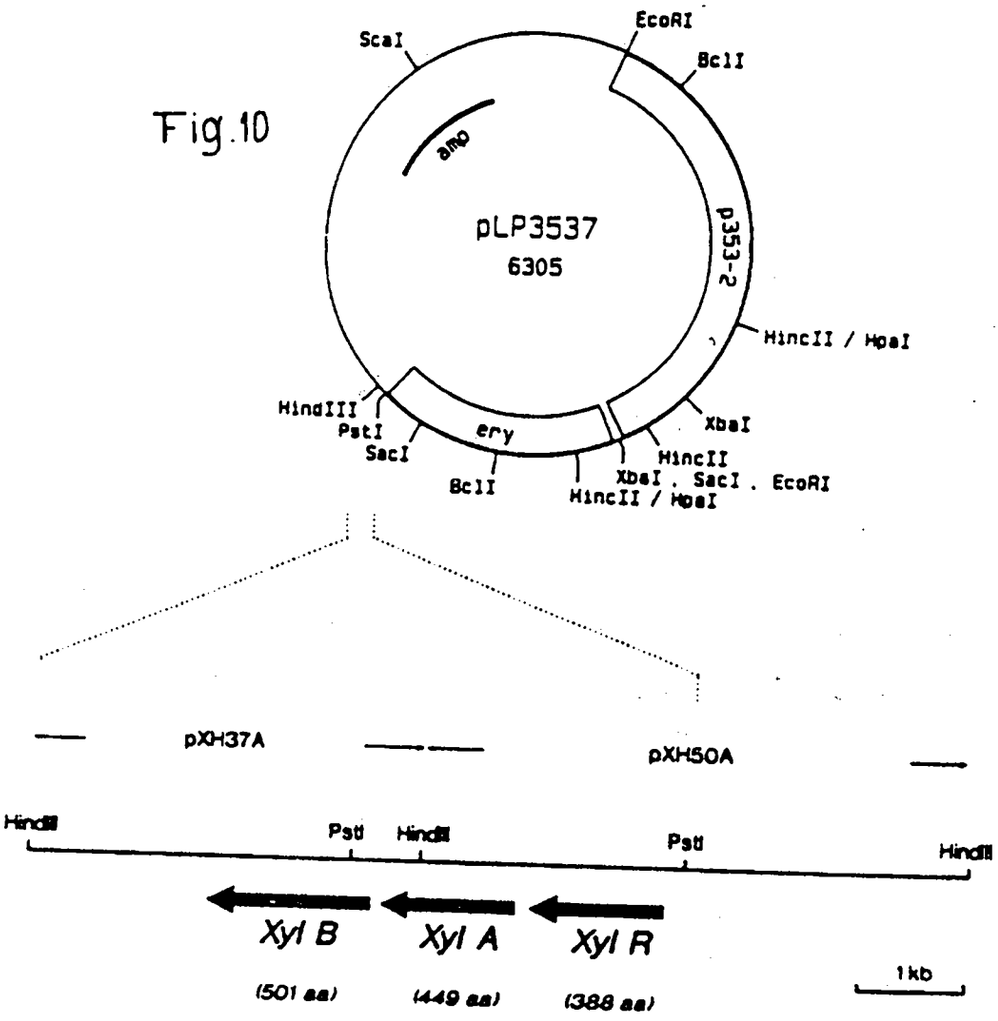
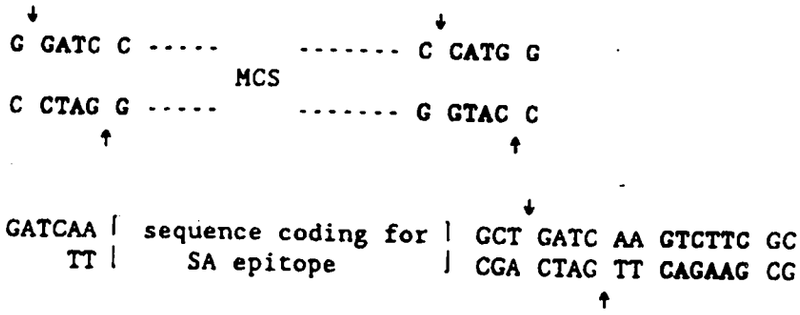
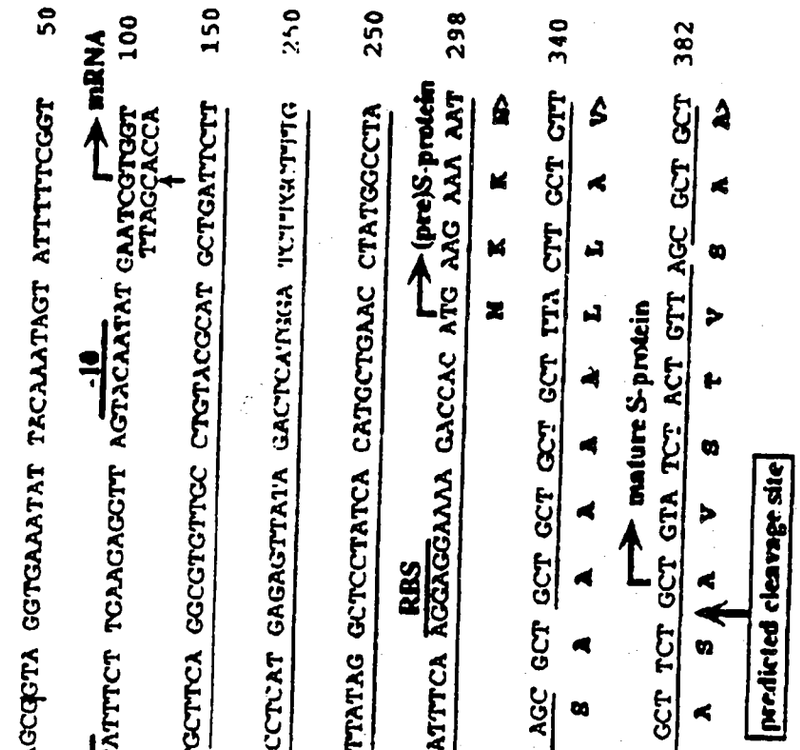


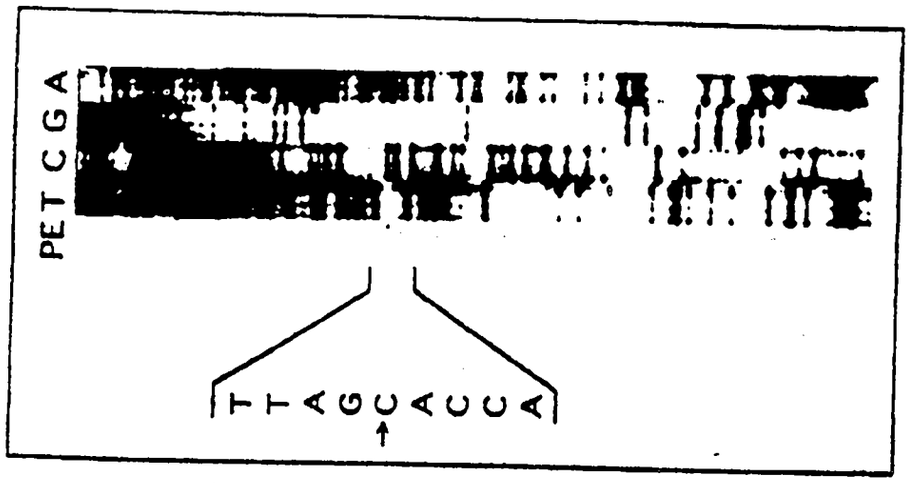
Fig.11



BNSDOCID: <WO__9632486A1_I_>

Fig.12





UNSDOCID: <WO___9632486A1_I_>

INTERNATIONAL SEARCH REPORT

Intern. al Application No
PCT/NL 95/00135

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/74 C12N15/62 C12N15/46 C12N15/44 C12N15/70
C12N15/31 C07K14/14 C07K14/11 //C12N1/21,C12R1:225

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KEYSTONE SYMPOSIUM ON MUCOSAL IMMUNITY: NEW STRATEGIES FOR PROTECTION AGAINST VIRAL AND BACTERIAL PATHOGENS, KEYSTONE, COLORADO, USA, JANUARY 16-23, 1995. JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT 0 (19A). 1995. 255. ISSN: 0733-1959, ABSTRACT NO. J1-203, BOERSMA W J A ET AL 'Lactobacillus as vectors with intrinsic adjuvanticity for safe live mucosal vaccines.' see abstract ---	1-12, 19-21, 25-27
X	WO,A,94 00581 (VIAGEN OY) 6 January 1994 see the whole document --- -/--	1-12,20, 26,27

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 December 1995	29.12.95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

NSDOCID: <WO___9632486A1_I_>

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/NL 95/00135

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 17117 (LYNXVALE LTD) 2 September 1993 see the whole document	1-12,20, 26,27
X	WO,A,92 04451 (GENESIT OY ;VALIO FINNISH CO OPERATIVE DAI (FI)) 19 March 1992 see the whole document	1-12,20, 26,27
X	EP,A,0 449 770 (CIBA GEIGY AG) 2 October 1991 see the whole document	1-12,20, 26,27
X	US,A,5 242 821 (PALVA ILKKA ET AL) 7 September 1993 see the whole document	1-12,20, 26,27
X	FEMS MICROBIOL. REVIEWS, vol. 88, 1992 ELSEVIER, AMSTERDAM, NL, pages 73-92, M. VAN DE GUCHTE ET AL. 'Gene expression in Lactococcus lactis' see the whole document	1-12,20, 26,27

INTERNATIONAL SEARCH REPORT

...ormation on patent family members

Interna...l Application No
PCT/NL 95/00135

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9400581	06-01-94	AU-B-	4329793	24-01-94
WO-A-9317117	02-09-93	EP-A-	0628083	14-12-94
		GB-A,B	2278358	30-11-94
		JP-T-	7504815	01-06-95
WO-A-9204451	19-03-92	AU-B-	652920	15-09-94
		EP-A-	0550428	14-07-93
		JP-T-	6503945	12-05-94
EP-A-0449770	02-10-91	AU-B-	643511	18-11-93
		AU-B-	7355891	03-10-91
		JP-A-	4211384	03-08-92
US-A-5242821	07-09-93	NONE		

BNSDOCID: <WO___9632486A1_I_>

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/74, 15/62, 15/31, C07K 14/36, 14/31, 14/33, C12N 1/21, A61K 39/08, 39/09, C12N 11/16, C12Q 1/02, G01N 33/68 // (C12N 1/21, C12R 1:225)

A1

(11) International Publication Number: **WO 97/09437**

(43) International Publication Date: 13 March 1997 (13.03.97)

(21) International Application Number: PCT/GB96/02195

(22) International Filing Date: 6 September 1996 (06.09.96)

(30) Priority Data:
9518323.2 7 September 1995 (07.09.95) GB

(71)(72) Applicants and Inventors: STEIDLER, Lothar [BE/BE]; Laboratory of Molecular Biology, Universiteit Gent, K L Ledeganckstraat 35, B-9000 Gent (BE). REMAUT, Erik [BE/BE]; Laboratory of Molecular Biology, Universiteit Gent, K L Ledeganckstraat 35, B-9000 Gent (BE). WELLS, Jeremy, Mark [GB/GB]; University of Cambridge, Dept. of Pathology, Tennis Court Road, Cambridge CB2 1QP (GB).

(74) Agents: CHAPMAN, Paul, William et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: MATERIALS AND METHODS RELATING TO THE ATTACHMENT AND DISPLAY OF SUBSTANCES ON CELL SURFACES

(57) Abstract

Methods for obtaning surface expression of a desired protein or polypeptide in Gram-positive host organisms are provided. In addition, vectors useful in such methods as well as Gram-positive host organisms tranformed with such vectors are disclosed.

BNSDOCID: <WO__9709437A1_I_>

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

ENSDOCID: <WO___9709437A1_I_>

Materials and Methods Relating to the Attachment and Display of Substances on Cell Surfaces

Field of the Invention

The present invention relates to materials and methods for the exportation, cell wall attachment and display of substances, in particular proteins and polypeptides, on the cell surface of Gram-positive bacteria such as Lactococcus lactis. Methods are disclosed providing for the surface expression of cell wall anchoring of proteins, including antigens, specific binding proteins and enzymes.

Background to the Invention

The display of polypeptides on the surface of bacteria has been a subject of investigation for several years. This interest has arisen because recombinant DNA technology makes it possible to use bacterial cells as factories for the inexpensive production of a wide range of different proteins. Work has centred on the display of polypeptides on the surface of Gram-negative bacteria, see for example the review provided in US patent 5348867. However, in Gram-negative bacteria it has proved difficult to find an adaptable method of displaying polypeptides on the surface of the cells so that they are securely attached to the surface at high density.

However, high density surface expression of recombinant products on the bacterial surface is a prerequisite for the use of these bacteria in certain industrial applications, and in the areas of vaccine development, the construction of peptide libraries and the production of whole bacterial cell adsorbents. There is no prior art known to the inventors which results in the consistent, stable, high density expression on the surface of bacteria, and more especially, on the cell surface of Gram-positive bacteria such as Lactococcus lactis and similar or related species.

BNSDOCID: <WO__9709437A1_I_>

Previous work on the bacterial surface presentation of polypeptides has focused mainly on the use of Gram-negative bacterial such as E. coli and Salmonella (Georgiou et al., 1993). In Gram-negative bacteria the peptidoglycan cell wall is bounded by two separate lipid membranes. The outer membrane is largely impermeable to molecules with a relative molecular mass greater than 500 (Mr) and as a result very few proteins are released into the growth medium. Owing to the presence of an outer membrane, surface exposed proteins of Gram-negative bacteria are either integral outer membrane proteins or protein appendages such as pili or flagella

The few proteins which are secreted by Gram-negative organisms are transported across the outer membrane by specialised transport systems which may involve up to 14 different gene products (eg pullulanase secretion; Kornacker and Pugsley 1990a).

The possibility of displaying foreign proteins by making C-terminal fusions to integral membrane proteins is complicated by the fact that the C-terminal regions of many integral membrane proteins appears to be necessary for targeting and correct assembly of the protein in the outer membrane. One way around this problem has been to use the N-terminal targeting sequence of the E. coli major lipoprotein (Lpp) to direct fusion proteins to the outer membrane. As Lpp does not transverse the outer membrane, the membrane spanning domain of an outer membrane protein (OMP; eg *E. coli* OmpA) is also required to localise a fusion protein to the cell surface. Nevertheless, the utility of these Lpp-OmpA fusions to display functional proteins such as β-lactamase, a single chain Fv antibody and a cellulose binding protein have been demonstrated (Georgiou et al., 1990).

Other approaches for the surface display of polypeptides

BNSDOCID: <WO___9709437A1_I_>

have involved making amino acid insertions within outer membrane loops of integral membrane proteins such as Lam B or in the pilin and flagellin proteins which do not contain transmembrane domains (Charbit et al., 1991; Thiry et al., 1989, Steidler et al, 1993 a,b). The major problem with this approach has been that large amino acid insertions/substitutions disrupt the proper folding and/or localisation of the fusion protein in such a way that when used to immobilise the bacterial cells, the fusion proteins are extracted from the outer membrane (Steidler, PhD Thesis, University of Gent, Belgium).

Although significant progress has recently been made in the development of methods from the surface display of proteins in Gram-negative bacteria, the major drawback to the use of integral membrane proteins for such purposes is that the fusion proteins are not firmly attached to the cell surface. Furthermore, the high level expression of membrane proteins is known to be detrimental to the cell, a factor which may limit the possible applications for such systems in cases where high density surface expression is required.

In contrast, Gram-positive bacteria do not possess an outer membrane and proteins which are displayed on the cell surface are thought to be linked to the cell surface via their C-terminus (Schneewind et al., 1992). Many surface proteins of Gram-positive bacteria possess certain common features which are unique to these organisms. Each molecule has at its N-terminal end a secretion leader peptide which acts as an export signal for cell secretion, a LPXTG motif, a C-terminal hydrophobic domain and charged amino acids at the very C terminus. Most work on the characterisation and function of these has been carried out in S. aureus using the cell wall sorting and anchoring domains of S. aureus protein A (Navarre and Schneewind, 1994). It has been shown that protein A is covalently

BNSDOCID: <WO__9709437A1_I_>

54

coupled to the cell wall of S. aureus by a mechanism which is yet to be elucidated.

Summary of the Invention

The present invention demonstrates that the firm attachment of foreign proteins to the surface of L. lactis and other Gram-positive bacteria is made possible by constructing chimeric genes encoding a fusion protein which contains nucleic acid encoding (1) an N-terminal secretion signal, (2) the protein to be surface expressed and (3) the cell wall sorting and anchoring domains of S. aureus protein A.

By comparison with E. coli, as relatively few heterologous proteins have been expressed at high level in L. lactis, before the present work it was impossible to predict with certainty whether the expression of any particular protein would be possible or whether such expression would be toxic to the cell. Such toxicity is likely to occur when a secreted or membrane associated protein is expressed at high level, as protein export may adversely affect the integrity of the cell membrane or the function of the general secretory pathway.

Thus, the present invention shows for the first time that proteins or polypeptides can be firmly attached to the cell wall of Gram-positive bacteria when expressed as fusions with a cell wall anchoring domain. As is shown below, it is not possible to remove the protein by boiling the cells in the presence of an ionic detergent which would normally release non-covalently bound proteins. While not wishing to be bound by any particular theory, we believe that L. lactis appears to possess the necessary enzyme(s) required to link (covalently) the expressed protein A fusion to the cell wall peptidoglycan. This binding could not have been readily predicted from the possession by protein A of the LPXTG motif and C-terminal hydrophobic domains common to

many surface proteins of Gram-positive bacteria, since these motifs are just necessary, but not sufficient conditions for the occurrence of covalent cell wall anchoring.

Accordingly, the present invention arises from the finding that it is possible to firmly attach proteins and polypeptides of interest (including but not restricted to adhesins, antigens, enzymes and ligands) to the outer face of the peptidoglycan cell wall of Gram-positive bacteria, such as Lactococcus lactis, using a chimeric gene comprising nucleic acid encoding a secretion signal sequence, a desired protein or polypeptide and a cell wall attachment domain, such as that derived from Staphylococcus aureus protein A.

Accordingly, in a first aspect, the present invention provides a recombinant vector including nucleic acid encoding a fusion protein comprising a secretion signal sequence, a desired protein or polypeptide and a cell wall attachment domain, so that when the vector is transformed in a Gram-positive bacterial host and the fusion protein is expressed, the desired protein or polypeptide is attached and displayed on the outer surface of the cell wall of the host.

In a further aspect, the present invention provides a Gram-positive host organism transformed with a recombinant vector as described above.

In a further aspect, the present invention provides a Gram-positive host organism described above wherein the desired protein or polypeptide has been expressed, and is covalently attached to the cell wall and is displayed on the outer face of the surface of the host organism.

In a further aspect, the present invention provides a

BNSDOCID: <WO___9709437A1_I_>

method of engineering a Gram-positive host organism to display a desired protein or polypeptide on its surface comprising transforming the host organism with recombinant nucleic acid encoding a fusion protein comprising a secretion signal sequence, a desired protein or polypeptide and a cell wall attachment domain, so that the host is capable of expressing the fusion protein, whereby the cell wall anchoring domain enables the host to attach and display the desired protein or polypeptide on the cell wall surface. This aspect also includes strains of Gram-positive host organisms as obtainable by this method.

Preferably, the Gram-positive host organism is a Lactococcus host, such as Lactococcus lactis, or a similar or related species having the properties of expressing the fusion protein, and attaching and stably displaying the desired protein or polypeptide on the cell surface. The unexpected finding that the protein A fusions are similarly attached to the cell wall in L. lactis and S. aureus suggests that this will also be the case with other cell wall binding domains in similar or related Gram-positive bacteria. Examples of other such Gram-positive bacteria include Bacillus subtilis, Streptococcus gordonii, Staphylococcus xylosus, Lactobacillus spec. and psychrophilic and psychrotropic species of the genera Bacillus spec., Clostridium spec. and Corynebacterium spec.

Preferably, the secretion signal sequence is capable of targeting the protein for secretion via the sec dependent pathway of secretion and is derived from a signal sequence of a naturally secreted protein such as usp45. However, while this secretion signal sequence is used in the examples, other secretion signal sequences may be employed, such as those derived from other secreted proteins.

Preferably, the cell wall anchoring domain comprises, at the C-terminal, anchoring domain derived from the

NSDOCID: <WO___9709437A1_I_>

Staphylococcus aureus protein A. However, other proteins may be used as the cell wall anchoring domain, provided they have the properties of being stably bound to the surface of the cell and are capable of representing the desired protein or polypeptide on the cell surface.

Preferably, the nucleic acid encoding the fusion protein is operably linked to control sequences to direct its expression. An example of this is a Lactococcus host organism further transformed with nucleic acid comprising a T7 or T7-like polymerase gene under the control of an inducible promoter effective in the Lactococcus host, wherein the control sequences comprise a promoter specific for said polymerase provided upstream of the nucleic acid encoding the fusion protein whereby the promoter directs transcription of the nucleic acid encoding the fusion protein selectively as a result of the expression of the polymerase (see GB-A-2278358).

The high level of heterologous protein expression which can be achieved using the T7 polymerase system has been found to provide high levels of expression of the Protein A-fusion polypeptide on the surface of the bacteria. For many of the applications envisaged for the invention, the economics of the associated processes will be materially favoured by achieving the highest possible surface density of fusion molecules on the bacteria. However, for some applications the use of constitutive, or less active, promoters may give useful results, especially where lower levels of expression are required.

Numerous variations on the disclosed vectors could be routinely prepared by those skilled in the art using known techniques and the disclosure provided herein. Further, the proteins or polypeptides encoded by the nucleic acid sequences above may be modified by varying their amino acid sequence without substantially altering their properties,

BNSDOCID: <WO__9709437A1_I_>

eg by manipulation of the nucleic acid or amino acid sequences through insertion, addition, deletion or substitution of one or more base pairs or amino acids. Such derivatives of the polypeptides disclosed herein are included within the present invention.

As mentioned above, the Gram-positive host organisms transformed with the nucleic acid encoding fusion proteins have a wide variety of applications as vaccines, to display biologically active molecules on cell surfaces, as adsorbents and to display enzymes on cell surfaces, eg for use as catalysts.

Thus, in a further aspect, the present invention provides Gram-positive host organisms wherein the desired protein or polypeptide includes at least one epitope to which it is desired to raise an immunological response. Preferably, the protein or polypeptide is an immunogen from a pathogen, an antigen or an adhesin.

In this aspect, the present invention also provides a vaccine comprising the above host organisms in admixture with a suitable carrier. The present invention also includes the use of the above Gram-positive host organisms in the preparation of a vaccine wherein the desired protein or polypeptide is an epitope to which it is desired to raise an immunological response. Preferably, the vaccines are administered intranasally or parenterally, or via other mucosal routes such as orally or vaginally.

In a further aspect, the present invention provides Gram-positive host organisms wherein the desired protein or polypeptide is a biologically active molecule. By way of example, the biologically active molecule may be a drug, a hormone, an enzyme, DNA/RNA binding protein, or a substance capable of performing secondary modifications (glycosylation, disulphide isomerisation) on other coexpressed and secreted proteins. Alternatively, the

BNSDOCID: <WO___9709437A1_I_>

desired protein or polypeptide can be a targeted group, for instance antibodies, or fragments thereof, or adhesins specific for receptors.

In this aspect, the present invention also includes a pharmaceutical composition comprising the Gram-positive host organisms, and their use in methods of medical treatment.

In a further aspect, the present invention provides Gram-positive host organisms wherein the desired protein or polypeptide is a specific binding partner of a ligand. In this aspect, the present invention also includes the use of Gram-positive host organisms in a method of purification or separation of a ligand wherein the desired protein or polypeptide is a specific binding partner of the ligand, whereby the display of the specific binding partner on the surface of the organism allows the binding partner to bind to the ligand.

The invention also provides a way of immobilising the Gram-positive host organisms on a solid phase, eg by expressing streptavidin fused to a cell wall anchoring domain so that the streptavidin represented on the cell surface can bind to biotin immobilised on a solid phase. Thus, the present invention provides a method of immobilising a Gram-positive host organism of the invention which comprises bringing a host organism of this aspect of the invention into contact with a solid surface which has the ligand immobilised thereon. The present invention also provides a method for detecting complexes with a specific ligand (e.g. biotin or an immunoglobulin) by coexpressing a reporter activity such as a luciferase or a fluorescent protein in cells which

also express on their surface the protein binding to the ligand to be detected (e.g. streptavidin or protein A).

In a further aspect, the present invention provides a Gram-positive host organism wherein the desired protein or polypeptide is an enzyme. This aspect of the invention also includes a composition comprising the Gram-positive host organism wherein the desired protein or polypeptide is an enzyme which has been expressed and displayed on the cell surface of the host, and the use of the composition in a reaction catalysed by the enzyme, for example a surface enzyme which can break down unwanted food components such as fat and cholesterol from the surrounding milieu.

In a further aspect, the present invention provides a method of screening a library of substances, such as antibodies or antigens or fragments thereof, comprising transforming Gram-positive host organisms with nucleic acid encoding a fusion of the substances and a cell wall anchoring domain, expressing the fusion so that the substance is displayed on the cell surface. The particular substance being screened for can then be detected using a recognition agent, eg labelled antibodies.

Brief Description of the Drawings

Figure 1 shows the construction of the gene fusions described below;

Figure 2 shows a Western blot of proteins recovered from (i) the growth medium, (ii) the supernatant of lyostaphin treated cells, (iii) intact cells boiled in Laemmli SDS

BNSDOCID: <WO___9709437A1_I_>

buffer "cracking released" and (iv) the residue of cell pellet of the lyostaphin treated cells. The proteins indicated (see arrow heads) were detected by immunoblotting with rabbit anti-streptavidin antiserum and a second antibody conjugate according to standard procedures. These results demostrate that the streptavidin protein fusion A protein produced in L. lactis is firmly attached to the cell surface;

Figure 3 shows that a biotin conjugate was able to specifically bind to the surface of intact cells expressing a streptavidin - protein A fusion, thus demonstrating that the protein is present on the surface of the cell. This figure shows a filter onto which cells expressing the strepavidin protein A fusion (pL2SAX) or streptavidin (pL2SA) have been collected and reacted with biotinylated horseradish peroxidase (B-POD) or B-POD which had been previously treated with streptavidin (SA) to block its binding sites (see wells labelled B-POD/SA). The cells binding B-POD were detected by incubation with a chromagenic substrate for horseradish peroxidase. Only the intact cells expressing the streptavidin protein A fusion protein on their surface bound the biotinylated enzyme conjugate B-POD;

Figure 4. The panel labelled pL2SAX shows a photograph of *L. lactis* cells expressing the streptavidin protein A fusion on their surface which have bound to a polystyrene surface coated with a biotinylated horseradish peroxidase conjugate. No cells are seen in the negative control which is representative of all the control samples described in the text.

Figure 5(A) shows physical and genetic maps of the

BNSDOCID: <WO___9709437A1_I_>

64

broad Gram-positive host range vector pTREX1, pTIL2TT and PTITT. The expression cassette, macrolides, lincosamides and streptogramin B (MLS) resistance determinant, TTFC gene, secretion signal sequence of *usp45* (L2), *S. aureus* protein A anchor (SPA-X) and the replicon (*Ori-* 'from pAMβ1') are depicted as thick lines;

Figure 5(B) is a schematic representation of the expression cassette in pTREX1 showing the various sequence elements involved in gene expression, the location of unique restriction endonuclease sites, Shine Dalgarno (SD) motif and translation initiation start codon (ATG);

Figure 5(C) shows the DNA sequence and restriction endonuclease site map of the pTREX1 expression cassette. The key expression elements are also indicated on the map;

Figure 6 shows the list of primers used for PCR;

Figure 7 shows a filter onto which intact cells expressing TTFC intracellularly (pTITT), secreting TTFC (pTIL2TT) or expressing a TTFC-protein A fusion protein (pTTA) have been incubated with rabbit TTFC polyclonal antisera, collected onto a membrane and then reacted with an antibody peroxidase conjugate and substrate to detect the binding of anti-TTFC antibody on the surface of the cells. Only the intact cells expressing the TTFC-protein A fusion displayed TTFC on their cell surface.

BNSDOCID: <WO___9709437A1_I_>

Detailed Description

Live or inactivated bacterial vaccines and tools for the production of anti-polypeptide antibodies

The invention may be used for the surface expression of antigens, adhesins and biologically active molecules in Gram-positive bacteria such as L. lactis. These bacteria are among those which have been proposed as vaccine delivery vehicles for mucosal immunisation (Wells et al, 1993). Antigens which are exposed on the bacterial cell surface may be more easily recognised by the immune system (than antigens which are only released following bacterial death and degradation) owing to the greater accessibility of surface antigens to their receptors on such antigen-recognising and processing cells as macrophages, dendritic cells and B lymphocytes. The surface expression of antigens and epitopes may therefore enhance the immunogenicity of antigens expressed by Gram-positive bacterial vaccines. Whole cells expressing antigen on their surface will also be useful immunogens for raising polyclonal and monoclonal antibodies in animals (Charbit et al, 1987).

Bacterial delivery of biologically active molecules

The surface expression of adhesins specific for receptors present on cells and/or tissues in the gut and other mucosal tissues (for example, the respiratory, uro-genital and nasal tracts), in addition to the expression of antigens and/or biologically-active molecules would enable the recombinant bacteria to be targeted to specific cells and tissues, eg neoplasia. Such targeting would increase the efficiency with which

BNSDOCID: <WO__9709437A1_I_>

these bacteria can be used to deliver antigens and/or biologically active molecules to mucosal surfaces. In cases where the bacteria being used do not normally adhere to, or proliferate on, a particular mucosal surface, such targeting would increase the contact time between the bacteria and the mucosal surface, thus facilitating delivery and/or uptake of a molecule or molecules synthesised by the bacteria.

The surface expression of numerous biologically active molecules would have a wide range of applications in the therapy of disease, by the delivery of any of a large number of hormones, cytokines or other immunomodulatory molecules, cell receptors and/or their ligands, enzymes, cell-specific toxins. Such molecules could be co-expressed with invasins or other proteins which enable bacteria to enter cells and/or to be released into the cytoplasm from the endosome; thus the targeting of bacteria to cells, and to compartments within cells, are rendered feasible by means of this invention.

Additionally, surface ligands with adequate binding affinity could be used to enable the bacteria to be coated with a wide variety of desired molecules; for example, surface expression of a DNA or RNA binding protein could be used to permit the organisms to be coated in DNA or RNA. Such a process could find applications in gene therapy or nucleic acid vaccination.

Surface display of specific antigens, antibodies and ligands

The recent development of phage display technology has

BNSDOCID: <WO__9709437A1_I_>

enabled specific antigens, antibodies and other ligands to be isolated from complex libraries by panning for recombinant bacteriophage expressing the counter-ligands which then adhere to an immobilised support containing the antigen, antibody or receptor of interest (Chiswell and McCafferty, 1992). An alternative approach would be to use Gram-positive bacteria for the surface presentation of proteins and peptides. The advantages to the bacterial display technology would be (i) bacteria expressing the desired ligand could be detected directly using a fluorescent counter ligand as a probe; these bacteria could then be obtained free of non-expressor cells using a FACS machine capable of sorting single bacteria. This procedure would avoid the need to propagate the phage to recover the cloned DNA sequence of interest (ii) higher density of surface protein expression is possible in bacteria compared to the phage systems and (iii) problems with the inability of certain recombinant phage to infect E. coli (so that they can be propagated) will not be encountered.

The use of bacteria as whole cell adsorbents

The use of affinity chromatography for the purification of biological products can frequently convert multi-step into single-step or near single-step procedures. This results in turn in a reduction in the complexity of downstream engineering processes associated with the recovery of biological products, and also means that large savings can be achieved in capital investment; operating costs can be reduced, and the products themselves can be recovered more rapidly. In fact, one of the best examples of this approach is the use of wild type Staphylococcus aureus to bind and purify antibodies.

BNSDOCID: <WO___9709437A1_I_>

Protein A found on the surface of the bacteria has a high affinity for the Fc domain of certain immunoglobulins and heat killed and chemically stabilised S. aureus cells have been routinely used for the purification of antibodies.

However, the need for an abundant supply of an immobilised form of the ligand can render affinity chromatography uneconomic.

The expression of the functional fragments of ligands or counter-ligands such as single chain antibodies, protein receptors, adhesion molecules etc on the surface of bacteria would enable these molecules to be replicated inexpensively by bacterial growth; entrapped bacterial cells could then be used to provide an inexpensive form of adsorbent.

Innocuous Gram-positive bacteria such as L. lactis do not contain the endotoxins present in E. coli which initially contaminate all recombinant products made in this bacterium, and which must be removed during the course of purification procedures. Hence, the use of Gram-positive bacteria for the surface display of ligands will not result in the contamination of products with endotoxins.

Enzyme coated bacteria as biocatalysts

Many enzymes made by Gram-positive bacteria and other organisms are secreted by these organisms into their environment, where they may mediate a variety of biochemical transformations. These chemical conversions can be essential for survival and growth, or they may enable the organisms to compete more effectively with

BNSDOCID: <WO___9709437A1_I_>

other organisms in their environment.

The enzymes needed for nutritional purposes include, for example, those which can degrade biological polymers such as carbohydrates, lipids, nucleic acids and proteins into their substituents, and provide nutrients for the growing organism.

Secreted enzymes also perform a range of different functions, such as the biosynthesis of slimes and mucins (glycoproteins or proteoglycans) which enable bacteria and other organisms to fasten themselves to favourable surfaces in their environment. They may also inactivate anti-microbial substances such as antibiotics or toxins emanating from other organisms; and they may degrade toxic or recalcitrant substances left in the environment by geological or industrial processes.

Many of these secreted enzymes have industrial utility, but ideally need to be present in a solid phase, in order that liquid reactants passed over this phase can be subjected to catalytic conversion by the immobilised enzyme.

It is another vital aspect of this invention that it will improve the feasibility of using enzymes in industrial processes by providing - in the form of bacteria able to manufacture the enzymes in immobilised form - the active components need for such biotransformation processes.

The use of immobilised cells for the production of secreted proteins

The invention provides a method for the production of

BNSDOCID: <WO___9709437A1_I_>

secreted proteins by Gram-positive bacteria which are attached to an immobilised support. For example, cells which are displaying streptavidin on their surface and also secreting an enzyme or other protein of interest could be attached to a biotin coated solid phase. One advantage of this approach is that the recovery of the growth medium containing the secreted protein of interest does not require centrifugation to remove the bacterial cells.

Assembly at the bacterial surface of heterodimeric proteins with bifunctional aminoterminal domains

In this aspect of the invention, one partner of the heterodimer, eg a fusion polypeptide comprising a signal peptide, a single chain antibody, the hinge and/or constant domains of the heavy chain and the cell wall anchoring domain of the Staphylococcus aureus protein A, is expressed at the bacterial surface and the other partner is secreted and composed of a similar fusion polypeptide, eg a different single chain antibody (recognizing a different antigen) or an enzymatic activity, but is devoid of the cell wall anchor. The constituent partner polypeptides may be synthesized either in the same engineered cell or in differently engineered cells grown in co-culture.

In this example, the bacterial surface acts as an assembly site for exclusively heterodimeric molecules having bifunctional aminoterminal domains. The assembled proteins may then be specifically released and purified from the collected bacteria, eg by the action of lysostaphin. Alternatively and preferentially, release may be obtained by the action on whole bacteria of a

BNSDOCID: <WO___9709437A1_I_>

specific protease, eg factor Xa, enterokinase, collagenase, Igase (from *Neisseria gonorrhoeae*, thrombine, TEV (Tobacco Etch Virus) protease, the recognition site of which was incorporated into the fusion polypeptide just upstream of the cell wall anchor. The desired proteins may be purified by conventional separation techniques.

Although methods for producing bifunctional heterodimers are known, there are at present no generally applicable methods available for separating them from the unavoidably co-produced homodimeric (hence monofunctional) molecules.

Additionally, this method could be used for the assembly of homo-multimeric proteins on the surface of a bacteria.

Example 1

Construction of expression plasmids

Stage 1: Cloning of S. aureus protein A anchoring domain

A DNA fragment encoding the Staphylococcus protein A (SPA) anchor of *S. aureus* strain Cowan I NCTC 8530 was obtained by the polymerase chain reaction (PCR) using primers based on the published sequence (Saiki et al. 1985). The PCR was performed with the thermostable Vent DNA polymerase (New England BioLabs) in a 100 µl of the buffer supplied by the manufacturer containing 2.5 mM each of dATP, dTTP, dCTP and dGTP, 1 µM of the sense (and antisense primers and 1 µg of genomic DNA isolated from S. aureus strain Cowan I NCTC 8530 by the method of Marmur et al., (1961). The sense and antisense primers

BNSDOCID: <WO__9709437A1_I_>

were designed to include restriction endonuclease sites for BamHI and XbaI at their 5' ends respectively to facilitate the subsequent cloning steps. After 20 cycles of denaturation (94°C for 45 seconds) primer annealing (68°C for 30 seconds) and DNA polymerisation (72°C for 45 seconds) a PCR amplified DNA product of the expected size (621 bp) was detected by agarose gel electrophoresis. The purified DNA fragment was then cloned and sequenced using standard procedures (Maniatis). The DNA sequence of the cloned DNA fragment encoding the C terminal region (nt 1043 to 2252) of S. aureus protein A was identical to that previously reported (Shuttleworth et al).

Stage 2: Cloning and manipulation of the streptavidin gene

Streptavidin was chosen as a reporter protein to demonstrate the feasibility of the present invention. Streptavidin is easily detected and is known to bind biotin for which antisera are commercially available. The streptavidin gene fragments were obtained from a series of plasmids in which the streptavidin gene had been mutated to incorporate different restriction endonuclease sites at the start and end of the coding sequence of the mature protein.

A gene fragment encoding the mature form of streptavidin was ligated into NaeI digested L. lactis expression plasmid pLET2N (Steidler et al., 1995) to generate in frame fusion of the usp45 signal secretion signal leader (present in pLET2N) and the streptavidin coding sequence (figure 1). The resulting plasmid, designated pL2SA, can be used to express and secrete streptavidin in L. lactis (see below). Plasmid pL2SAX containing the S. aureus

SNSDOCID: <WO___9709437A1_I_>

protein A C terminal anchoring domain (referred to as the X domain) fused to the 3' end of the streptavidin gene in pL2SA was then constructed by cloning the PCR amplified DNA fragment encoding the protein A anchor region (see stage 1 and Figure 1). A lactococcal expression strain harbouring pL2SAX expresses a streptavidin - protein A C-terminal domain fusion protein which is firmly attached to the cell wall (see below).

Expression of streptavidin and streptavidin protein A fusion proteins in L. lactis

Plasmids pL2SA and pL2SAX were isolated from E. coli strain MC1022 and used to transform the L. lactis T7 expression host strain MG1820 harbouring pILPol (strain UCP1000; Wells et al., 1993) by electroporation. Expression of the streptavidin and streptavidin - protein A fusion protein was induced in L. lactis by replacing lactose for glucose in the growth medium (Steidler et al., 1995). In order to detect expression of the streptavidin protein products in L. lactis, cellular fractionation and immunoblotting were performed with cells which had been induced for 3 hours. Ten ml of cells were first pelleted by centrifugation and the supernatant centrifuged a second time at 100 000 g for 1 hour to remove any insoluble material. Protein factions were then recovered from the high speed supernatant by phenolic extraction as previously described (Steidler et al., 1995). The cell pellets obtained in the first step were washed three times in Tris buffered saline (TBS: 0.15 M NaCl, 0.02 M Tris-HCl pH 7.5) and resuspended in 250 µl of 10% sucrose, 20mM Tris-HCl (pH 7.5) containing 0.6 mg of lysostaphin (Sigma, St. Louis, USA) and incubated for 1 hour at 37°C to enzymatically digest the

BNSDOCID: <WO___9709437A1_I_>

74

cell wall.

After treatment with lysostaphin, the cells (referred to as the residue) were separated from the enzymatically released cell wall material by centrifugation and lysed by boiling in Laemmli cracking buffer. The supernatants referred to as the "lysostaphin released protein fractions" were similarly treated with cracking buffer. The proteins recovered from the growth medium, lysostaphin released fractions and cell residues were analysed by immunoblotting with antisera to streptavidin using standard procedures. Lysostaphin has been shown to specifically release proteins which are linked to the pentaglycine peptide present in the peptidoglycan of the cell wall (Schneewind et al, 1993).

The results of cell fractionation and immunoblotting of proteins recovered from L. lactis expression strain UCP1000 harbouring pL2SA showed that a protein of the expected size for streptavidin was produced and secreted into the growth medium by L. lactis. Streptavidin was not detected in the total cell protein residues of cells treated with lysostaphin and only trace amounts of protein were detected in the lysostaphin released cell wall material (see figure 2). In this strain, the relatively small proportion of the total amount of secreted streptavidin released by treatment of the cells with lysostaphin may be a consequence of streptavidin which has not diffused through the cell wall after transport across the cell membrane.

In contrast to these findings, the streptavidin - protein A fusion protein produced by the strain carrying pL2SAX was not secreted into the growth medium. This fusion protein was however, present in substantial amounts in

BNSDOCID: <WO__9709437A1_I_>

the material released from the cell wall by treatment with lysostaphin indicating that it had become associated with the cell wall (Figure 2). The fusion protein was also detected in the pellet (residue) of lysostaphin treated cells suggesting that a proportion of the released protein had not diffused out of the cell wall during the incubation with lysostaphin. When washed cells expressing the streptavidin protein A fusion protein were boiled in Laemmli cracking buffer the protein detected by immunoblotting had an apparent molecular weight which was higher than the protein detected in the supernatant of the fraction of cells treated with lysostaphin (Figure 2). This higher molecular weight polypeptide may represent an intermediate in the pathway which sorts and anchors the fusion protein to the cell wall. Both forms of the protein are found in the boiled cell pellet (residue) fraction of the lyostaphin treated cells, indicating that the more gentle enzymatic treatment of the cells with lyostaphin does not release the higher molecular weight form of the fusion protein into the supernatant.

Display of streptavidin on the surface of L. lactis

In order to show that the fusion of streptavidin to the C terminal domain of S. aureus protein A would result in the display of the expressed protein on the cell surface, strains of L. lactis expressing streptavidin or the streptavidin protein A fusion product were washed three times in TBS containing 1% bovine serum albumin (BSA) and then incubated with biotinylated horseradish peroxidase (1 μg) for 1 hour at room temperature. Control samples of cells were also incubated with biotinylated horseradish peroxidase in the presence of soluble

BNSDOCID: <WO__9709437A1_I_>

streptavidin to show that the binding to cells could be blocked by competition with the free substrate. The cells were then washed three times with TBS containing 1% BSA and harvested onto a cellulose nitrate filter by means of a vacuum apparatus. The binding of biotin to the cell surface was detected by incubating the filter in a solution of TMB (horseradish peroxidase substrate) according to the manufacturer's recommendations. The results in Figure 3 show that the biotin horseradish peroxidase conjugate bound the surface of intact cells expressing the streptavidin protein A fusion protein but not to cells expressing streptavidin. The possibility that the biotin - horseradish peroxidase conjugate binds non-specifically to the cell surface of L. lactis expressing the streptavidin protein A fusion protein can be ruled out as binding was inhibited in the presence of soluble streptavidin.

Specific immobilisation of L. lactis cells expressing streptavidin on their cell surface

To provide a solid surface for the specific binding of the bacterial cells expressing streptavidin on their cell surface Petri dishes (20 mm, Maxisorp, Nunc, Denmark) were coated with 1 µg/ml of biotinylated alkaline phosphatase (Boehringer Mannheim, Germany) for 1 hour in 1 ml of TBS at room temperature. Non-specific binding sites were then blocked by incubating the plates with 2 ml of TBS containing 1% BSA for 2 h at room temperature. 10 ml of cells expressing either streptavidin or the streptavidin protein A fusion protein were washed three times in TBS and resuspended in 1 ml of TBS containing 1% BSA for 1 hour at room temperature. During this incubation step the cells were kept in suspension by

BNSDOCID <WO___9709437A1_I_>

shaking for 6 seconds every minute. Finally, the cell suspension was diluted to 10 ml with TBS and 1 ml of this suspension added to the pre-treated petri dishes (see above) for 1 hour at room temperature. The petri dishes were then washed three times with 1 ml of TBS for ten min on an orbital shaker before they were examined by light microscopy. The results (Figure 4 & Table 1) show that L. lactis expressing the streptavidin protein A fusion protein bind to the surface of the petri dish coated with biotin-alkaline phosphatase but not to petri dishes which were untreated with this conjugate. Cells expressing and secreting streptavidin did not bind to the surface of coated or uncoated petri dishes. The binding of cells expressing the streptavidin protein A fusion product was blocked by the addition of soluble streptavidin indicating that the binding was specific and mediated through the binding of biotin to the streptavidin present on the surface of the cells (Table 1).

Table 1: results of surface binding assays

	Surface treatments		
Strain of *L. lactis*	(i) 1% BSA	(i) 1 µg/ml B-AP; (ii) 1% BSA	(i) µg/ml B-AP; (ii) 30 µg/ml SA (iii) 1% BSA
MG1820 (pILPol,pL2SA)	ND	ND	ND
MG1820 (pILPol,pL2SAX)	ND	2×10^5 m m^{-1}	ND

Legend to Table 1. The expression strains of *L. lactis* either carried plasmid pL2SAX and produced the streptavidin protein A fusion product or plasmid pL2SA and produced

BNSDOCID: <WO___9709437A1_I_>

streptavidin, ND; none detected, B-AP; biotin-alkaline phosphatase conjugate, BSA; bovine serum albumin, SA; streptavidin.

Example 2

Display of tetanus toxin fragment C (TTFC) antigen on the surface of *L. lactis*

This additional example provides evidence that protein antigens can be firmly attached to the surface of *L. lactis* by constructing chimeric genes encoding a fusion protein which contains nucleic acid encoding (1) an N-terminal secretion signal (2) the protein antigen to be surface expressed and (3) the cell wall sorting and anchoring domain of *S. aureus* protein A.

Stage 1: Cloning and genetic manipulation of TTFC and the *S. aureus* protein A anchoring domain.

(1a) Construction of pTREX and pTREX1

Synthetic oligonucleotides encoding a putative RNA stabilising sequence, a translation initiation region and a multiple cloning site for target genes were annealed by boiling 20ug of each oligonucleotide in 200ul of 1 x TBE, 150mM NaCl and allowing to cool to room temperature. Annealed oligonucleotides were extended using Tfl DNA polymerase in 1 x Tfl buffer containing 250μM deoxynucleotide triphosphates and 1.5mM MgCl2 at 35°C, 45°C, 55°C and 65°C each for 1 minute followed by 10 minutes at 72°C. The sense and antisense oligonucleotides contained the recognition sites for NheI and BamHI at their 5′ ends respectively to facilitate further cloning.

BNSDOCID: <WO__9709437A1_I_>

The resulting ds DNA fragment was cut with NheI and BamHI, and cloned between the XbaI and BamHI sites in pUC19NT7, a derivative of pUC19 which contains the T7 expression cassette from pLET1 (Wells *et al.*, 1993c) cloned between the EcoRI and HindIII sites. The resulting construct was designated pUCLEX. The complete expression cassette in pUCLEX was then removed by cutting with HindIII and blunting followed by cutting with EcoRI before cloning into the EcoRI and SacI (blunted) sites of pIL253 to generate the vector pTREX. In order to construct the expression vector pTREX1 a PCR amplified DNA fragments containing the lactococcal promoter P1 was cloned between the EcoRI and BglII sites present in the expression cassette of vector pTREX (Figure 5). This promoter had been previously isolated using the promoter probe vector pSB292 and characterised by primer extension and DNA sequencing analysis (Waterfield *et al.*, 1995).

(1b) Construction of plasmid pT1L2TT

A DNA fragment encoding the tetanus toxin fragment C gene fused to the lactococcal signal secretion leader sequence of the usp45 gene was obtained by the polymerase chain reaction (PCR) using plasmid pLET2-TTFC as a template and the published sequences (Wells *et al.*, 1993b). The PCR was performed with the thermostable vent DNA polymerase (New England Biolabs) in a 100 µl of the reaction buffer supplied by the manufacturer containing 2.5 mM each of dATP, dTTP, dCTP, and dGTP , 1 µm of the sense and antisense primers (Figure X) and approximately 50 ng of plasmid pLET2-TTFC as template DNA. The antisense primer was designed to include a BamHI site at its 5' end to facilitate the subsequent cloning steps. After 30 cycles of denaturation (94°C for 45 seconds), primer annealing (50°C for 30 seconds) and DNA polymerisation (72°C for

BNSDOCID: <WO__9709437A1_I_>

83

120 seconds) a PCR amplified DNA product of the expected size (1478 bp of double stranded DNA) was detected by agarose gel electrophoresis. The purified DNA fragment was then digested with restriction endonuclease BamHI and cloned into lactococcal expression plasmid pTREX1 which had first been cut with SphI blunted and then cut with BamHI. The resulting plasmid designated pT1L2TT is shown in Figure 5.

(1c) Construction of pT1TT

A fragment of tetanus toxin fragment C (TTFC) was generated by PCR (using standard conditions; see above) which encoded XbaI and BamHI sites at the 5' and 3' ends respectively. A stop codon was introduced at the 3' end of the TTFC sequence before the BamHI site. The TTFC PCR fragment was first cloned between the XbaI and BamHI sites of pUCLEX and then removed by cutting with SphI-BamHI and recloned intoSphI and BamHI digested pTREX1. The resulting plasmid designated pT1TT is shown in Fig 5.

(1d) Construction of pTTA

A DNA fragment encoding the *Staphylococcus* protein A (SPA) anchor of *S.aureus* strain Cowan I NCTC 8530 was obtained by PCR using primers based on the published sequence (Figure X). The sense and antisense primers were designed to include restriction endonuclease sites for BamHI and BglII at their 5' ends respectively to facilitate the subsequent cloning steps. The PCR was performed with the thermostable vent DNA polymerase (New England Biolabs) in a 100 µl of the reaction buffer supplied by the manufacturer containing 2.5 mM each of dATP, dTTP, dCTP, and dGTP , 1 µm of the sense and antisense primers (Figure 6) and approximately 50 ng of

GNSDOCID: <WO___9709437A1_I_>

plasmid pL2SAX (further described in this patent) as template DNA. After 30 cycles of denaturation (94°C for 45 seconds), primer annealing (55°C for 30 seconds) and DNA polymerisation (72°C for 35 seconds) a PCR amplified DNA product of the expected size was detected by agarose gel electrophoresis. The purified DNA fragment was then digested with restriction endonucleases BamHI and BglII and cloned into plasmid pT1L2TT which had first been cut with BamHI and dephosphorylated using calf intestinal phosphatase (Boehringer Mannheim) according to the methods recommended by the manufacturer. The resulting plasmid designated pTTA contains the *S.aureus* protein A C terminal domain (referred to as the X domain) fused to the 3' end of the TTFC gene (Figure 5).

Stage 2: Expression and surface display of the TTFC-protein A fusion protein in *L. lactis*.

In order to show that the fusion of TTFC to the C terminal domain of *S.aureus* protein A would result in the display of the expressed protein on the cell surface, strains of *L. lactis* expressing TTFC intracellularly (i.e. carrying plasmid pT1TT), secreting TTFC (i.e. carrying plasmid pT1L2TT) or theTTFC protein A fusion product (i.e. carrying plasmid pTTA) were harvested by centifugation, washed three times in TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 1% BSA and then incubated with 1 μg of rabbit-anti-TTFC for 30 min at room temperature. The cells were then washed three times with 1 ml of TBS and resuspended in 1 ml of TBS. The cells in 100 μl of this cell suspension were harvested onto a cellulose nitrate membrane (0.22 μm) by means of a vacuum apparatus. The membrane was then incubated for 1 hour at room temperature in 5 ml of PBS-2.5% skim milk powder

BNSDOCID: <WO__9709437A1_I_>

85

containing 5 μg of anti rabbit IgG conjugated to horse raddish peroxidase (Boehringer Manheim). The binding of the anti rabbit peroxidase to rabbit anti TTFC antibodies bound on the surface of intact cells of *L.lactis* carrying pTTA but not pT1L2TT and pT1TT was detected by incubating the filter in a solution of of TMB (horseradish peroxidase substrate) according to the manufacturer's recommendations. The results shown in Figure 7 indicate that TTFC is only detected on the surface of intact cells expressing the TTFC protein A fusion protein.

BNSDOCID: <WO___9709437A1_!_>

References:

Charbit et al, J. Bacter., 173(1): 262-275, 1991

Charbit et al, J. Immunol. 139: 1658-1664, 1987

Chiswell and McCafferty, Trends Biotechnol. 10: 80-84, 1992

Georgio, G et al, TIBTECH 11: 6-10, 1993

Klauser et al, EMBO J. 9: 1991-1999, 1990

Kornacker and Pugsley Mol. Micro. 7: 1101-1109, 1990b

Kornacker and Pugsley Annu Rev. Genet. 24: 59-72, 1990a

Mamur, J. Mol. Biol., 3:208-218, 1961

Maniatis et al, Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press

Navarre and Schneewind Mol. Micro. 14(1): 115-121, 1994

Pohlner et al, Nature 352: 458-462, 1987

Saki et al, Science 230: 1350-1354, 1985

Schneewind et al, EMBO J 12(12): 4803-4811, 1993

Shuttleworth et al, Gene 56:283-295, 1987

Simon, D. and Chopin, A., *Biochimie* 70: 559-567 (1988)

Steidler et al, Mol. Gen. Genet. 236: 187-192, 1993a

BNSDOCID: <WO__9709437A1_I_>

87

Steidler et al, J. Bact. 175(23): 7639-7643, 1993b

Steidler et al, Appl. Environ. Microbiol. 61: 1627-1629, 1995

Thiry et al, Appl. Environ. Microbiol. 55:984-993, 1989

Waterfield, N. R., Le Page, R. W. F., Wilson, P. W. and Wells, J. M., *Gene*. **165**: 9-15 (1995).

Wells et al, Mol. Microbiol. 8: 1155-1162, 1993

Wells, J. M., Wilson, P. W., Norton, P. M., Gasson, M. J. and Le Page, R. W. F., *Molec. Microbiol.* **8**: 1155-1162 (1993a)

Wells, J. M., Wilson, P. W., Norton, P. M. and Le Page, R. W. F., *Environ. Microbiol.* **59**: 3954-3959 (1993b)

Wells, J. M., Wilson, P. W. and Le Page, R. W. F., *J. Appl. Bact.* **74**: 629-636 (1993c)

BNSDOCID: <WO___9709437A1_I_>

CLAIMS:

1. A recombinant vector including nucleic acid encoding a fusion protein comprising a secretion signal sequence, a desired protein or polypeptide and a cell wall attachment domain, so that when the vector is transformed in a Gram-positive bacterial host and the fusion protein is expressed, the desired protein or polypeptide is attached and displayed on the outer surface of the cell wall of the host.

2. A recombinant vector as claimed in claim 1 wherein the Gram-positive host organism is *Lactococcus spec., Bacillus subtilis, Streptococcus gordonii, Staphylococcus xylosus, Lactobacillus spec.,* psychrophilic and psychotropic species of the genera *Bacillus spec., Clostridium spec. or Corynebacterium spec..*

3. A recombinant vector as claimed in claim 2 wherein the Gram-positive host organism is a *Lactococcus* host, preferably *Lactococcus lactis*.

4. A recombinant vector as claimed in any one of claims 1 to 3 wherein the secretion signal sequence is capable of targeting the protein or polypeptide for secretion via the sec dependent pathway of secretion and is derived from a signal sequence of a naturally secreted protein.

5. A recombinant vector as claimed in claim 4 wherein the secretion signal peptide is derived from the signal sequence of the protein usp45.

6. A recombinant vector as claimed in any one of claims

BNSDOCID: <WO___9709437A1_I_>

89

1 to 5 wherein the cell wall attachment domain comprises at least the C-terminal anchoring domain derived from the *Staphylococcus aureus* protein A.

7. A recombinant vector as claimed in any one of claims 1 to 6 wherein the nucleic acid encoding the fusion protein is operably linked to control sequences which direct its expression.

8. A recombinant vector as claimed in claim 7 wherein the nucleic acid further comprises a T7 or T7-like polymerase gene under the control of an inducible promoter provided upstream of the nucleic acid encoding the fusion protein.

9. A Gram-positive host organism transformed with a recombinant vector as defined in any one of claims 1 to 8.

10. A Gram-positive host organism as defined in claim 9 wherein the desired protein or polypeptide has been expressed, and is covalently attached to the cell wall and is displayed on the outer face of the surface of the host organism.

11. A method of engineering a Gram-positive host organism to display a desired protein or polypeptide on its surface comprising transforming the host organism with recombinant nucleic acid encoding a fusion protein comprising a secretion signal sequence, a desired protein or polypeptide and a cell wall attachment domain, so that the host is capable of expressing the fusion protein, whereby the cell wall attachment domain enables the host to attach and display the desired protein or polypeptide

BNSDOCID: <WO___9709437A1_I_>

on the cell wall surface.

12. A method as claimed in claim 11 modified by any one or more of the features of claims 2 to 8.

13. A Gram-positive host organism obtainable by a method as claimed in claim 11 or claim 12.

14. A gram-positive host organism as defined in claim 9 or claim 10 wherein the desired protein or polypeptide includes at least one epitope to which it is desired to raise an immunological response.

15. A Gram-positive host organism as claimed in claim 14 wherein the desired protein or polypeptide is an immunogen from a pathogen, an antigen or an adhesin.

16. A vaccine comprising Gram-positive host organisms as defined in claim 14 or claim 15 in admixture with a suitable carrier.

17. The use of Gram-positive host organisms as defined in claim 14 or claim 15 in the preparation of a vaccine.

18. A Gram-positive host organism as defined in claim 9 or claim 10 wherein the desired protein or polypeptide is one for which it is desired to elicit tolerance.

19. The use of a Gram-positive host organism as defined in claim 18 in eliciting tolerance for the protein or polypeptide.

20. A gram-positive host organism as defined in claim 9 or claim 10 wherein the desired protein or polypeptide is

BNSDOCID: <WO__9709437A1_I_>

a biologically active molecule.

21. A Gram-positive host organism as claimed in claim 20 wherein the biologically active molecule is a drug, a hormone, an enzyme, DNA/RNA binding protein or a substance capable of performing secondary modifications on other coexpressed and secreted proteins.

22. A Gram-positive host organism as defined in claim 9 or claim 10 wherein the desired protein or polypeptide is a targeting group capable of directing binding of the host organism to a target site.

23. A Gram-positive host organism as claimed in claim 20 wherein the targeting group is an antibody, or fragment thereof, which is capable of binding to a target antigen or hapten or is an adhesin.

24. A pharmaceutical composition comprising a Gram-positive host organism as defined in any one of claims 9, 10, or 18 to 23.

25. A Gram-positive host organism as defined in any one of claims 9, 10, 13-15, or 18 to 23 for use in medicine.

26. A gram-positive host organism as defined in claim 9 or claim 10 wherein the desired protein or polypeptide is a specific binding partner of a ligand.

27. A Gram-positive host organism as claimed in claim 26 wherein the ligand is bound to a solid surface such that the host organism will be immobilised thereon.

28. A Gram-positive host organism as claimed in claim 27

BNSDOCID: <WO___9709437A1_I_>

wherein the desired protein or polypeptide is streptavidin and the ligand is Biotin or a Biotin conjugate, eg a Biotinylated enzyme such as Biotinylated horseradish peroxidase.

29. A method for immobilising a Gram-positive host organism as defined in any one of claims 26 to 28 which comprises bringing the host organism into contact with a surface on which the ligand has been immobilised.

30. The use of a Gram-positive host organism as defined in any one of claims 26 to 28 in the purification or separation of a ligand.

31. The use of a Gram-positive host organism as defined in claim 9 or claim 10 in the detection of complexes having a specific ligand, wherein the nucleic acid further comprises a gene which when expressed gives rise to a reporter activity.

32. The use as claimed in claim 31 wherein the reporter activity is a fluorescent protein or luciferase and the desired protein is streptavidin.

33. A Gram-positive host organism as defined in claim 9 or claim 10 wherein the desired protein or polypeptide is an enzyme.

34. A composition comprising a Gram-positive host organism as defined in claim 33 wherein the enzyme has been expressed and is displayed on the cell surface of the host organism.

35. The use of a composition as defined in claim 34 in

BNSDOCID: <WO__9709437A1_I_>

a reaction catalysed by the reaction.

36. A method of screening a library of substances which comprises the step of transforming Gram-positive host organisms with nucleic acid encoding a fusion of the substances and a cell wall anchoring domain and expressing the fusion on the cell surface.

37. A method as claimed in claim 36 wherein the substances are antibodies or antigens or fragments thereof.

BNSDOCID: <WO___9709437A1_I_>

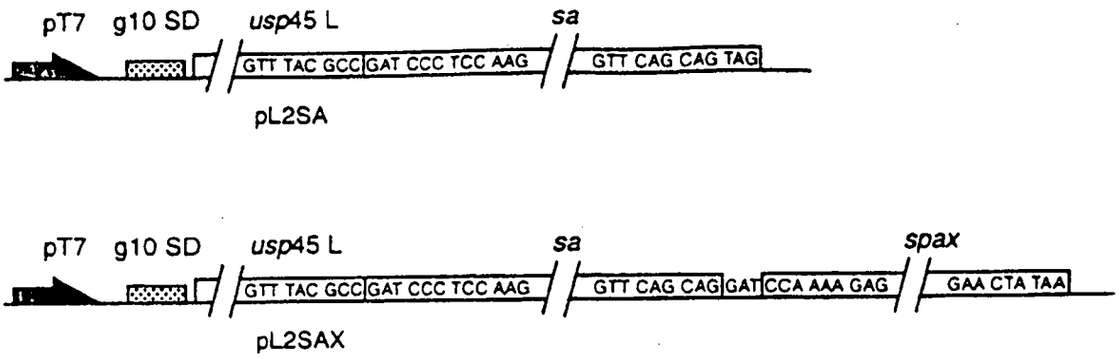


FIG. 1

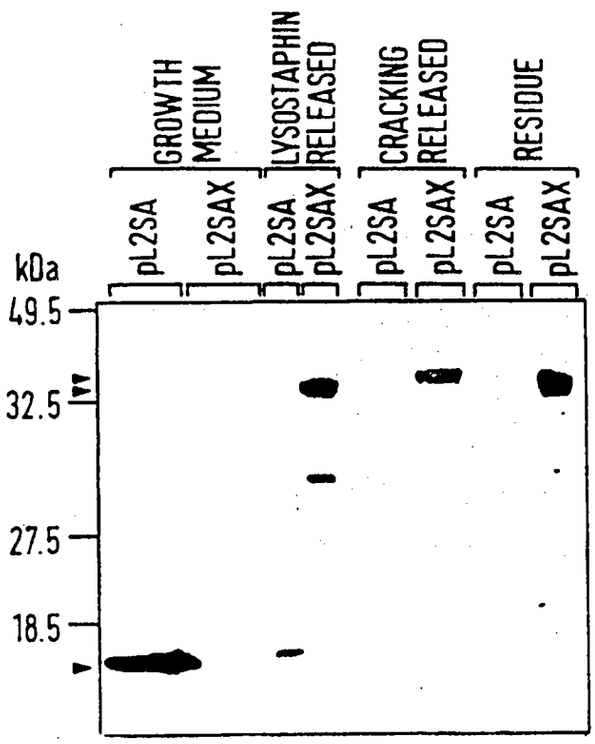


FIG. 2

BNSDOCID: <WO__9709437A1_I_>

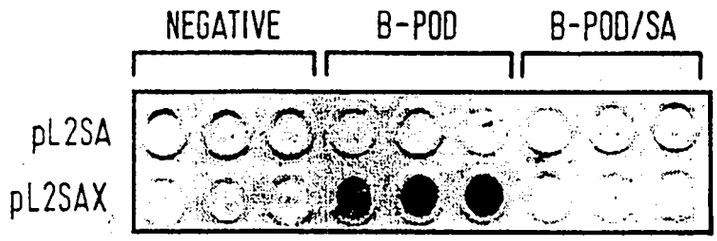


FIG. 3

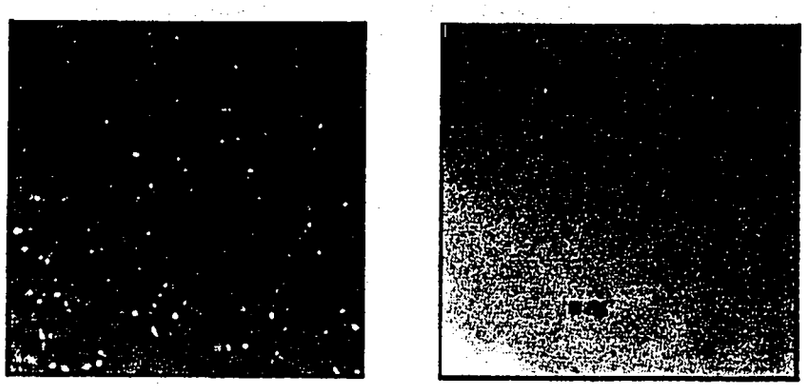


FIG. 4

BNSDOCID: <WO___9709437A1_I_>

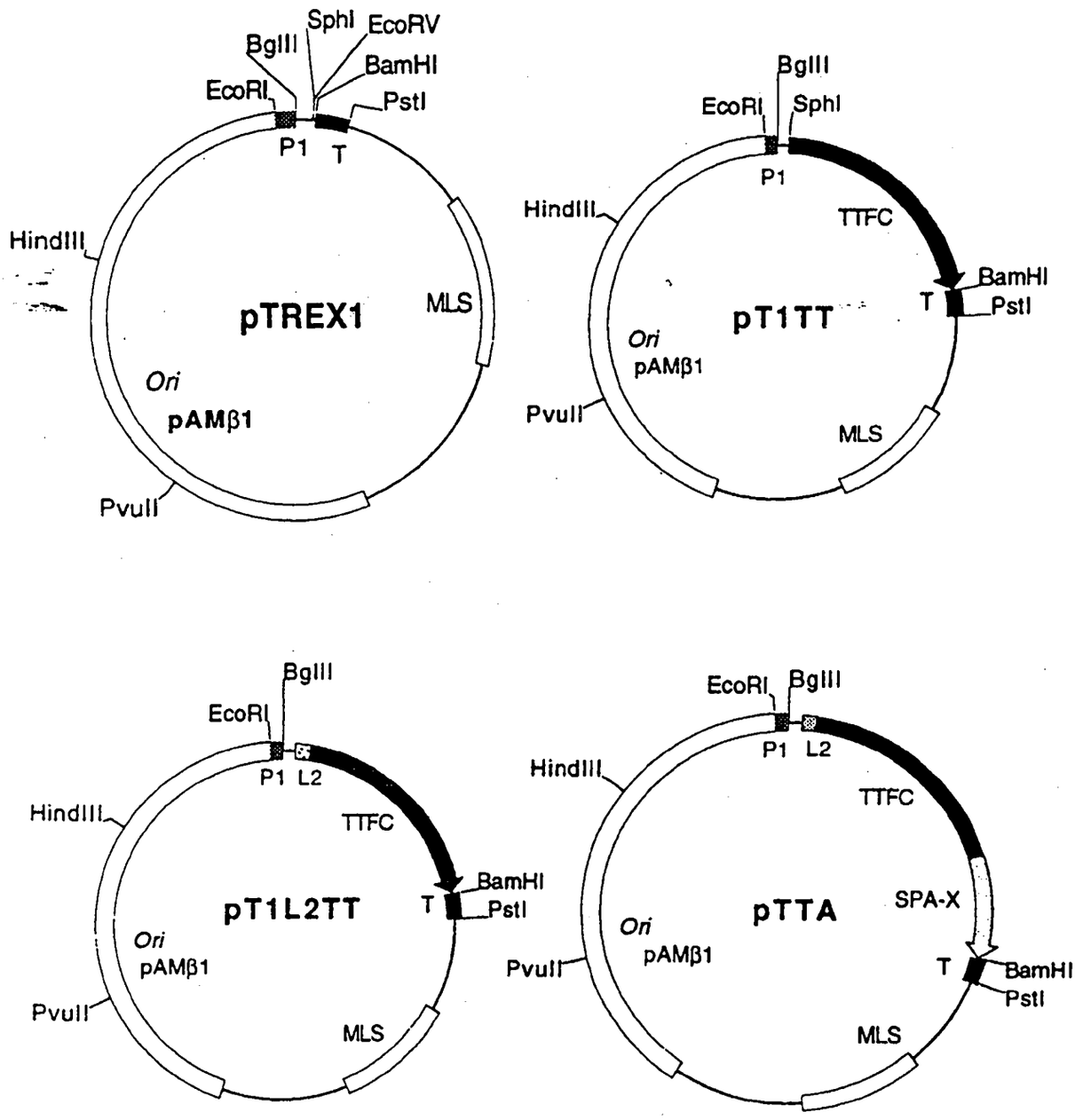


FIG. 5A

BNSDOCID: <WO___9709437A1_I_>

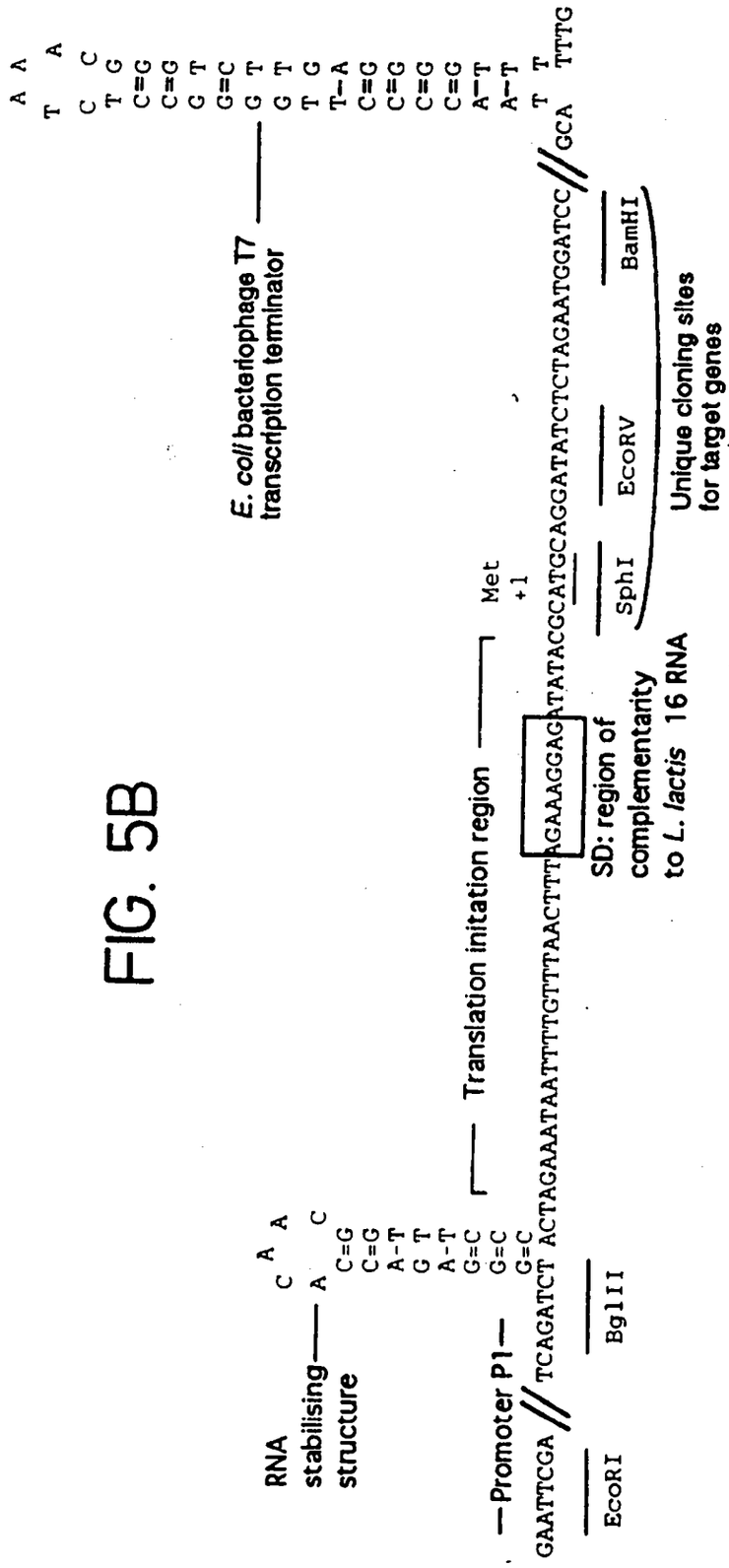
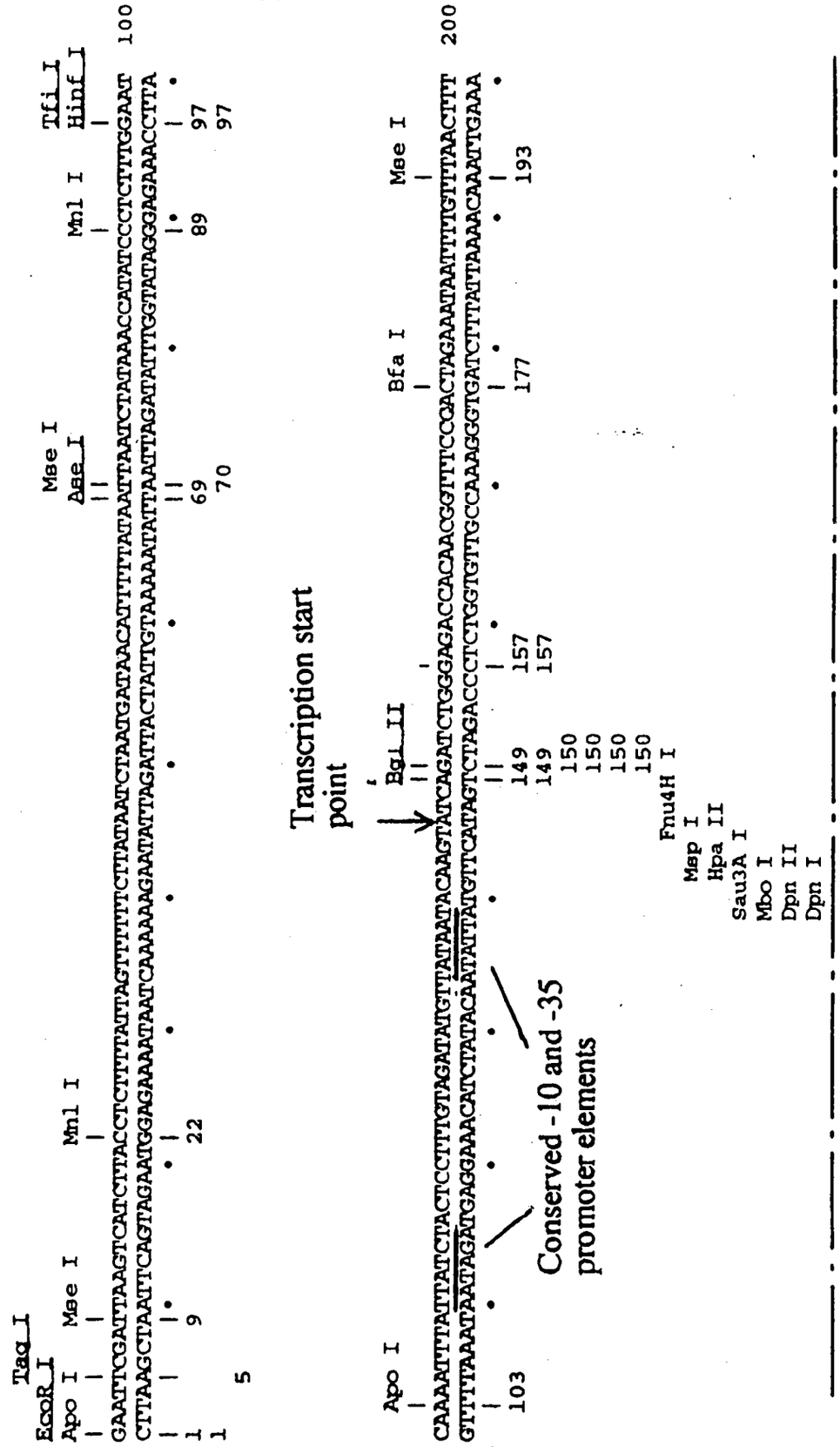


FIG. 5B

BNSDOCID: <WO___9709437A1_I_>

FIG. 5C(I)

FEATURES OF THE pTREX1 EXPRESSION CASSETTE



BNSDOCID: <WO__9709437A1_I_>

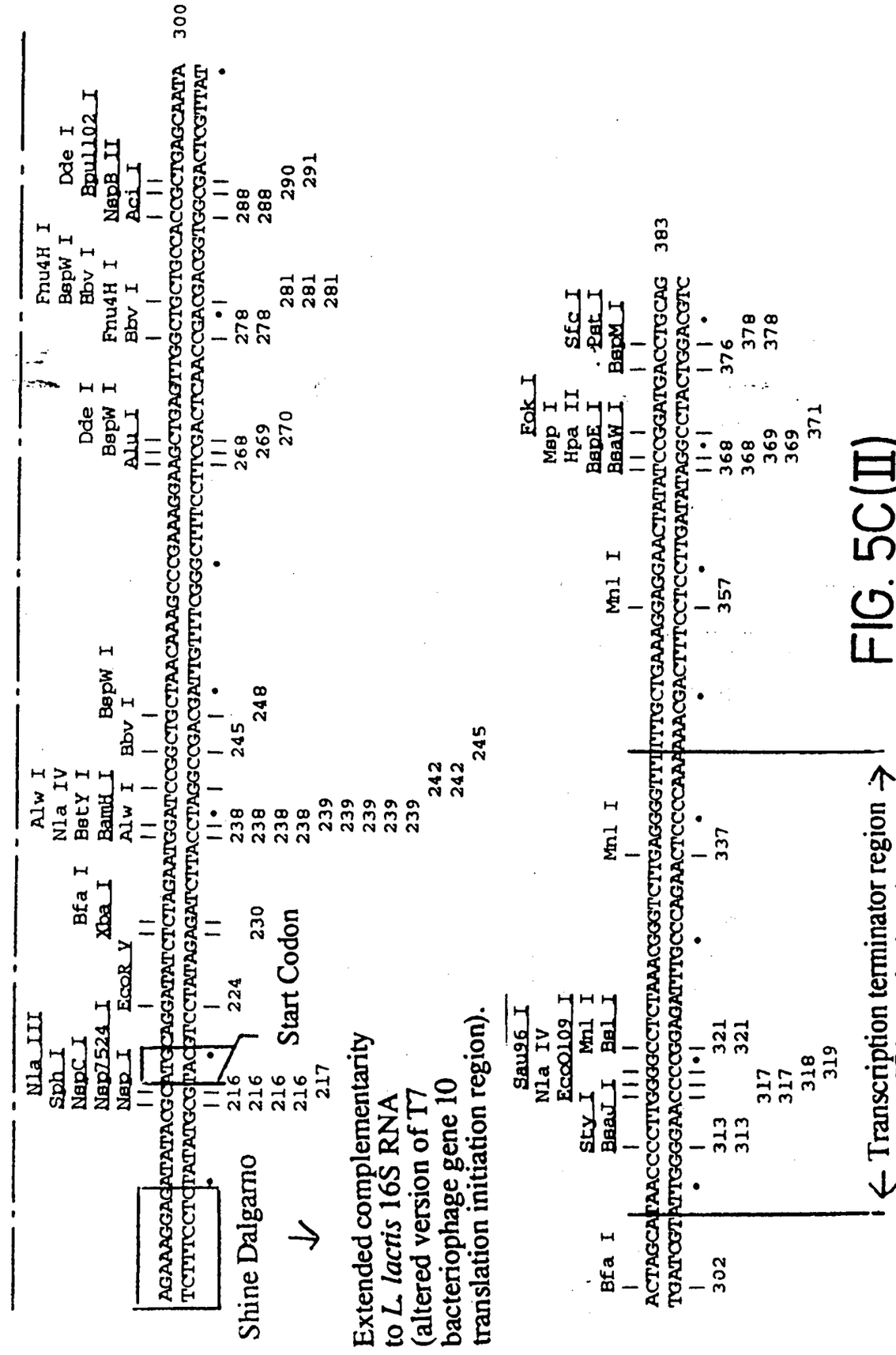


FIG. 5C(II)

BNSDOCID: <WO___9709437A1_I_>

List of primers used for PCR

Sense primer for PCR of *usp45*-TTFC gene fusion
5' CATGAAAAAAAAGATTATCTCAGCTATTT 3'

Antisense primer for *usp45*-TTFC gene fusion including a BamHI site for in-frame fusion to the *S. aureus* protein A anchoring domain
5' CGGGATCCTGATCATTTGTCCATCCTTCATCTG 3'

Sense primer for *S. aureus* protein A anchoring domain
5' CGGGATCCAAAAGAGGAAGACAACAACAAG 3'

Antisense primer for *S. aureus* protein A anchoring domain
5' GAAGATCTACTAGTTATAGTTCGCGACGACG 3'

FIG. 6

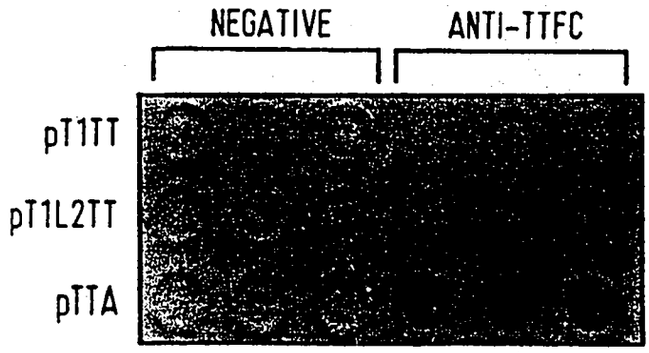


FIG. 7

BNSDOCID: <WO___9709437A1_I_>

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 96/02195

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/74 C12N15/62 C12N15/31 C07K14/36 C07K14/31
C07K14/33 C12N1/21 A61K39/08 A61K39/09 C12N11/16
C12Q1/02 G01N33/68 //(C12N1/21,C12R1:225)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GENE, vol. 128, 1993, AMSTERDAM NL, pages 89-94, XP000561818 NGUYEN TN ET AL: "Cell-surface diplay of heterologous epitopes on Staphylococcus xylosus as a potential delivery system for oral vaccination" see page 91; figure 1 ---	1,2,6,7, 9-27
X	JOURNAL OF BACTERIOLOGY, vol. 177, no. 6, March 1995, pages 1470-1476, XP000612670 SAMUELSON P ET AL.: "Cell surface diplay of recombinant proteins on Staphylococcus carnosus" see abstract; figures 1,3	1,2,6,7, 9-27
Y	--- -/--	3-5,8, 28-37

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 December 1996	07. 01. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Espen, J

Form PCT/ISA/210 (second sheet) (July 1992)

BNSDOCID: <WO___9709437A1_I_>

INTERNATIONAL SEARCH REPORT

Int'l Application No PCT/GB 96/02195

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VACCINE, 13 (8) P775-9, June 1995, ENGLAND, XP000612017 OGGIONI MR ET AL: "Immunization of mice by oral colonization with live recombinant commensal streptococci." see the whole document ---	1,2,6,7, 9-27
Y	APPL ENVIRON MICROBIOL, APR 1995, 61 (4) P1627-9, UNITED STATES, XP002021060 STEIDLER L ET AL: "Secretion of biologically active murine interleukin-2 by Lactococcus lactis subsp. lactis." cited in the application see the whole document ---	3-5,8, 28-37
X	WO,A,93 18163 (UNIV ROCKEFELLER) 16 September 1993 see figures 1-5; table 1 -----	1,2,6,7, 9-27

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

BNSDOCID: <WO__9709437A1_I_>

INTERNATIONAL SEARCH REPORT

Inte 'onal Application No
PC[/GB 96/02195

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9318163	16-09-93	CA-A-	2131995	16-09-93
		EP-A-	0646176	05-04-95
		HU-A-	70306	28-09-95

BNSDOCID: <WO___9709437A1_I_>

D1 119

ELSEVIER

Vaccine 17 (1999) 2117–2128

Vaccine

Instruments for oral disease-intervention strategies: recombinant *Lactobacillus casei* expressing tetanus toxin fragment C for vaccination or myelin proteins for oral tolerance induction in multiple sclerosis

C.B.M. Maassen[a,b], J.D. Laman[a,b,*], M.J. Heijne den Bak-Glashouwer[a], F.J. Tielen[a], J.C.P.A. van Holten-Neelen[a], L. Hoogteijling[a], C. Antonissen[c], R.J. Leer[c], P.H. Pouwels[c], W.J.A. Boersma[d], D.M. Shaw[a]

[a] *Division of Immunological and Infectious Diseases, TNO-Prevention and Health (TNO-PG), P.O. Box 2215, 2301 CE Leiden, Netherlands*
[b] *Department of Immunology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, Netherlands*
[c] *Department of Molecular Genetics and Gene Technology, TNO-Nutrition and Food Research Institute, P.O. Box 360, 3700 AJ Zeist, Netherlands*
[d] *Institute for Animal Science and Health (ID-DLO), Department of Immunology, P.O. Box 65, 8200 AB Lelystad, Netherlands*

Received 11 November 1998; received in revised form 15 December 1998; accepted 29 December 1998

Abstract

Lactobacillus strains possess properties that make them attractive candidates as vehicles for oral administration of therapeutics. In this report we describe the construction and analysis of recombinant *Lactobacillus casei* applicable in oral vaccination against an infectious disease (tetanus) and in oral tolerance induction for intervention in an autoimmune disease, multiple sclerosis.

Recombinant *L. casei* which express surface-anchored tetanus toxin fragment C (TTFC) were generated. Quantitative analysis by flow cytometry demonstrated a high level of cell wall-bound expression of TTFC and immunogenicity was demonstrated by parenteral immunization with whole cell extracts of the recombinants.

A series of expression vectors was constructed to secrete human myelin basic protein (hMBP) or hMBP as a fusion protein with β-glucuronidase from *Escherichia coli*. These heterologous products produced by *L. casei* were detected in the growth medium and parenteral immunization with this medium evoked antibodies against hMBP, confirming that secretion indeed had occurred.

Based on the different localization of the heterologous proteins, lactobacilli expressing surface-anchored TTFC are ideally suited for the induction of antibody responses, whereas lactobacilli that secrete myelin proteins can be used for the induction of peripheral T-cell tolerance. In conclusion, the specific technology described here allows the construction of a wide array of safe live recombinant lactobacilli which may prove to be useful in oral intervention strategies for the prevention of infectious diseases or treatment of autoimmune diseases. © 1999 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Experimental autoimmune/allergic encephalomyelitis (EAE); Mucosal vaccines; Heterologous gene expression

1. Introduction

Among the acquired disorders of the central nervous system (CNS) are infectious and autoimmune diseases. For the prevention of infectious diseases, oral vaccines possess a variety of advantages over parenterally administered vaccines [1]. Similarly, as an intervention in autoimmune diseases, autoantigens can be administered orally to restore peripheral T-cell tolerance. Active vaccination as opposed to the induction of T-cell tolerance following oral administration of antigens

* Corresponding author. Tel.: +31-715-181542; fax: +31-715-181901.
E-mail address: jd.laman@pg.tno.nl. (J.D. Laman)

0264-410X/99/$20.00 © 1999 Published by Elsevier Science Ltd. All rights reserved.
PII: S0264-410X(99)00010-9

BNSDOCID: <XP__4164996A__1_>

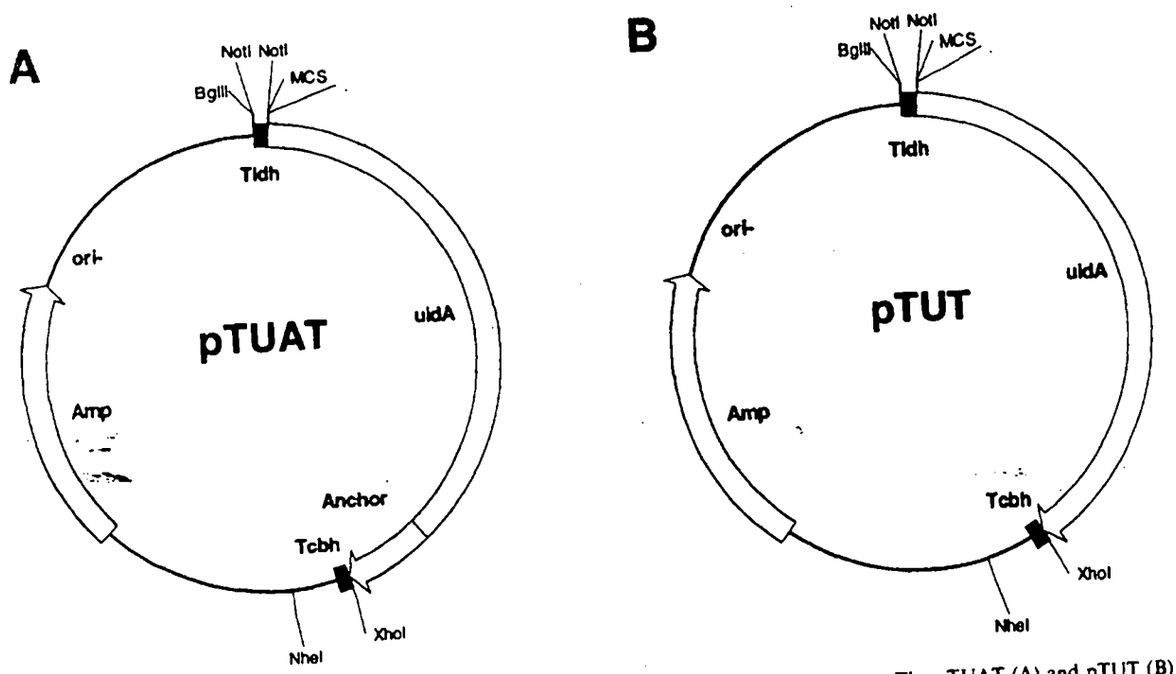


Fig. 1. pTUT/pTUAT vectors to simplify cloning DNA fragments into *Lactobacillus* expression vectors. The pTUAT (A) and pTUT (B) vector series are based on the pGEM-3 vector (Promega, Madison, WI). In brief, the multiple cloning site (MCS) of pGEM-3 was replaced by sequences encompassing a fragment flanked by *NotI* sites containing the *ldh* gene transcription terminator sequences, the *uidA* gene encoding β-glucuronidase and the transcription terminator sequence of the *L. plantarum cbh* gene. In case of pTUAT the β-glucuronidase open reading frame (orf) sequence was fused in frame with that of a sequence encoding the sequences responsible for the anchoring of *prtP* of *L. casei ATCC 393*. The MCS, which is direct in front of the *uidA* gene sequence, contains several restriction sites including *BamHI* and *NcoI*.

is considered to be crucially dependent on the particulate or soluble nature of the antigen delivered to the intestinal tract [2,3]. Therefore, in the current study, we have developed various recombinant *Lactobacillus* strains which differ in the form of expression of the antigen, facilitating either surface-anchored or secreted heterologous antigen expression.

It has long been recognized that feeding of soluble proteins can result in a state of immunological non-responsiveness termed oral tolerance [4,5]. This natural feature of the gut mucosa can be exploited to generate tolerance towards autoantigens. We have previously shown that oral tolerance to β-galactosidase can be induced with *L. plantarum* transformed to express β-galactosidase [6]. In this context, it has been reported that oral administration of MBP can result in inhibition of experimental autoimmune encephalomyelitis (EAE) in Lewis rats, a model for multiple sclerosis (MS) [7,8].

The mucosal surfaces of the intestinal tract are designed for the uptake of nutrients. Immune responses towards dietary antigens are unwanted and therefore suppressed and as a result it is very difficult to evoke systemic immune responses following delivery of antigens to the mucosal surfaces. Enhanced immunogenicity following oral administration is achieved by presenting antigen to the immune system in association with inert particles, bacteria [9] or live viral vectors (reviewed for vaccinia by Moss) [10]. Due to the many advantages of oral vaccination, the 'Children's Vaccine Initiative' (CVI) of the World Health Organization (WHO) supports the development of a new generation of vaccines that can be administered orally.

Lactobacillus species are members of the diverse group of Gram-positive lactic acid bacteria that are generally recognized as safe (GRAS). These bacteria are potentially food grade, a status corroborating their particular suitability for oral therapeutical applications. Presentation of antigens on the surface of lactobacilli is attractive for vaccine design, especially because the peptidoglycan layer of some strains appears to exhibit natural immuno-adjuvanticity [11–14]. The chemical coupling of the hapten trinitrophenyl (TNP) to the surface of *Lactobacillus* has resulted in the induction of significant TNP-specific antibody responses following oral immunization [15]. In contrast to immunogenic applications of the strains expressing the antigen at the cell surface, recombinant lactobacilli that secrete (auto)antigens locally in the gastrointestinal tract may also be used for the induction of peripheral T-cell tolerance following oral administration.

Here we describe the construction of recombinant *L. casei* expressing tetanus toxin fragment C (TTFC) on the cell surface. Tetanus is an infectious disease affect-

toxin heavy chain which is capable of inducing protective antibody responses, and hence TTFC is a rational choice for inclusion in a subunit [16,17]. In this study we examine the expression of TTFC by recombinant *L. casei* using both immunochemistry and flow cytometry analysis. The immunogenicity of the transformed cells was evaluated following parenteral immunization.

For oral tolerance induction, *L. casei* recombinants expressing myelin antigens were constructed. Multiple sclerosis is a T cell mediated autoimmune disease of the CNS that affects the myelin sheath, which insulates the nerve axons. EAE (experimental autoimmune encephalomyelitis), a model for MS, can be induced in several animal species by immunization with myelin antigens such as MBP. Two vectors were created directing secretion of the encephalitogenic/tolerogenic compound hMBP or hMBP fused to β-glucuronidase. In this study we report the analysis of the MBP recombinants and the immunogenicity of the hMBP secreted by transformed *L. casei* following immunization of mice with concentrated growth medium.

Consequently, based on this generic technology a wide array of recombinant lactobacilli can be designed for application in oral intervention strategies for the prevention and treatment of infectious as well as autoimmune diseases.

2. Materials and methods

2.1. Bacterial strains and vector construction

The *E. coli* strain DH5α [18] was used as host strain for the construction of shuttle vectors. *L. casei* (ATCC 393) was cultured in MRS broth (Difco Laboratories, Detroit, MI). General molecular cloning, PCR techniques and transformation of *Lactobacillus casei* were carried out essentially as described elsewhere [19,20].

A series of TTFC expression vectors was constructed, based upon the constitutive *ldh* (L-(+)-*lactate dehydrogenase* gene) promoter of *L.casei* and regulatable *amy* (α-*amylase* gene) promoter of *L. amylovorus*, as described previously [14]. The TTFC gene, kindly provided by Dr. A. Mercenier (Transgene, Strasbourg, France), containing a *Bam*HI site at its 5′ end was extended by PCR at the 3′ end using designed primers in order to obtain *Xho*I and *Nco*I sites. A *Bam*HI–*Nco*I DNA fragment was cloned into the multiple cloning site (MCS) of pTUAT (Fig. 1). The pTUT/pTUAT vector series were developed for convenient analysis of DNA sequences and to simplify construction of the desired DNA fragment into the pLP400/500 series of *Lactobacillus/E.coli* shuttle vectors [14]. Subsequently, the *Bam*HI/*Nhe*I fragment containing TTFC-*uidA* was exchanged with the *Bam*HI/*Nhe*I fragment of the expression–secretion vectors of the pLP400/500 series. In the pLP401-TTFC/U expression vector TTFC is fused to the marker gene *uidA*, which encodes β-glucuronidase. This expression vector is under the transcriptional control of the promoter and secretion signal sequence of the regulatable *amylase* gene of *L. amylovorus* (*amy*). The fusion TTFC-*uidA* open reading frame is in frame with a sequence coding for the cell wall anchor sequence of the *prtP* gene of *L. casei* in order to expose the synthesized product at the cell surface of the bacterium. The pLP501 expression vector resembles the pLP401 expression vector, however expression is under the transcriptional control of the constitutive *ldh* promoter and translocation is facilitated by fusion of the orf to the secretion signal sequences of the *prtP* gene of *L. casei*. By digestion with *Xho*I the *uidA* gene sequence was removed resulting in expression vectors pLP401-TTFC and pLP501-TTFC (Fig. 2).

A series of expression vectors was constructed to secrete human MBP or hMBP fused to *uidA* under transcriptional control of the *amy* promoter, in a similar fashion as used for the TTFC vectors. The 513 bp DNA coding for human MBP (ATCC clone M13577) was extended at its 3′ end with a *Bsp*HI site and at the 5′ end with a *Bgl*II site using PCR. The *Bgl*II–*Bsp*HI MBP DNA fragment was cloned in pTUT (Fig. 1), following digestion of pTUT with *Bam*HI and *Nco*I. Subsequently, the *Bgl*II/*Nhe*I fragment containing the insert was exchanged with the *Bam*HI/*Nhe*I fragment of pLP402 creating pLP402-hMBP/U (Fig. 2D). Digestion with *Xho*I removed the *uidA* gene, resulting in the vector containing only hMBP (Fig. 2C). The pLP400/500 vectors are stable in *E. coli* due to the presence of the *ldh* terminator (T_{ldh}) [14]. After removal of the T_{ldh} terminator by *Not*I digestion, ligation of the vectors juxtaposed the heterologous protein encoding sequences in frame with the first few codons of the translation initiation region present downstream of the promoter sequences, allowing heterologous gene expression in *Lactobacillus* species (Fig. 2).

2.2. Expression analysis of heterologous gene products

For expression analysis, recombinant *L. casei* containing the vectors pLP401 and pLP501 expressing TTFC (designated pLP401-TTFC and pLP501-TTFC) or containing the vector pLP402 expressing hMBP or hMBP fused to β-glucuronidase (designated pLP402-hMBP and pLP402-hMBP/U) were routinely prepared from glycerol stocks by two consecutive overnight (o/n) cultures without aeration of a 1:50 dilution in MRS medium containing 5 μg/ml erythromycin.

Transformants with expression vectors containing the constitutive *ldh* promoter were subsequently grown

BNSDOCID: <XP__4164996A__I_>

105

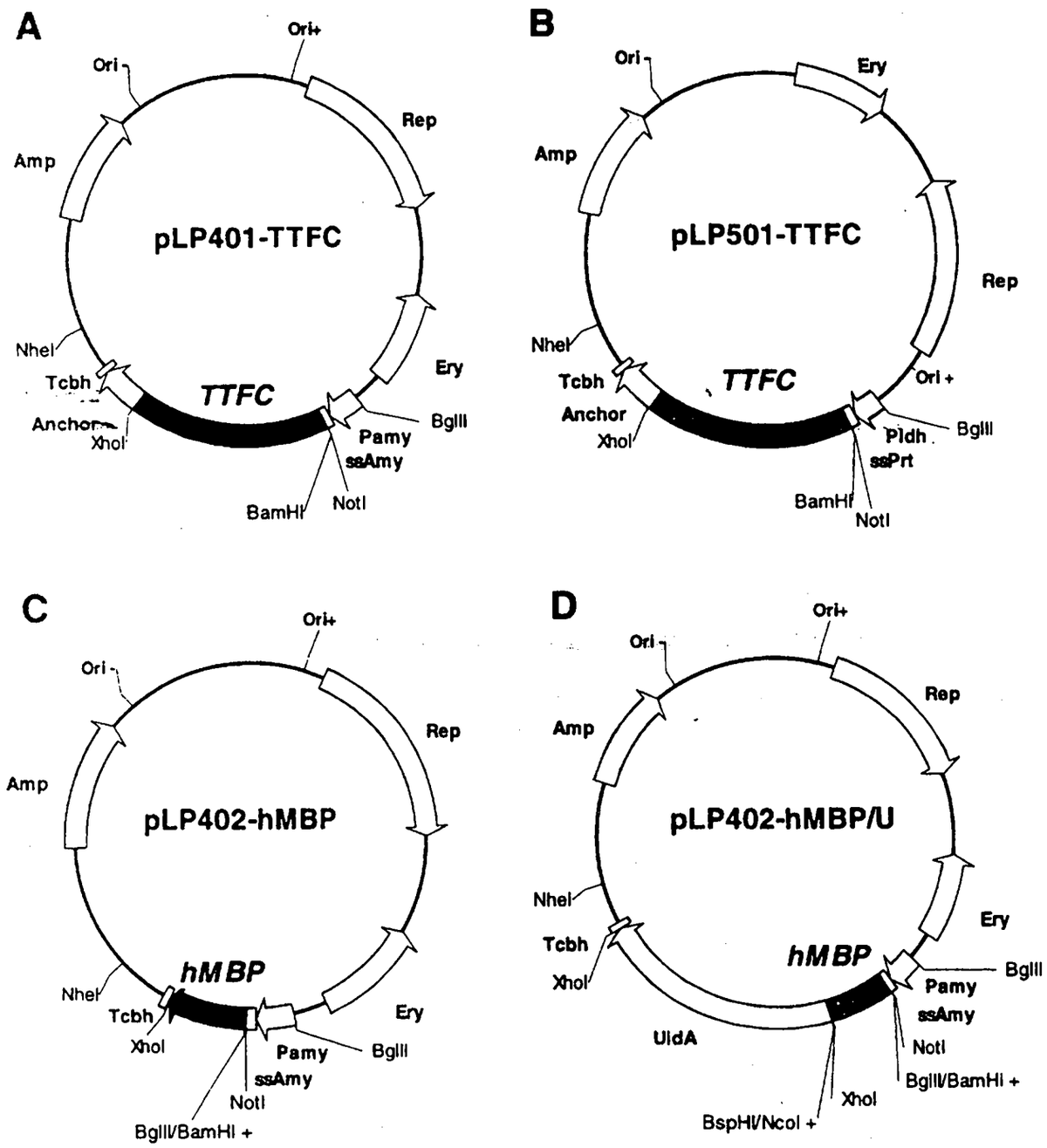


Fig. 2. *Lactobacillus* expression vectors for secretion or surface-anchored expression of heterologous proteins. (A) pLP401-TTFC vector mediating surface-anchored expression of TTFC under control of the regulatable *amylase* promoter. (B) pLP501-TTFC vector mediating surface-anchored expression of TTFC under control of the constitutive *lactate dehydrogenase* promoter. (C) pLP402-hMBP vector mediating secretion of hMBP under control of the regulatable *amylase* promoter. (D) pLP402-hMBP/U vector mediating secretion of hMBP fused to β-glucuronidase under control of the regulatable *amylase* promoter. P_{amy} = promoter sequence of the α-*amylase* gene of *L. amylovorus*. ssAmy = sequences encoding secretion signal (36aa) of the α-*amylase* gene of *L. casei* and the N-terminus (26aa) of α-*amylase* gene of *L. casei*. P_{ldh} = promoter sequences of the *ldh* gene of *L. casei*. ssPrt = sequences encoding secretion signal (33aa) of the *prt* P gene of *L. casei* and the N-terminus (36aa) of the *prt* P gene of *L. casei*. Anchor = anchor peptide (117aa) encoding sequence of *L. casei*. T_{cbh} = transcription terminator sequence of the *cbh* gene of *L. plantarum 80*. UidA = sequences encoding β-glucuronidase. Rep = *repA* gene of plasmid p353-2 from *L. pentosis*. Ery = *erythromycin resistance* gene. Amp = *ampicillin resistance* gene. Ori = origin of replication (ori + = ori *E. coli*, ori− = ori *Lactobacillus*). + = disappeared restriction site.

ing the CNS of which over 400.000 children die worldwide each year. Infection with *Clostridium tetani* results in the production of tetanus toxin, which enters the CNS via the blood, leading to muscle cramping and eventually to death. TTFC is a non-toxic but immunogenic 47 kDa fragment of the tetanus holo-

BNSDOCID: <XP__4164996A__1_>

in antibiotic selective MRS medium or *Lactobacillus* carrying medium (LCM) [21] containing 1% glucose (w/v) at 37°C, using a 1:50 dilution of the last o/n culture. In order to induce expression of the heterologous proteins in transformants containing plasmids under the transcriptional control of the regulatable *α-amy* promoter, cells were grown in mannitol (1% w/v) supplemented LCM or modified MRS medium (mMRS) (1% w/v proteose peptone, 0.5% w/v yeast extract, 0.2% w/v meat extract, 0.1% v/v Tween 80, 37 mM sodium acetate, 0.8 mM magnesium sulfate, 0.24 mM manganese sulfate, 8.8 mM diammonium citrate in 0.1 M potassium phosphate buffer [pH 7.0]). Cells were harvested at defined time points, pelleted by centrifugation and suspended in phosphate buffered saline (PBS). Cell extracts were obtained from the bacteria by sonicating the cells four times on a 30 s on/30 s off cycle using an MSE Soniprep 150 sonicator at 10 amplitude microns (Sanyo Gallenkamp PLC, Leicester, UK), to release both cytoplasmic and cell wall-bound proteins. After centrifugation the soluble fraction was used for immunoblot analysis.

To detect heterologous proteins secreted into the medium, the culture medium was collected after spinning down the cells and filtered through a 0.2 μm filter to remove the remainder of cells. After addition of 0.2 mg/ml trypsin inhibitor (Sigma Chemical, La Jolla, CA) the culture medium was freeze-dried and concentrated approximately 200-fold in PBS. Dilutions of these concentrated media are used for immunoblot analysis. Proteins in cell extracts or culture medium were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)(7.5, 10 or 14% acrylamide, 400 mM Tris pH 8.8) and run in a 25 mM Tris, 192 mM glycine buffer (pH 8.3) at 200 V for 45 min. Protein was transferred electrophoretically onto nitrocellulose using a Bio-Rad Miniprotean II blotting unit (Bio-Rad Laboratories, Hercules, CA). Immunoblots were incubated with optimally diluted rabbit anti-TTFC (Calbiochem–Novabiochem International, San Diego, CA), rabbit anti-hMBP or rabbit anti-β-glucuronidase antiserum. Antibodies against hMBP and β-glucuronidase were induced by immunization of New Zealand White rabbits with human MBP and β-glucuronidase in complete adjuvant containing *Mycobacterium tuberculosis H37Ra* (Difco Laboratories). The second incubation step was performed with swine anti-rabbit Ig-HRP (Dako A/S, Glostrup, Denmark). The immunoblots were incubated with ECL detection reagents (Amersham Life Science Ltd, Buckinghamshire, UK). A light sensitive film (Kodak X-omat) was exposed to the blots for a variety of time periods before development. In all immunoblots, recombinant TTFC antigen (List Biological Laboratories, Campbell, CA), bovine MBP (purified according to Deibler et al.) [22] or β-glucuronidase (Sigma Chemical) were used as references for the immuno-reagents. Culture medium of various recombinant *L. casei* strains expressing fusion proteins with β-glucuronidase were used as controls for secretion or lack of secretion of heterologous proteins into the culture medium in immunoblot experiments. pLP402-HA/U, which secretes the 111–120 peptide of hemagglutinin of influenza (HA111-120) fused to β-glucuronidase, was used as a positive control for secretion [23]. Surface-expressed TTFC fused to β-glucuronidase (pLP401-TTFC/U) and intracellularly expressed TTFC fusion protein (pLP503-TTFC/U) are examples of heterologous proteins that are not directed to the medium and were utilized as negative controls. The amount of soluble heterologous hMBP-β-glucuronidase was calculated by comparison of several dilutions of extracts of transformants with a reference of purified β-glucuronidase in immunoblot analysis making use of Bio-1D V6.32 software (Vilber Lourmat, Marne la Vallée, France).

2.3. Flow cytometry analysis

At defined time points bacteria were prepared for analysis by FACScan. Cells were washed twice with FACS fluid (FF), Facsflow® (Becton–Dickinson, San Jose, CA) with 1% BSA and 0.1% sodium azide. Cells were resuspended in 200 μl FF containing optimal dilutions of antibodies specific for TTFC, MBP or β-glucuronidase and incubated for 1 h at 4°C. Cells were again washed twice in FF. Bound antibody was detected by incubation with fluorescein isothiocyanate-conjugated (FITC) labeled goat anti-rabbit immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, PA) at a dilution of 1:1500 for 1 h at 4°C. Cells were then washed twice prior to analysis on a flow cytometer (FACScan®, Becton–Dickinson). CellQuest® software was used for analysis of the data. A gate was set for events representing lactobacilli as determined by cytograms of forward and side scatter to enable exclusion of debris. Control strains were wild type *L. casei* or recombinant strains expressing an irrelevant protein harvested in the same growth phase. For control stainings normal rabbit serum was used or the antigen-specific primary antibody was omitted. For each sample, data was collected for 10,000 or 20,000 gated events. The fluorescence obtained from bacterial cell suspensions was represented by fluorescence histograms and mean channel intensities were calculated.

For quantification of surface expression, *L. casei* pLP401-TTFC cells were analyzed by examining TTFC antibody binding at the point of antibody saturation. This precise determination of fluorescence levels at the point of saturation of binding sites was achieved empirically following the generation of a titration curve for the TTFC-specific polyclonal antibody.

BNSDOCID: <XP__4164996A_1_>

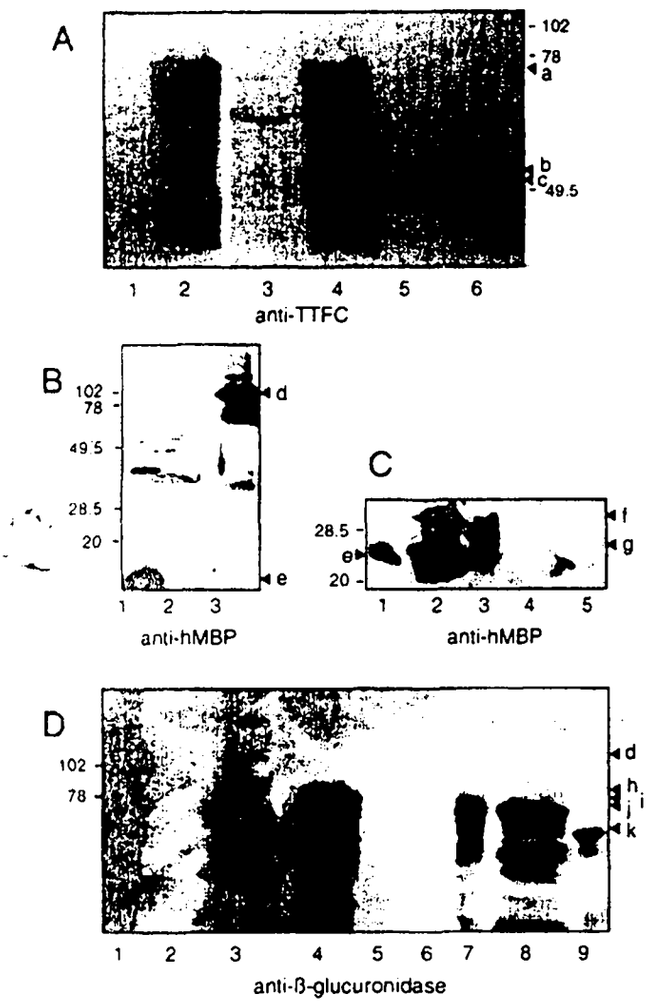


Fig. 3. Immunoblot analysis of *Lactobacillus casei* recombinants. (A) Expression of TTFC by recombinants carrying pLP401-TTFC (lane 4) or pLP501-TTFC (lane 2). When the culture reached an optical density of 0.6 at 690 nm after derepression of the α-*amy* promoter, cell extracts were prepared and analyzed by immunoblot. Nitrocellulose membranes were stained with serum specific for TTFC. Purified TTFC was used as positive control (lane 5) and cell extracts of wild type *L. casei* carrying an pLP501 vector with an irrelevant insert (lane 1) or a pLP401 vector with an irrelevant insert (lane 3) were used as negative controls. pLP503-TTFC, which retains the TTFC product intracellularly, is shown in lane 6. (B) Expression of hMBP fused to β-glucuronidase by recombinants carrying pLP402-hMBP/U (lane 3). At an optical density of 1.1 at 690 nm, cell extracts were prepared and analyzed by immunoblot. Nitrocellulose membrane was stained with serum specific for hMBP. Purified bovine MBP was used as positive control antigen (lane 1) and a cell extract of *L. casei* expressing β-glucuronidase (pLP402-U)(lane 2) was used as a negative control. (C) Expression of hMBP in cell extracts and in culture medium. Cell extracts (lane 3) and culture medium (lane 2) of *L. casei* recombinants pLP402-hMBP were analyzed by immunoblot for the presence of heterologous hMBP. Nitrocellulose membrane was stained with serum specific for hMBP. Purified bovine MBP was used as positive control antigen (lane 1) and a cell extract (lane 5) and concentrated culture medium (lane 4) of *L. casei* with pLP402-U were used as negative controls. (D) Detection of secreted β-glucuronidase fusion products. At an optical density of 1.1 at 690 nm, cell extracts and culture medium were prepared for analysis by immunoblot. Nitrocellulose membrane was stained with serum specific for β-glucuronidase. Lane 1 showed hMBP-β-glucuronidase in a cell extract from pLP402-hMBP/U. Lane 2 is kept empty, to be sure that the secreted product in medium from pLP402-hMBP/U in lane 3 can not be due to overflow. Lane 4 and lane 8 show the secreted products in medium from pLP402-HA/U and pLP402-U respectively. Lane 5 and 6 showed the concentrated media of pLP503-TTFC (intracellular TTFC) and pLP401-TTFC (anchored TTFC) respectively. In lane 7, pLP402-U extract was analyzed. Purified β-glucuronidase was used as positive control antigen in lane 9. a = heterologous anchored TTFC, b = intracellular TTFC, c = purified TTFC, d = hMBP-β-glucuronidase, e = purified MBP, f = non-secreted hMBP, g = secreted hMBP, h = secreted HA-β-glucuronidase, i = non-secreted β-glucuronidase, j = secreted β-glucuronidase, k = purified β-glucuronidase. Sizes of the low range marker are indicated in kDa.

The conversion of measured fluorescence intensities into values representing the number of TTFC molecules accessible for binding on the surface (ABC) was undertaken using the Quantum25® FITC Kit calibration beads (Flow Cytometry Standards, Leiden, The Netherlands). To enable quantitative estimates of the numbers of antibodies bound per cell, peak channel data generated from the calibration beads was regressed against the defined values of molecules of equivalent soluble fluorochrome (MESF) possessed by each bead population. Because of the broader distribution of channel intensities for stained cells, extrapolation of the MESF value for pLP401-TTFC vectors was then undertaken using the mean channel fluorescence intensity obtained at antibody saturation of the lactobacilli presenting TTFC on the cell surface. Following corrections for fluorochrome/protein (F/P) ratios in the anti-rabbit FITC conjugate (second) step an estimation of numbers of surface molecules available for binding on the surface of the recombinant lactobacilli was made.

2.4. Parenteral immunization with *L. casei* recombinants

In order to demonstrate the immunogenicity of the expressed heterologous proteins, mice were immunized parenterally with cell extracts or concentrated growth medium of the *L. casei* recombinants. Cell extracts of the recombinants pLP401-TTFC and pLP501-TTFC were obtained as described above. Three BALB/c mice per group were immunized s.c. with an emulsion of 112.5 μl total cell extract and 137.5 μl Difco's complete adjuvant with *M. tuberculosis* H37Ra (Difco Laboratories). Concentrated culture medium from the recombinants pLP402-hMBP and pLPhMBP/U was used for immunization. Three SJL/J mice per group were immunized s.c. with 250 μl concentrated culture medium emulsified in Difco's complete adjuvant or i.p. in incomplete adjuvant (Difco Laboratories). The immunization procedure was repeated 28 d after the first

BNSDOCID: <XP__4164996A_I_>

immunization. Serum was collected for analysis in ELISA every seven days.

2.5. TTFC and MBP specific ELISA

To test serum for the presence of anti-TTFC or anti-MBP antibodies, Nunc immuno plates (Life Technologies, Gaithersburg,MD) were coated with tetanus toxoid (RIVM, Bilthoven, The Netherlands) (5 μg/ml, 50 μl/well) in 0.05 M carbonate–bicarbonate buffer (pH 9.6) or guinea pig MBP (5 μg/ml, 50 μl/well) in PBS overnight at 4°C. Non-specific binding was blocked by incubation with 3% BSA in PBS (150 μl/well) for 1 h at 37°C. Subsequently the plates were incubated for 1 h at 37°C with dilutions of sera of mice immunized with cell extracts or culture medium of *L. casei* recombinants and preimmunization sera to correct for background reactivity. For the detection of bound antibodies specific for TTFC or MBP, alkaline phosphatase-labelled goat anti mouse IgG (γ) (KPL, Gaithersburg, MD) was used (1 h, 37°C). After addition of the substrate 4-nitrophenylphosphate, the absorbance at 405 nm was read at several time points. Endpoint titer was defined as the highest dilution which gives an absorbance above twice the absorbance with normal mouse serum.

3. Results

3.1. TTFC-expressing recombinant L. casei

3.1.1. Construction of TTFC-expressing L. casei transformants

To enable the construction of TTFC-expressing *L. casei* vectors, the 1329 bp DNA coding for TTFC was cloned into the *Lactobacillus* expression vectors pLP401 and pLP501 (Fig. 2). The pLP401 vectors enable expression via the regulatable α-*amy* promoter and include the secretion signal and N-terminus of the *amylase* gene of *L. amylovorus*. The pLP501 vectors contain the promoter sequence of the *ldh* gene of *L. casei* and include the secretion signal and N-terminus of the *prtP* gene of *L. casei*. Both the pLP401 and the pLP501 vectors contain the anchor sequence of the *prtP* gene of *L. casei*, resulting in expression of the TTFC-anchor protein at the cell surface. Candidate vectors were constructed in *E. coli DH5α* hosts and after removal of the *ldh* terminator introduced in *L. casei* by electroporation.

3.1.2. Immunoblot analysis of TTFC-expressing L. casei transformants

Expression of TTFC was demonstrated by immunoblot analysis using a rabbit polyclonal TTFC-specific antiserum (Fig. 3A). Cell extracts were prepared of the *L. casei* transformant carrying pLP401-TTFC approximately 8 h (log phase) after inoculation in LCM supplemented with mannitol at $OD_{690}=0.6$. Expression of TTFC in the pLP501-TTFC strain, in which expression is driven by P*ldh*, is not particularly time dependent (data not shown). This confirmed the constitutive expression by pLP501-TTFC during growth, whereas in transformants containing pLP401-TTFC, TTFC synthesis was induced by derepression of the α-*amy* promoter following replacement of glucose in the growth medium by mannitol. Expression was demonstrated to be optimal at the end of the log phase ($OD_{690}=0.6$). Fig. 3A demonstrates that the amount of TTFC product present in pLP501-TTFC (lane 2) is higher than in pLP401-TTFC (lane 4). The TTFC products of both vectors are larger than the purified TTFC (lane 5) due to additional elements such as the anchor sequence. The apparent molecular weight of these TTFC products on immunoblot is approximately 75 kDa. In extracts of either of the transformants types a variety of breakdown products was observed. This in contrast with pLP503-TTFC transformants which retain the TTFC product intracellularly. The breakdown products of this recombinant are less abundant (Fig. 3A, lane 6). This difference in breakdown products between anchored/secreted products and intracellular products holds true for all pLP400/500 vectors constructed so far. *L. casei* transformed with the pLP401/pLP501 vectors with irrelevant sequences demonstrated no corresponding heterologous product expression (Fig. 3A, lane 1 and 3).

3.2. L. casei producing MBP and MBP fusion products

For oral tolerance induction *Lactobacillus* transformants containing expression vectors with which secretion of heterologous proteins can be achieved were constructed. DNA encoding human MBP was cloned into the expression vector pLP402 in frame with or without the sequence of β-glucuronidase (Fig. 2C–D). These pLP402 plasmids drive the expression via the regulatable α-*amy* promoter and contain the secretion signal and N-terminus of the *amylase* gene of *L. amylovorus*.

Expression of both fusion products was demonstrated in extracts by immunoblot analysis using antiserum against hMBP (Fig. 3B, lane 3 and Fig. 3C, lane 3). HMBP fused to β-glucuronidase was also detected after incubation of the immunoblots with anti-β-glucuronidase serum (Fig. 3D, lane 1). Semi-quantitative analysis yielded a calculated amount of 1–1.5% MBP-β-glucuronidase fusion protein as a percentage of total protein.

BNSDOCID: <XP__4104996A__I_>

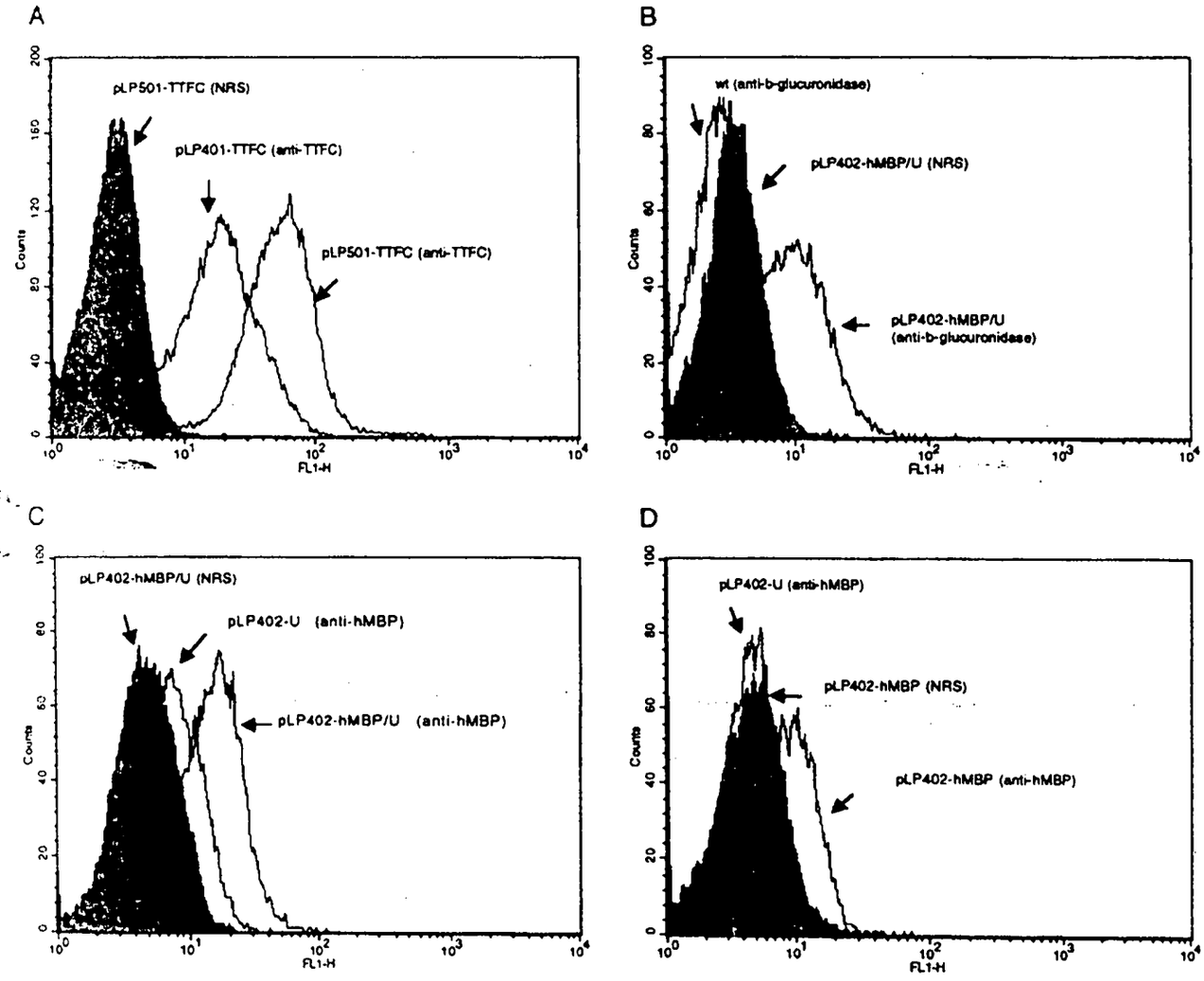


Fig. 4. Flow cytometry analysis of *Lactobacillus casei* recombinants. (A) Surface-association of TTFC. TTFC was detected on the cell surface of *Lactobacillus casei* recombinants with pLP401-TTFC (α-*amy* promoter) or pLP501-TTFC (*ldh* promoter) by incubation of cells with rabbit antibodies specific for TTFC and stained with FITC labelled goat anti-rabbit serum. Recombinant pLP501-TTFC incubated with normal rabbit serum (NRS) was used as negative control. (B) Surface-association of hMBP-β-glucuronidase using antiserum against β-glucuronidase. *Lactobacillus casei* recombinants secreting hMBP-β-glucuronidase (pLP402-hMBP/U) showed surface-association when the cells were incubated with rabbit antibodies against β-glucuronidase and stained with FITC labelled goat anti-rabbit serum. Wild type *Lactobacillus casei* (wt) incubated with serum specific for β-glucuronidase and pLP402-hMBP/U incubated with normal rabbit serum (NRS) were used as negative controls. (C) Surface-association of hMBP-β-glucuronidase using hMBP specific antiserum. pLP402-hMBP/U recombinants were incubated with rabbit antibodies directed against MBP and stained with FITC labelled goat anti-rabbit serum. Recombinant *L. casei* secreting β-glucuronidase only (pLP402-U) incubated with serum specific for hMBP and pLP402-MBP/U incubated with normal rabbit serum (NRS) were used as negative controls. (D) Surface-association of hMBP using hMBP specific antiserum. pLP402-hMBP recombinants were incubated with rabbit antibodies directed against hMBP and stained with FITC labelled goat anti-rabbit serum. Recombinant *L. casei* secreting β-glucuronidase only (pLP402-U) incubated with serum specific for hMBP and pLP402-hMBP incubated with normal rabbit serum (NRS) were used as negative controls.

3.3. Secretion of MBP and MBP fusion products into the medium

Culture medium used for the propagation of the recombinant lactobacilli was analyzed in order to detect whether the cells secreted MBP or MBP fusion proteins. Using immunoblot analysis of cell extracts, the time point of optimal expression was determined to be 12 h after initiation of growth in mannitol-supplemented mMRS medium (OD_{690} = 1.1). The fusion protein hMBP fused to β-glucuronidase was detectable in 50-fold concentrated medium (= 750 μl medium) in low concentration using the β-glucuronidase specific anti-serum (Fig. 3D, lane 3). The breakdown products, which are abundantly present, are specific for β-glucuronidase. This indicates that the product is, at least partially, secreted. The fusion product of hMBP with β-glucuronidase could not be detected in the media when

BNSDOCID: <XP__4164996A_1_>

Table 1
Quantitation of the number of TTFC molecules expressed on the cell surface of recombinant *L. casei*

Cells	Mean channel fluorescence	MESF	*F/P* ratio	Surface-molecules/ cell
pLP401-TTFC	[21]	4282	[3.]1	[1.4×10^3]
pLP501-TTFC	[55]	12170	[3.1]	[3.9×10^3]

anti-hMBP serum is used (data not shown). Other secreted fusion proteins could be detected in the medium, such as the influenza peptide fused to β-glucuronidase of *L. casei* pLP402-HA/U (Fig. 3D, lane 4). That this product is probably intact can be concluded from the fact that the MW of this product is higher than secreted β-glucuronidase from the *L. casei* pLP402/U recombinant (Fig. 3D, lane 8). During the secretion process the signal sequence is cleaved off. Therefore, the secreted products are smaller than the heterologous proteins detected in the cell extracts, as can be seen by comparison of the cell extract (Fig. 3D, lane 7) with medium of *L. casei* pLP402/U (Fig. 3D, lane 8). In concentrated culture medium from two control recombinant *L. casei* strains which expressed irrelevant antigens fused to β-glucuronidase either intracellular (pLP503-TTFC/U) (Fig. 3D, lane 5) or anchored to the bacterial surface (pLP401-TTFC/U)(Fig. 3D, lane 6), no heterologous proteins were detected using antiserum specific for β-glucuronidase, as expected. This indicated that the positive signals obtained with the medium samples of the recombinants are unlikely to result from leakage or lysis of cells.

Although the large hMBP-β-glucuronidase protein is not efficiently secreted, the much smaller hMBP product from the pLP402-hMBP transformant was secreted well. This product was clearly demonstrated in 25 fold-concentrated culture medium on a immunoblot incubated with anti-hMBP serum (Fig. 3C, lane 2). All medium samples have the property that they run in a wide pattern (Fig. 3D, lane 3, 4 and 8), but they show very wide and diffuse bands in the region of the smaller proteins (Fig. 3C, lane 2). As a negative control pLP402/U, which secretes only β-glucuronidase, did not show a specific reactivity, as expected (Fig. 3C, lane 4).

3.4. Flow cytometric analysis and selection

3.4.1. Flow cytometry analysis of surface association of TTFC

Presentation of the TTFC antigen on the surface of *L. casei* transformed with plasmids pLP401-TTFC or pLP501-TTFC was demonstrated by FACScan analysis of recombinant lactobacilli. TTFC gene expression by *L. casei* was analyzed using a polyclonal rabbit TTFC-specific antiserum in the period of 4–9 h following the onset of growth. Data obtained 7 h after inoculation are presented (Fig. 4A). Mean fluorescence levels observed with pLP501-TTFC after staining with non-immune rabbit serum were used as control (Fig. 4A). A higher level of surface-anchored exposure of TTFC under control of the *ldh* promoter (pLP501-TTFC) in comparison to that of the surface-anchored exposure of TTFC under control of the α-*amy* promoter (pLP401-TTFC) [24] was indicated by the distinct mean channel fluorescence intensities of the two recombinants at an equivalent time point. Similar results were obtained at other time points (results not shown).

3.4.2. Quantitation of the number of TTFC molecules present on the cell surface

To estimate the mean number of TTFC molecules present on the surface of the cell, mean channel intensities obtained following staining of *L. casei* pLP401-TTFC and *L. casei* pLP501-TTFC with anti-TTFC were used for calculation. As shown in Table 1, after 7 h of growth approximately 3.9×10^3 and 1.4×10^3 TTFC molecules were found on the surface of the pLP501-TTFC and pLP401-TTFC recombinant *L. casei*, respectively.

3.4.3. Analysis of secreted hMBP and hMBP fusion products

In order to determine whether it is possible to detect epitope-specific sequences associated with the cell wall of heterologous proteins which are directed to be secreted, transformants secreting MBP and MBP fusion proteins were analyzed by flow cytometry, at time points of optimal expression as determined by immunoblot analysis (data not shown). The cells were incubated with specific antiserum against β-glucuronidase or against MBP. Wild type *L. casei* as well as *L. casei* transformed with the vector pLP402-U, which secreted β-glucuronidase, were used as controls. As shown in Fig. 4B and C, it was possible to detect myelin proteins fused to β-glucuronidase associated with the cell surface by flow cytometry. Significant levels of hMBP-β-glucuronidase (mean channel fluorescence = 13.5 channels) could be detected using anti-β-glucuronidase serum as compared to the wild type *L. casei* (mean channel fluorescence ≈2.5). Using anti-MBP serum, the hMBP fusion protein (Fig. 4C) and hMBP (Fig. 4D) only could also be detected, although the level of

BNSDOCID: <XP__4164996A_1_>

111

surface expression of hMBP only was very low (mean channel fluorescence = 8.1 versus 5.1 of pLP402-U). The increase in fluorescence intensity of pLP402-MBP/U compared to the control recombinant pLP402-U, which only secretes β-glucuronidase (mean fluorescence = 15.5 respectively 7.7 channels) (Fig. 4C) was slightly less than the increase in fluorescence intensity of the control strains and the recombinant pLP402-MBP/U detected with anti-β-glucuronidase (Fig. 4B).

3.5. Immunogenicity of heterologous proteins expressed by L. casei

To determine whether the heterologous TTFC proteins expressed by *L. casei* recombinants pLP401-TTFC and pLP501-TTFC were immunogenic, BALB/c mice were immunized with total cell extracts of these recombinants. Immunogenicity of TTFC construct pLP501 was demonstrated in a tetanus toxoid specific ELISA within 14 d after the second immunization with an IgG endpoint titer of $10^{3.2}$ ($\log_{10}$) for the pooled mice sera. The TTFC construct pLP401-TTFC showed an endpoint titer of $10^{2.9}$ ($\log_{10}$) 21 d after the second immunization.

SJL/J mice were immunized with cell-free culture medium of *L. casei* recombinants pLP402-hMBP and pLP402-hMBP/U to demonstrate immunogenicity as well as secretion of the heterologous proteins into the medium. Immunogenicity of secreted MBP was shown by IgG endpoint titers of $10^{2.3}$ ($\log_{10}$) for pLP402-hMBP and $10^{2.9}$ ($\log^{10}$) for pLP402-hMBP/U after 21 d with negligible values at 14 d. None of the selected control recombinants (i.e. *L. casei* pLP402/U) induced MBP or TTFC antibodies, as expected.

4. Discussion

In this study we describe the construction and characterization of *Lactobacillus* expression/delivery vectors as potential vaccines or therapeutics for diseases of the central nervous system. Essentially, similar vectors are designed for use of two distinct applications: live oral vaccines to actively immunize versus live oral therapeutics to restore peripheral T cell tolerance in autoimmune disease. The distinct effects are facilitated through the precise location of the antigen; surface-anchored antigen-expression for the vaccine application versus antigen secretion into the local environment for tolerance induction [2,3].

Two plasmids were constructed to enable surface-anchored expression of TTFC. The expression is under transcriptional control of the constitutive *ldh* promoter (pLP501-TTFC) or under the control of the α-*amy* promoter (pLP401-TTFC), which is repressed in the presence of glucose. For application in vaccine strategies, the number of molecules of the antigen present on the cell-surface is considered important for immunogenicity. Flow cytometry has previously been used to determine the composition of bacterial cultures, in which each bacterial species has its own characteristic 'signature' [25]. In addition, heterologous proteins have also been demonstrated on the surface of other recombinant Gram-positive bacteria such as *Staphylococcus xylosus* [26]. Flow cytometry, to our best knowledge, has not been used before to detect heterologous proteins associated with the *Lactobacillus* surface. We have shown that this technology allows an estimation of the relative number of molecules expressed on the cell wall by individual cells. In the utilization of a polyclonal antibody we assumed that each TTFC molecule is bound by only one antibody molecule, enabling the relative measurements between recombinant lactobacilli. With this strategy it was demonstrated that for *L. casei* pLP501-TTFC, under control of the constitutive *ldh* promoter, the number of TTFC molecules per cell, present on the *Lactobacillus* cell wall is three times higher than observed for *L. casei* pLP401-TTFC which expresses TTFC under the control of the regulatable α-*amy* promoter. The difference in expression level can be explained by the fact that the promoter sequences used have different transcription activities [14]. The different secretion signals (proteinase versus amylase) inherent to the vectors may also lead to higher translocation efficiencies in the pLP501-TTFC recombinant and may result in elevated levels of TTFC on the cell wall accessible for binding by antibody [24,27]. The difference in level of expression/surface association may also account for the higher levels of TTFC specific antibodies found after parenteral immunization with total cell extracts of *L. casei* pLP501-TTFC as compared to pLP401-TTFC. Although both recombinant strains appear to be immunogenic, the results argue for the use in vaccine applications of recombinant strains utilizing the constitutive promoter. The breakdown products present in extracts obtained from either the pLP401 or pLP501 recombinants, which are not as abundantly present in extracts obtained from pLP503 recombinants maintaining the heterologous proteins intracellular, may well be due to activity of proteases present at the surface of the bacterium or in the medium.

Vectors for the use in oral tolerance studies preferably should secrete the tolerogen [2,3]. Therefore, two vectors that secrete hMBP (pLP402-hMBP) or hMBP fused to β-glucuronidase were constructed and analyzed. Although secretion of both products was demonstrated in immunoblot analysis using protein samples prepared from the culture medium, there was a clear difference in the levels of secretion. The quantity of hMBP-β-glucuronidase fusion protein actually

BNSDOCID: <XP___4104996A__I_>

present in the culture medium was very low, and the product apparently was digested into two main products. However, this fusion product was detectable by flow cytometry using either anti-MBP or anti-β-glucuronidase antibodies. This suggests that although the protein is translocated over the membrane, it did not undergo signal peptide cleavage or was degraded by extracellular proteases. When cleavage does not occur within the encephalitogenic epitopes, the secreted protein could still maintain its tolerogenicity. Association of the mature form of translocated heterologous proteins with the cell wall has been described for Gram-positive bacteria [28–30]. The smaller hMBP protein without β-glucuronidase is much more efficiently secreted than hMBP fused to β-glucuronidase. Following analysis of this recombinant by flow cytometry, we only found a very low surface-association of hMBP. Apparently, this product is indeed quite efficiently secreted into its environment. We have shown that protein secretion into the medium from recombinants transformed with vectors using the same secretion signals (pLP402-hMBP, pLP402-hMBP/U, pLP402-HA/U, pLP402-U) does not occur at similar levels. These differences probably arise from biochemical properties of the protein itself (e.g. size, hydrophobicity, conformation). In addition, varying sensitivities of each protein for proteases may affect the amount of protein found in the medium. Introduction of pLP402-hMBP/U in alternative *Lactobacillus* strains may result in an increase of the level of secretion of this particular protein.

Immunization of mice with concentrated culture medium of the hMBP recombinants evoked MBP-specific antibodies, from which it could be concluded that the heterologous proteins were immunogenic and secreted into the medium. However, the antibody levels induced by medium containing the hMBP-β-glucuronidase fusion protein were higher than the levels induced by medium containing hMBP.

Host strain selection is a factor that may impact significantly on the capacity to successfully induce antibodies or restore peripheral T-cell tolerance by oral administration. Recently, *Lactobacillus* strains have been identified that persist for extended periods in the intestinal tract of mice (D.M. Shaw, unpublished) as well as in humans in comparison to the prototype *L. casei*, and this property may improve efficacy of oral vaccination and of tolerance induction considerably. We have previously shown that induction of oral tolerance can be induced with *L. plantarum* transformed to express β-galactosidase [6] and mucosal antibody responses can be induced with orally administered trinitrophenyl-*Lactobacillus acidophilus* conjugates [31].

In conclusion, the generic technology described here allows the construction of a multitude of safe recombinant lactobacilli which may prove advantageous in oral intervention strategies for the prevention and treatment of infectious and autoimmune diseases of the central nervous system. Currently, recombinant lactobacilli expressing TTFC or viral antigens are tested in animal models for their ability to induce specific antibodies after oral administration. Whether orally administered recombinant lactobacilli secreting myelin antigens are capable of inducing peripheral T-cell tolerance is currently investigated in mouse and rat EAE-models for multiple sclerosis.

Acknowledgements

The authors thank Eric Claassen for critically reading the manuscript and Lizet Groenewegen and Chantal Smittenaar for their expert technical assistance. This work was financially supported by contract number B104-CT96-0542 of EC-Biotech and by grant number MS93-147 from The Netherlands Foundation for the support of Multiple Sclerosis Research.

References

[1] Czerkinsky C, Holmgren J. The mucosal immune system and prospects for anti-infectious and anti-inflammatory vaccines. The Immunologist 1995;3:97–103.

[2] Brandtzaeg P. History of oral tolerance and mucosal immunity. Ann NY Acad Sci 1996;778:1–27.

[3] Gebert A, Rothkotter HJ, Pabst R. M cells in Peyer's patches of the intestine. Int Rev Cytol 1996;167:91–159.

[4] Strobel S, Mowat AM, Drummond HE, Pickering MG, Ferguson A. Immunological responses to fed protein antigens in mice. II. Oral tolerance for CMI is due to activation of cyclophosphamide-sensitive cells by gut-processed antigen. Immunology 1983;49:451–6.

[5] Mowat AM. The role of antigen recognition and suppressor cells in mice with oral tolerance to ovalbumin. Immunology 1985;56:253–60.

[6] Maassen CBM, Gerritse K, Leer RJ, Heemskerk D, Boersma WJA, Claassen E. *Lactobacillus* as a vector for oral delivery of antigens: The role of intrinsic adjuvanticity in modulation of the immune response. In: Husband A, et al., editorsMucosal solutions, Advances in Mucosal Immunology, part 2. Sydney, Australia: The University of Sydney, 1998. p. 235–47.

[7] Bitar DM, Whitacre CC. Suppression of experimental autoimmune encephalomyelitis by the oral administration of myelin basic protein. Cell Immunol 1988;112:364–70.

[8] Higgins PJ, Weiner HL. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein and its fragments. J Immunol 1988;140:440–5.

[9] Challacombe SJ, Tomasi TBJ. Systemic tolerance and secretory immunity after oral immunization. J Exp Med 1980;152:1459–72.

[10] Moss B. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc Natl Acad Sci USA 1996;93:11341–8.

[11] Perdigon G, Alvarez S, Pesce de Ruiz Holgado AA. Immunoadjuvant activity of oral *Lactobacillus casei*: influence of dose on the secretory immune response and protective capacity in intestinal infections. J Dairy Res 1991;58:485–96.

BNSDOCID: <XP__4164096A__1_>

[12] Boersma WJA, Zegers ND, Van den Bogaerd A, Leer RJ, Bergmans A, Pouwels PH, Posno M, Claassen E. Development of safe oral vaccines based on *Lactobacillus* as a vector with adjuvant activity. In: Proc. ICHEM 2nd Int. Congr. Biotech., UK, Brighton, 1994. p. 43–6.
[13] Link-Amster H, Rochat F, Saudan KY, Mignot O, Aeschlimann JM. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. FEMS Immunol Med Microbiol 1994;10:55–63.
[14] Pouwels PH, Leer RJ, Boersma WJA. The potential of *Lactobacillus* as a carrier for oral immunization: development and preliminary characterization of vector systems for targeted delivery of antigens. J Biotechnol 1996;44:183–92.
[15] Gerritse K, Posno M, Schellekens MM, Boersma WJA, Claassen E. Oral administration of TNP-*Lactobacillus* conjugates in mice: a model for evaluation of mucosal and systemic immune responses and memory formation elicited by transformed lactobacilli. Res Microbiol 1990;141:955–62.
[16] Fairweather NF, Chatfield SN, Makoff AJ, Strugnell RA, Bester J, Maskell DJ, Dougan G. Oral vaccination of mice against tetanus by use of a live attenuated Salmonella carrier. Infect Immun 1990;58:1323–6.
[17] Robinson K, Chamberlain LM, Schofield KM, Wells JM, Le Page RW. Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. Nat Biotechnol 1997;15:653–7.
[18] Hanahan D. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 1983;166:557–80.
[19] Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor University Press, 1989.
[20] Posno M, Leer RJ, van Luijk N, van Giezen MJF, Heuvelmans PTHM, Lokman BC, Pouwels PH. Complementation of the inability of *Lactobacillus* strains utilize D-xylose with D-xylose catabolism-encoding genes of *Lactobacillus pentosus*. Appl Environ Microbiol 1991;57:1822–8.
[21] Efthymiou C, Hansen PA. Antigenic analysis of *Lactobacillus acidophilus*. J Infect Dis 1962;110:258–67.
[22] Deibler GE, Martenson RE, Kies MW. Large scale preparation of myelin basic protein from central nervous tissue of several mammalian species. Prep Biochem 1972;2:139–65.
[23] Hackett CJ, Hurwitz JL, Dietzschold B, Gerhard W. A synthetic decapeptide of influenza virus hemagglutinin elicits helper T cells with the same fine recognition specificities as occur in response to whole virus. J Immunol 1985;135:1391–4.
[24] Pouwels PH, Leer RJ. Genetics of lactobacilli: plasmids and gene expression. Antonie van Leeuwenhoek 1993;64:85–107.
[25] Pinder AC, Purdy PW, Poulter SA, Clark DC. Validation of flow cytometry for rapid enumeration of bacterial concentrations in pure cultures. J Appl Bacteriol 1990;69:92–100.
[26] Samuelson P, Hansson M, Ahlborg N, Andreoni C, Gotz F, Bachi T, Nguyen TN, Binz H, Uhlen M, Stahl S. Cell surface display of recombinant proteins on *Staphylococcus carnosus*. J Bacteriol 1995;177:1470–6.
[27] Hols P, Slos P, Dutot P, Reymund J, Chabot P, Delplace B, Delcour J, Mercenier A. Efficient secretion of the model antigen M6-gp41E in *Lactobacillus plantarum* NCIMB 8826. Microbiology 1997;143:2733–41.
[28] Saunders CW, Schmidt BJ, Mallonee RL, Guyer MS. Secretion of human serum albumin from *Bacillus subtilis*. J Bacteriol 1987;169:2917–25.
[29] Simonen M, Palva I. Protein secretion in *Bacillus* species. Microbiol Rev 1993;57:109–37.
[30] Wells JM, Wilson PW, Norton PM, Le PR. A model system for the investigation of heterologous protein secretion pathways in *Lactococcus lactis*. Appl Environ Microbiol 1993;59:3954–9.
[31] Gerritse K, Posno M, Fasbender MJ, Schellekens MM, Boersma WJA, Claassen E. Mucosal immune responses and systemic immunological memory after oral administration of trinitrophenyl *Lactobacillus* conjugates in mice. Lymphatic Tissues and in vivo Immune Responses 1991;84:497–504.

BNSDOCID: <XP__4164996A__I_>

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/01, A23J 3/34, A23C 9/123, 9/12 — A1

(11) International Publication Number: **WO 97/00078**

(43) International Publication Date: 3 January 1997 (03.01.97)

(21) International Application Number: PCT/FI96/00350

(22) International Filing Date: 12 June 1996 (12.06.96)

(30) Priority Data:
952926 14 June 1995 (14.06.95) FI

(71) Applicant *(for all designated States except US)*: VALIO OY [FI/FI]; Meijeritie 6, FIN-00370 Helsinki (FI).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: ISOLAURI, Erika [FI/FI]; Tallinnenkuja 6, FIN-21120 Raisio (FI). METSÄNIITTY, Leena [FI/FI]; Kuusmiehentie 44 C, FIN-00670 Helsinki (FI). KORHONEN, Hannu [FI/FI]; Lemmenmäentie 28 A 3, FIN-11120 Riihimäki (FI). SALMINEN, Seppo [FI/FI]; Puutarhakatu 2 C 32, FIN-20100 Turku (FI). SYVÄOJA, Eeva-Liisa [FI/FI]; Tonttutytönkuja 3 D 21, FIN-02200 Espoo (FI).

(74) Agent: OY JALO ANT-WUORINEN AB; Iso Roobertinkatu 4-6 A, FIN-00120 Helsinki (FI).

(81) Designated States: AU, BG, BR, CA, CN, CZ, EE, IL, JP, KE, KP, KR, LT, LV, MX, NO, NZ, PL, RO, RU, SG, SK, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHODS OF PREVENTING OR TREATING ALLERGIES

(57) Abstract

The present invention relates to methods and means of suppressing food-induced hypersensitivity reactions in patients suffering from food allergy. Particularly the invention provides methods of making a protein hydrolysate formula for downregulating hypersensitivity reactions and for promoting gut immune barrier, and methods of preventing or treating allergies, especially cow's milk allergy in infants by fortifying the cow's milk elimination diet with suitable probiotic bacteria derived from gastrointestinal tract, especially Lactobacilli. In addition, administering fragments of an allergen modified by gastrointestinal bacteria immune response can be "turned around" from sensitization to systemic hyporesponsiveness. A preferable bacterium to be used in this invention is *Lactobacillus* GG (ATCC 53103).

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

SNSDOCID: <WO___9700078A1_I_>

Methods of preventing or treating allergies

Field of the invention

The present invention relates to methods and means of suppressing food-induced hypersensitivity reactions in patients suffering from food allergy. Particularly the invention provides methods of preventing or treating allergies, especially cow's milk allergy in infants. The invention also relates to development of specific formulae for allergic infants with impaired gut barrier function.

Cow's milk allergy (CMA) is defined as an immune-mediated adverse reaction to cow's milk proteins. The present treatment of choice is the complete elimination of cow's milk antigens. In infants with CMA, it is necessary to use a substitute formula when human milk is unavailable. Hydrolysed formulae, based on cow's milk-derived whey or casein, are used to provide adequate nutrition with a reduced antigenic load. The preliminary heat treatment of cow's milk mainly affects the conformation of proteins and facilitates their hydrolysis. Subsequent enzymatic hydrolysis with pepsin, trypsin, pancreatic extracts and extracts from the intestinal mucosa causes progressive destruction of sequential epitopes and refines the formulae into the least antigenic and allergenic form.

In most cases, extensively hydrolysed cow's milk-derived formulae can be safely introduced and these are efficient and clinically and metabolically well tolerated. Enzymatic hydrolysis, however, does not necessarily make the formula nonallergenic, as the optimal extent of hydrolysis is not known and traces of the original protein are detected in the hydrolysate. Therefore, introduction of these substitutes to children with cow's milk allergy must be cautious.

The approach to control allergic inflammation by antigen elimination has not been satisfactory, particularly in patients with multiple food allergies (Sampson *et al.*, 1992). These patients frequently show increased intestinal permeability and dysfunction of the intestine's defence barrier (Majamaa *et al.*, 1996, Majamaa and Isolauri, 1996). This enhances the risk for growth disorders and sensitization to multiple foods. New approaches are urgently needed for the treatment of cow's milk allergy to improve the substitute formulae in CMA.

Intestinal antigen handling determines subsequent immune response to the antigen. In health, antigens are absorbed across epithelium along two functional pathways. The main pathway is degradative reducing the immunogenicity of the antigen. A minor pathway allows the transport of intact proteins which is crucial for antigen-specific immune responses. Aberrant antigen absorption enhances the sensitization process (Fargeas *et al.*, 1995).

Differential production of cytokines by T-helper (Th) cells during an immune reaction has important regulatory effects on the nature of the immune response. The cytokine profile of the natural immune response determines the phenotype of the subsequent specific immune response. Apart from controlling IgE synthesis, IL-4 is crucial for the development and maturation of the Th2 phenotype, characterized for allergic inflammation.

This process appears crucial for the development of tolerance to ingested protein. Oral tolerance is a state of antigen-specific systemic non-responsiveness characterized by local antigen-specific IgA response.

An isolated human intestinal strain, *Lactobacillus* strain GG (*Lactobacillus* GG, ATCC 53103) has recently been shown to promote local IgA responses against dietary antigens

encountered by the enteric route and may therefore aid in immune elimination (Isolauri *et al.*, 1993). It is not as yet known whether particular strains of intestinal bacteria could directly modify the immunogenicity of the food antigens and consequently downregulate hypersensitivity reactions.

Lactobacilli are included in the microbial flora of healthy intestines. It has been assumed that Lactobacilli act in the intestinal tract by competing for receptors and nutrients against pathogenic microbes on the intestinal mucosa.

Probiotics are viable microbial preparations which promote health by maintaining the natural microflora in the gut. A microbial preparation can be acknowledged as a probiotic if the functioning microbes thereof and their mode of action are known. The probiotics attach on the intestinal mucosa, colonize the human intestinal tract and prevent attachment of harmful microbes. A crucial presumption is that they get up to the gut's mucosa and do not get destroyed in the upper part of the gastrointestinal tract. *Lactobacillus* GG is one of known bacteria having probiotic characteristics.

Description of the invention

The present inventors have now found that certain bacteria of the gastrointestinal tract, especially Lactobacilli, and especially bacteria having probiotic characteristics, can be used to enhance the efficacy of elimination diets and to improve the oral tolerance, in preventing or treating food-induced hypersensitivity reactions in a patient.

One aspect of the invention is that protein hydrolysates obtained by hydrolysis of proteins with above mentioned

119

gastrointestinal bacteria can also be used to said purpose. We show here that protein hydrolysates obtained according to this invention have an immunological effect promoting hypoallergenicity. The hydrolysates downregulate hypersensitivity reactions thus promoting the gut immune barrier function, i.e. stabilising the gut mucosal barrier.

The present invention provides an improved protein hydrolysate formula downregulating hypersensitivity reactions and promoting the gut immune barrier function. The hydrolysate formula is obtainable by hydrolysing proteins with enzymes derived from probiotic gastrointestinal bacteria, especially Lactobacilli, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103), and with trypsin and/or pepsin.

Alternatively, a protein hydrolysate formula of the invention can be obtained by hydrolysing proteins with trypsin and/or pepsin, and adding to the hydrolysate so obtained a bacterial preparation comprising probiotic gastrointestinal bacteria, especially Lactobacilli, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103). The bacteria are added into the hydrolysate formula preferably as a lyophilized preparation.

While administering such a hydrolysate formula to a patient, it is to be expected that the hydrolysing enzymes of the bacteria present are released *in vivo* whereby the same effect is achieved as with an improved hydrolysate formula as defined above. In addition, the viable bacteria stabilize the gut mucosal barrier enhancing the local defence.

BNSDOCID: <WO___9700078A1_I_>

An embodiment of this invention is a method of preventing or treating food-induced hypersensitivity reactions in an infant, which method comprises the step of administering to an infant at risk the improved protein hydrolysate formula of the invention, or alternatively a protein hydrolysate formula together with a bacterial preparation comprising probiotic gastrointestinal bacteria, especially Lactobacilli, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103).

A further embodiment of this invention is a method of treating cow's milk allergy in a patient, comprising administering to the patient the improved protein hydrolysate formula of the invention or, alternatively administering a protein hydrolysate formula together with a bacterial preparation comprising probiotic gastrointestinal bacteria, especially Lactobacilli, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103).

The invention also provides a method of promoting tolerogenic immune responses to food antigens in a patient, comprising oral administration of the improved protein hydrolysate formula of the invention to the patient, or, alternatively, a bacterial preparation comprising probiotic gastrointestinal bacteria, especially Lactobacilli, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103), or, alternatively, a protein hydrolysate formula together with a bacterial preparation comprising probiotic gastrointestinal bacteria, especially Lactobacilli, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103).

BNSDOCID: <WO__9700078A1_I_>

In the methods of the invention as defined above, when a protein hydrolysate formula is used in combination with selected probiotic bacteria as defined above, such a formula can be any suitable protein hydrolysate formula, known or novel. It can thus be either a partial protein hydrolysate, or alternatively an extensively hydrolysed formula. Preparation of a protein hydrolysate suitable for this purpose is disclosed e.g. in EP patent application No. 601 802.

Preferred bacterium to be used in the formulae and methods of the invention is *Lactobacillus* GG (ATCC 53103).

In this specification and in the appended claims the phrase "bacteria, especially Lactobacilli, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103)" should be understood to define bacteria whose adhesive and colonizing characteristics are comparable to those of the LGG strain, e.g. as defined in EP-patent 199 535. These bacteria are also supposed to have same kind of enzyme system as LGG, which means that enzymes derived from such bacteria are able to degrade proteins to produce hydrolysation products with same effects as those obtained using enzymes derived from LGG.

Abbreviations

CI	confidence interval
IFN-γ	Interferon-γ
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IL-4	Interleukin-4
LGG	*Lactobacillus* GG (ATCC 53103)
OKT3	anti-CD3 antibody
PBMC	peripheral blood mononuclear cells

BNSDOCID: <WO___9700078A1_I_>

TNF-α	tumour necrosis factor α
WF	test group receiving extensively hydrolysed Whey Formula
WF-GG	test group receiving extensively hydrolysed Whey Formula and LGG preparation

P/T-casein = casein hydrolysed by pepsin and trypsin
P/T-α_{s1}-casein = α_{s1}-casein hydrolysed by pepsin and trypsin

Brief description of the drawings

Fig. 1A-1D Mitogen-induced proliferative responses of PBMCs *in vitro* to P/T-casein (1A), P/T-α_{s1}-casein (1B), P/T-casein additionally hydrolysed with LGG derived enzymes (1C) and P/T-α_{s1}-casein additionally hydrolysed with LGG derived enzymes (1D). Results are expressed as mean counts per minute for cultures without hydrolysed product (PHA-RPMI 1640) and with hydrolysed product at three concentrations (0.1, 10 and 1000 µg/ml). Horizontal lines represent the geometric mean of counts per minute of the six experiments, enumerated the same for each individual.

Fig. 2 The clinical score of atopic dermatitis (SCORAD) in each patient and the median score for extent (A), intensity (B) and subjective score (C) for atopic dermatitis before management (0) and one month later (I) in infants receiving extensively hydrolysed whey formula without (WF) or the same with *Lactobacillus* GG (WF-GG).

Fig. 3 The effect of casein and *Lactobacillus* GG-degraded casein on the production of IL-4 and IFN-γ by PBMC in atopic patients (a,b) and in nonatopic healthy children (c,d). White columns represent the median cytokine production in control cultures; black columns, in cultures containing purified casein; and hatched columns, in cultures containing *Lactobacillus* GG-degraded casein. Inter-

secting lines represent the upper and lower quartiles. *Statistically significant pairwise comparison to control cultures.

Fig. 4 The effect of α_{s1}-casein and *Lactobacillus* GG-degraded α_{s1}-casein on the production of IL-4 and IFN-γ by PBMC in atopic patients (a,b) and in nonatopic healthy children (c,d). White columns represent the median cytokine production in control cultures; black columns, in cultures containing α_{s1}-casein; and hatched columns, in cultures containing *Lactobacillus* GG-degraded α_{s1}-casein. Intersecting lines represent the upper and lower quartiles. *Statistically significant pairwise comparison to control cultures.

The following examples illustrate the invention further. Methods for preparing the improved hydrolysate are described, as well as experiments showing the beneficial therapeutic effect observed in the present studies.

BNSDOCID: <WO___9700078A1_I_>

Experimental

Example 1 Suppression of lymphocyte proliferation *in vitro* by bovine caseins hydrolysed with *Lactobacillus* GG derived enzymes

1.a. Hydrolysis of Cow's Milk Proteins

Bovine total casein and casein components (α_{s1}-casein) were purified from cow milk (Syväoja, 1992). The hydrolysis with the enzyme mixture derived from *Lactobacillus* GG was applied separately to casein and α_{s1}-casein. The enzymes were isolated using the modified method of Exterkate and de Veer, 1985. Briefly, the enzymes were released by sonication of frozen bacterial cells. The supernatant of the centrifuged cells was separated and used for the hydrolysis of casein and α_{s1}-casein. The hydrolysis was carried out for 24 h at 34°C.

The GG-hydrolysates so obtained were further hydrolysed with pepsin and trypsin. Briefly, the samples were hydrolysed first with 0.1 % pepsin in 0.1 mol/l HCl for 3 hours at 37°C, pH 2.5. After pH adjustment with 250 mg $NaHCO_3$ and 2 mol/l NaOH, 0.1 % trypsin was added to the samples and these were hydrolysed for 5 hours at 37°C, pH 8.0.

P/T-casein and P/T-α_{s1}-casein samples were obtained by hydrolysing the purified casein and α_{s1}-casein only with pepsin and trypsin as described above, without GG-hydrolysation.

1.b. Lymphocyte transformation test

A modification of the whole blood micromethod for mitogen-induced lymphocyte transformation was used. Six to eight healthy individuals volunteered as blood donors for the experiment. Briefly, heparinized venous blood was obtained and diluted 1:7 with RPMI 1640 culture medium

(Grand Island Biological Co. NY, USA) containing antibiotics. Phytohemagglutinin (PHA) (Difco Laboratories, Detroit, Mich., USA) was diluted with RPMI 1640 containing antibiotics, with ranges of final concentrations in cultures from 5 to 1250 µg/ml. Lyophilized hydrolysates of casein and α_{s1}-casein were diluted in RPMI 1640 to their final concentrations of 0.1 µg/ml (low), 10 µg/ml (medium) and 1000 µg/ml (high) as proven to be nontoxic on T cells in dye exclusion studies with eosin.

Two sets of experiments were undertaken to investigate the mitogen-induced proliferative responses of peripheral blood mononuclear cells (PBMCs) to (A) casein hydrolysed with pepsin and trypsin (=P/T-casein), (B) α_{s1}-casein hydrolysed with pepsin and trypsin (=P/T-α_{s1}-casein), (C) P/T-casein additionally hydrolysed with *Lactobacillus* GG derived enzymes, and (D) P/T-α_{s1}-casein additionally hydrolysed with *Lactobacillus* GG derived enzymes.

Experiment consisted of four control cultures with different dilutions of the culture medium and the mitogen, as well as corresponding test cultures with 25 µl of hydrolysed products at three different concentrations. The assay was carried out as described in Sütas *et al.*, 1996.

The mitogen-induced proliferation of PBMCs was expressed as counts per minute, background excluded. The results were presented for control cultures containing 125 µg/ml PHA and RPMI 1640 and for the three test cultures containing 125 µg/ml PHA and hydrolysed products at the low, medium, and high concentrations.

1.c. Statistical analysis

A nonparametric pairwise test (Wilcoxon signed-rank test) was used to compare the change of the values in counts per minute of each of the test cultures with those of the control cultures. The level of significance was $p < 0.05$.

BNSDOCID: <WO__9700078A1_I_>

Significant results of pairwise comparisons were presented as suppression or stimulation according to decrease or increase of the values in counts per minute in the test culture.

1.d. Results

In the experiments done with P/T-casein and P/T-α_{s1}-casein additionally hydrolysed with *Lactobacillus* GG derived enzymes, the mitogen induced proliferation of PBMCs was significantly suppressed at 0.1, 10 and 1000 µg/ml ($p=0.03$, $p=0.03$, and $p=0.03$) (Figs 1C and 1D) compared to P/T-casein and P/T-α_{s1}-casein (Figs 1A and 1B).

Example 2. The treatment of infants with atopic dermatitis with protein hydrolysate formula supplemented with lactic acid bacteria

2.a. Patients and study design

The study comprised 31 infants, aged 2.5 to 15.7 (mean age 8) months, fulfilling the Hanifin criteria (Hanifin, 1987) for atopic eczema in children. They had been referred to a paediatric clinic on the basis of atopic eczema and suspected cow's milk allergy. The mean age at onset of symptoms was 2.4 months. Duration of total breast-feeding was 5.9 months. A positive family history of atopic diseases (asthma, atopic eczema and allergic rhinitis) or food allergy in first-degree relatives was noted in 26 (84 %) patients. The eczematous lesions were treated with emollients and topical corticosteroids. No patient was receiving systemic corticosteroid therapy. Besides atopic eczema, gastrointestinal disturbances such as loose stools, vomiting or diarrhea were seen in 9 (19 %) patients. After the study periods the patients were allocated to double-blind placebo-controlled cow's milk challenge. Only those having a positive reaction (27/31) were included in the final study population.

The patients were randomized to two groups for one month. One group (WF, n=14) received extensively hydrolysed whey formula (Valio Ltd, Helsinki, Finland, EP-A-601802) and another group (WF-GG, n=13) received the same formula with additional *Lactobacillus* GG preparation ($5x10^8$ cfu/g) (supplied by Valio Ltd, Helsinki, Finland). After the one-month therapy with their assigned formulae; both groups received an extensively hydrolysed whey formula (Valio Ltd) for one additional month. The serum total IgE concentration, cow's milk-specific IgE (RAST, Pharmacia, Uppsala, Sweden) and skin prick tests were determined from all patients before dietary intervention. Prick testing was done on the volar aspect of the forearm, using a commercially available cow milk allergen ALK (Allergologisk Laboratorium, Horsholm, Denmark) and test formula diluted to normal feed concentration. A 1 mm single-peak lancet with shoulder to prevent deeper penetration (ALK) was used, with histamine dihydrochloride 10 mg per milliliter (ALK) as positive control. Reactions were read after 15 minutes, and a response half of the histamine reaction size or more was recorded as positive on condition that the mean diameter of the wheal was at least 3 mm and the negative control at the same time 0 mm.

Faecal samples for the determination of α-1 antitrypsin and TNF-α were collected before the commencement of the management and 1 (corresponding to the study period) and 2 months later.

The severity of atopic dermatitis was scored according to the SCORAD method, established by the European Task Force on Atopic Dermatitis (1993). Briefly, the extent (score A) of the dermatitis was estimated using the rule of nines. The intensity (score B) of the dermatitis was the sum of the individual scores (0-3) for erythema, oedema and/or papules, excoriation, lichenification and dryness. The subjective (score C) manifestations (Scored 1-10),

BNSDOCID: <WO___9700078A1_I_>

including pruritus and sleep loss, were assessed from parents' estimations. SCORAD was obtained with the calculation: A/5+3.5xB+C.

The study protocol was approved by the Ethical Review Committee of Tampere University Hospital. An informed consent was obtained from the parents.

2.b. Determination of α-1 antitrypsin in faecal specimens

Frozen faecal specimens were thawed at room temperature and homogenized. Approximately 1 g was transferred to a glass tube and lyophilized. The resulting dry material was ground and 50 mg transferred to an Eppendorf tube. One ml of 0.15 M NaCl solution was added, and α-1 antitrypsin was extracted by vigorous mixing in a Vortex mixer for 20 minutes at room temperature. The resulting suspension was centrifuged at 25,000 g for 10 minutes to remove the debris, and the supernatant was used for the determination of α-1 antitrypsin using a Behring BNA nephelometer according to the manufacturer's instructions. The results are given as mg/g dry weight of lyophilized faeces.

2.c. Determination of TNF-α in faecal specimens

Deep-frozen faecal specimens were thawed at room temperature, suspended 1:1 (w/v) in physiological saline and allowed to sediment. Of the supernatant, 0.5-1.0 ml was transferred to an Eppendorf tube and centrifuged at 25,000 g for 10 minutes. The supernatant was then used for the determination of TNF-α. A commercial enzyme immunoassay (Human TNF-α ELISA Kit, Endogen Inc., Boston, Massachusetts, USA) was used, as instructed for serum specimens, for the determinations of faecal TNF-α.

2.d. Statistics

By reason of the skewed distributions of serum IgE concentrations, logarithmic (ln) transformation was used and data are presented as means with 95 % confidence intervals (CI). The concentrations of inflammatory parameters are presented with medians with lower and upper quartiles. The Wilcoxon signed rank test and the Mann-Whitney U-test were used in statistical comparisons.

2.e. Results

2.e.1. Clinical data

The mean (95 % CI) serum total IgE was 31 (15-61) kU/l in patients receiving the hydrolysate formula. RAST for cow's milk was positive (>0.4 kU/l) in 10/31 (37 %) atopic eczema patients. Skin prick test for cow's milk was positive in 8/31 (30 %) patients.

The severity of atopic dermatitis in each patient before the commencement of the managements and one month later, i.e. after the study period are presented in Figure 2. The median (lower quartile-upper quartile) SCORAD score was 21(14-31) in group WF and 26 (17-38) in group WF-GG before management (p=0.33). There was a significant improvement of SCORAD score after one month's intervention in those receiving *Lactobacillus* GG (p=0.008), but not in those receiving extensively hydrolysed formula without *Lactobacillus* GG (p=0.89). The SCORAD score was then 19 (13-31) in group WF and 15 (7-28) in group WF-GG. The decrease in the SCORAD score in WF-GG was due to the reduction of the extent (score A, p=0.004), intensity (score B, p=0.05) and subjective score (score C, p=0.01) for atopic dermatitis (Figure 2). The improvement of SCORAD score was achieved by 2 months in the WF group, and in group WF-GG in remained unchanged after cessation of *Lactobacillus* GG. At 2 months, the median (lower quartile-upper quartile) SCORAD score in group WF was 14(2-38) and in group WF-GG 16(6-25).

BNSDOCID: <WO__9700078A1_I_>

2.e.2. The concentrations of α-1 antitrypsin and TNF-α in faeces

In healthy controls (n=9), the median (lower quartile-upper quartile) concentration of α-1 antitrypsin was 0.5 (0.5-1.7) mg/g. The concentration of α-1 antitrypsin was comparable between groups WF anf WF-GG before management (p=0.22). As indicated in Table 1, the concentration of α-1 antitrypsin decreased significantly in group WF-GG (p=0.03), but not in group WF (p=0.68) during the one month study period. At two months, the concentration of α-1 antitrypsin was 1.2 (0.5-1.6) in WF and 0.5 (0.5-0.7) in WF-GG.

The concentration of faecal TNF-α was 0 (0-0.08) pg/g in healthy controls. The concentration of faecal TNF-α was significantly higher in atopic children, p<0.0001 (Table 1). The concentration of TNF-α was comparable between the groups WF and WF-GG before management (p=0.57). The concentration of faecal TNF-α decreased significantly in WF-GG (p=0.003) but not in WF (p=0.38) during the one month study period (Table 1). A reduction in TNF-α concentration was achieved by 2 months in group WF, while in group WF-GG who were also given the extensively hydrolysed formula without *Lactobacillus* GG a tend to increased TNF-α was detected. The concentration of TNF-α was then 84 (25-129) in WF and 144 (20-338) in WF-GG.

Table 1. The concentrations of faecal α-1 antitrypsin and TNF-α before management (0) and one month later (I) in infants receiving extensively hydrolysed whey formula (WF) or the same formula containing *Lactobacillus* GG bacteria (WF-GG). Data denote median (lower quartile-upper quartile).

	WF	WF-GG
α-1 antitrypsin 0 (mg/g)	1.7 (1.5-2.3)	1.4 (0.5-1.9)
α-1 antitrypsin I (mg/g)	1.7 (1.1-2.8)	0.5 (0.5-1.0)
TNF-α 0 (pg/g)	632 (126-1880)	709 (91-1131)
TNF-α I (pg/g)	494 (147-1009)	34 (19-103)

Example 3. Downregulation of cytokine production by peripheral blood mononuclear cells in atopic children

3.a. Patients and methods

Altogether, 14 patients aged 5-29 (mean, 16) months who fulfilled the Hanifin criteria for atopic dermatitis (Hanifin, 1987) and eight age-matched non-atopic healthy children were studied. None were receiving systemic corticosteroids at the time of the study.

OKT3 (anti-CD3-antibody) containing ascites fluid was a kind gift from Dr M. Kaartinen, Department of Bacteriology and Immunology, University of Helsinki, Helsinki, Finland. Bovine total casein had been purified from bovine milk as described in Syväoja (1992). Purified casein was hydrolysed with *Lactobacillus* GG-derived enzymes as described in Example 1. Purifi d casein or *Lactobacillus* GG-degraded casein were lyophilized and stored at room temperature. Before experiments, they were diluted in

BNSDOCID <WO___9700078A1_I_>

RPMI 1640 and filter sterilization (0.1 µm, Millipore corporation, Bedford, MA, USA) was applied.

Complete culture medium consisted of RPMI 1640 with 10 % foetal calf serum, 10 mM Hepes buffer, 2 mM L-glutamin (Gibco Life Technologies, Paisley, UK), 50 U/ml benzylpenicillin (Sigma, St. Louis, MO, USA), 10 mg/ml gentamycin (Roussel Laboratories Ltd, Uxbridge, Middlesex, UK). Peripheral venous blood (5 ml) was obtained. PBMC containing 90 % lymphoid cells was isolated by FicollPaque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation and suspended at 1 x 10^6 cells/ml in complete culture medium. Culture wells were precoated with anti-CD3 antibody containing ascites fluid at a pretested optimal dilution. The test culture additionally contained dilution of casein or *Lactobacillus* GG-degraded casein at a final concentration of 1 mg/ml. These experiments were repeated for purified bovine α_{s1}-casein or *Lactobacillus* GG-degraded α_{s1}-casein. After 24 h of incubation in a humidified 5 % CO_2 atmosphere at 37°C, supernatants were collected and stored at -70°C for cytokine assays. IL-4 and IFN-γ in culture supernatants were determined by commercially available ELISA kits according to manufacturers' instructions (IL-4: CLB, Compact Human Interleukin-4 ELISA kit, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands; IFN-γ. EIFNG, Endogen Inc., Cambridge, MA, USA). The results from different runs were equalized employing the comparison of standard curves and were expressed as pg/ml. The sensitivity of the assays for IL-4 was 2.3 pg/ml and for IFN-γ, 5 pg/ml. Wilcoxon signed-rank test was used in statistical comparisons of the test cultures to the control cultures. The level of significance was $P < 0.05$.

3. b. Results

In atopic patients, both IL-4 and IFNγ production were increased in cultures containing purified casein when compared to control cultures, $P = 0.008$ and $P = 0.008$, respectively (Fig. 3a,b). No such effect of bovine casein was observed when degraded by enzymes of *Lactobacillus* GG. Conversely, the IL-4 production in cultures containing *Lactobacillus* GG-degraded casein was significantly less than in control cultures, $P = 0.003$ (Fig. 3a), and the IFN-γ production in these cultures was comparable to control cultures, $P = 0.10$ (Fig. 3b).

In healthy children, the production of IL-4 and IFN-γ in cultures containing purified casein were comparable to control cultures, $P = 0.10$ and $P = 0.10$ (Fig. 3c,d). In parallel to the findings in atopic patients, healthy children had significantly less IL-4 production in cultures containing *Lactobacillus* GG-degraded casein than in control cultures, $P = 0.01$ (Fig. 3c) and the IFN-γ production in these cultures remained comparable to control cultures, $P = 0.50$ (Fig. 3d). Parallel results were obtained with α_{s1}-casein and *Lactobacillus* GG-degraded α_{s1}-casein (Fig. 4).

BNSDOCID: <WO__9700078A1_I_>

References

European task force on atopic dermatitis. Severity scoring of atopic dermatitis: the SCORAD index. Dermatology 1993; 186:23-31.

Exterkate F and de Veer G. Partial isolation and degradation of caseins by cell wall proteinase(s) of *Streptococcus cremoris*. Appl Environ Microbiol 1985; 49:328-32.

Fargeas MJ, Theodorou V, More J, Wal JM, Fioramonti J, Bueno L. Boosted systemic immune and local responsiveness after intestinal inflammation in orally sensitized guinea pigs. Gastroenterology 1995; 109:53-62.

Hanifin JM. Epidemiology of atopic dermatitis. Monogr Allergy 1987; 21:116-131

Isolauri E, Majamaa H, Arvola T, Rantala I, Virtanen E, Arvilommi H. *Lactobacillus casei* strain GG reverses increased intestinal permeability induced by cow milk in suckling rats. Gastroenterology 1993; 105:1643-1650.

Majamaa H, Isolauri E. Evaluation of gut mucosal barrier: evidence for increased antigen transfer in children with atopic eczema. J Allergy Clin Immunol 1996, in press.

Majamaa H, Miettinen A, Laine S, Isolauri E. Intestinal inflammation in children with atopic eczema: faecal eosinophil cationic protein and tumor necrosis factor-α as noninvasive indicators of food allergy. Clin Exp. Allergy 1996; 26:181-187.

Sampson HA, James MJ and Bernhisel-Broadbent J. Safety of an amino acid-derived infant formula in children allergic to cow milk. Pediatrics 1992; 90:463-465.

Sütas Y, Soppi E, Korhonen H, Syväoja EL, Saxelin M, Rokka T, Isolauri E. Suppression of lymphocyte proliferation *in vitro* by bovine caseins hydrolyzed with *Lactobacillus casei* GG derived enzymes. J Allergy Clin Immunol 1996, in press.

Syväoja EL. Quantitative determination of the main casein components and purification of α_{s2}- and κ-casein from bovine milk. Milchwissenschaft 1992; 47:563-566

Claims

1. A method of making a protein hydrolysate formula for downregulating hypersensitivity reactions and for promoting gut immune barrier, comprising the steps of

hydrolysing proteins with enzymes derived from a bacterial preparation comprising probiotic gastrointestinal bacteria, especially Lactobacilli, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103), and with pepsin and/or trypsin.

2. The method as defined in claim 1 wherein the enzymes are derived from a bacterial preparation comprising *Lactobacillus* GG (ATCC 53103).

3. A method of making a protein hydrolysate formula for downregulating hypersensitivity reactions and for promoting gut immune barrier, comprising the steps of

hydrolysing proteins with pepsin and/or trypsin, and adding to the hydrolysate a bacterial preparation comprising probiotic gastrointestinal bacteria, especially Lactobacilli, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103).

4. The method as defined in claim 3 wherein the bacterial preparation comprises *Lactobacillus* GG (ATCC 53103).

5. A protein hydrolysate formula for downregulating hypersensitivity reactions and for promoting gut immune barrier, which hydrolysate is obtainable by the method as defined in any one of claims 1 to 4.

6. A method of preventing or treating food-induced hypersensitivity reactions in an infant, comprising

BNSDOCID: <WO___9700078A1_I_>

administering to an infant at risk the protein hydrolysate formula according to claim 5.

7. A method of treating cow's milk allergy in a patient, comprising administering to the patient the protein hydrolysate formula made according to claim 1.

8. A method of promoting tolerogenic immune responses to food antigens in a patient, comprising oral administration of the protein hydrolysate formula according to claim 5.

9. A method of preventing or treating food-induced hypersensitivity reactions in an infant, comprising administering to an infant at risk a protein hydrolysate formula made according to claim 1 together with a bacterial preparation comprising probiotic gastrointestinal bacteria, especially Lactobacilli, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103).

10. A method of treating cow's milk allergy in a patient, comprising administering to the patient a protein hydrolysate formula together with a bacterial preparation comprising probiotic gastrointestinal bacteria, especially Lactobacilli, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103).

11. A method of promoting tolerogenic immune responses to food antigens in a patient, comprising oral administration of a bacterial preparation comprising probiotic gastrointestinal bacteria, especially Lactobacilli, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103), or a protein

hydrolysate formula together with a bacterial preparation comprising probiotic gastrointestinal bacteria, especially Lactobacilli, which have adhesive and colonizing characteristics and a protease enzyme system which are similar to those of the strain *Lactobacillus* GG (ATCC 53103).

12. The method according to any one of claims 9 to 11 wherein the bacterial preparation comprises *Lactobacillus* GG (ATCC 53103).

BNSDOCID: <WO___9700078A1_I_>

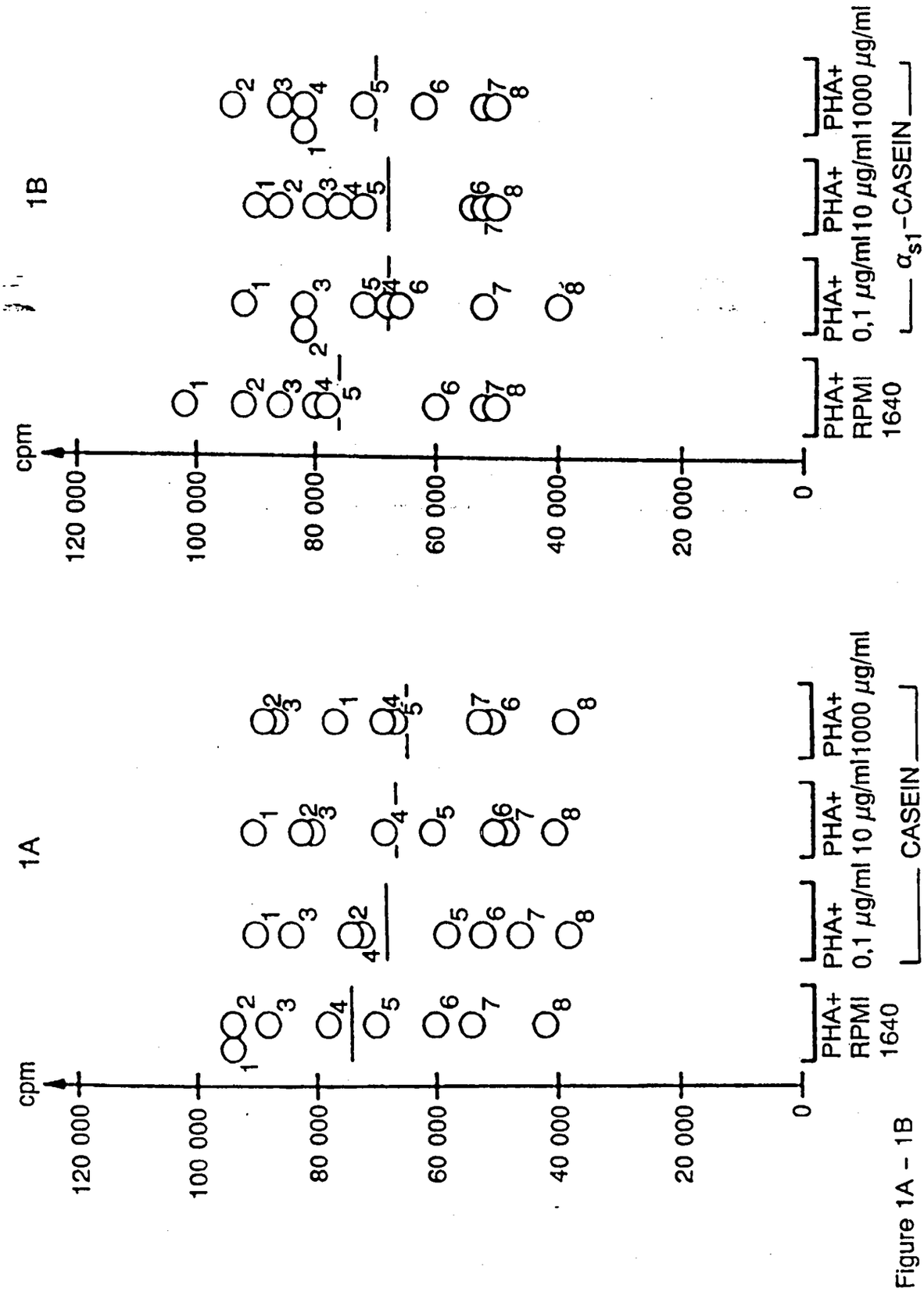

1A
1B
cpm
120 000
100 000
80 000
60 000
40 000
20 000
0
PHA+
RPMI 1640
PHA+
0,1 µg/ml
PHA+
10 µg/ml
PHA+
1000 µg/ml
CASEIN
α_{s1}-CASEIN

Figure 1A - 1B

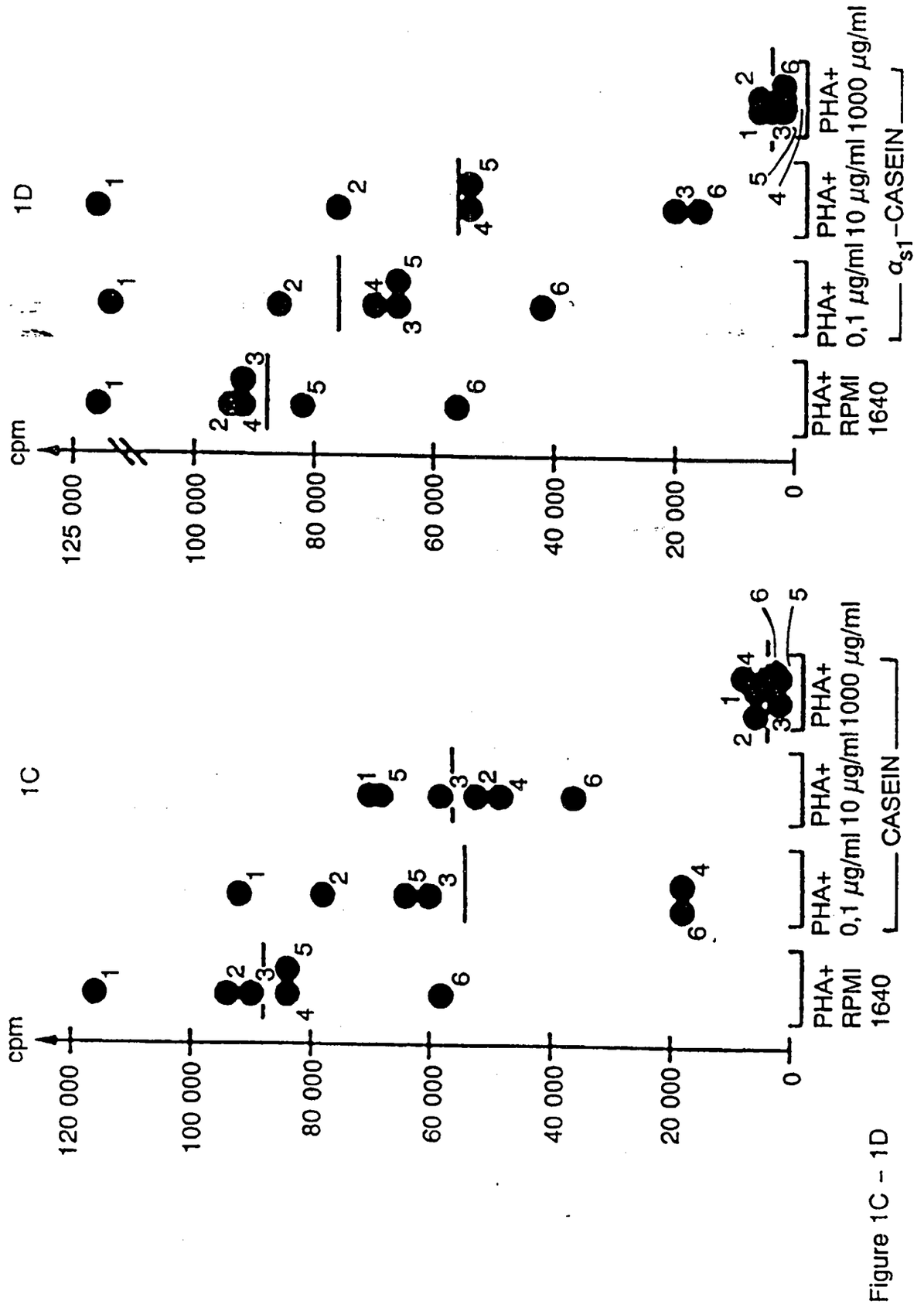


Figure 1C – 1D

BNSDOCID: <WO___9700078A1_I_>

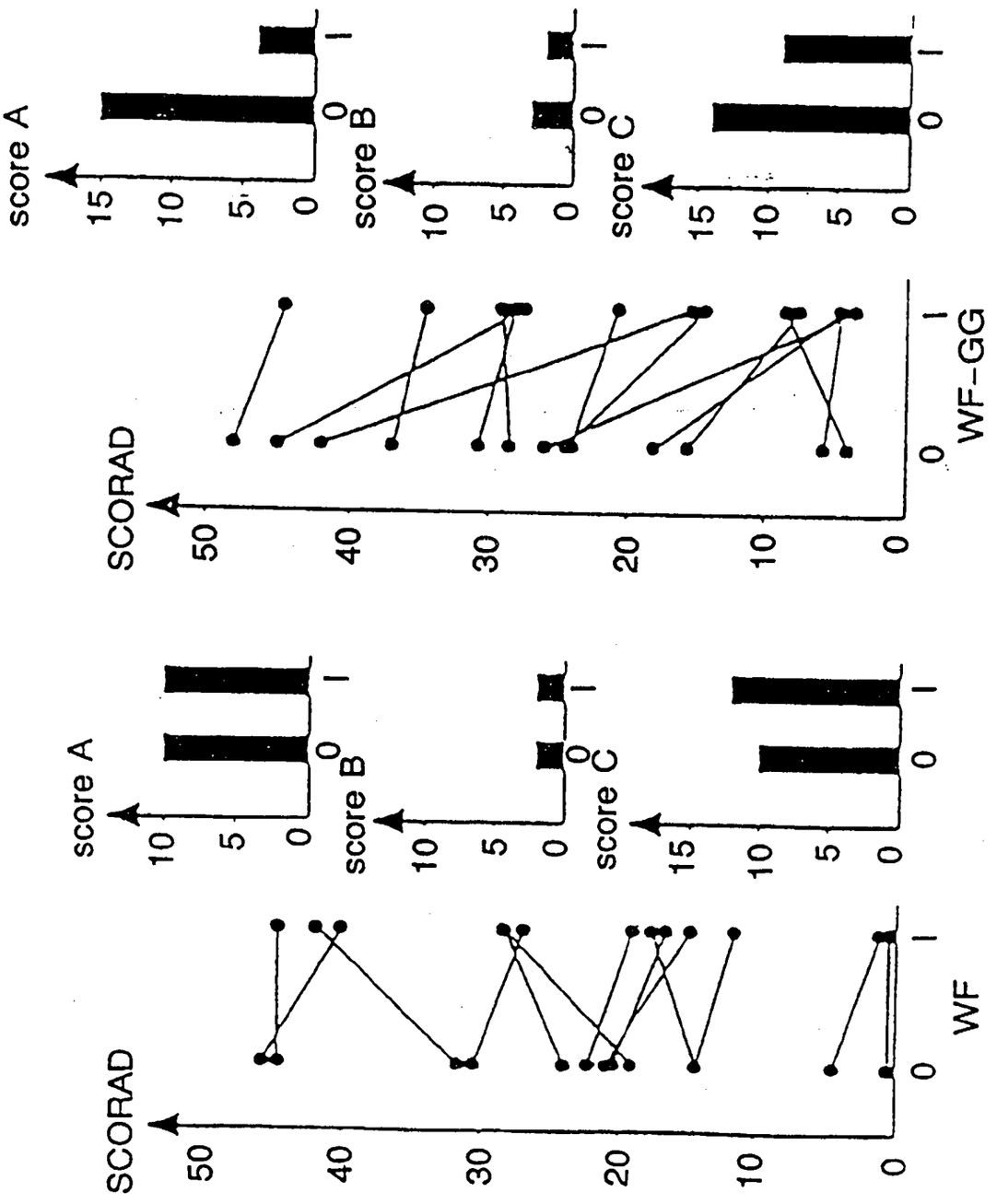


Figure 2

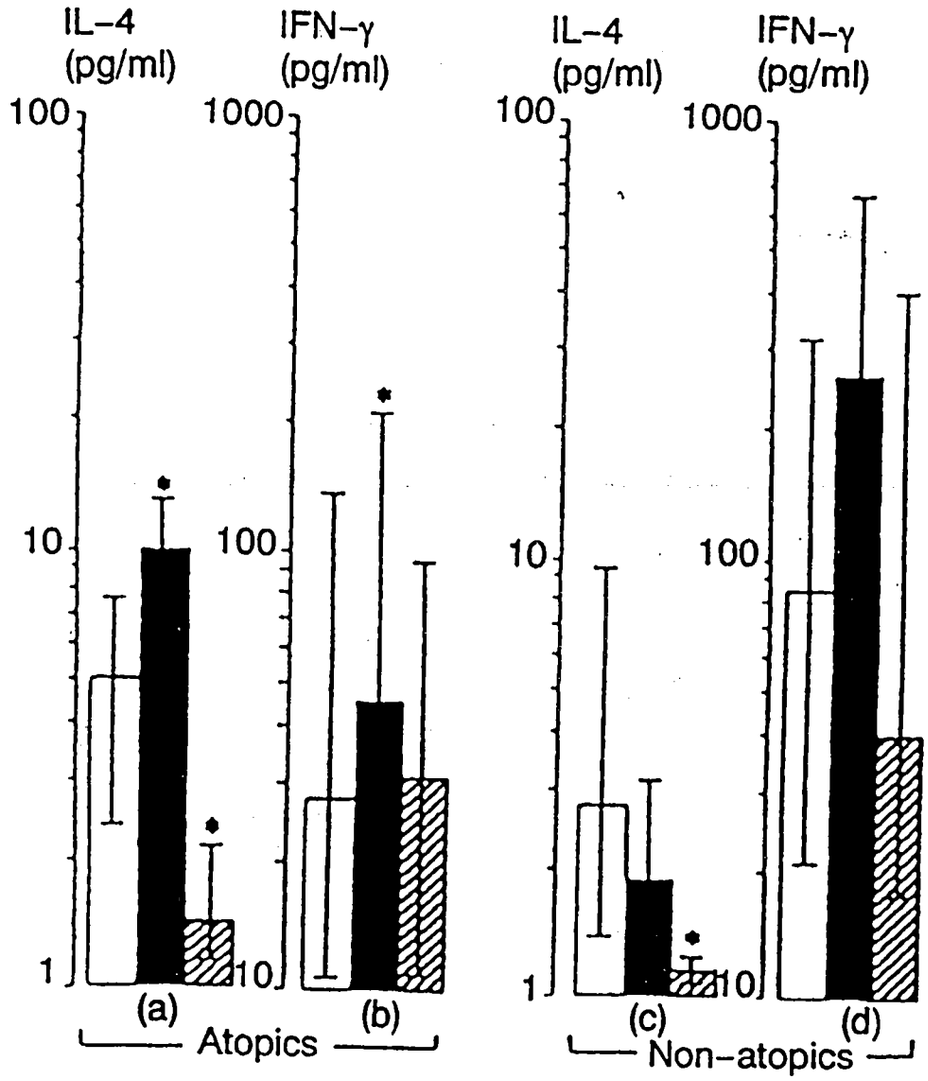


Figure 3

BNSDOCID: <WO___9700078A1_I_>

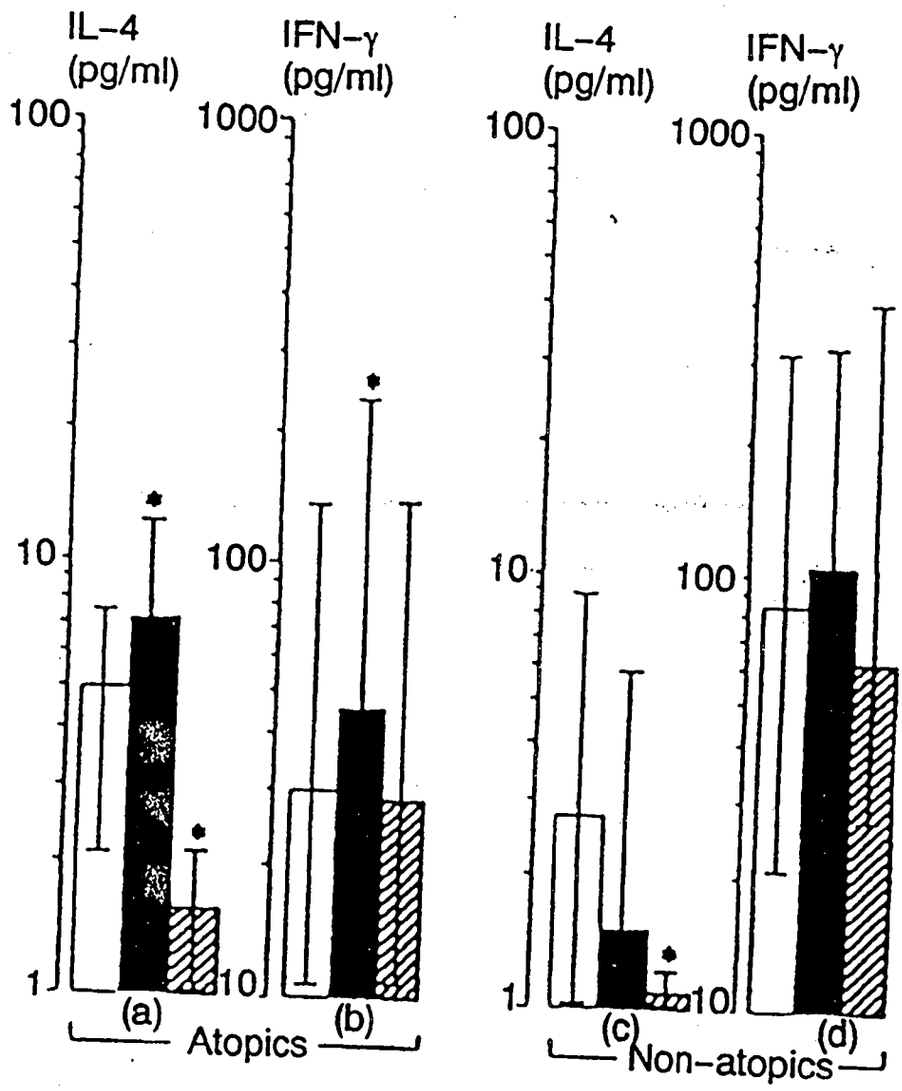

IL-4
(pg/ml)
IFN-γ
(pg/ml)
IL-4
(pg/ml)
IFN-γ
(pg/ml)
100
10
1
1000
100
10
100
10
1
1000
100
10
(a)
(b)
Atopics
(c)
(d)
Non-atopics

Figure 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 96/00350

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 38/01, A23J 3/34, A23C 9/123, A23C 9/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, A23J, A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, WPI, WPIL, FSTA, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 350,WPI, Dialog accession no. 001837412, WPI accession no. 77-58412y/33, Morinaga Milk KK: "Protein decomposition product prepn. - having no bitter taste and no antigen activity"; & JP,A,52079083, 770702, 7733 (Basic) --	1-12
X	DD 281540 A5 (VEB BERLIN-CHEMIE), 15 August 1990 (15.08.90), see the claims --	1-12

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 Sept 1996	18 -09- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

BNSDOCID: <WO___9700078A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 96/00350

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, file 351, WPIL, Dialog accession no. 010305519, WPI accession no. 95-206779/27, Wado Doctors Group KK et al: "Compsn. contg. live enteral bacteria as anti-allergic agent - useful for improving intestinal flora, for treating atopic dermatitis, allergic rhinitis etc."; & WO,A1,9514485, 950601, 9527 (Basic)	11-12
A	--	9-10
A	EP 0629350 A1 (SANDOZ NUTRITION LTD.), 21 December 1994 (21.12.94), page 3, line 1 - line 2, the claims	1-12
	--	
A	Dialog Information Services, file 155, Medline, Dialog accession no. 08759844, Medline accession no. 94074844, Isolauri E. et al: "Lactobacillus casei strain GG reverses increased intestinal permeability induced by cow milk in suckling rats", Gastroenterology (UNITED STATES) Dec 1993, 105 (6) p1643-50	1-12
	--	

INTERNATIONAL SEARCH REPORT

Int...ational application No.
PCT/FI96/00350

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 6-12
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 6-12 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition (c.f.PCT Rule 39.1(iv).

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
[ ] The additional search fees were accompanied by the applicant's protest.
[ ] No protest accompanied the payment of additional search fees.

46
BNSDOCID: <WO___9700078A1_I_>

INTERNATIONAL SEARCH REPORT

Information on patent family members

31/07/96

International application No.
PCT/FI 96/00350

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DD-A5- 281540	15/08/90	NONE	
EP-A1- 0629350	21/12/94	AU-A- 6471494	22/12/94
		CA-A- 2125793	17/12/94
		JP-A- 7101873	18/04/95

Form PCT/ISA/210 (patent family annex) (July 1992)

164
A02 63WO
D5

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A23J 3/34, A23L 1/305, A61K 38/01 — A1

(11) International Publication Number: **WO 00/42863**

(43) International Publication Date: 27 July 2000 (27.07.00)

(21) International Application Number: PCT/EP00/00334

(22) International Filing Date: 17 January 2000 (17.01.00)

(30) Priority Data:
99200130.5 19 January 1999 (19.01.99) EP

(71) Applicant *(for all designated States except US)*: SOCIETE DES PRODUITS NESTLE S.A. [CH/CH]; P.O. Box 353, CH-1800 Vevey (CH).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: FRITSCHE, Rodolphe [CH/CH]; Ch. de Chaumeny 48, CH-1814 La Tour-de-Peilz (CH). PECQUET, Sophie [FR/CH]; Chemin de Beau Val, 22, CH-1012 Lausanne (CH). BOVETTO, Lionel [FR/FR]; St. Thomas-Larringes, F-74500 Larringes (FR). MAYNARD, Françoise [FR/CH]; Leiserenweg 8, CH-3122 Kehrsatz (CH).

(74) Common Representative: SOCIETE DES PRODUITS NESTLE S.A.; Chautard, Cécile, P.O. Box 353, CH-1800 Vevey (CH).

(81) Designated States: AU, BR, CA, CN, ID, IL, IN, JP, KR, MX, NO, NZ, PL, RU, SG, TR, US, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A HYPOALLERGENIC COMPOSITION CONTAINING TOLEROGENIC PEPTIDES INDUCING ORAL TOLERANCE

(57) Abstract

A hypoallergenic composition for the induction of protein tolerance in at risk individuals of protein allergy, consisting of (i) a "non allergenic" protein extensively hydrolysed basis and/or of (ii) a free amino acid basis, said composition comprising as the active ingredient at least one tolerogenic peptide of the allergenic protein.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

A hypoallergenic composition containing tolerogenic peptides inducing oral tolerance

Field of the invention

This invention relates to a hypoallergenic composition containing specific tolerogenic peptides of proteins, said composition can induce oral tolerance to the native proteins. It also relates to the use of tolerogenic peptides of milk protein for the preparation of a composition inducing immulogical tolerance to milk proteins.

Background of the invention

During the common process of nutrition, dietary proteins are presented to the immune system via the gut, followed by an immune unresponsiveness to the ingested nutrients. This vital phenomenon, called oral tolerance, is efficient for the large majority of people. If oral tolerance fails, food allergy occurs, requiring the strict avoidance of the incriminated food.

Targeted food avoidance represents a hard task for adult patients with food allergy. For example, to strictly remove cow's milk from the diet of allergic infants might be even more difficult, especially if breastfeeding is not possible or desired.

The allergies to cow's milk and to the formulas containing cow's milk adapted to the needs of infants are due to the fact that the proteins of cow's milk differ from the proteins of mother's milk and can constitute allergens. Among whey proteins, β-lactoglobulin is the major component and is a strong allergen.

Besides breastfeeding, the primary recommendation for prevention, hypoallergenic formulae are systematically prescribed to "at risk" newborns, namely asymptomatic infants with atopic parents.

In contrast to adapted formulae, cow's milk proteins have been hydrolyzed in hypoallergenic formulae, to decrease the potential allergenicity. This approach has been demonstrated to be efficient in order to prevent sensitization by native proteins present in the adapted formulae.

Thus, in US 4,293,571 a protein hydrolysate was prepared by pancreatic hydrolysis, coagulation of non-hydrolysed proteins by a heat treatment and ultrafiltration to eliminate the coagulated residual proteins and the macropeptides

which could constitute allergens. Also, US 5,039,532 provides an improved process for the preparation of a hydrolysate of animal milk proteins substantially reduced in allergens, in which a whey product was subjected to enzymatic hydrolysis.

Two different types of hypoallergenic formulae are proposed to high-risk babies: partially and extensively hydrolyzed formulae, differentiated by the extent of hydrolysis of the native proteins.

But as is clear from the art cited above, the primary focus to date in dealing with allergies to cow's milk has been to find preparations which will not induce an allergic response, i.e. to provide non-allergenic formulations.

Nonetheless, while such formulations have permitted a person allergic to cow's milk to avoid an allergic response, they do not solve the problem which is to enable a person to drink unaltered milk products.

More recently, it has been investigated that partially hydrolysed milk formulations are not only of reduced allergenicity but can induce immunological tolerance to milk proteins. Extensively hydrolysed formulae are specially designed for treating patients allergic to cow's milk proteins but their ability to induced long-term oral tolerance is questioned.

Thus, EP 0 629 350 discloses the use of non-allergenic whey protein hydrolysates which are said to be capable of inducing cow's milk protein tolerance. Although this patent application indicates that whey protein hydrolysates substantially free of allergenic proteins could be used to induce cow's milk protein tolerance in children at risk of cow's milk allergy, the present inventors found on analysing other non-allergenic whey protein hydrolysates that non-allergenicity did not necessarily translate into the ability to induce cow's milk protein tolerance. Indeed, some of the formulations exhibiting the highest degree of non-allergenicity were found to be unsuitable for inducing cow's milk protein tolerance.

Thus, it is clear that the art lacked the information to provide formulae that would be the most suitable for tolerance induction.

Summary of the invention

This invention provides a hypoallergenic composition for the induction of protein tolerance in at risk individuals of protein allergy containing (i) a "non-

DNSDOCID: <WO_ 00:42863A1_I_>

allergenic" extensively hydrolysed proteins basis and/or (ii) a free amino acid basis, said composition comprising as the active ingredient at least one tolerogenic peptide of the allergenic protein.

In a preferred embodiment, said tolerogenic peptides are present in the form of (i) isolated tolerogenic peptidic fractions of hydrolysis of proteinaceous material containing the allergenic protein and/or (ii) synthetically prepared tolerogenic peptides.

This composition contains a source of nitrogen which may provide 7 to 25% of the total energy, a source of carbohydrates which may provide at least 28 to 66% of the total energy, a source of lipids which may provide at least 25 to 60% of the total energy and at least one tolerogenic peptide of the different proteins.

A major advantage of this composition is to induce oral tolerance in "at risk" individuals, in order to avoid eventual sensitization by use of native tolerogens. Moreover, the tolerogenic peptides derived from protein hydrolysis offer both hypoallergenic and tolerogenic properties and induce oral tolerance at the humoral and cellular levels.

This composition is particularly intended for individuals at risk of milk protein allergy.

Another aspect of the present invention is the use of tolerogenic peptides of milk proteins for the preparation of a hypoallergenic composition intended for mammals susceptible to cow's milk allergy.

In a prefered embodiment tolerogenic peptides are from milk origin and particularly from β-Lactoglobulin (β-LG), α-lactalbumin, bovin serum albumin or casein origin.

For the preparation of said composition, tolerogenic peptides may be used in the form of peptidic fraction containing the following peptides : H_2N-I-D-A-L-N-E-N-K-COOH, H_2N-V-L-V-L-D-T-D-Y-K,-K-COOH or H_2N-T-P-E-V-D-D-E-A-L-E-K-F-D-K-COOH from β-Lactoglobulin.

In another aspect, the invention provides a method for the preparation of tolerogenic peptides useful in the induction of protein tolerance in at risk individuals to protein allergy, wherein:

(i) a proteinaceous material containing the allergenic protein is hydrolysed to a degree of hydrolysis of about 10 to 50 %;

(ii) then treated to inactivate residual enzyme activity;

(iii) the protein hydrolysate solution is clarified and submitted to precipitation treatment or passed into a chromatography colum filled with appropriated resin to extract tolerogenic peptidic fractions.

Detailed description of the invention

In the present invention, the term tolerance is to be understood as a state of specific immunological unresponsivness. Both humoral (antibodies) and cell-mediated (lymphocyte ....) pathways of the immune response may be suppressed by tolerance induction. A breakdown of oral tolerance is considered to be the underlying cause of food allergy.

The term allergen is to be understood as a protein or macropeptide capable of initiating allergic reactions in humans, particularly at risk infants or nurslings. Infants are considered being "at risk" of protein allergy when either one, two parents or one sibling is atopic.

The term tolerogenic peptides is to be understood as proteic fragments, corresponding to parts of the native protein, sized from 200 to 6000 Da (3 to 50 amino acids), and preferably between 500 to 3000 Da and being able to induce specific oral tolerance to native proteins.

The term "non-allergenic" basis is to be understood as a nitrogen source containing a well-balanced amino-acids composition. The "non-allergenicity" is defined for milk proteins as residual allergenicity of individual whey proteins not exceeding 1 ppm and as residual allergenicity of total caseins not exceeding 10 ppm.

The hypoallergenic composition may contain as a source of nitrogens, peptides or free amino acids and particularly from milk proteins such as whey

NSDOCID: <WO_ 0042863A1_I_>

proteins, α-lactalbumin, β-lactoglobulin, bovine serum albumin, casein acid, caseinates, or α, β, κ-casein, for example. The source of nitrogen can provide at least 7 to 25 % of the total energy.

As a source of carbohydrates, lactose, saccharose, starch or maltodextrin may be used. Carbohydrates may provide at least 28 to 66 % of the total energy.

Vegetable oils or butter oil are preferably used as a source of lipids which may provide at least 25 to 60 % of the total energy.

Vitamins, oligoelements and minerals can be added in an amount sufficient to meet daily requirements.

The composition according to the present invention comprises as the active ingredient at least one tolerogenic peptide of the allergenic protein, said tolerogenic peptide has been selected for its ability to induce oral tolerance.

The tolerogenic peptides can be obtained by enzymatic hydrolysis of proteinaceous material containing the allergenic proteins that are responsible for allergies in at risk individuals, followed by isolation of tolerogenic peptidic fractions. These peptidic fractions enriched in said tolerogenic peptides can be obtained by separation of the protein hydrolysate. The tolerogenic peptides may also be present in the composition in the form of synthetically prepared tolerogenic peptides.

The composition contain an amount sufficient to induce oral tolerance which is preferably the one which allows a complete oral tolerance induction, namely the one which prevents from any reaction after DBPCFC (double blind placebo controlled food challenge) performed with cow's milk. Accordingly, tolerogenic peptides may be present in an amount of about 0.01% to 10% (nitrogen source of the protein), for example and preferably about 0.1 to 0.2 % of total peptides.

In the particular case of tolerance to milk proteins, the composition may contain tolerogenic peptides from milk origin such as β-lactoglobulin or caseins, for example. Tolerogenic peptides may thus be in the form of a peptidic fraction comprising at least one of the following peptides : H_2N-I-D-A-L-N-E-N-K-COOH,

H_2N-V-L-V-L-D-T-D-Y-K,-K-COOH or H_2N-T-P-E-V-D-D-E-A-L-E-K-F-D-K-COOH from β-lactoglobulin.

In a preferred embodiment, a method for the preparation of tolerogenic peptides comprises the following steps:

(i) a proteinaceous material containing the allergenic protein is hydrolysed to a degree of hydrolysis of about 10 to 50 %;

(ii) then treated to inactivate residual enzyme activity;

(iii) the protein hydrolysate solution is clarified and submitted to precipitation treatment or passed into a chromatography colum filled with appropriated resin and tolerogenic peptidic fractions are recovered.

This preferred method is well suited to treatment of hydrolysates prepared from various protein concentration (Ntot %= N * 6.38) for modifying the ratio of tolerogenic activity by residual antigenicity from proteinaceous material. If one defines arbitrarily the antigenicity of a native protein to be 10^6 (as 10^6 µg/g of protein), and the tolerogenic response to be 1, then, for a native protein, this ratio is 10^{-6}. Therefore, the ratio qualifying the tolerogenic activity of one given fraction or tolerogenic peptide should be at least 2×10^{-2}.

The proteinaceous material to be treated may be any composition containing protein material and in particular solution or dispersion of milk proteins : whey proteins, acid whey protein, sweet whey proteins, whey protein concentrates, whey protein isolate, demineralized whey powder or caseinates, for example.

In general, the protein content may vary within the range of about 70 to 95% by weight but the starting material is preferably as rich in protein as possible.

The proteins present in the proteinaceous material can be modified with proteolytic enzymes into protein hydrolysate having a degree of hydrolysis (α-amino-N/Ntot) of preferably about 10-50 %.

The proteolytic enzymes may be for example, from animal or vegetable origins (pepsin, chymotrypsin, trypsin, intestinal mucosa extract, pancreatic extracts, chymosin, papaïn, bromelaïn, ficin), bacterial or fungi origins (serine and metalloproteases from *Bacillus subtilis, Bacillus licheniformis, Aspergillus orysae,*

3NSDOCID: <WC___0042863A1_I_>

Aspegillus wentii and acidic proteases from *Aspergillus orizae, Aspergillus wentii, Mucor miehei, Mucor pusillus, Endothia parasitica)* or a combination of these.

During hydrolysis, concentration of proteinaceous material in solution or in suspension is preferably around 5-20% by weight and could be pasteurised before introducing proteases. The ratio enzyme/protein may be 0.1-10% weight/weight and preferably of about 0.25 to 4%.

Hydrolysis may be conducted at a temperature of about 35°C to 65°C, during 30 minutes to 10 hours, preferably 30 min to 4 hours at pH values within the range 2.5 to 11, preferably 4.5, 7.0, 8.0, and 8.5. If desired the pH of the solution can be adjusted and regulated with citric acid, food grade HCl or NaOH, NH_4OH, KOH, Ca $(OH)_2$ for instance at a concentration of 2N pure or in blend.

Then, the protein hydrolysate may be submitted to a heat treatment of about 0.1 to 10 min at a temperature of about 70 to 110°C to inactivate residual enzymes (i.e. proteases).

The protein hydrolysate solution thus obtained can be clarified by centrifugation and/or ultrafiltration to remove insoluble and intact proteins respectively, and the clear solution is recovered. It is possible to use at industrial scale different type of membranes (spiral, tubular, flat, allow fibbers) made with different materials (minerals, polysulfone, ...) and having different cut off limits between 1.000 and 100.000 Daltons. Depending on the type of enzyme, the hydrolysis conditions and the type of membranes the modification of the tolerogenic fractions might be sufficient at this step.

The recovered clear hydrolysate solution can, if desired, be concentrated by evaporation to a dry solid content of 10-50% for a subsequent treatment or spray dried if enrichment in tolerogenic peptides is sufficient.

The protein hydrolysate solution thus obtained can be submitted to precipitation treatment by solvent, acid, or salts, for example, followed by a centrifugation. In the precipitation treatment, concentration of hydrolysate solution increases the yield and reduces the quantities of solvent. For example, ethanol may be added to obtain a final concentration within 15-60% volume/volume at a

temperature of about 4°C to 25°C. After one hour of incubation, a centrifugation (30 min at 4500 g) may allow to separate soluble and insoluble peptides. Depending on the proteolysate we can use acid (phosphoric or chlorhydric, for example) or phospho-calcic precipitation. Then, solvents can be removed by evaporation and salts by electrodialysis for instance.

The clear solution and the insoluble fraction are preferably recovered.

The protein hydrolysate solution thus obtained may be passed into a column filled with adsorption, ion exchange or hydrophobic resin at a flow rate of 0.1-4 column volumes per hour at a temperature of about 4°C to 60°C. Before the chromatography treatment, the protein hydrolysate can be concentrated to provide a solution having a dry solid content of 8-35% by weight.

During chromatography, a fraction of peptide is absorbed into the resin by passing the hydrolysate solution into a column filled with the convenient support at a rate of 0.1-4 column volumes per hour. It is possible to use at industrial scale the different types of chromatography as: ions exchange, hydrophobic interactions, reverse phases, adsorption (hydroxyapatite, active charcoal, polystyrene base hydrophobic resins...) or covalent chromatography, for example.

In the chromatography treatment, the amount of hydrolysate solution per litre of resin filled column can be as high as 5 litres with the respect to dry solids of 10%. Preferably, a hydrolysate solution having 20-1000g of dry solid per litre of resin is passed into the resin filled column. The chromatography treatment may be carried out at a pH of about 2 to 10 preferably 6-8, for the clarified hydrolysate solution. The chromatography treatment can be conducted at a temperature of about 4°C to 60°C.

For example, the chromatography treatment to select tolerogenic fractions from β-lactoglobulin may consist in using:

- a strong cationic resin equilibrated with 0.1 N HCl at a flow rate of 1volume/hour. The non-retained fraction was eluted with 3 volumes of water, the second fraction (fraction containing tolerogenic peptides) was eluted with 0-0.5 N NaOH, and the third fraction was eluted with 0.1 N HCl.

3NSDOCID <WO 0042863A1_i_>

- a reverse phase (C18) resin equilibrated with pure water. Non retained fraction was eluted with water, then step by step (20% and 40% of ethanol) the second and the third were recovered.
- a strong anionic resin equilibrated with 0.1N NaOH. Non retained fraction was eluted by 3 volumes of water. The second fraction was eluted with 0.5N HCl, the third one with 0.1 N NaOH.

The most preferred method is to treat with resin a neutral solution, in that case, no pH adjustment is required-after hydrolysis step and the salt content of the product will be lower .

To conclude the chromatography treatment, the column can be eluted with pure water, then water containing salts, buffer, acids, bases, or organic solvents at a temperature of 4-60°C. Elution is realised step by step or by a gradient of concentration. The solutions that have passed through the column are recovered. If necessary, salts, solvents, acids, bases, are removed from the recovered solution, and the recovered solutions can be concentrated to a dry solids content of 35-65% and spray dried.

These peptides are then specific fragments corresponding to a part of the native protein sequence or to a part of the specific tryptic peptides of hydrolysed protein.

These tolerogenic peptides can be used for the preparation of a composition inducing oral tolerance to native proteins, said composition is intended for mammals susceptible to protein allergy and particularly human and pets.

The following examples are given by way of illustration only and in no way should be construed as limiting the subject matter of the present application.

Figures

Figure 1 represents specific oral tolerance induction in mice fed once with either TTH β-LG, F2 or F(7+9). A: Humoral (dark bars) and mucosal (gray bars) anti-β-LG IgE response. B: β–LG-specific DTH response (•) are individual foot pad increments and (-) are means from 10 mice per group. C: Specific (β-LG) and non

specific (PHA) proliferative responses of splenic cells. Splenic lymphocytes from mice fed with either saline (♦), TTH β-LG (■), F2 (▲), or F (7+9) (•) were isolated and subsequently stimulated with decreasing concentrations of antigens. ^{3}H thymidine incorporation was measured after 120 hours of culture. (^{3}H)Tdr incorporation results were expressed in cpm, as a mean of triplicate cultures, the blank-subtracted mean was then plotted respectively against β-LG or PHA concentration.

Figure 2 represents primary sequences assigned to tryptic peptides identified in β-Lactoglobulin hydrolyzate. Vertical bar represents disulfide bond.

Examples

Example 1: Selection and characterization of the tolerogenic peptides of β-Lactoglobulin

In the first following exemple, experiments were performed to select the tolerogenic peptides of β-LG.

β-LG was digested by TPCK treated trypsin in conditions selected to provoke its mild digestion. The size and the molecular weight distribution of the resulting peptides from our β-LG hydrolysis model, respectively ranged from two to twenty-three aminoacids, and from 247 to 2719 Da. The tolerogenic properties of the resulting peptides have been assayed in an oral tolerance experimental mouse model

Methods

a) Animals

Female Balb/c mice were obtained from IFFA-Crédo (L'Abresle, France). They were all bred and raised on a milk free diet. The mice were 3 weeks old at the start of the experiments.

b) Preparation of tolerogenic fractions

RNSDOCID: <WO 0042863A1_I_>

In order to be digested, β-LG (220 grams) was dissolved in bi-distilled water at 5% (w/w) final concentration. The β-LG was digested by TPCK treated trypsin, using an Enzyme/Substrate (E/S) ratio of 1/100(w/w) at 40°C, pH 7.6 under constant stirring. After one hour hydrolysis, same amount of enzyme was added to give a 2/100 (w/w) final E/S ratio. After 4 hours hydrolysis, the reaction was stopped by inactivation of trypsin at 85°C for 5min. Total tryptic hydrolysate of β-lactoglobulin (TTH β-LG) was then lyophilized. The digested β-LG products were separated by preparative chromatography on a cationic resin.

15 different fractions were obtained, individually nanofiltered to concentrate and to eliminate salts, diafiltered, dialyzed, lyophilized and dry stored at room temperature until *in vivo* experiments.

Each fraction was further characterized by its peptide content using reverse phase High Performance Liquid Chromatography. Considering TTH β-LG as reference, enrichment and impoverishment of peptides in each fraction was appreciated by area detected at 214 nm using iso nitrogen injections.

c) Oral tolerance induction and immunization procedure

Gavages were orally administered to the mice at age 22 days, by gastric feeding of native β-LG (5 mg/g of body weight), various amounts of TTH β-LG, or various amounts of the different β-LG peptidic fractions. Control mice were fed saline water. 5 days later, all mice were immunized β-LG and OVA (Ovalbumin, gradeV, Sigma), as a non-related antigen for testing the specificity of the immune response, 21 days after systemic challenge, a Delayed Type Hypersensitivity (DTH) evaluation was done by duplicate thickness measures of the left, rear footpad prior to, and following, immunization with β-LG.

d) Evaluation of the immune response

24 hours after DTH immunization, individual increases in foot pad thickness of those injected the day before were measured. Differences between these two measurements were expressed in Δ thickness (millimeters) and used in group comparisons. Then, from all mice, blood samples were obtained, spleens were taken and pooled according to group of treatment. Splenocyte specific proliferation assays

were performed for each group. Intestinal contents were individually collected. Serum and intestinal samples were rapidly frozen at -80°C, until assays. Specific IgE against β-LG and against OVA were determined both in seric and intestinal samples.

e) Specific IgE antibody assays

Serum and intestinal fluid dilutions were assayed in duplicate for anti-β-LG and anti-OVA IgE antibodies by ELISA. Pooled samples from twenty non-immunized female mice were used as negative controls in each plates. Titers were determined by calculating the dilution of the sample which gave twice the absorbance of the negative control. Titers were expressed as the $\log_{10}$ of the reciprocal of the dilution.

f) Cell cultures

Spleen cell solutions were homogenized and purified. Cells were cocultured in the presence of β-LG or of phytohaemagglutinin A. (^{3}H)Tdr (Amersham, Zurich) was added in the final 6 hours of culture and the plates were harvested and analyzed by scintillation counting. Stimulation indices were calculated as the ratio of blank-subtracted test and control values expressed as the mean cpm (^{3}H)Tdr incorporation by triplicate cultures.

g) Determination of β-LG IgG binding capacity

An ELISA inhibition was used to determine β-LG epitopes in β-LG hydrolysate and in β-LG fractions. β-lactoglobulin titers were calculated from a 5-fold dilution β-LG standard curve run on each plate.

DTH responses, seric and intestinal IgE responses were compared using ANOVA single factor tests.

Results

a) Characterization of fractions obtained from TTH β-LG

BNSDOCID <WO 0042863A1_1_>

In order to isolate the tolerogenic peptides from the TTH β-LG, large amounts of β-LG tryptic fractions have been produced by preparative chromatography. From each of them, 15 fractions (F1-F15) were collected and respectively pooled. The assigned sequences of β-LG tryptic peptides have been previously determined (fig.2).

- No specific peptide enrichment could be detected in fractions F1, F5 and F15.
- 3 different peptides T6, T17, and T18present in fraction F2 were enriched:, but were not concentrated in any of the other fractions.
- Similarly, T7 was specifically enriched in fraction F6.

Apart from these peptides, concentrated in only one given fraction, some others were enriched in several fractions. This was the case for T21, for instance, clearly detected at high rates in fractions F7, F9, and F10 (Table 1).

Fractions	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**
Masses (g)	52	13.9	11	11.8	2.57	1.94	4.37
Significantly enriched peptides	-	T6 T17 T18	T23	T23	-	T7 T9	T20 T21 T24
F8	**F9**	**F10**	**F11**	**F12**	**F13**	**F14**	**F15**
13.1	5.01	6.53	1.05	3.46	1.9	2.67	28.8
T9 T12 T24	T12 T21	T10 T21	T11	T10	T10 T11	T10 T11	-

Table 1: β-LG fraction masses and significant peptide enrichment within fractions.

b) Two different fractions of the β-LG tryptic hydrolysate presented tolerogenic properties:

Fractions were pooled according to their enriched peptides similarities and in proportion to their respective masses, and then tested *in vivo*.

- F1, F2, F5, and F15 were considered to be fed individually because they did not share peptide enrichment with the others fractions.
- F3 and F4 were pooled based on high concentration of T23;
- F8 and F9 were mixed to test the tolerogenic activity of T12;
- F7 and F9 were pooled according to enrichment of T20 and T21;
- F6, F11, F12, F13, and F14 were pooled together, each enriched in T9, T10, and T11.

Qualitative responses of the tolerogenic properties generated from feeding the different fraction groups at two different concentrations (0.5 or 0.125 mg/g of body weight) were evaluated. Induction or absence of induction of tolerogenic responses to β-LG has been determined by the statistical significance of the immune response.

Two of the eight tested fractions appeared to be active both in reducing seric anti-β-LG IgE responses and β-LG specific splenocyte proliferation:

- F2, when given orally at 0.125 mg/g of body weight,
- F(7+9), at both tested doses.

c) **Humoral, mucosal and cellular tolerance induced by oral administration of β-LG tryptic fractions F2 and F(7+9)**

According to our quantitative criteria, seric and intestinal anti-β-LG IgE responses, DTH, and specific splenocyte proliferation, oral tolerance has been induced in mice fed either with F2 or with F(7+9) (Fig. 1).

For the two fraction fed groups, the seric anti-β-LG IgE titers, 3.58 +/- 0.18 and 3.21 +/- 0.29 respectively for F2 and F(7+9), were not as low as when induced by TTH β-LG feeding (2.68 +/- 0.05) (Fig 1A). However, specific levels of IgE were significantly lower than titers of control mice (4.16 +/- 0.05)($p<0.05$). Specific intestinal IgE was also decreased in mice fed TTH β-LG (1.66 +/- 0.32) or fed the two β-LG tryptic fractions (1.1 +/- 0.34 and 1.9 +/- 0.21 respectively for F2 and F(7+9)), assessing the orally induced down-regulation compared to the control group (2.32 +/- 0.64). Fig. 1B shows that specific DTH was clearly reduced in mice fed with either TTH β-LG (0.074 mm) or each of the two tolerogenic fractions (0.063 mm and 0.062 mm respectively for F2 and F(7+9)) in comparison to local hypersensitivity measured in control mice fed only with saline (0.164 mm).

SDOCID <WO__0042863A1_I_>

To confirm the achieved induction of oral tolerance on cellular immunity, specific and non-specific splenocyte proliferation assays were performed. Fig. 1C and D show that feeding mice either of the two elected β-LG fractions as well as TTH β-LG provoked a severe decrease of the proliferative response to β-LG in comparison to the one observed from splenocytes isolated from saline fed control mice. Indeed, stimulation indices were 0.225, 0.174, and 0.341, respectively for TTH β-LG, F2, and F(7+9) fed mice. As expected, the non-specific proliferative response due to PHA stimulation was not affected by the different feeding regimen.

d) Antigenicity of the β-LG hydrolysate and β-LG fractions

Measurement of residual β-LG epitopes performed for the different fractions by ELISA inhibition showed that amounts of β-LG epitopes present per g of proteins were highly different from one fraction to another, ranging from 15 µg/g of protein for F1 up to 9240 µg/g of protein for F13. These results also indicate that antigenic and tolerogenic sites might be distinctly located, suggesting that allergenicity and tolerogenicity could be uncoupled. Schematically, three different type of fractions can be described :

- a first type, represented by fraction F2, including basic peptides with a high tolerogenic potential associated to a very low antigenicity,
- at the other end, an F13 type fraction containing acidic peptides, highly antigenic and devoid of tolerogenic activity and, finally,
- in a median position, the peptides present in fractions F7 and F9 inducers of tolerance but still having notable antigenicity.

Fractions F2 and the mixture F(7+9) were both tolerogenic. Antigenicity of the tolerogenic fractions F2, F7 and F9 were lower than that of the total hydrolysate. Remarkably, the antigenicity of the tolerogenic F2 was found to be 53 times lower than the antigenicity of TTH β-LG. In these two tolerogenic fractions, the sizes of the potentially tolerogenic peptides were distributed between 8 amino acids for T6, and 23 amino acids for T21, given respective molecular weights between 915 and 2719 Da.

To be tolerogenic, peptides seem to require a precise balance between dose, size, sequence and structure. However, until now, correlation between peptide structure and tolerogenic properties has not been described in the literature.

Example 2 : Obtention of tolerogenic peptides from whey protein

In order to obtain tolerogenic peptides, 1 kg of whey protein isolate was dissolved in 7 litres of water at 50°C. The pH was adjusted at 7.5 with 2N KOH. The volume was adjusted to 14 litres with water at 50°C to obtain a 7% solution with respect of powder.

10 g of enzyme Trypsin+Chymotrypsin were added and the solution was allowed to hydrolysis during 120 min. The pH was maintained with an alkaline solution of 2N KOH and 2N NaOH. After the hydrolysis, the solution was heated to 80°C during 10 min. The solution was cooled to 40°C and ultrafiltred with 40,000 Da cut off membranes.

The clear permeate was treated by cantionic resin conducted as followed in a final resine volume of about 180 ml. The resin was washed and equilibrated with 0.1N NaOH at a flow rate of 300 ml/h. 100 ml of clear permeate 40,000 Da were passed into the column. The non retained fraction was eluted by 540 ml of pure water. The second and the third were eluted by 900 ml of 0.5N HCl and 540 ml of 0.1N NaOH respectively.

The tolerogenic peptides are present in fraction F2 for example, in an amount sufficient to prevent from any reaction after DBPCFC performed with cow's milk.

Example 3 : obtention of tolerogenic peptides from caseinates

1 kg of natrium caseinate was dissolved in 7 litres of water at 50°C. The pH was adjusted at 7.5 with 2N KOH. The volume was adjusted to 10 litres with water at 50°C to obtain a 10% solution with respect of powder.

40 g of Alcalase enzyme were added and the solution was allowed to hydrolysis during 120 min. The pH was maintained with an alkaline solution of 2N KOH and 2N NaOH. Subsequent to the hydrolysis, the solution was heated to 80°C during 10 min. The solution was cooled to 40°C and ultrafiltred with 10,000 Da cut off polyethylsulfone membranes.

SUBSTITUTE SHEET (RULE 26)

Reverse phase chromatography: 300 ml of the clear permeate were passed into the column equilibrated at a flow rate of 3 ml / min. The non retained fraction was eluted with 300 ml of water, then the second fraction was eluted with 200 ml of ethanol gradient 0-20%. Finally the third fraction was eluted with 200 ml of ethanol gradient 20-40 %. The column was regenerated by 400 ml gradient ethanol 40-80 % v/v and equilibrated with 500 ml of pure water.

Example 4

The procedure was as described in example 3, except that the enzyme quantity and membrane cut off were changed by Alcalase 20 g and 40,000 Da respectively. The subsequent chromatography on cationic resin was conducted as followed : 150 ml of resin were washed 3 times by 500 ml of pure water then poured in a column which final resin volume was 180 ml. The resin was washed and equilibrated by 0.1N NaOH at a flow rate of 300 ml/h. 100 ml of clear permeate 40,000 Da were passed into the column at a flow rate of 300 ml/h. The non retained fraction was eluted by 540 ml of pure water. The second and the third ones were eluted by 900 ml of 0.5N HCl and 540 ml of 0.1N NaOH respectively.

Example 5

The procedure was as described in example 3, except that the reverse phase treatment was changed by a chromatography on a strong anionic resin as followed: 150 g of resin were washed 3 times by 500 ml of pure water, then poured in a column where the resin was washed and equilibrated with 0.1N HCl at a flow rate of 180 ml/h. 100 ml of clear ultra filtrate were passed into the column at a flow rate of 180 ml / h. The non retained fraction was eluted with 540 ml of water. The second and the third ones were eluted by 3 column volumes of 0.5N NaOH and 5 column volumes of 0.1N HCl respectively.

Example 6: Tolerogenic infant formula

The tolerogenic peptides T6 and T17 contained in tolerogenic fraction F2 were synthetically prepared. T13 contained in a non tolerogenic fraction was also synthetized as a negative control. Oral tolerance induction of these petides were

161

tested in mice as described in example 1c) except that the dose is of 20 mcg/g of body weight.

Results :

Peptides	tolerized anti-β-LG IgE / control	Stimulation index (rate)	Stimulation index (GLM)
T17	2.67/3.39	0.305	0.405
T6	3.24/3.39	0.53	0.285
T6-T17(linked)	3.40/3.39	0.92	0.81
T13 (negative control)	3.20/3.39	1.7	0.87

The oral administration of T6 or T17 induces a cellular tolerance (specific inhibition of lymphocyte proliferation). T17 also induces a humoral tolerance by significantly decreasing specific anti-b-LG IgE.

The oral administration of T13 (negative control) do not induce any tolerance.

These two peptides T6 (H_2N-I-D-A-L-N-E-N-K-COOH) and T17 (H_2N-V-L-V-L-D-T-D-Y-K,-K-COOH) have a high tolerogenic potential associated to a very low antigenicity. They can be used in food or pharmaceutical composition for inducing a protein tolerance in at risk individuals of protein allergy.

Example 7: Tolerogenic infant formula

The composition for 100 g of powder contains 12.5 % of peptides (synthetized peptides T6 or T17 or tolerogenic peptides as prepared in example 2, represent about 0.1 to 0.2% of total peptides), 26 % of fats, 56 % carbohydrates (including lactose 39 %, maltodextrin 11 %, starch 6 %), traces of vitamins and oligoelements to meet daily requirements, 2.5 % minerals and 3 % moisture.

13 g of this powder is mixed in 100 ml of water. The obtained composition is an infant formula particularly intended for at risk infant to cow's milk allergy.

INSDOCID: <WO_ 0042863A1_I_>

Example 8: tolerogenic infant formula

In order to obtain a tolerogenic infant formula, we prepare the following mixture containing for 100 ml of formula, 1.6 % peptides (tolerogenic peptides as prepared in example 2, represent about 0.1 to 0.2 % of total peptides), 3.4 % fat, 7.4 % carbohydrates (including lactose 5.2 %, maltodextrin 1.4 %, starch 0.8 %), traces of vitamins and oligoelements to meet daily requirements, 0.3 % of minerals and 79.9 % of water.

Exemple 9:

In order to obtain a tolerogenic preparation of a small volume, we prepare the following mixture containing for 30ml of preparation, from about 0.003% to about 0.015% of tolerogenic peptides (tolerogenic peptides as prepared in example 2, represent 100% of total peptides), 7.4% of carbohydrates (including maltodextrin 1.4% and saccharose 6%), 92.5% of water and aroma. In order to induce oral tolerance, this preparation is to be administered 1 to 5 times daily , to newborns « at risk » of cow's milk allergy either fed hypoallergenic formula or breastfed.

Claims

1. A hypoallergenic composition for the induction of protein tolerance in at risk individuals of protein allergy, containing (i) a "non allergenic" protein material extensively hydrolysed basis and/or of (ii) a free amino acid basis, said composition comprising as the active ingredient at least one tolerogenic peptide of the allergenic protein.

2. A composition according to claim 1, wherein said tolerogenic peptides are present in the form of (i) isolated tolerogenic peptidic fractions of the hydrolysis of proteinaceous material containing the allergenic protein and/or (ii) synthetically prepared tolerogenic peptides.

3. A composition according to any of claims 1 to 2, which contains tolerogenic peptides or selected tolerogenic peptidic fractions of hydrolysis in such an amount that the ratio of tolerogenic activity by residual antigenicity is at least of about 2×10^{-2}.

4. A composition according to any of claims 1 to 3, which contains an amount of tolerogenic peptide of about 0.01 % to 10%, and preferably about 0.1 % to 0.2 % of the total nitrogen source of the proteins in the composition.

5. A composition according to any of claims 1 to 4, intended for mammals susceptible to cow's milk allergy, which contains a peptidic fraction comprising H_2N-I-D-A-L-N-E-N-K-COOH, H_2N-V-L-V-L-D-T-D-Y-K,-K-COOH or H_2N-T-P-E-V-D-D-E-A-L-E-K-F-D-K-COOH from β-lactoglobulin.

6. A composition according to any of claims 1 to 5, which contains a source of nitrogen providing 7 to 25% of the total energy, a source of carbohydrates which provides 28 to 66% of the total energy, a source of lipids which provides 25 to 60% of the total energy, minerals and vitamins to meet daily requirements and at least one of said tolerogenic peptides that is added in an efficient quantity so as to induce oral tolerance.

7. The use of tolerogenic peptide of milk protein selected for its ability to induce oral tolerance to milk proteins, for the preparation of a hypoallergenic composition intended for mammals susceptible to cow's milk allergy.

8. The use according to claim 6 wherein tolerogenic peptide is from milk origin and particularly from β-lactoglobulin, α-lactalbumin, bovin serum albumin or casein origin.

9. The use according to any of claims 6 to 7, wherein tolerogenic peptide comprises H_2N-I-D-A-L-N-E-N-K-COOH, H_2N-V-L-V-L-D-T-D-Y-K,-K-COOH or H_2N-T-P-E-V-D-D-E-A-L-E-K-F-D-K-COOH from β-lactoglobulin.

10. The use of tolerogenic peptide according to any of claims 6 to 8 in an amount of about at least 0.01% to 10% (Nitrogen source of the protein).

11. A method of preparation of tolerogenic peptide useful in the induction of protein tolerance in at risk individuals to protein allergy, wherein :
    (i) a proteinaceous material containing the allergenic protein is hydrolysed to a degree of hydrolysis of about 10 to 50 %;
    (ii) then treated to inactivate residual enzyme activity;
    (iii) the protein hydrolysate solution is clarified and submitted to precipitation treatment or passed into a chromatography colum filled with appropriated resin to extract tolerogenic peptidic fractions

12. A method according to claim 11, wherein tolerogenic peptidic fractions are concentrated to a dry solids content of about 35-65 % and spray dried.

171

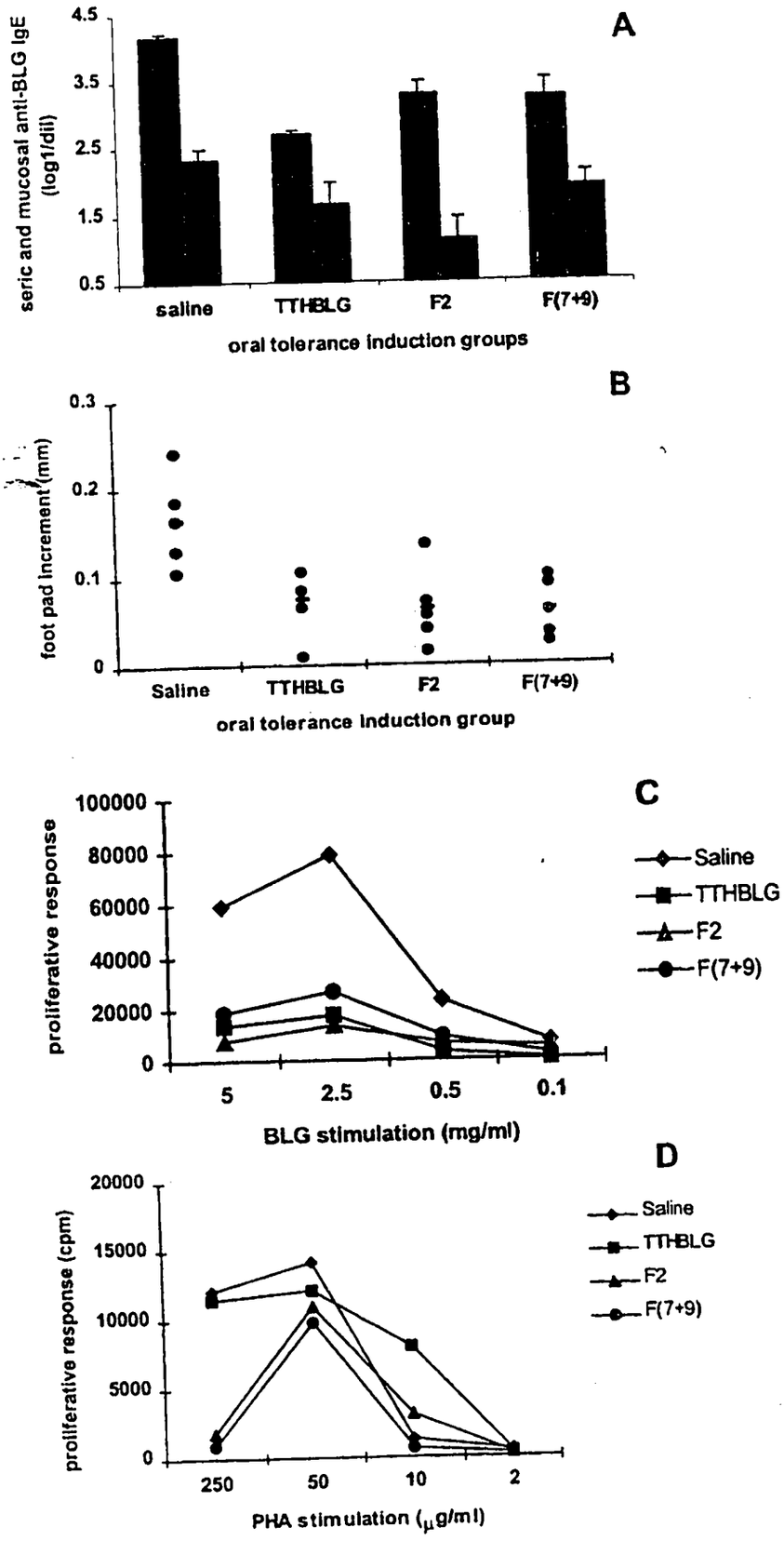

A
seric and mucosal anti-BLG IgE (log1/dil)
4.5
3.5
2.5
1.5
0.5
saline
TTHBLG
F2
F(7+9)
oral tolerance induction groups
B
foot pad increment (mm)
0.3
0.2
0.1
0
Saline
TTHBLG
F2
F(7+9)
oral tolerance induction group
C
proliferative response
100000
80000
60000
40000
20000
0
5
2.5
0.5
0.1
BLG stimulation (mg/ml)
Saline
TTHBLG
F2
F(7+9)
D
proliferative response (cpm)
20000
15000
10000
5000
0
250
50
10
2
PHA stimulation (µg/ml)
Saline
TTHBLG
F2
F(7+9)

Figure 1

Peptide T1 H_2N-A-L-K-COOH (139-141)

Peptide T2 H_2N-H-I-R-COOH (146-148)

Peptide T3 H_2N-F-D-K-COOH (136-138)

Peptide T4 H_2N-I-I-A-E-K-COOH (71-75)

Peptide T5 H_2N-E-N-G-E-C-A-Q-K-COOH (62-69)

Peptide T6 H_2N-I-D-A-L-N-E-N-K-COOH (84-91)

Peptide T7 H_2N-G-L-D-I-Q-K-COOH (9-14)

Peptide T8 H_2N-A-L-P-M-COOH (142-145)

Peptide T9, T10, T11 H_2N-W-E-N-G-E-C-A,-Q,-K,-COOH (61-67,-68,-69)

Peptide T12 H_2N-T-P-E-V-D-D-E-A-L-E-K-COOH (125-135)

Peptide T13 H_2N-L-I-V-T-Q-T-M-K-COOH (1-8)

Peptide T14 H_2N-A-L-P-M-H-I-R-COOH (142-148) = T8 (142-145) + T2 (146-148)

Peptide T15 H_2N-I-P-A-V-F-K-COOH (78-82)

Peptide T16 H_2N-V-A-G-T-W-Y-COOH (15-20)

Peptide T17 H_2N-V-L-V-L-D-T-D-Y-K,-K-COOH (92-99,100)

Peptide T18 H_2N-T-P-E-V-D-D-E-A-L-E-K-F-D-K-COOH (125-138) = T12 (125-135) + T3 (136-138)

Peptide T19 H_2N-A-A-S-D-I-S-L-L-D-A-Q-S-A-P-L-R-COOH (25-40)

Peptide T20, T21 (61, 62-69):S-S:(149-162)

H_2N-W,-E-N-G-E-C-A-Q-K-COOH
|
H_2N-L-S-F-N-P-T-Q-L-E-E-Q-C-H-I-COOH

Peptide T22 H_2N-S-L-A-M-A-A-S-D-I-S-L-L-D-A-Q-S-A-P-L-R-COOH (21-40)

Peptide T23 H_2N-V-Y-V-E-E-L-K-P-T-P-E-G-D-L-E-I-L-L-Q-K-COOH (41-60)

Peptide T24 H_2N-L-S-F-N-P-T-Q-L-E-E-Q-C-H-I-COOH (149-162)

Figure 2

BNSDOCID: <WO___0042863A1_I_>

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/00334

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A23J3/34 A23L1/305 A61K38/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A23J A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, FSTA, EPO-Internal, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 629 350 A (SANDOZ NUTRITION LTD) 21 December 1994 (1994-12-21) cited in the application page 6, line 20-35; claims 1-28	1-4
X	EP 0 827 697 A (NESTLE SA) 11 March 1998 (1998-03-11) claims 1-8	1-4
Y	LO C.W. AND KLEINMAN R.E.: "Infant formula, past and future: opportunities for improvement" AMERICAN JOURNAL OF CLINICAL NUTRITION, vol. 63, no. 4, 1996, page 646S-650S XP002102415	1-10
X	abstract	11,12

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 June 2000	26/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	De Jong, E

Form PCT/ISA/210 (second sheet) (July 1992)

BNSDOCID: <WO__0042863A1_I_>

INTERNATIONAL SEARCH REPORT

rnational Application No
PCT/EP 00/00334

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	FRITSCHÉ R.: "Induction of oral tolerance to cow's milk proteins in rats fed with a whey protein hydrolysate" NUTRITION RESEARCH, vol. 18, no. 8, 1998, page 1335-1341 XP002102881 page 1340 ---	1-10
A	DATABASE WPI Section Ch, Week 9530 Derwent Publications Ltd., London, GB; Class B04, AN 95-228640 XP002102416 & JP 07 138187 A (NIPPON HAM KK), 30 May 1995 (1995-05-30) abstract ---	1,7
A	US 5 039 532 A (JOST ROLF ET AL) 13 August 1991 (1991-08-13) cited in the application claim 1; examples 1-3 ---	1
A	NICODEMUS C., ET AL.: "Integrated clinical experience with tolerogenic peptides" INTERNATIONAL ARCHIVES OF ALLERGY AND IMMUNOLOGY, vol. 113, no. 1-3, 1997, page 326-328 XP002102867 page 328 -----	1,7

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

BNSDOCID: <WO__0042863A1_I_>

INTERNATIONAL SEARCH REPORT

Information on patent family members

rnational Application No
PCT/EP 00/00334

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0629350 A	21-12-1994	AU 6471494 A	22-12-1994
		CA 2125793 A	17-12-1994
		JP 7101873 A	18-04-1995
EP 0827697 A	11-03-1998	NONE	
JP 7138187 A	30-05-1995	NONE	
US 5039532 A	13-08-1991	EP 0321603 A	28-06-1989
		AT 84680 T	15-02-1993
		AU 2659688 A	29-06-1989
		CA 1334064 A	24-01-1995
		DE 3877733 A	04-03-1993
		DE 3877733 T	09-06-1993
		EP 0322589 A	05-07-1989
		ES 2053690 T	01-08-1994
		JP 2002319 A	08-01-1990
		JP 6077504 B	05-10-1994
		MX 169602 B	14-07-1993
		PH 26140 A	18-03-1992
		PT 89325 A,B	29-12-1989
		ZA 8809280 A	27-09-1989

D:\ISDOCID: <WO__ 0042863A1_I_>

XP-000921209

1998
155-167 (13)

D2 192

ELSEVIER

International Journal of Food Microbiology 41 (1998) 155–167

International Journal of Food Microbiology

Lactic acid bacteria as antigen delivery vehicles for oral immunization purposes

Peter H. Pouwels[a,*], Rob J. Leer[a], Michael Shaw[b], Marie-Joan Heijne den Bak-Glashouwer[b], Frans D. Tielen[b], Egbert Smit[a], Beatriz Martinez[a], Jan Jore[a], Patricia L. Conway[c]

[a] *TNO Nutrition and Food Research Institute, P.O. Box 360, Zeist, The Netherlands*
[b] *Department of Immunological and Infectious Diseases, TNO Prevention and Health, P.O. Box 2215, Leiden, The Netherlands*
[c] *Department of Microbiology, University of New South Wales, Sydney, Australia*

Accepted 6 March 1998

Abstract

In vaccination programmes in which large numbers of subjects are involved, the oral route of administration is more convenient as compared to the more frequently used parenteral route. This is particularly relevant when vaccines are to be applied in less industrialized countries. Lactic acid bacteria in general and strains of *Lactobacillus* in particular have a variety of properties which make them attractive candidates for oral vaccination purposes, e.g. GRAS status, adjuvant properties, mucosal adhesive properties and low intrinsic immunogenicity. An overview is given of current research aimed at unravelling the relationship between structure and properties of surface proteins of lactobacilli and in vivo colonization, in particular of species capable of adhering to epithelial cells in vitro. Secondly, the state of the art will be discussed with respect to antigen presentation by lactic acid bacteria. Finally, some preliminary immunological data of recombinant lactic acid bacterial strains expressing antigens from pathogens will be presented. © 1998 Elsevier Science B.V.

Keywords: Adherence; Expression vector; *Lactobacillus*; Surface protein; Vaccine

1. Introduction

Lactic acid bacteria (LAB) have been involved since time immemorial in food processing and food preservation, and are applied in particular for the manufacturing of dairy products, fermented meat, vegetables, bread and ensilage. To obtain fermentation products of a reproducible and high quality, present large-scale fermentations are initiated by the addition of well-defined LAB starter cultures. Research carried out in recent years has led to the conviction that certain strains of LAB, in particular strains from the genera *Lactobacillus* and *Bifidobacterium*, may promote health in man and animals. It has been suggested that lactobacilli or products made

*Corresponding author. Tel.: + 31 30 69 44 462; fax: + 31 30 69 44 466; e-mail: pouwels@voeding.tno.nl

0168-1605/98/$19.00 © 1998 Elsevier Science B.V. All rights reserved.
PII: S0168-1605(98)00048-8

EN:DOCID: <XP___921209A__1_>

by these organisms have antagonistic effects against undesirable intestinal infections and certain forms of cancer (Matsuzaki, this volume). Lactobacilli or their products may also have an anticholesteremic effect by lowering the blood serum cholesterol level (Holzapfel et al., this volume).

Some strains of *Lactobacillus* are found as natural commensals of the gastro-intestinal tract (GIT), the oral cavity and the female uro-genital tract of animals and humans. The ability to adhere to specific regions of the GIT is a property which has prompted research aimed at the use of lactobacilli as vehicles for the delivery of biologically active compounds, such as pharmaca, antibodies, enzymes and vaccines. This review will focus on the potential of lactobacilli as vehicles for the delivery of oral vaccines. The first part of the review deals primarily with the interaction between lactobacilli and mucosal tissues of the host. Special emphasis is given to the structure and properties of surface proteins of lactobacilli in relation to adherence and colonization of mucosal tissues. In the second part of the review an overview will be given of novel molecular biological developments aimed at the efficient expression of antigens from pathogenic organisms by lactobacilli. Finally, some preliminary results of the immunological analysis of such antigen producing bacteria will be given, as well as a perspective for future developments.

2. Oral immunization

Oral presentation of antigens offers a number of advantages over other routes (parenteral administration). Oral administration of vaccines is convenient, can be carried out on a large scale and is relatively inexpensive. In addition, oral immunization may yield, for a number of pathogens, herd immunity, which means that immunity is spread among the members of the community following immunization of only a small proportion of individuals. These advantages are particularly relevant when vaccines are to be applied in less industrialized countries (Bloom, 1989; Husband, 1993). Furthermore, many bacterial, viral and parasitic pathogens enter the body via the mucosal surfaces of the body. The first line of defense for humans and animals is, not surprisingly, found in the intestines, which form the largest immunological organ of the human body. More than 60% of antibodies produced daily are secreted into the GIT. Finally, oral immunization frequently evokes both local and systemic immune responses, resulting in an effective elimination of foreign invaders.

3. Lactic acid bacteria as vaccine delivery vehicles

Lactic acid bacteria possess a number of properties which make them attractive candidates for oral vaccination purposes. These bacteria have been used for centuries in the fermentation and preservation of food, and are considered to be 'safe' organisms with a GRAS (Generally Recognized As Safe) status. This is in contrast to other live vaccine carriers used (e.g., *Salmonella*, *Escherichia coli*, Vaccinia) which cannot be classified as 'safe'. Furthermore, in contrast to LAB, the latter type of carriers are themselves highly immunogenic. This may prevent repetitive use of the carriers in multi-schedule immunizations with the same or other antigens. A particularly attractive feature of lactobacilli, but not of lactococci, is their capacity to colonize certain regions of the mucosa which permits, in principle, the induction of a local immune response. An additional benefit of the use of lactobacilli is that some strains are considered to show health-promoting (probiotic) activities for humans and animals (cf. Holzapfel et al., this volume).

4. Features of lactobacilli related to their use as delivery vehicles

4.1. Molecular-biological and biochemical properties

The genus *Lactobacillus* comprises more than 50 species widely differing in biochemical, ecological, molecular-biological and immunological properties. This large variation is reflected, for example, by the large differences in the G + C content of their DNA. Whereas species such as *Lactobacillus acidophilus* and *Lactobacillus helveticus* contain DNA with a G + C content of approximately 36 mol%, that of *Lactobacillus delbrueckii* and *Lactobacillus fermentum* is more than 50 mol%. Control of transcription

and translation may differ greatly from one species to the other, implying that knowledge generated for one organism may not simply be transferred to another. This means, for example, that genes that are efficiently expressed in one *Lactobacillus* species are not necessarily expressed in (all) other species, or are expressed with a different efficiency and/or with a different regulation of the gene expression (Pouwels and Leer, 1993).

Lactobacilli derive the energy necessary for growth largely from the fermentation of carbohydrates. The capacity to grow on specific energy sources and the fermentation pathways of these sugars differ considerably in different *Lactobacillus* species. In addition, many differences in surface structure and surface properties have been noted for different *Lactobacillus* strains. Sugar metabolism and surface properties are both highly relevant factors for the application of lactobacilli as delivery vehicles. As lactobacilli require complex media for growth, the availability of specific energy sources in the intestines, in addition to the capacity of the bacteria to make use of such energy sources, will determine their competitive advantage over other bacteria. Similarly, differences in surface properties will contribute greatly to the capacity of bacteria to survive and adhere to food particles and/or to the mucosa (vide infra).

4.2. Colonization

Of the many *Lactobacillus* species that have been taxonomically distinguished, 11 have been detected in gastro-intestinal and faecal material from pigs, mice or rats (Tannock, 1992). An additional 10 species were isolated from human and animal intestines (Mitsuoka, 1992), illustrating the wide diversity of species inhabiting the GIT. Efficient colonization of, and adhesion to, human tissue surfaces is often considered important for the probiotic effects of lactobacilli (Blomberg et al., 1993; Coconnier et al., 1992). It seems plausible to assume that colonization and adhesion may also play a major role in both non-specific and specific immune stimulation.

Mucosal colonization in vivo appears to be host specific for the systems studied (Lin and Savage, 1984; Tannock et al., 1982). In a recent study aimed at determining the applicability of in vitro selection criteria, for example adhesion to epithelial cells, to the selection of *Lactobacillus* isolates from human ileum origin that could colonize human mucosa in vivo, it was found that several strains were stably maintained in the human GIT (K. Collins, personal communication). Repopulation of the host mucosa is, however, not a guaranteed property of lactobacilli that have been collected from faecal samples or biopsies. Intestinal strains may rapidly lose the capacity to colonize intestinal tissue following subcultivation outside the body, in fermentation broth or on agar plates. For the purpose of exploiting lactobacilli as delivery vehicles for specific hosts it is therefore of paramount importance to know the origin of the *Lactobacillus* strain and whether the strain remains capable of adhering to the tissue from which it originated.

4.3. *In vitro* adherence

Colonization of intestinal tissues requires the incoming bacteria to adhere to and be maintained at mucosal tissues. The difficulties involved in performing studies on bacterial adherence in vivo have led to the development of in vitro model systems for this purpose. A number of human adenocarcinoma cell lines with properties similar to those of enterocytic intestinal cells have been isolated. Cell lines used for this purpose include Caco-2 cells, HT-29 and HeLa cells. Such cells are morphologically similar to normal small intestinal cells and express several characteristic markers. Therefore, they are highly suited for studies on the interaction between commensal bacteria and the gut intestine, and that between intestinal cells and pathogenic bacteria that are competing for the same tissues. In vitro adhesion has in some cases been demonstrated to be host specific. For example, adhesion to chick crop epithelial cells is observed only with chicken isolated *Lactobacillus* (Fuller, 1975). Similarly, only lactobacilli isolated from rats are able to attach to rat squamous epithelial cells (Suegara et al., 1975). In contrast, *L. fermentum* 104-R and *L. fermentum* KLD, of porcine and human origin, respectively, demonstrate no detectable specificity in adhesion for porcine tissue. However, the human strain adheres better to human epithelium as compared to the porcine strain (Conway and Henriksson, 1993).

The mechanism of adhesion of *Lactobacillus* to the *pars oesophagea* of different mammals and birds

BNSDOCID: <XP___921209A__I_>

has been studied extensively in vitro and has led to the conclusion that adhesion is mediated by polysaccharides (Brooker and Fuller, 1975). In a study examining the adhesion to urinary and intestinal epithelial cells, it was observed that adhesion to urinary cells correlated with bacterial hydrophilicity, whereas an extracellular protein and a non-proteinaceous factor were involved in adherence to Caco-2 cells (Reid et al., 1993). In addition, other studies have shown that proteinaceous as well as carbohydrate moieties are involved in adherence of lactobacilli to the mucosa. For example, Coconnier et al. (1992) concluded that *L. acidophilus* BG2FO4 adheres to Caco-2 cells by means of a secreted protein, a surface-bound protein and carbohydrate factors. In a similar study, it was found that *L. acidophilus* strain LB adhered to Caco-2 cells through a cell-surface factor that was protease insensitive and through an extracellular factor that was protease sensitive (Chauviere et al., 1992a). These authors also showed that *L. acidophilus* LB, in the presence of spent culture supernatant, inhibited the attachment of *E. coli* to Caco-2 cells (Chauviere et al., 1992b).

Despite numerous investigations of in vitro adherence, no clear picture can be given of the mechanism of interaction between the surface structures of lactobacilli and their tissue receptors. One possibility to reconcile the sometimes seemingly conflicting results concerning the importance of polysaccharide vs. proteinaceous compounds might be found by assuming that the proteins serve as lectins connecting bacterial polysaccharide receptors with the host receptor (Conway and Kjelleberg, 1989). Very limited information is available concerning the receptor structures of the host mucosal tissues for lactobacilli. Fibronectin is a glycoprotein secreted by the gastric glandular mucosa with affinity for several Gram-positive bacteria, including lactobacilli. There are a number of other proteins present in the extra-cellular matrix, such as collagen, elastin, laminin and proteoglycans, to which lactobacilli may also bind.

No clear correlation was found between in vitro adherence and in vivo colonization of lactobacilli in the porcine digestive tract (Pedersen and Tannock, 1989). It was concluded that both adhering and non-adhering *Lactobacillus* strains showed a similar ability to colonize the stomach epithelium. Consequently, the conclusions that have been presented in the literature concerning specific interaction of *Lactobacillus* strains with a given tissue, based on in vitro adhesion experiments, have to be validated in vivo, if one wishes to use the strain for targeting of an antigen to a specific region of the body.

4.4. Immunogenicity and adjuvanticity

Administration of lactobacilli by the parenteral route induces the formation of antibodies of the class IgG in experimental animals. In contrast, oral immunization with lactobacilli induces, in general, only low titers of antibodies. This is not entirely surprising as these bacteria are normally found in the gut and our immune system has developed a tolerance to most food components and bacteria that are common to the gut. In previous experiments it was demonstrated that lactobacilli can non-specifically increase antigen-specific immune responses after intraperitoneal (i.p.) injection, if administered with the antigen (Perdigón et al., 1993; Link-Amster et al., 1994; Isolauri et al., 1995). We have confirmed and extended these results by investigating the adjuvant properties of various *Lactobacillus* strains. To determine the adjuvant function, the non-specific immune enhancement was analyzed using selected *Lactobacillus* strains which were injected i.p. together with a model antigen. The responses were evaluated as delayed type hypersensitivity (DTH, T-cell responses). As shown in Fig. 1, DTH responses are clearly different for different lactobacilli. *L. casei* var. Shirota and *L. plantarum* ATCC 8014 showed strong adjuvant effects, equivalent to that of the common water-in-oil emulsion adjuvant, Specol (Boersma et al., 1992). However, two *L. fermentum* strains used in this study showed only negligible adjuvant effect (Pouwels et al., 1996). The adjuvant effect of *L. casei* Shirota was confirmed in an experiment in which experimental encephalitis was evoked using a standard protocol with a peptide as the inductive agent (Gerritse, 1995). To further demonstrate the potential of specific strains as vaccine carriers, *L. casei* was also administered orally to mice, which were concomitantly injected i.p. with UV-irradiated Chikungunya virus. A similar adjuvant effect of the *L. casei* strain was observed under these conditions as observed after i.p. injection. Thus, our results indicate that even with different routing of antigen and adjuvant, a systemic immune response is

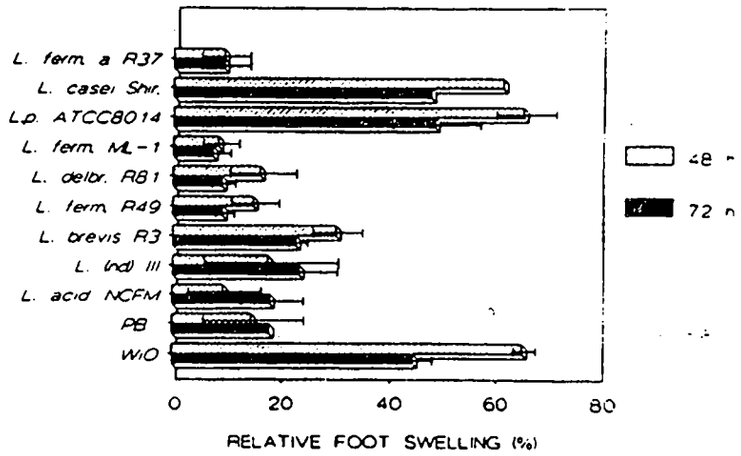


Fig. 1. Adjuvant enhanced DTH responses in mice. Groups of four mice were immunized intraperitoneally (i.p.) with trinitrophenylated chicken globulin (50 μg per mouse). Live *Lactobacillus* (5×10^8) were simultaneously administered i.p. to test the adjuvant character of the microorganisms. As a control, mice were either administered a physiological salt solution (PBS) or Specol, a water-in-oil emulsion adjuvant (Boersma et al., 1992). Four weeks later, mice received a local subcutaneous challenge with the antigen in the foot. The response was measured as a relative footswelling at 48 and 72 h after challenge. (Reprinted, with permission, from J. Biotechnol. 44, 183–192 (1996).)

supported. The immune stimulating effect of orally administered *L. casei* was significantly higher than that of *L. acidophilus*, in accordance with the results obtained when the bacteria were injected i.p.

5. Surface layer proteins

More than 300 different species of Eu- and Archaebacteria, including bacteria of the genus *Lactobacillus*, have been reported to possess a regular (S-layer) structure (short for Surface-layer) on the outside of the cell envelope (Messner and Sleytr, 1992). The mono-molecular S-layer is the result of the secretion and subsequent crystallization of a single protein (S-protein). S-protein contains all the information necessary to enable crystallization into a regular structure. In this context, crystallization of isolated S-proteins into authentic S-layers in vitro has been demonstrated.

S-layers are found on the surfaces of bacterial species living in almost every known biotope, signifying the importance of S-layers. Although some particular functions could be assigned to specific S-proteins, such as barriers between the cell and the environment and attachment structures for extracellular enzymes, no general function has been described which is applicable for all S-layers. S-proteins have been shown to be involved in adherence and pathogenesis. Loss of the S-layer of *Aeromonas salmonicida*, a pathogen for salmon, resulted in significant reduction of the virulence (Munn et al., 1982). Besides *A. salmonicida*, other pathogenic bacterial species have been reported to possess an S-layer, e.g. *Campylobacter* sp. (Blaser and Gotschlich, 1990; Borinski and Holt, 1990). For several of these pathogenic bacteria it has been shown that the S-layer plays a role in circumventing the immune response of the infected host.

5.1. Adherence of S-layer containing bacteria

An S-layer has also been found on bacteria of several *Lactobacillus* species known to inhabit the GIT, in particular strains of species belonging to the acidophilus group of bacteria, e.g. *L. acidophilus* and *L. crispatus* (Masuda and Kawata, 1983; Johnson et al., 1987). Coconnier et al. (1992) have reported that the *L. acidophilus* S-layer functions as a non-fimbrial adhesin to human Caco-2 cells. Greene and Klaenhammer (1994) challenged this conclusion by presenting evidence that the pH rather than the presence of a secreted protein was responsible for the high level of adherence of the S-layer bearing strain

BNSDOCID: <XP ___921209A_I_>

BG2FO4 of *L. acidophilus*. Adhesion of *L. acidophilus* to chicken epithelial cells was shown to be mediated by the S-layer protein (Schneitz et al., 1993). Since in these types of experiments different bacterial isolates were used and different methods to assess the role in adherence of S-layer protein to epithelial cells of different origin, no firm conclusions can be drawn regarding the role of the S-layer protein of *L. acidophilus*. The role of S-layer protein in adherence to proteins of the mammalian extracellular matrix was studied by Toba et al. (1995), who observed that *L. crispatus* JCM 5810 adhered efficiently to immobilized type IV and I collagens, laminin, and, with a lower affinity, to type V collagen and fibronectin. *L. crispatus* also adhered to the extracellular matrix prepared from human intestinal 407 cells. The adhesiveness was found to depend on the presence of an S-layer protein on the bacteria. *L. acidophilus* ATCC 4356 did not exhibit adhesiveness in this assay (Toba et al., 1995).

5.2. S-layer protein of Lactobacillus acidophilus

To investigate the role of the S-layer or S-layer protein of lactobacilli in adhesion to epithelial tissues, we initiated a research project aimed at the characterization of S-proteins of *Lactobacillus* and their mode of expression. The *L. acidophilus* ATCC 4356 type strain, which originates from the human pharynx, was initially selected because of its presumed adhering properties and because it has been shown to contain an S-layer. The amino acid composition of purified S-protein of *L. acidophilus* is typical for S-proteins, i.e. a relative abundance of threonine, serine and hydrophobic amino acids, and absence of cysteine and methionine residues (Boot et al., 1993). The isoelectric point of the *L. acidophilus* S-protein, calculated from the deduced amino acid sequence, is 9.4. The S-proteins of *L. crispatus*, *L. brevis* and *L. helveticus* display similarly high values. These high values are in contrast to those of S-proteins of other species which are, in general, negatively charged (pI 3–4).

5.3. Isolation and characterization of the S-layer protein encoding gene of Lactobacillus acidophilus

The gene encoding the S-layer protein was cloned and expressed in *E. coli* as measured by the formation of a protein that specifically reacted with antibodies raised against purified S-protein, with the same size as that of S-protein. When the gene coding for the S-protein (*slpA*) was used to probe the chromosome for the presence of related genes, a second gene (*slpB*) was found that strongly hybridized with *slpA*. Subsequent analysis of the nucleotide sequence revealed that the two genes contained highly conserved sequences comprising the signal sequence encoding region including the 5′ untranslated region, and the 3′ region encoding the C-terminal one-third of the mature protein (Fig. 2). In contrast to the *slpA* gene, the *slpB* gene lacked a promoter and was found not to be expressed under different physiological conditions (Boot et al., 1995). The S-protein encoding gene and the silent gene are located near each other on the chromosome, in opposite orientation (Boot et al., 1996a). Nucleotide sequence analysis of the region flanked by the two *slp* genes revealed the presence of two open reading frames (ORF), with the same polarity as *slpB*. Since no promoter sequence could be identified in this region, we assume that expression of *slpB* and the two downstream genes is dependent on a chromosomal rearrangement that places the DNA fragment behind a promoter. Measurement of RNA encoded by DNA sequences from this region indicated that the genes that are located between the two *slp* genes are transcribed with very low efficiency, if at all (Boot, 1996).

By a series of PCR experiments, Boot et al. (1996a) demonstrated that in a fraction of the bacterial population the 6 kb fragment spanning the two *slp* genes is inverted, placing *slpB* and the two downstream genes under the control of the promoter of the *slpA* gene. The frequency of inversion was high (1/300), yet all attempts to demonstrate expres-

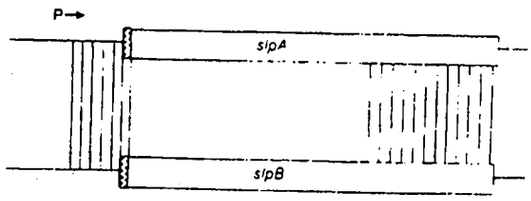


Fig. 2. Schematic drawing of the chromosomal *slpA* and *slpB* regions of the *L. acidophilus* type strain. Strongly conserved or even identical regions of 50 nucleotides are indicated by vertical open boxes. The arrow indicates the promoter of the *slpA* gene.

198

sion of the *slpB* gene have so far been unsuccessful. Apparently, under the experimental conditions used there is a strong bias for the presence of the SlpA protein. In fact, attempts to isolate *L. acidophilus* mutants lacking the S-protein have as yet failed. It is tempting to speculate that expression of *slpB* is favoured under environmental conditions different from that of the test tube, for example those prevailing within the GIT. Phase variation or antigenic variation, as a result of inversion of the *slp* segment, might enable *L. acidophilus* bacteria to better adhere to specific regions of the mucosa.

5.4. Occurrence of two S-layer genes in related Lactobacillus species

Previously, it was shown that strains belonging to the acidophilus A group (*L. acidophilus*, *L. crispatus*, *L. amylovorus* and *L. gallinarum*) contain an S-layer, whereas strains belonging to the acidophilus B group (*L. gasseri* and *L. johnsonii*) lack an S-layer (Johnson et al., 1987). We have confirmed and extended these results at the chromosomal level. Southern blot analysis of chromosomal DNA indicated that all strains of species of the acidophilus group A bacteria contain two S-protein encoding genes, whereas strains from the B group of bacteria lack an S-protein encoding gene (Boot et al., 1996b). The 5' and 3' regions of the different S-protein encoding genes were found to be highly conserved, implying important functions for the two regions. The 5' region, which codes for 5' untranslated sequences and for the signal sequence, probably comprises sequences involved in *L. acidophilus* in inversion of the *slp* fragment (vide infra). The finding that a similar structure also occurs in the S-protein gene of related species suggests that inversion of the *slp* segment also takes place in other *Lactobacillus* species. Preliminary data, indicating that the *slp* genes of *L. crispatus* are organized in a manner similar to that of *L. acidophilus*, support this hypothesis. Interestingly, *L. helveticus*, a species that is evolutionary related to bacteria of the acidophilus group, contains in addition to the S-protein encoding gene a truncated *slp* gene that lacks the 5' conserved region. In the presence of a conserved 5' region upstream of the *slpB* gene, inversion cannot take place, consistent with the notion that *L. helveticus* does not normally adhere to mucosal tissues. Conservation of the 3' region encoding the C-terminal region of the S-protein might indicate that this region is involved in binding of the S-protein to the underlying cell wall or in S-protein monomer interaction.

The N-terminal regions of S-proteins of acidophilus group A bacteria are quite distinct (Fig. 3). This observation corroborates the results of antigenicity determinations, which indicate that antibodies raised against the *L. acidophilus* S-protein react differently with S-proteins of the different group A bacteria and with S-protein from *L. helveticus* (Boot et al., 1996b). The suggestion which emerges from these findings is that the N-terminal region of S-proteins of lactobacilli is exposed to the environment. The presence of phage receptor sites in the variable region of the S-protein of *L. helveticus* (Morelli, personal communication) supports this hypothesis. If the N-terminal region of S-proteins is indeed exposed to the environment, it seems plausible to assume that the determinants for adhesion of S-proteins to mucosal tissues are also located in this region. Preliminary data indicate that differences in adherence properties for extra-cellular matrix proteins of different strains of *L. crispatus* correspond to differences in structure of the N- but not of the C-terminal regions of the S-proteins. Furthering our knowledge concerning the relationship between structure and function of S-proteins of lactobacilli may help to unravel the relationship between adherence and adjuvanticity and to develop novel screening methods for the isolation of strains with improved colonization properties.

By virtue of their presence in a highly ordered

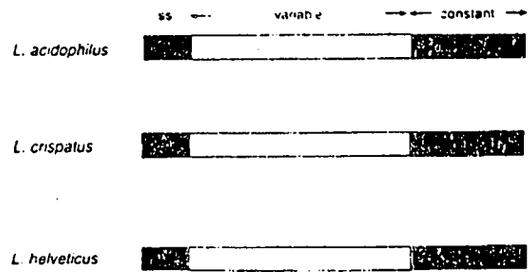


Fig. 3. Structure of the SLP proteins of *L. acidophilus*, *L. crispatus* and *L. helveticus*. The signal sequences (ss; black) are identical. The N-terminal regions (variable; open box) display a strongly reduced similarity compared to the C-terminal one-third of the proteins that are strongly conserved (> 80% identity).

BNSDOCID: <XP___921309A__L>

183

structure and their capacity to reassemble in vitro into a structure with the same periodicity, bacterial surface proteins are ideally suited for several nanotechnological applications, such as ultrafiltration, bio-membranes and affinity chromatography. The periodic structure of the S-layer and the vast number of molecules present at the bacterial surface ($\sim 10^5$ per bacterium) also make S-proteins attractive candidates for the presentation of antigens or antibodies at the bacterial surface. Our present research is aimed at determining in which regions of the S-protein of *L. acidophilus* foreign antigens can be inserted and which regions can be replaced by foreign antigens, in order to construct bacteria that can present biotechnologically interesting proteins such as vaccine epitopes, single chain antibody fragments or enzymes at the bacterial surface. Preliminary data indicate that the N-terminal end can be extended and that foreign antigens can be inserted at a number of internal positions in the S-protein without disturbing the capacity to form the paracrystalline structure.

6. Surface related adhesion protein of *L. fermentum*

Adhesion and colonization most likely involve more than one type of molecular interaction as it has been shown that different types of protein as well as different types of saccharides play a role. Previously, it has been shown that the capacity of *L. fermentum* 104R, a strain with probiotic properties for pigs, to adhere to pig gastric mucosa was caused by the presence of a surface related protein. The protein has a MW of 29 kD, is loosely associated with the bacterial surface, in contrast to S-layer proteins, and can bind to the mucus in vitro. It is thought that, in vivo, the protein functions to form a bridge between the bacteria and epithelial cells (Conway and Kjelleberg, 1989). The gene encoding the adhesion protein was isolated from a gene library made in *E. coli* and was sequenced. Comparison of the deduced amino acid sequence with sequences from the database revealed, quite remarkably, that the mucus adhesion promoting protein was structurally similar to a surface protein from *Pasteurella haemolytica*, to a virulence protein from *Campylobacter jejuni* and to the *Mycobacterium tuberculosis* or *M. leprae* 85K complex proteins A, B and C. Western blot experiments have shown the presence of mucus adhesion protein-like proteins in some but not all *Lactobacillus* strains. It will be interesting to see whether the presence of such proteins can be correlated with probiotic properties of lactobacilli and more specifically to investigate the functional relationship between the adhesion protein and virulence proteins of pathogenic organisms.

7. Genetic engineering of lactobacilli: Expression of foreign antigens

In the remaining part of this review we will discuss the construction and properties of recombinant lactobacilli expressing foreign epitopes of pathogenic organisms aimed to be used as delivery vehicles for mucosal immunization purposes. For a successful immune response against an antigen, it is necessary that the antigen is present in sufficient quantities and in a form that can be recognized by antigen presenting cells of the immune system. The immunogenicity of soluble proteins is, in general, low when administered orally but can be considerably enhanced by coupling of the protein to a carrier, e.g. bacteria, or by genetic engineering of bacteria resulting in the production of the desired antigen. Production of antigens by bacteria can, in principle, occur in three different ways: intracellularly, extracellularly and surface-bound. Previous experiments have shown that a peptide epitope that was coupled to the surface of lactobacilli, but not a mixture of peptide and lactobacilli, yielded antibodies of the desired class (IgG) (Gerritse et al., 1990; Gerritse, 1995). Therefore, we have selected surface exposure of the antigen, rather than extracellular production, as a means of presenting antigens. For the purpose of comparison, the intracellular production of antigen and subsequent immunological evaluations were also carried out.

In initial experiments using *L. casei* and *L. plantarum* as host organisms and expression vectors with the *E. coli* β-galactosidase gene under the control of the *xylR* or *cbh* promoter of *Lactobacillus*, it was found that the levels of intracellular production were rather low (generally less than 0.2% of total soluble protein). Moreover, fusion of an epitope to the N-terminal end of the β-galactosidase resulted in a considerable reduction of the expression levels.

BNSDOCID: <XP____921209A__1_>

Intraperitoneal injection of mice with *L. casei* that intracellularly expressed β-galactosidase led to significant serum immune responses against the antigen. However, after oral immunization with the recombinant bacteria, only weak and inconsistent IgA responses in gut washes were obtained.

7.1. Novel high-efficiency Lactobacillus expression vectors

To overcome these obstacles, we have constructed a series of expression vectors with which any antigen of choice can be efficiently expressed (Fig. 4). The vectors were designed in such a way that antigens can be expressed either intracellularly, extracellularly or secreted and anchored to the surface. All vectors are comprised of cassettes, enabling easy exchange of different expression signals between different vectors. The expression signals and replication elements of the vectors are all derived from *Lactobacillus* DNA sequences. Replacement of current selection markers by *Lactobacillus* sequences will result in a completely food-grade vector. The use of food-grade vectors is expected to facilitate the application, as a food additive, of the products made by lactobacilli by minimizing safety and environmental concerns to regulatory authorities.

The expression vectors have been successfully used to construct different *Lactobacillus* strains expressing antigens. Full-size proteins such as the tetanus toxin fragment C (TTFC), several rota virus surface proteins with sizes ranging from 37 to 87 kD, urease A and B subunits from *Helicobacter pylori*, and some small peptide epitopes (e.g., from influenza virus and encephalitogenic myelin peptides; Kitty Maassen, personal communication) that were fused to a carrier protein (β-glucuronidase) have all been successfully expressed. For example, production in *L. casei* of antigens that were targeted to the cytosol yielded levels of production ranging from 1 to 5%. Efficient production levels were also obtained in this and other *Lactobacillus* species when the products were directed to the bacterial surface. Using fluorescence-activated cell sorter (FACS) analysis we could demonstrate that the heterologous antigens were exposed to the environment at the surface of the bacteria, by staining recombinant bacteria with specific antisera (Fig. 5). It was estimated, using S-protein (the number of which is known per bacterium) containing bacteria as a standard, that the

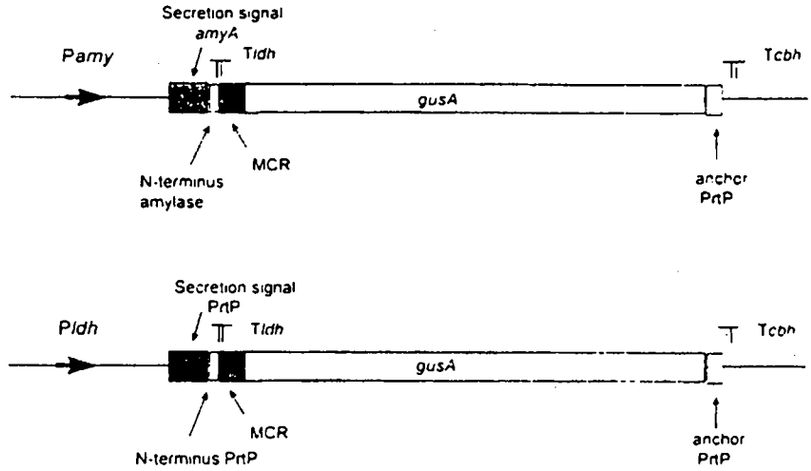


Fig. 4. Structure of the relevant part of the expression vectors. The expression region consists of the following cassettes, each separated by unique restriction enzyme sites: promoter, secretion signal sequence including the first few codons of the corresponding gene, the transcription terminator of the *ldh* gene of *L. casei*, a multi-cloning region (MCR) comprising more than 10 unique restriction enzyme sites, *gusA* encoding the β-glucuronidase gene, the anchor sequence of the proteinase gene of *L. casei*, and the transcription terminator of the *cbh* gene of *L. plantarum*. Before transfer of the expression vector to *Lactobacillus*, the transcription terminator (T*ldh*) is removed by digestion of the plasmid with *Not*I, and subsequent ligation.

BNSDOCID: <XP____921209A__I_>

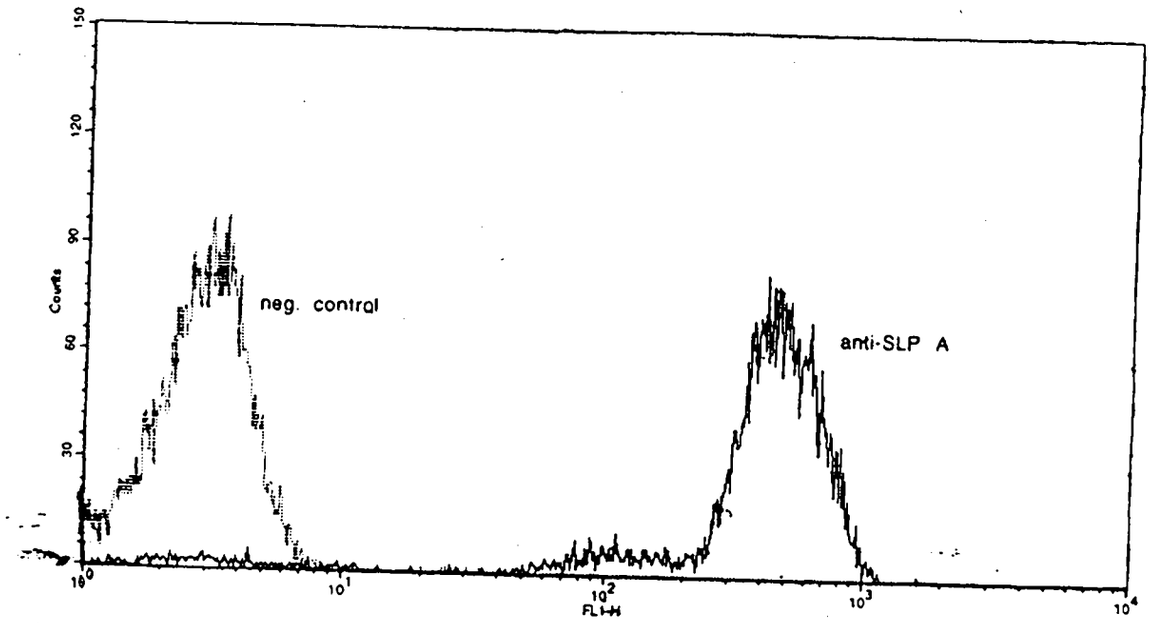


Fig. 5. Fluorescence-activated cell sorter (FACS) analysis of the S-layer of *L. acidophilus*. *Lactobacillus* cells were gated on the basis of forward- (size) and side- (granularity) scatter cytograms and bacterial cells were probed with a murine *slpA*-specific antiserum optimally diluted. Bound antibody was detected with optimally diluted FITC-conjugated anti-mouse antibody and gated cells analyzed for fluorescence by FACScan analysis. Mean channel fluorescence levels (= 450) were demonstrated with cells stained 6 h following initiation of culture and are shown in histogram form and presented in relation to mean channel fluorescence (= 3) obtained with *L. acidophilus* cells stained with non-specific immune serum (neg. control). 10 000 bacteria were analyzed in each experiment.

number of antigen molecules at the surface of the bacteria was between 10^3 and 10^4 per cell. In similar studies where *Lactococcus lactis* was used as host bacterium, equivalent or higher levels of production were reported when antigens were produced intracellularly (Wells et al., 1993). However, the levels of antigen production in this organism decreased drastically when the antigens were directed to the bacterial surface. Moreover, the antigen used for this study, TTFC, was not correctly transported over the membrane, as suggested by the inability to detect it at the bacterial surface (Norton et al., 1996).

7.2. Immunological evaluation of recombinant lactic acid bacteria expressing foreign antigens

To determine whether LAB strains expressing a foreign antigen intracellularly or at the surface of the bacterium could evoke an immune response, mice were immunized by administration of live recombinant bacteria by the parenteral, oral or nasal routes. Levels of antibodies induced following immunization (IgG in serum; IgA in faeces) were measured by ELISA. Significant immune responses against foreign antigens such as TTFC following mucosal administration were found only when the priming reaction was followed by a booster immunization. Parenteral immunization, however, resulted in detectable antibodies following priming immunizations only.

L. casei or *Lactococcus* bacteria that intracellularly express foreign antigens induced a significant immune response (measured as IgG antibodies) after i.p. or subcutaneous injection. Similar results were obtained with recombinant bacteria expressing antigens at the surface of the bacteria. These results indicate that the quantities that are produced by genetically engineered LAB strains are sufficient for eliciting an immune response. Although antigen production at the bacterial surface is in general less

efficient compared to intracellular production, equivalent or even higher immune responses were observed with cell wall associated antigens (Norton et al., 1996). After oral or nasal priming followed by a booster immunization via the same or the parenteral route, significant levels of IgA and IgG were observed for antigens expressed by recombinant *Lactobacillus*, *Lactococcus* and *Streptococcus gordonii* strains (for a review, see Wells et al., 1996). Only very low levels of antibodies against the host bacteria were observed, even after repeated immunizations. In studies using TTFC as the antigen, it was shown that the levels of antibody produced were sufficiently high to protect the animals against a 20-fold lethal dose of tetanus toxin (Wells et al., 1993).

Recombinant *L. casei* bacteria expressing TTFC induced high levels of IgG following i.p. immunization but very low antibody levels were observed after oral immunization. This result may be explained by the poor viability of this strain in the gut of mice. After feeding of mice with doses of 10^9 bacteria, virtually no live bacteria could be detected in the faeces after 24 h. The persistence of LAB strains in the gut may therefore be an important parameter in the selection of strains that can be used for immunization experiments. In initial experiments to evaluate a variety of *Lactobacillus* strains originating from intestinal material, we have identified two strains that could persist in mice for 5–7 days and one strain that was still present in large numbers after 20 days. Such strains may prove more suitable for immunization experiments than the *L. casei* strain used to date.

8. Perspectives

By the end of the eighties the directives contained in the 'Children's Vaccine Initiative' of the WHO/UNDP aimed at the eradication of a number of infectious diseases such as malaria, measles, etc. by application of a single-dose oral vaccine against a large number of pathogens. The European Community has followed up this initiative and started a multidisciplinary programme within BIOTECH Cell Factory to coordinate and stimulate research within Europe, directed at the development of an oral vaccine based on lactic acid bacteria as delivery vehicles. During the last few years significant progress has already been made towards this goal. By combining the knowledge generated in the different disciplines, molecular biology, immunology, ecology and gastro-enterology, even more progress is expected to be made towards an understanding of the underlying principles of oral immunization and the importance of live bacterial carriers therein. The elucidation and characterization at the DNA level of bacterial surface proteins involved in adherence to mucosal tissues has provided new and challenging opportunities which will allow us to better understand the interaction between intestinal lactobacilli and mucosal tissues. The recent development of molecular biological techniques that permit construction of lactic acid bacteria presenting foreign antigens in different ways to the immune system, offers for the first time opportunities to further explore the potential of these food bacteria as vaccine vehicles.

Despite the rapid advancement of our knowledge and concurrent development of techniques, a number of problems will have to be solved before the first LAB vaccine can be marketed. Although it is possible to accurately determine the levels of antigen expression in the test tube, we cannot measure gene expression in the gut. Therefore, methods will have to be developed to analyze expression of the antigen encoding genes in situ so as to control the efficiency and duration of expression. In addition, how long colonizing bacteria are present in the gut and what the effects are of continuous expression of a LAB-associated antigen remain to be determined. Will tolerance be induced? If so, then ways will have to be explored to temporarily or permanently interfere with expression, for example by induction of a system that triggers elimination of the antigen encoding gene or kills the bacteria. Finally, lack of survival of recombinant LAB strains in the environment and lack of transfer of antigen encoding genetic material are prerequisites necessary to be validated prior to the clinical application of these strains.

Acknowledgements

Part of the research described in this review was supported by the European Community (contract BIO2-CT943055).

BNSDOCID: <XP____921209A__I_>

References

Blaser, M.J., Gotschlich, E.C., 1990. Surface array protein of *Campylobacter fetus*. J. Biol. Chem. 265, 14529–14535.

Blomberg, L., Henriksson, A., Conway, P.L., 1993. Inhibition of adhesion of *Escherichia coli* K88 to piglet ileal mucus by *Lactobacillus* spp. Appl. Environ. Microbiol. 59, 34–39.

Bloom, B.R., 1989. New approaches to vaccine development. Rev. Infect. Dis. 11, 460–466.

Boersma, W.J.A., Bogaerts, W.J.A., Bianchi, A.T.J., Claassen, E., 1992. Adjuvant properties of stable water-in-oil emulsions: Evaluation of the experience with Specol. Res. Immunol. 143, 503–512.

Boot, H.J., 1996. The surface layer protein genes of *Lactobacillus acidophilus*. Thesis, University of Amsterdam.

Boot, H.J., Kolen, C.P.A.M., van Noort, J.M., Pouwels, P.H., 1993. S-layer protein of *Lactobacillus acidophilus* ATCC 4356: Purification, expression in *Escherichia coli*, and nucleotide sequence of the corresponding gene. J. Bacteriol. 175, 6089–6096.

Boot, H.J., Kolen, C.P.A.M., Pouwels, P.H., 1995. Identification, cloning and nucleotide sequence of a silent S-layer protein gene of *Lactobacillus acidophilus* ATCC 4356 which has extensive similarity with the S-layer protein gene of this species. J. Bacteriol. 177, 7222–7230.

Boot, H.J., Kolen, C.P.A.M., Pouwels, P.H., 1996a. Interchange of the active and silent S-protein genes of *Lactobacillus acidophilus* by inversion of the chromosomal *slp* segment. Mol. Microbiol. 21, 799–809.

Boot, H.J., Kolen, C.P.A.M., Pot, B., Kersters, K., Pouwels, P.H., 1996b. The presence of two S-layer protein encoding genes is conserved among species related to *Lactobacillus acidophilus*. Microbiology 142, 2375–2384.

Borinski, R., Holt, S.C., 1990. Surface characteristics of *Wolinella recta* ATCC 33238 and human clinical isolates: Correlation of structure with function. Infect. Immun. 58, 2770–2776.

Brooker, B.E., Fuller, R., 1975. Adhesion of lactobacilli to the chicken crop epithelium. J. Ultrastruct. Res. 52, 21–31.

Chauviere, G., Coconnier, M.H., Kerneis, S., Fourniat, J., Servin, A.L., 1992a. Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. J. Gen. Microbiol. 138, 1689–1696.

Chauviere, G., Coconnier, M.H., Kerneis, S., Darfeuille-Michaud, A., Joly, B., Servin, A.L., 1992b. Competitive exclusion of diarrheagenic *Escherichia coli* (ETEC) from human enterocyte-like Caco-2 cells by heat-killed *Lactobacillus*. FEMS Microbiol. Lett. 70, 213–217.

Coconnier, M.H., Klaenhammer, T.R., Kerneis, S., Bernet, M.F., Servin, A.L., 1992. Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell lines in culture. Appl. Environ. Microbiol. 58, 2034–2039.

Conway, P.L., Henriksson, A., 1993. Strategies for the isolation and characterisation of functional probiotics. In: Gibson, S.A.W. (Ed.), Human Health: The Contribution of Microorganisms. Springer, London, pp. 75–93.

Conway, P.L., Kjelleberg, S., 1989. Protein-mediated adhesion of *Lactobacillus fermentum* strain 737 to mouse stomach squamous epithelium. J. Gen. Microbiol. 135, 1175–1186.

Fuller, R., 1975. Nature of the determinant responsible for the adhesion of lactobacilli to crop epithelial cells. J. Gen. Microbiol. 87, 245–250.

Gerritse, K., 1995. *Lactobacillus* as a vector for oral delivery of antigens: The role of intrinsic adjuvanticity in enhancement of immune responses or tolerance induction. In: Antibody Formation and Immunomodulation in Experimental Allergic Encephalomyelitis and Multiple Sclerosis. Thesis, Erasmus University, Rotterdam.

Gerritse, K., Posno, M., Schellekens, M.M., Boersma, W.J.A., Claassen, E., 1990. Oral administration of TNP-*Lactobacillus* conjugates in mice: A model for evaluation of mucosal and systemic immune responses and memory elicited by transformed lactobacilli. Res. Microbiol. 141, 955–962.

Greene, J.D., Klaenhammer, T.R., 1994. Factors involved in adherence of lactobacilli to human Caco-2 cells. Appl. Environ. Microbiol. 60, 4487–4494.

Husband, A.J., 1993. Novel vaccination strategies for the control of mucosal infection. Vaccine 11, 107–112.

Isolauri, E., Joensuu, J., Suomalainen, H., Luomala, M., Vesikari, T., 1995. Improved immunogenicity of oral D x RRV reassortant rotavirus vaccine by *Lactobacillus casei* GG. Vaccine 13, 310–312.

Johnson, M.C., Ray, B., Bhowmik, T., 1987. Selection of *Lactobacillus acidophilus* strains for use in 'acidophilus products'. Anthonie van Leeuwenhoek 53, 215–231.

Lin, J.H-C., Savage, D.C., 1984. Host specificity of the colonization of murine gastric epithelium by lactobacilli. FEMS Microbiol. Lett. 24, 67–71.

Link-Amster, H., Rochat, F., Saudan, K.Y., Mignot, O., Aeschlimann, J.M., 1994. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. FEMS Immunol. Med. Microbiol. 10, 55–64.

Masuda, K., Kawata, T., 1983. Distribution and chemical characterization of regular arrays in the cell walls of strains of the genus *Lactobacillus*. FEMS Microbiol. Lett. 20, 145–150.

Messner, P., Sleytr, U.B., 1992. Crystalline bacterial cell-surface layers. Adv. Microb. Physiol. 33, 213–275.

Mitsuoka, T., 1992. The human gastrointestinal tract. In: Wood, B.J.B. (Ed.), The Lactic Acid Bacteria: The Lactic Acid Bacteria in Health and Disease. Applied Science, Vol. 1. Elsevier, Amsterdam, pp. 69–114.

Munn, C.B., Ishiguro, E.E., Kay, W.W., Trust, T.J., 1982. Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. Infect. Immun. 36, 1069–1075.

Norton, P.M., Brown, H.W.G., Wells, J.M., Macpherson, A.M., Wilson, P.W., Le Page, R.W.F., 1996. Factors affecting immunogenicity of tetanus toxin fragment C expressed by *Lactococcus lactis*. FEMS Immunol. Med. Microbiol. 14, 167–177.

Pedersen, K., Tannock, G.W., 1989. Colonization of the porcine gastrointestinal tract by lactobacilli. Appl. Environ. Microbiol. 55, 279–283.

Perdigón, G., Medici, M., Bibas Bonet de Jorrat, M.E., Valverde de Budeguer, M., Pesce de Ruiz Holgado, A., 1993. Immunomodulating effects of lactic acid bacteria on mucosal and tumoral immunity. Int. J. Immunother. IX, 29–52.

BNSDOCID: <XP____921209A__1_>

Pouwels, P.H., Leer, R.J., 1993. Genetics of *Lactobacilli*. Plasmids and gene expression. Antonie van Leeuwenhoek 65, 85–107.

Pouwels, P.H., Leer, R.J., Boersma, W.J.A., 1996. The potential of *Lactobacillus* as a carrier for oral immunization: Development and preliminary characterization of vector systems for targeted delivery of antigens. J. Biotechnol. 44, 183–192.

Reid, G., Servin, A.L., Bruce, A.W., Busscher, H.J., 1993. Adhesion of three *Lactobacillus* strains to human urinary and intestinal epithelial cells. Microbios 75, 57–65.

Schneitz, C., Nuotio, L., Lounatma, K., 1993. Adhesion of *Lactobacillus acidophilus* to avian intestinal epithelial cells mediated by the crystalline bacterial cell surface layer (S-layer). J. Appl. Bacteriol. 74, 290–294.

Suegara, N., Morotomi, M., Watanabe, T., Kawai, Y., Mutai, M., 1975. Behaviour of microflora in the rat stomach: Adhesion of lactobacilli to the keratinized epithelial cells of the rat stomach in vitro. Infect. Immun. 12, 173–179.

Tannock, G.W., 1992. Lactic microflora of pigs, mice and rats. In: Wood, B.J.B. (Ed.), The Lactic Acid Bacteria: The Lactic Acid Bacteria in Health and Disease. Applied Science, Vol. 1. Elsevier, Amsterdam, pp. 21–48.

Tannock, G.W., Szylit, O., Duval, Y., Raibaud, P., 1982. Colonisation of tissue surfaces in the gastrointestinal tract of gnotobiotic animals by *Lactobacillus* strains. Microb. Ecol. 5, 35–42.

Toba, T., Virkola, R., Westerlund, B., Björkman, Y., Sillanpää, J., Vartio, T., Kalkkinen, N., Korhonen, T.K., 1995. A collagen-binding S-layer protein in *Lactobacillus crispatus*. Appl Environ. Microbiol. 61, 2467–2471.

Wells, J.M., Wilson, P.W., Norton, P.M., Gasson, M.J., Le Page, R.W.F., 1993. *Lactococcus lactis*: High-level expression of tetanus toxin fragment C and protection against lethal challenge. Mol. Microbiol. 8, 1155–1162.

Wells, J.M., Robinson, K., Chamberlain, L.M., Schofield, K.M., Le Page, R.W.F., 1996. Lactic acid bacteria as vaccine delivery vehicles. Antonie van Leeuwenhoek 70, 317–330.

BNSDOCID: <XIP____921209A__1_>

XP 000608165

JOURNAL OF BACTERIOLOGY, June 1996, p. 3059–3065 (7)
0021-9193/96/$04.00+0
Copyright © 1996, American Society for Microbiology
Vol. 178, No. 11

A New Cell Surface Proteinase: Sequencing and Analysis of the *prtB* Gene from *Lactobacillus delbrueckii* subsp. *bulgaricus*

CHRISTOPHE GILBERT,[1] DANIÈLE ATLAN,[1]* BRIGITTE BLANC,[1] RAYMOND PORTALIER,[1] JACQUES EDOUARD GERMOND,[2] LUCIANE LAPIERRE,[2] AND BEAT MOLLET[2]

Laboratoire de Microbiologie et Génétique Moléculaire, UMR CNRS 106, Université Claude Bernard—Lyon I, F-69622 Villeurbanne Cedex, France,[1] and Nestlé Research Centre, Nestec Ltd., Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland[2]

Received 27 November 1995/Accepted 18 March 1996

Investigation of the chromosomal region downstream of the *lacZ* gene from *Lactobacillus delbrueckii* subsp. *bulgaricus* revealed the presence of a gene (*prtB*) encoding a proteinase of 1,946 residues with a predicted molecular mass of 212 kDa. The deduced amino acid sequence showed that PrtB proteinase displays significant homology with the N termini and catalytic domains of lactococcal PrtP cell surface proteinases and is probably synthesized as a preproprotein. However, the presence of a cysteine near the histidine of the PrtB active site suggests that PrtB belongs to the subfamily of cysteine subtilisins. The C-terminal region strongly differs from those of PrtP proteinases by having a high lysine content, an imperfect duplication of 41 residues, and a degenerated sequence compared with the consensus sequence for proteins anchoring in the cell walls of gram-positive bacteria. Finally, the product of the truncated *prtM*-like gene located immediately upstream of the *prtB* gene seems too short to be involved in the maturation of PrtB.

Lactobacillus delbrueckii subsp. *bulgaricus* is one of the lactic acid bacteria used in industrial milk fermentation. The proteolytic system is essential to ensure rapid growth in milk and supply auxotrophic lactic acid bacteria with amino acids from caseins, the major milk proteins. This system is complex and has been extensively studied in lactococci (21, 35). The first step in milk casein degradation is performed by a cell surface proteinase, PrtP. Two types of PrtP enzymes have been distinguished on the basis of their substrate specificities. PI-type proteinases preferentially cleave β-casein, whereas PIII-type enzymes degrade α, β, and κ caseins (10). Lactococcal PrtPs are serine proteases and show extensive homology with subtilisins secreted by bacilli. Lactococcal proteinases are synthesized as inactive preproproteins. An N-terminal signal peptide of residues is removed during translocation through the cytoplasmic membrane, and the C terminus remains anchored to the cell envelope. Then, a maturation process mediated by a membrane-located lipoprotein, PrtM, leads to the removal of the pro region (154 residues). The 33-kDa PrtM protein is encoded by a gene located immediately upstream and in the opposite direction of *prtP* (13, 49). Incubation of lactococci cells in a calcium-free buffer promotes self-digestion of the carboxy terminus of PrtP and its release into the extracellular medium.

Lactobacilli have been investigated to a lesser extent but display a high cell surface proteinase activity with substrate specificity differing from that of lactococci (4). Cell surface proteinases have been purified from *Lactobacillus paracasei* subsp. *paracasei* NCDO151, *L. helveticus* CNRZ303, and L89, and *L. delbrueckii* subsp. *bulgaricus* CNRZ397 (24, 27, 33, 52). The sequence of the gene encoding proteinase from *L. paracasei* subsp. *paracasei* was determined; the deduced amino acid sequence shows 1,902 residues and a high degree of identity (96%) with lactococcal PrtP (15). The presence of a *prtM* gene suggests a maturation process similar to lactococcal PrtP maturation. In contrast, proteinases from thermophilic lactobacilli appear to be different from those of lactococci. The *L. helveticus* CNRZ303 proteinase is characterized by a unique specificity toward α_{s1} casein (1-23 fragment). The proteinases of *L. delbrueckii* subsp. *bulgaricus* CNRZ397 and *L. helveticus* L89 cannot be released from the cell wall by a procedure using calcium-free buffer. Moreover, the *L. delbrueckii* subsp. *bulgaricus* enzyme is sensitive to serine and cysteine protease inhibitors.

Spontaneous lactose-negative mutants of *L. delbrueckii* subsp. *bulgaricus* NCDO1489 were characterized by large chromosomal deletions of several kilobases including the *lacS* and *lacZ* genes, coding for a permease and β-galactosidase, respectively (11, 32). We took advantage of the availability of some mutants with a proteinase-deficient phenotype to identify and localize the structural gene (*prtB*) coding for the cell surface proteinase. A comparison with PrtP proteinases highlighted new characteristics of the *L. delbrueckii* subsp. *bulgaricus* cell envelope proteinase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *L. delbrueckii* subsp. *bulgaricus* NCDO1489 and mutants N310 and LFi5.F6 (spontaneous *lacZ* derivatives from NCDO1489) were from the Nestec collection (32), and strain CNRZ 397 was obtained from the Institut National de la Recherche Agronomique (Jouy en Josas, France). *Lactococcus lactis* subsp. *lactis* NCDO763 was a gift of A. Hirashima (20). *Escherichia coli* DH5α [*supE44 ΔlacU169* (φ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was purchased from Gibco BRL (Eragny, France). Plasmids pUC18 and pCU19 (51) were used for cloning PCR fragments and DNA sequencing.

L. delbrueckii subsp. *bulgaricus* strains were routinely cultured at 40°C in MRS broth (7), and *Lactococcus lactis* subsp. *lactis* were grown at 30°C in M17 broth supplemented with 0.5% glucose (45). Reconstituted 10% Gamma milk from skim milk powder (Prulait, Niort, France) was centrifuged (15,000 × *g*, 15 min, 6°C) and immediately used. *E. coli* was grown with shaking at 37°C in L broth (30). *E. coli* transformants were selected in the presence of ampicillin (100 μg ml^{-1}) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 0.1 mM) to detect β-galactosidase activity.

Analysis of casein hydrolysis. Bacterial cells were collected in exponential phase as described previously (2) and treated by the procedure of Hill and Gasson (14) as modified (24). Peptides resulting from casein degradation were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (23) on

* Corresponding author. Phone: 33 72 43 13 66. Fax: 33 72 43 11 81. Electronic mail address: lmgm@biomserv.univ-lyon1.fr.

BNSDOCID: <XP___608165A__I_>

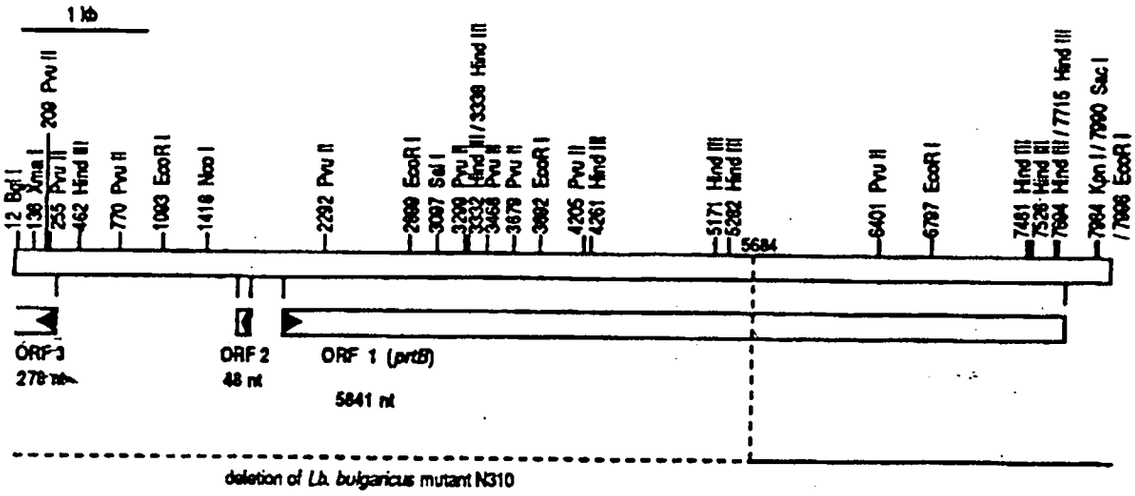


FIG. 1. Restriction map of a chromosomal DNA sequence of 8,071 nucleotides (nt) from *L. delbrueckii subsp. bulgaricus* NCDO1489. Directions of transcription of three ORFs are indicated by arrows. Deletion of mutant N310 within the *prtB* gene is shown by dashed lines.

12% acrylamide gels and stained with Coomassie blue as described previously (24).

Molecular techniques and transformation. Genomic DNA of *L. delbrueckii* subsp. *bulgaricus* was purified as described previously (6). Reverse PCR was performed from self-ligated *Eco*RI fragments of chromosomal DNA as described previously (11). PCR-amplified DNA fragments were blunt ended with T4 polymerase and cloned in plasmid pUC18 or pUC19. Plasmid extraction, restriction enzyme digestion, ligation, and *E. coli* transformation were performed according to standard procedures (37). Stringent conditions were used for Southern blot hybridizations (44). Primers were made by Schmihcini (Windisch, Switzerland) and the Molecular and Cellular Genetics Centre of the University Lyon I (Villeurbanne, France).

DNA sequencing and analysis. DNA sequences of plasmid inserts were determined by the dideoxy-chain termination method with the T7 and DeazaT7 sequencing kits (Pharmacia) and universal and reverse primers (38). Alternatively, the Fmol DNA sequencing system (Promega Corp., Madison, Wis.) was used for direct sequence determination of some DNA fragments.

Nucleotide and amino acid sequences data were analyzed by using the Bisance databases (8), and the sequence alignments were performed with the FASTA program (26).

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence reported in this paper is L48487.

RESULTS AND DISCUSSION

***L. delbrueckii* subsp. *bulgaricus* mutant with a cell wall-bound proteinase deficiency.** Mutant N310, with a large chromosomal deletion (12 kb) from the *lacZ* gene (11), was unable to develop normally in milk supplemented with glucose; rapid growth was restored by addition of a protein digest (proteose peptone) (data not shown). Kinetics of β-casein hydrolysis by whole cells of *L. delbrueckii* subsp. *bulgaricus* CNRZ397, NCDO1489, and LFi5.F6 (a mutant carrying downstream of the *lacZ* gene a deletion shorter than that detected in mutant N310) (11) showed the same major products (our unpublished results). β-Casein was not hydrolyzed by mutant N310. Consequently, this mutant is deficient in a cell surface proteinase activity, and genes involved in biosynthesis or biosynthesis regulation or in maturation of the proteinase were suspected to be located near the *lac* region. The β-casein hydrolysis pattern obtained with *Lactococcus lactis* subsp. *lactis* NCDO763 showed additional peptides. This result suggests that the cleavage specificity of cell surface proteinases from *L. delbrueckii* subsp. *bulgaricus* and *L. lactis* subsp. *lactis* are different.

Identification, DNA sequencing, and analysis of the *prtB* region. The DNA region located downstream of the deletion in the *lacZ* gene from mutant N310 was isolated by reverse PCR, and its DNA sequence was determined (Fig. 1). Search for protein homology in protein libraries revealed an identity of 27.8% between the deduced amino acid sequence and the sequence of PrtP from *L. paracasei* subsp. *paracasei* NCDO 151. The region downstream of *lacZ* and including the entire *prtP*-like gene was further isolated and sequenced in a cascade PCR strategy using synthetic primers as the known sequence progressed. A restriction map and partial sequence of 8,071 identified nucleotides are presented in Fig. 1 and 2 and showed three relevant open reading frames (ORFs).

The largest ORF (ORF1) displays homology with lactococcal *prtP* genes and displays three putative start ATG codons in frame at positions 1862, 1886, and 1940. However, the protein alignment with PrtP and the presence of a Shine-Dalgarno sequence (at position 1930) suggest the ATG at position 1940 as a valid start codon. We will refer to the *L. delbrueckii* subsp. *bulgaricus* proteinase as PrtB and its gene as *prtB*. The putative *prtB* is 5,841 nucleotides long and is preceded by promoter-like sequences; −35 (TTCAGA) and −10 (TTTGAT) boxes with a spacing of 16 nucleotides are in accordance with the promoter consensus sequences of *E. coli* (TTGACA and TATAAT, respectively). A UP element, characterized by oligo(A) and T stretches, is detected 5 nucleotides upstream the −35 box. Such an element is known to enhance transcription in bacteria (36) and has been found upstream of promoters of lactococcal *prtP* genes and several peptidase genes of *L. lactis* (22, 28). The stop codon (TAA) at position 7778 is followed by a dyad symmetry. A hairpin, detected between nucleotides 7784 and 7818, is consistent with a terminator structure and displays a stem of 11 bp (with two mismatches) and a loop of 13 nucleotides ($\Delta G = -18.1$ kcal [ca. −75.7 kJ]/mol). The GC content of the *prtB* gene is 47.6%, which is very similar to the values calculated for the *prtP* genes of *L. paracasei* subsp. *paracasei* and *Lactococcus lactis* subsp. *lactis* NCDO763 (47.5 and 47.2%, respectively). Consequently, the GC contents of proteinase genes differ significantly from the GC contents of their respective genomes: 54% for *L. delbrueckii* subsp. *bulgaricus* and 38% for *L. lactis* subsp. *lactis* (19). Other genes (*lacZ*, *lacS*, and *pepIP*) from *L. delbrueckii* subsp. *bulgaricus* show GC contents within the expected ranges (52.5, 53.1, and 56.9%, respectively) (1, 25).

BNSDOCID: <XP____608165A__1_>

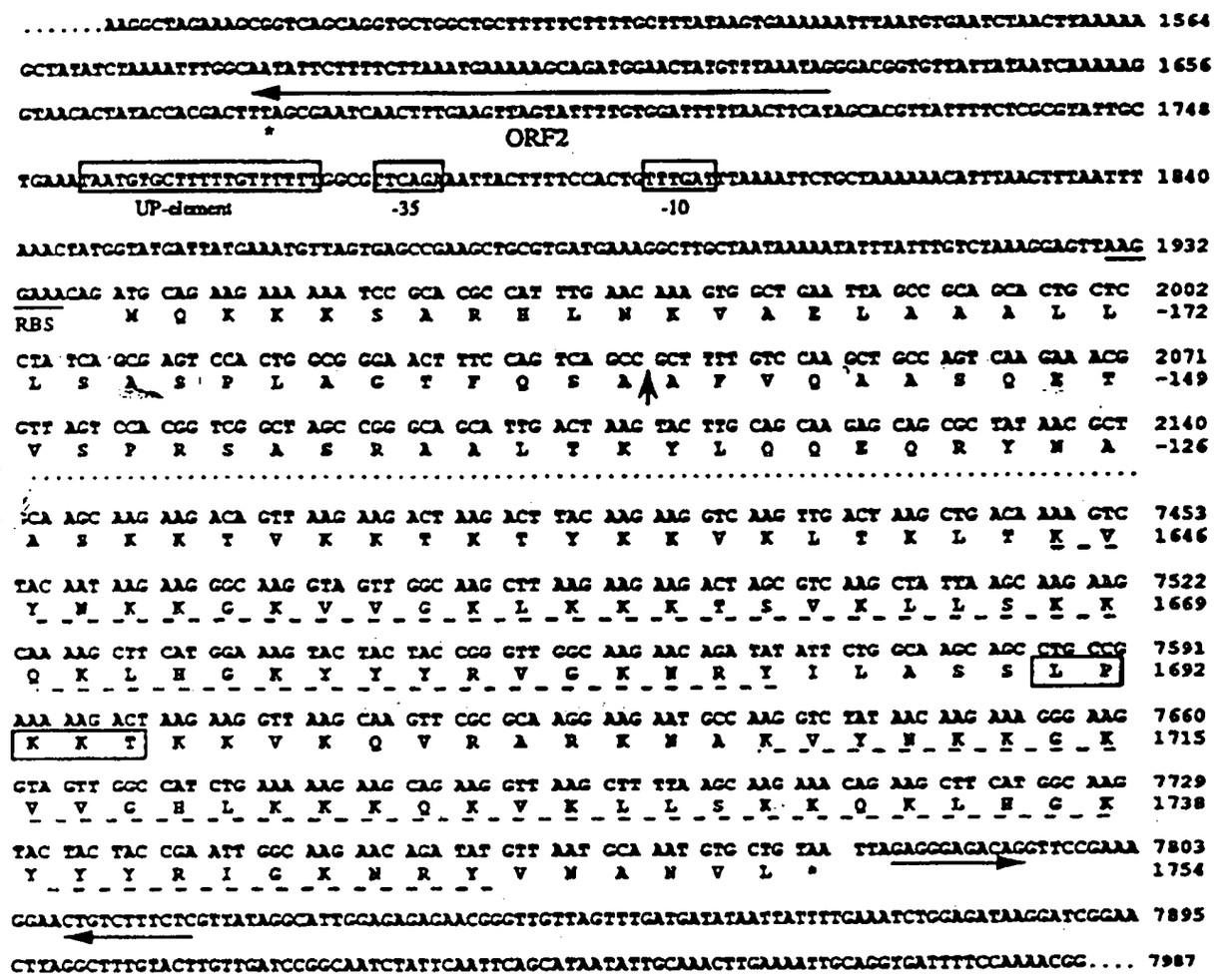


FIG. 2. Partial nucleotide and deduced amino acid sequences of the *L. delbrueckii* subsp. *bulgaricus prtB* gene and its flanking regions. The predicted −35 and −10 regions of the putative promoter and the UP element of the *prtB* gene are boxed. The proposed ribosome binding site (RBS) is underlined. The dyad symmetry of the terminator structure is indicated by convergent arrows. The amino acid sequence deduced from the *prtB* gene is numbered from the first amino acid of the putative proteinase. The putative cleavage site of the signal sequence is shown by a vertical arrow. The duplicated C-terminal stretches are underlined with dotted lines, and the LPKKT motif is boxed. ORF2 is shown by an arrow above the sequence indicating the direction of transcription. Stop codons are indicated with asterisks.

A DNA region (1711 to 1750) located upstream of the *prtB* gene displays 73.8% identity with the *L. paracasei* subsp. *paracasei prtM* gene. ORF2 is divergent from the *prtB* gene, partially overlaps this region homologous to *prtM*, and spans positions 1723 (ATG) to 1678 (TAA). The resulting polypeptide (MKLKIHKILTSRLIR) is very too short to act as a functional chaperone. No sequences homologous to *prtM* or ORF2 were found downstream of *prtB*.

A truncated ORF (ORF3) is located 1,663 nucleotides upstream and in the opposite direction of *prtB*. The start codon (ATG) is at position 278, and the deduced polypeptide shows no homology with a known protein and is devoid of signal peptide at the N terminus.

Amino acid content of PrtB and comparison with PrtP and other lactobacilli proteins. The putative PrtB proteinase consists of 1,946 amino acids and is characterized by a predicted M_r of 212,271. This length is consistent with those of previously described PrtP proteinases: 1,902 residues in *L. paracasei* subsp. *paracasei*, *Lactococcus lactis* subsp. *cremoris* Wg2, and *L. lactis* subsp. *lactis* NCDO763 and 1,962 residues in *Lactococcus lactis* subsp. *cremoris* SK11, associated with a duplication of 60 amino acids at the C-terminal end (15, 20, 22, 48). PrtB shows 27% identity with PrtP of *L. paracasei* subsp. *paracasei* over the first 1,806 residues and up to 39.5% when only the first 820 residues are compared. The C terminal part of PrtB shows no homology with PrtP proteinases.

The major amino acid of PrtB is lysine (12%), compared with leucine for other *L. delbrueckii* subsp. *bulgaricus* enzymes

TABLE 1. Comparison of domains surrounding the cleavage sites of pro regions from cell surface proteinases

Surface proteinase	Cleavage site of the pro region[a]
PrtB from *L. delbrueckii* subsp. *bulgaricus*	VKNVTPVKVYHPTDESADQMAQVQDVW
PrtPs[b]	VKTVTLAKVYYPTDAKANSMANVQAVW

[a] The cleavage site of PrtPs is indicated by an arrow.
[b] PrtP sequences are related to those of *L. paracasei* subsp. *paracasei* NCDO151, *Lactococcus lactis* subsp. *cremoris* Wg2 and SK11, and *Lactococcus lactis* subsp. *lactis* NCDO763.

BNSDOCID: <XP___608165A_I_>

TABLE 2. Comparison of C termini of *L. delbrueckii* subsp. *bulgaricus* PrtB, PrtPs and some proteins harboring the anchoring signal LPxTG, protein A from *S. aureus*, and protein M6 from *Streptococcus pyogenes*

Protein	C terminus[a]
L. delbrueckii subsp. *bulgaricus* PrtB	LPKKTKKVKQVRARKSAKVYNKKGKVVGHL--KKKQKVKKLLSK..[b]
L. paracasei subsp. *paracasei* NCDO151 PrtP	LPKTAETTERPAFGFLGVIVVSLMGVLGL--KRKQREE
Lactococcus lactis subsp. *cremoris*	↓
Wg2 PrtP	LPKTGETTERPAFGFLGVIVVILMGVLGL--KRKQREE
SK11 PrtP	LPKTGETTERPAFGFLGVIVVSLMGVLGL--KRKQREE
Lactococcus lactis subsp. *lactis* NCDO763 PrtP	LPKTGETTERPAFGFLGVIVVSLMGVLGL--KRKQREE
S. aureus protein A	LPETGE--ENPLIGTTVFGGLSLAGAALLAGRRREL
Streptococcus pyogenes protein M6	LPSTGETA-NPFFTAAALTVMATAGVAAVV-KRKEEN

[a] LPxTG sorting signal, the proline (P) preceding the α-helix, and the charged tail are indicated in boldface. A vertical arrow shows the cleavage site between threonine (T) and glycine (G).
[b] An extension of 23 residues is present without any E.

(LacS, β-galactosidase, and PepIP) or threonine for other lactococcal and *L. paracasei* subsp. *paracasei* proteinases and S-layer protein (SlpA) of *L. brevis* (47). Codon usage is similar to that of other *L. delbrueckii* subsp. *bulgaricus* enzymes for lysine and glycine, but serine, aspartic acid, and valine are preferentially encoded as in lactococci (data not shown).

PrtB and cell envelope location. The sequence homology between N-terminal regions of PrtB with PrtP proteinases suggests that PrtB is probably synthesized as a preproprotein. PrtB exhibits a positively charged N extremity followed by a predicted membrane-spanning helix with a high content in leucine and alanine. This domain closely resembles those of signal peptides of exported proteins in gram-positive bacteria (42). The putative cleavage site compared with those that of other PrtP proteinases (Fig. 2) is most probably located between Ala-159 and Ala-158. A second cleavage site between Ala-154 and Ala-153 cannot be ruled out. The consensus recognition sequence is Ala-X-Ala, with a cleavage after the carboxy-terminal alanine, but a cleavage between two alanines was also reported (neutral proteases from *Bacillus amyloliquefaciens* and *B. subtilis*) (42). Consequently, the expected signal peptide might be 34 (or 39) residues long, which is in agreement with the lengths of the signal peptides from gram-positive bacteria. The signal sequence is followed by a region homologous to pro regions of lactococcal and *L. paracasei* subsp. *paracasei* PrtP proteinases. The cleavage site of pro regions of lactococcal PrtPs was determined between residues 186 (T) and 187 (D) (Table 1). A domain with high identity was found in *L. delbrueckii* subsp. *bulgaricus* PrtB, and we assume that a cleavage may occur between residues 192 (T) and 193 (D at position 1). The resulting mature PrtB protein would contain 1,754 residues.

The C extremity of PrtB is quite different from the corresponding domains described for PrtP proteinases. The PrtB C terminus is characterized by a high lysine content and displays an imperfect duplication of a stretch of 41 amino acids, residues 1645 to 1685 and 1708 to 1748, with different amino acids at four positions (Fig. 2). The carboxy end of PrtP proteinases shows a consensus sequence detected in several cell envelope-associated proteins of gram-positive bacteria, e.g., protein A from *Staphylococcus aureus* and protein M6 from *Streptococcus pyogenes* (16, 46, 48) (Table 2). A predicted hydrophobic α helix of 18 to 20 amino acids is preceded by a proline residue and followed by a charged tail. Recent data showed that a conserved motif, LPxTG, located 10 amino acids upstream of the proline, acts as a sorting signal anchoring the protein in the cell wall (40). A mechanism was proposed for all surface proteins harboring this sorting signal. A specific cleavage occurs between the threonine and glycine, and a peptide bond would occur between the liberated carboxy terminus of the threonine and the N terminus of a residue from the peptidoglycan cross bridge (34). PrtB displays a degenerated sorting signal (LPKKT, 1691 to 1695) immediately followed by a charged region; this signal is located between both duplicated stretches of 41 residues. In contrast to PrtP, the region preceding the sorting motif shows a high lysine content. These data suggest that PrtB is attached to the cell envelope differently from the other proteinases.

TABLE 3. Comparison of sequences surrounding the amino acids involved in the active site of cell surface proteinases and other subtilisin family proteases

Protein	Sequence[a]			
	D-30,[b] D-30[c]	H-94, H-94	N-189, N-196	S-425, S-433
Surface proteinase				
L. delbrueckii subsp. *bulgaricus*	VISIIDTGIDS	GCGEMHGQHVA	VISAGNSGVAG	MSGTSMASP
L. paracasei subsp. *paracasei* NCDO151	VVSVIDTGIDP	TVDEQHGMHVA	VISAGNSGTSG	MSGTSMASP
Lactococcus lactis subsp. *cremoris*				
Wg2	VVSVIDSGIDP	TVDEQHGMHVA	VISAGNSGTSG	MSGTSMASP
SK11	VVSVIDSGIDP	KVDEQHGMHVA	VISAGNSGTSG	MSGTSMASP
Lactococcus lactis subsp. *lactis* NCDO763	VVSVIDSGIDP	TVDEQHGMHVA	VISAGNSGTSG	MSGTSMASP
B. subtilis Carlsberg subtilisin	KVAVLDTGIQA	TDGNSHGTHVA	VAAAGNSGNSG	LNGTSMASP
Saccharomyces cerevisiae Protease B	TSYVIDTGVNI	LDGNGHGTHCA	AVAAGNENQDA	LSGTSMASP
Tritirachium album Proteinase K	CVYVIDTGIEA	RDGNGHGTHCA	AVAAGNNNADA	ISGTSMATP
T. vulgaris Thermitase	KIAIVDTGVQS	QNGNGHGTHCA	VAAAGNAGNTA	LSGTSMATP

[a] Cysteines and amino acids involved in the catalysis and stabilization of the oxyanion hole are shown in boldface.
[b] Numbering corresponds to the *L. delbrueckii* subsp. *bulgaricus* mature PrtB.
[c] Numbering corresponds to the mature PrtPs of *L. paracasei* subsp. *paracasei* NCDO 151 and *Lactococcus lactis* subsp. *cremoris* SK11.

BNSDOCID: <XP___608165A__I_>

TABLE 4. Comparison of sequences surrounding several substrate-binding sites in cell surface proteinases and other subtilisin family proteases

Protein	Sequence at indicated site[a]					
	124[b] 131[c]	131 138	135–137 142–144	159 166	215 222	728–729 747–748
Surface proteinase						
L. delbrueckii subsp. *bulgaricus*	AMKVFSNNAKNSGAYDDDIISA		MSLGSVSSDVG	ELSTVGTPGT		GVGKEGTKDYYS
L. paracasei subsp. *paracasei* NCDO151	AMKVFTNSDTSATTGSATLVSA		MSLGSDSGNQT	DNEMVGTPGT		SSSTNQTKTYYD
Lactococcus lactis subsp. *cremoris*						
Wg2	AMKVFTNSDTSATTGSSTLVSA		MSLGSDSGNQT	DNEMVGTPGT		SSSTNLTKTYYN
SK11	AMKVFSNSDTSAKTGSATVVSA		MSLGSNSGNQT	DNEMVGSPGT		SSSTNRKKTYYN
Lc. lactis NCDO763	AMKVFTNSDTSATTGSATLVSA		MSLGSDSGNQT	DNEMVGTPGT		SSSTNRKKTYYN
B. subtilis Carlsberg subtilisin	AVKVLNSSGSGS---YSGIVSG		MSLGGPSGS--	-----GYPAK		
T. vulgaris thermitase	AVRVLDNSGSGT---WTAVANG		LSLGGTVGN--	-----NYPAY		

[a] Amino acids different between PI and PIII lactococcal proteinases are indicated in boldface.
[b] Numbering corresponds to the *L. delbrueckii* subsp. *bulgaricus* mature PrtB.
[c] Numbering corresponds to the mature PrtPs of *L. paracasei* subsp. *paracasei* NCDO151 and *Lactococcus lactis* subsp. *cremoris* SK11.

Putative active site of PrtB. Comparison of the PrtB amino acid sequence with those of other PrtP proteinases shows a strong homology of the stretches surrounding the three amino acids (Asp-30, His-94, and Ser-425) involved in the catalytic site and the asparagine (N-189) providing electrostatic stabilization of the oxyanion hole (50) (Table 3). The known PrtPs and subtilisins are devoid of cysteine residues (43). In contrast, a single cysteine has been found at position 90 of PrtB, i.e., closely upstream of the histidine of the active site. Interestingly, PrtB is known to be sensitive to cysteine and serine protease inhibitors (24). These characteristics prompted us to propose PrtB as a member of the cysteine-containing subgroup of subtilisins, exhibiting a cysteine residue four amino acids downstream of the histidine. The identified enzymes of this subgroup are the eukaryotic proteinase K (*Tritirachium album*) and protease B (*Saccharomyces cerevisiae*) and thermitase, an extracellular proteinase from *Thermoactinomyces vulgaris* (18, 29, 31). Thermitase displays 44% identity with subtilisins, and the most striking homologies are observed in the regions involving the active site; the overall tertiary structure of its active site is not thought to be modified by the presence of a cysteine residue.

Putative substrate binding regions of PrtB. Two regions of lactococcal PrtP proteinases are known to play a role in the specific hydrolysis of caseins (41). The lactococcal region from 130 to 225 is homologous to the substrate-binding region of subtilisins. In a three-dimensional structure, lactococcal residues 138 and 166 are located on either side of the substrate-binding cleft. They correspond to threonine (T) or lysine (K) (at position 138) and aspartic acid (D) or asparagine (N) (at position 166) in *Lactococcus lactis* subsp. *cremoris* Wg2 or SK11, respectively (Table 4). In *L. delbrueckii* subsp. *bulgaricus*, a glycine (G) corresponds to the lactococcal residue 138 and a valine (V) to residue 166. Furthermore, the PrtB domain corresponding to lactococcal residues 133 to 145 is quite different, and the substitutions of residues 137 to 139 in *Lactococcus lactis* subsp. *cremoris* SK11 are known to modify the substrate specificity and activity of PrtP (41).

The second substrate-binding region of PrtP proteinases is located in the remote C extension (with no equivalent in subtilisin) and characterized by the adjacent residues 747 and 748: RK in *Lactococcus lactis* subsp. *cremoris* SK11 and *Lactococcus lactis* subsp. *lactis* NCDO763, LT in *Lactococcus lactis* subsp. *cremoris* Wg2, and QT in *L. paracasei* subsp. *paracasei*. The PrtP domain including residues 747 and 748 shows homology with a region of PrtB, but the amino acids corresponding to lactococcal residues 747 and 748 are different: glycine (G-728) and threonine (T-729) (Table 4).

These results are in accordance with the biochemical data of casein degradation and strengthen the hypothesis of different

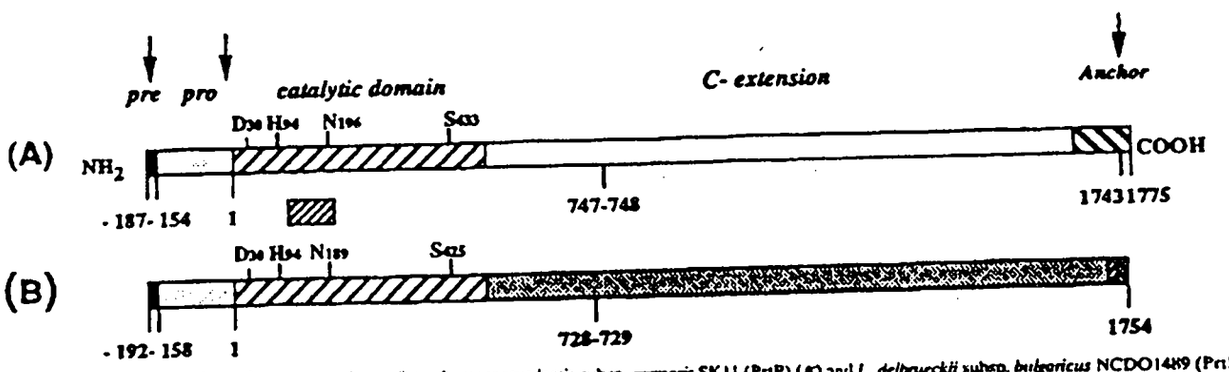


FIG. 3. Schematic comparison of preproproteinases from *Lactococcus lactis* subsp. *cremoris* SK11 (PrtP) (A) and *L. delbrueckii* subsp. *bulgaricus* NCDO1489 (PrtB) (B). The various domains are indicated, and arrows correspond to the cleavage sites identified in PrtP. The active-site triad (aspartic acid [D], histidine [H], and serine [S]) and asparagine (N) necessary for oxyanion binding are noted. The region from 130 to 225 (▨) is located in the catalytic domain and is homologous to the substrate-binding sites of subtilisins. Residues 747 and 748 of PrtP are also thought to take part in this substrate-binding site and are located in the long C extension. This latter region, and especially the C extremity, is significantly different in the two proteinases: PrtP is characterized by a 60-amino-acid duplication followed by a consensus sequence containing the sorting signal LPxTG; this motif is probably cleaved (at position 1743), causing the cell wall anchoring of PrtP. With regard to PrtB, a duplication of 41 residues is identified but the LPxTG motif is degenerated.

BNSDOCID: <XP____608165A__I_>

substrate specificities for proteinases from lactobacilli and lactococci.

In conclusion, the cell surface proteinase PrtB of *L. delbrueckii* subsp. *bulgaricus* is probably synthesized as a preproprotein of 1,946 amino acids but strongly differs from PrtP proteinases (Fig. 3). The absence of a *prtM*-like gene located immediately upstream or downstream of the *prtB* gene and expressing a functional chaperone gives rise to two hypotheseses. The maturation and export of PrtB could be processed by a PrtM-like chaperone encoded by a gene located elsewhere on the chromosome. However, it cannot be ruled out that a general chaperone could act on different extracytoplasmic proteins, PrtB included, as does the PrsA protein involved in the maturation and export of subtilisins and α-amylase of *B. subtilis* (17).

In contrast to the PrtP proteinases, PrtB is not recovered in the culture medium and consequently is not able to remove its C extremity by self-digestion. The difference of amino acid sequence in this domain suggests an absence of essential cleavage sites or a three-dimensional structure not consistent with the recognition of these sites. Assuming that the LPXTG signal and the closed α helix are essential to the anchorage of PrtPs in the cell wall, the attachment of PrtB to the envelope likely depends on a different mechanism.

The peptides released from β-casein by PrtB action are different from those resulting from the hydrolysis by PrtP. Other steps of the proteolytic system of *L. delbrueckii* subsp. *bulgaricus* are also suspected to be different. For instance, *L. delbrueckii* subsp. *bulgaricus* exhibits high peptidase activities toward substrates containing proline (5, 12, 39).

Comparison of PrtB and PrtP proteinases showed that these serine proteases probably evolved from two closely related enzyme families, subtilisin and cysteine subtilisin, respectively. A divergence is also observed with the *lac* system, essential to the growth of lactic acid bacteria in milk. In most mesophilic lactococci, the lactose degradation system (phosphoenolpyruvate-dependent phosphotransferase system and phospho-β-galactosidase) (9) and the *prtP* and *prtM* genes are often harbored on different plasmids. It is interesting that although the two systems are different in *L. delbrueckii* subsp. *bulgaricus*, they are located in the same chromosomal region.

ACKNOWLEDGMENTS

We thank M. Delley for providing the strains N310 and LFiS.F6.

This work was supported by research funds from the Centre National de la Recherche Scientifique (UMR 106) and Université Claude Bernard-Lyon I.

REFERENCES

1. Atlan, D., C. Gilbert, B. Blanc, and R. Portalier. 1994. Cloning, sequencing and characterization of the *pepIP* gene encoding a proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ397. Microbiology 140:527–535.
2. Atlan, D., P. Laloi, and R. Portalier. 1989. Isolation and characterization of aminopeptidase-deficient *Lactobacillus bulgaricus* mutants. Appl. Environ. Microbiol. 55:1717–1723.
3. Boot, H., C. Kolen, J. van Noort, and P. Pouwels. 1993. S-layer protein of *Lactobacillus acidophilus* ATCC4356: purification, expression in *Escherichia coli*, and nucleotide sequence of the corresponding gene. J. Bacteriol. 175: 6089–6096.
4. Bosman, B., M. Sasaki, and P. Tan. 1993. Comparative study on proteinase activity in lactobacilli. FEMS Microbiol. Rev. 12:P72D9.
5. Casey, M., and J. Meyer. 1985. Presence of X-prolyl-dipeptidyl-peptidase in lactic acid bacteria. J. Dairy Sci. 68:3212–3215.
6. Delley, M., B. Mollet, and H. Hottinger. 1990. DNA probe for *Lactobacillus delbrueckii*. Appl. Environ. Microbiol. 56:1967–1970.
7. de Man, J., M. Rogosa, and E. Sharpe. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23:130–135.
8. Dessen, P., C. Fondrat, C. Valencien, and C. Mugnier. 1990. Bisance: a French service for access to biomolecular sequence databases. CABIOS 6:335–336.
9. de Vos, W., and E. Vaughan. 1994. Genetics of lactose utilization in lactic acid bacteria. FEMS Microbiol. Rev. 15:217–237.
10. Exterkate, F., A. Alting, and P. Bruinenberg. 1993. Diversity of cell envelope proteinase specificity among strains of *Lactococcus lactis* and its relationship to charge characteristics of the substrate-binding region. Appl. Environ. Microbiol. 59:3640–3647.
11. Germond, J. E., L. Lapierre, M. Delley, and B. Mollet. 1995. A new mobile element in *Lactobacillus delbrueckii* subsp. *bulgaricus*. Mol. Gen. Genet. 248:407–416.
12. Gilbert, C., D. Atlan, B. Blanc, and R. Portalier. 1994. Proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ397: purification and characterization. Microbiology 140:537–542.
13. Haandrikman, A., J. Kok, J. S. Soemitro, A. Ledeboer, W. Konings, and G. Venema. 1989. Identification of a gene required for maturation of an extracellular lactococcal serine proteinase. J. Bacteriol. 171:2789–2794.
14. Hill, S., and M. Gasson. 1986. A quantitative screening procedure for the detection of casein hydrolysis by bacteria, using sodium dodecyl sulphate polyacrylamide gel electrophoresis. J. Dairy Res. 53:625–629.
15. Holck, A., and H. Naes. 1992. Cloning, sequencing and expression of the gene encoding the cell-envelope-associated proteinase from *Lactobacillus paracasei* subsp. *paracasei* NCDO151. J. Gen. Microbiol. 138:1353–1364.
16. Hollingshead, S., V. Fishetti, and J. Scott. 1986. Type 6M protein of the group A *Streptococcus*. J. Biol. Chem. 261:1677–1686.
17. Jacobs, M., J. Andersen, V. Kontinen, and M. Sarvas. 1993. *Bacillus subtilis* PrsA is required *in vivo* as an extracytoplasmic chaperone for secretion of active enzymes synthesized with or without prosequences. Mol. Microbiol. 8:957–966.
18. Jany, K.-D., G. Lederer, and B. Mayer. 1986. Amino acid sequence of proteinase K from the mold *Tritirachium album* Limber. FEBS Lett. 199: 139–144.
19. Klipper-Balz, R., G. Fisher, and K. Schleifer. 1982. Nucleic acid hybridation of group N and D streptococci. Curr. Microbiol. 7:245–250.
20. Kiwaki, M., H. Ikemura, M. Shimizu-Kadota, and A. Hirashima. 1989. Molecular characterization of a cell-wall associated proteinase gene from *Streptococcus lactis* NCDO763. Mol. Microbiol. 3:359–369.
21. Kok, J. 1990. Genetics of the proteolytic system of lactic acid bacteria. FEMS Microbiol. Rev. 87:15–42.
22. Kok, J., K. Leenhouts, A. Haandrikman, A. Ledeboer, and G. Venema. 1988. Nucleotide sequence of the cell-wall proteinase gene of *Streptococcus cremoris* Wg2. Appl. Environ. Microbiol. 54:231–238.
23. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
24. Laloi, P., D. Atlan, B. Blanc, C. Gilbert, and R. Portalier. 1991. Cell-wall associated proteinase of *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ397: differential extraction, purification and properties of the enzyme. Appl. Microbiol. Biotechnol. 36:196–204.
25. Leong-Morgenthaler, P., M. Zwahlen, and H. Hottinger. 1991. Lactose metabolism in *Lactobacillus bulgaricus*: analysis of the primary structure and expression of the genes involved. J. Bacteriol. 173:1951–1957.
26. Lipman, D., and W. Pearson. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA 85:2444–2448.
27. Martin-Hernandez, C., A. Alting, and F. Exterkate. 1994. Purification and characterization of the mature, membrane associated cell-envelope proteinase of *Lactobacillus helveticus* L89. Appl. Microbiol. Biotechnol. 40:828–834.
28. Matera, H., J. Klein, J. Heinrich, and R. Plapp. 1995. Determination and comparison of *Lactobacillus delbrueckii* subsp. *lactis* DSM7290 promoter sequences. FEMS Microbiol. Lett. 122:121–128.
29. Meloun, B., M. Dandys, V. Kostka, G. Hausdorf, C. Frömmel, and W. Höhne. 1985. Complete primary structure of thermitase from *Thermoactinomyces vulgaris* and its structural features related to the subtilisin-type proteinases. FEBS Lett. 183:195–199.
30. Miller, J. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. Moehle, C., R. Tizard, S. Lemmon, J. Smart, and E. Jones. 1987. Protease B of the lysomelike vacuole of the yeast *Saccharomyces cerevisiae* is homologous to the subtilisin family of serine proteases. J. Bacteriol. 7:4390–4399.
32. Mollet, B., and M. Delley. 1990. Spontaneous deletion formation within the β-galactosidase gene of *Lactobacillus bulgaricus*. J. Bacteriol. 172:5670–5676.
33. Naes, F., and J. Nissen-Meyer. 1992. Purification and N-terminal amino acid sequence determination of the cell-wall-bound proteinase from *Lactobacillus paracasei* subsp. *paracasei*. J. Gen. Microbiol. 138:313–318.
34. Navarre, W., and O. Schneewind. 1994. Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in gram-positive bacteria. Mol. Microbiol. 14:115–121.
35. Pritchard, G., and T. Coolbear. 1993. The physiology and biochemistry of the proteolytic system in lactic acid bacteria. FEMS Microbiol. Rev. 12:179–206.
36. Ross, W., K. Gosink, J. Salomon, K. Igarashi, C. Zou, A. Ishihama, K. Severinov, and R. Gourse. 1993. A third recognition element in bacterial promoters: DNA binding by the α subunit of RNA polymerase. Science 262:1407–1413.

196

BNSDOCID: <XP___608165A__I_>

37. Sambrook, J., E. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. Sanger, F., S. Nicklen, and A. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.
39. Sasaki, M., B. Bosman, and P. Tan. 1993. Characterisation of exopeptidase profiles of *Lactobacillus* species. FEMS Microbiol. Rev. 12:P75D16.
40. Schneewind, O., D. Mihaylova-Petkov, and P. Model. 1993. Cell wall sorting signals in surface proteins of Gram-positive bacteria. EMBO J. 12:4803–4811.
41. Siezen, R., P. Bruinenberg, P. Vos, L. van Alen-Boerrigter, M. Nijhuis, A. Alting, F. Exterkate, and W. de Vos. 1993. Engineering of the substrate-binding region of the subtilisin-like, cell-envelope proteinase of *Lactococcus lactis*. Protein Eng. 6:927–937.
42. Simonen, M., and I. Palva. 1993. Protein secretion in *Bacillus* species. Microbiol. Rev. 57:109–137.
43. Smith, E., R. Delange, W. Evans, M. Landon, and F. Markland. 1968. Subtilisin carlsberg. V. The complete sequence; comparison with subtilisin BPN'; evolutionary relationships. J. Biol. Chem. 243:2184–2191.
44. Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.
45. Terzaghi, B., and W. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Environ. Microbiol. 29:807–813.
46. Uhlen, M., B. Guss, B. Nilsson, S. Gatenbeck, L. Philipson, and M. Lindberg. 1984. Complete sequence of the staphylococcal gene encoding Protein A. J. Biol. Chem. 259:1695–1702.
47. Vidgrén, G., I. Palva, R. Pakkanen, K. Lounatmaa, and A. Palva. 1992. S-layer gene of *Lactobacillus brevis*: cloning by polymerase chain reaction and determination of the nucleotide sequence. J. Bacteriol. 174:7419–7427.
48. Vos, P., G. Simons, R. Siezen, and W. de Vos. 1989a. Primary structure and organization of the gene for procaryotic, cell envelope-located serine proteinase. J. Biol. Chem. 264:13579–16585.
49. Vos, P., M. van Asseldonk, F. van Jeveren, R. Siezen, G. Simons, and W. de Vos. 1989b. A maturation protein is essential for production of active forms of *Lactococcus lactis* SK11 serine proteinase located or secreted from the cell envelope. J. Bacteriol. 171:2795–2802.
50. Wells, A., and D. Estell. 1988. Subtilisin, an enzyme designed to be engineered. Trends Biochem. Sci. 13:291–297.
51. Yanisch-Perron, C., J. Vieira, and J. Messing. 1991. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119.
52. Zevaco, C., and J. C. Gripon. 1988. Properties and specificity of a cell-wall proteinase from *Lactobacillus helveticus*. Lait 68:393–408.

BNSDOCID: <XP____608165A__I_>

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/63, 15/86, 5/16, A61K 39/00	A1	(11) International Publication Number: WO 95/21926
		(43) International Publication Date: 17 August 1995 (17.08.95)

(21) International Application Number: PCT/US95/01671

(22) International Filing Date: 10 February 1995 (10.02.95)

(30) Priority Data:
08/195,874 14 February 1994 (14.02.94) US

(71) Applicant: UNIVERSITY OF ROCHESTER [US/US]; 518 Hylan Building, Rochester, NY 14627 (US).

(72) Inventors: SCOTT, David, W.; 49 Hunters Pointe, Pittsford, NY 14534 (US). ZAMBIDIS, Elias, T.; 5 Selden Street, Rochester, NY 14605 (US).

(74) Agent: RAASCH, Kevin, W.; Schwegman, Lundberg & Woessner, 3500 IDS Center, 80 South Eighth Street, Minneapolis, MN 55402 (US).

(81) Designated States: CA, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: INDUCING TOLERANCE WITH TOLEROGENIC FUSION PROTEINS

(57) Abstract

The invention provides methods and compositions for inducing and maintaining tolerance to epitopes or antigens containing the epitopes. The compositions include expression cassettes and vectors including DNA sequences coding for a fusion immunoglobulin operably linked to transcriptional and translational control regions functional in a hemopoietic or lymphoid cell. The fusion immunoglobulin includes at least one heterologous tolerogenic epitope at the N-terminus variable region of the immunoglobulin. Cells stably transformed with the expression vector are formed and used to produce fusion immunoglobulin. The invention also provides methods for screening for novel tolerogenic epitopes and for inducing and maintaining tolerance. The methods of the invention are useful in the diagnosis and treatment of autoimmune or allergic immune responses.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

BNSDOCID: <WO___9521926A1_I_>

INDUCING TOLERANCE WITH TOLEROGENIC FUSION PROTEINS

Background of the Invention

Self-nonself discrimination is one of the cornerstones of immunology. Normally, individuals develop tolerance to self constituents during the early development of the immune system. However, the maintenance of this unresponsive state requires the persistence of antigen, a fact which implies that tolerance induction is a lifelong process. Smith, Advances in Immunology, 1:67 (1961). Indeed, the breakdown of tolerance in older individuals explains the increased incidence of autoimmunity in aging populations.

Isologous or heterologous gamma globulins have been used as tolerogenic carrier molecules (primarily IgG's). Scott, Immunol. Rev., 43:241 (1979). Although different sources of IgG's may vary in their persistence and/or mechanism of tolerance induction, by far, IgG carriers have been the most efficacious at tolerance inductions in adults to haptens, nucleosides and peptides. Borel, Immunological Reviews, 50:71 (1980); and Scott, Cell Immunol., 22:311 (1976). These carriers owe their superior tolerogenicity to their persistence in vivo and the ability of epitopes chemically attached to IgG's to crosslink mIgM with B-cell Fc receptors. However, chemical crosslinking of epitopes to IgG carriers is limited by the availability of free amino groups and the uncontrolled targeting of the added determinant to different portions of the IgG.

Recombinant DNA technology can be used to genetically engineer molecules having heterologous epitopes. For example, heterologous oligopeptide epitopes of biological interest have been expressed in bacterial flagellin (Jennings et al., Protein Eng., 2:365 (1989)); hepatitis B surface antigen (Rutgers et al., Biotechnology, 6: 1065 (1988)); and in the complementarity determining regions of immunoglobulins (Zanetti et al., Nature, 355:476 (1992)). Some attempts have been made to test the ability of recombinant proteins to serve as antigens to immunize animals and generate immune responses to the heterologous oligopeptide. However, induction and

maintenance of tolerance to oligopeptides presented to the immune system has not been demonstrated. The ability to maintain tolerance to an antigen or epitope requires persistence of the epitope in vivo.

Therefore, there is a need to develop a method of inducing stable and long lasting tolerance to an epitope. There is a need to develop a vector that can provide for persistence of the epitope in vivo so that tolerance is maintained. There is a need to develop a recombinant vector which codes for a recombinant polypeptide that has a heterologous epitope and that can be used to induce and maintain tolerance in individuals.

Summary of the Invention

The invention provides for methods and compositions for inducing and maintaining tolerance to epitopes and antigens containing those epitopes. The methods and compositions are useful to identify novel tolerogenic epitopes or antigens containing such epitopes. The methods and composition are also useful for inducing and maintaining tolerance to epitopes or antigens containing the epitopes associated with autoimmune or allergic immune responses.

The compositions include an expression cassette and a vector. The expression cassette and vector can be used to form transformed cells. The expression cassette comprises a DNA sequence coding for a fusion immunoglobulin operably linked to transcriptional and translational control regions functional in a hemopoietic or lymphoid cell. The fusion immunoglobulin has at least one heterologous tolerogenic epitope at the N-terminus variable region of the immunoglobulin molecule. A vector includes the expression cassette and is a vector that can provide for stable maintenance, i.e. provide for gene expression of the expression cassette, in the hemopoietic or lymphoid cell throughout the lifetime of the cell. Hemopoietic or lymphoid cells are stably transformed with a vector to provide transformed cells expressing the fusion immunoglobulin.

The invention also includes pharmaceutical compositions. A pharmaceutical composition comprises an amount of a fusion immunoglobulin

sufficient to induce and/or maintain tolerance combined with a pharmaceutically acceptable excipient. The fusion immunoglobulin includes at least one heterologous tolerogenic epitope at the N-terminus variable region of the immunoglobulin.

The invention also provides methods for identifying epitopes or antigens containing epitopes that can serve as novel tolerogens. The methods involve stably transforming cells with an expression cassette coding for a fusion immunoglobulin to form a population of transformed cells producing or expressing the fusion immunoglobulin. The fusion immunoglobulin having one or more than one epitope from an antigen suspected of being capable of inducing tolerance can be screened for the ability to induce tolerance to the epitope in a variety of ways. One method of determining whether the fusion immunoglobulin can induce tolerance is to administer a tolerogenic amount of the fusion immunoglobulin to an animal. In another method, the transformed cells expressing the fusion immunoglobulin can be administered to an animal to determine whether tolerance to the epitope can be induced and/or maintained. In a third method, epitopes or antigens containing the epitope can be identified by reactivity with allergic or autoimmune immune serum or lymphocytes.

The invention also includes methods of inducing and maintaining tolerance to an epitope in an animal. One of the methods involves administering a tolerogenic amount of a fusion immunoglobulin sufficient to induce and/or maintain tolerance to the heterologous epitope on the fusion immunoglobulin. In another method, transformed cells expressing a fusion immunoglobulin are administered to an animal to induce and maintain tolerance. In another method, a pharmaceutical composition including a fusion immunoglobulin is administered to induce tolerance to the heterologous epitope and transformed cells expressing the fusion immunoglobulin are then administered to maintain tolerance to the heterologous epitope.

BNSDOCID: <WO___9521926A1_I_>

Brief Description of the Figures

FIGURE 1: Strategy for preparation of a murine DNA construct coding for a fusion immunoglobulin including the 12-26 epitope of λ-CI repressor protein at the N-terminus of IgG1: (A) A map of plasmid pSNR containing the genomic sequence for a γ1 H chain, modified as described in Example 1. (B) Restriction map and sequence showing the DNA sequence coding for the 12-26 epitope as combined with the DNA sequence coding for the variable region of the heavy chain.

FIGURE 2: Detection of a heterologous epitope on the 12-26-IgG fusion protein. The 12-26-IgG1 construct (Q3), as well as the control pSNR construct (P6) were electroporated into J558L myeloma cells, which synthesize only λ light chains. Recombinant IgG's were purified from bulk supernatants of transformed cells with anti-mouse IgG-sepharose or protein-A-sepharose columns. Western blotting: samples were electrophoresed on 10% SDS-PAGE. Gels were transferred to nitrocellulose and probed with anti-mouse IgG (left lanes) or with anti-12-26 monoclonal antibody B3.11 (right lanes) plus alkaline phosphatase-conjugated antibodies as secondary reagents.

FIGURE 3: ELISA inhibition curves. Pre-titrated monoclonal antibody B3.11 was mixed with increasing amounts of 12-26 peptide, 12-26 peptide chemically coupled to rabbit gamma globulin (RGG/12-26), or Q3 (recombinant fusion protein 12-26 IgG1).

FIGURE 4: Tolerance induction by 12-26-IgG fusion protein as determined in vitro. Spleen cells were cultured for 18 hours with increasing amounts of 12-26 peptide or 12-26-IgG fusion protein (Q3.13) or a 12-26-rabbit gamma globulin (RGG) conjugate. Cells were then washed and challenged with an antigen containing the 12-26 epitope (12-26-fagellin) and ELISA assays were done on day 4 supernatants.

FIGURE 5: In vivo tolerance induction with 12-26-IgG. Balb/c mice were injected with a tolerizing dose of control IgG (P6) at 1 mg/mouse [solid bars], the 12-26 peptide at 100 μg/mouse [open bar], the chemical conjugate of 12-26 chemically conjugated to rabbit gamma globulin (12-26-RGG) at 1 mg/mouse [stripped bar] and the fusion immunoglobulin

BNSDOCID: <WO___9521926A1_I_>

(Q3.13) at 1 mg/mouse [dash-dot bar]. After 7 days, spleen cells were evaluated for responsiveness to in vitro challenge with an antigen containing the 12-26 epitope as described in Figure 4.

FIGURE 6A: Western blot showing expression of the 12-26 peptide in supernatants from A20.2J cells infected with MBAE-12-26 vector. Supernatants were slot-blotted on nitrocellulose and probed with anti-12-26 monoclonal antibody B3.11. MBpepA, MBpepB, MBpepC, and MBpepD represent individually infected A20.2J clones producing the 12-26 peptide coding for MBAE-12-26 vector.

FIGURE 6B shows proliferation of a T-cell anti-12-26-IgG TH1 clone in response to incubation with supernatants from A20 cells infected with MBAE-12-26 vector or control supernatants.

FIGURE 7 shows construction of an MBAE retroviral vector containing the DNA sequence coding for the 12-26 epitope.

FIGURE 8 shows a Southern blot of cDNA prepared from reverse transcribed polymerase chain reaction (PCR) products from MBAE-12-26 infected bone marrow cells after maturation in irradiated recipients. Peripheral blood cells were obtained from mice 2 weeks after receiving infected bone marrow cells. RNA was reverse-transcribed and PCR performed with V_H and 12-26 primers. The gels were probed with an oligonucleotide probe complementary to the DNA sequence coding for the 12-26 epitope. The experiment demonstrates expression of mRNA coding for the 12-26 epitope based on RT-PCR of RNA from peripheral blood cells at 2 weeks after bone marrow transplantation.

Detailed Description of the Invention

The invention provides for compositions and methods for inducing and maintaining tolerance to antigens. The compositions include an expression cassette and vector comprising a DNA sequence that codes for a fusion immunoglobulin operably linked to transcriptional and translational control regions functional in a hemopoietic cell or lymphoid cell. The fusion immunoglobulin has at least one heterologous epitope located at the N-

terminus of the variable region of the immunoglobulin chain. The vectors are preferably those vectors that can provide for stable integration of the expression cassette into a hemopoietic cell. The invention also includes cells transformed with the vectors. Fusion immunoglobulins having a heterologous epitope at the N-terminus can be used in a pharmaceutical composition that provides for induction of tolerance to the epitope and/or its antigen. The invention also provides for methods of identifying novel tolerogenic antigens and epitopes, as well as methods for inducing and maintaining tolerance to an antigen.

As used herein, the term "antigen" refers to an agent that is capable of eliciting an immune response in an animal.

An "epitope" is a portion of the antigen that is capable of eliciting an immune response and combines with an antibody specific for that portion of the antigen.

A "heterologous epitope" is an epitope that is not normally associated with the immunoglobulin carrier molecule. It is obtained or derived from an antigen that is not the same as the immunoglobulin carrier molecule.

A "hemopoietic cell" is a cell that can form blood cells include lymphocytes and macrophages from such tissues as bone marrow cells and other extramedullary tissues.

An "expression cassette or vector" is stably maintained in a hemopoietic or other cell type when it is either integrated into the chromosome so that the expression cassette or vector is replicated and transmitted to progeny cells or is maintained in the cell without loss of functional activity, i.e. gene expression, over the lifetime of the cell.

A "tolerogenic epitope" is an epitope that can induce immunological unresponsiveness to the epitope and/or an antigen containing an epitope. A tolerogenic epitope is selected because of a desire to induce immunological unresponsiveness to the epitope and/or an antigen containing the epitope. A tolerogenic epitope can be identified as an epitope that can stimulate an immune response if appropriately presented to the immune

106
BNSDOCID: <WO___9521926A1_I_>

system or it can be an auto- or self-antigen which may not normally elicit an immune response. A tolerogenic epitope can interact with T cells or B cells or both. Suitable tolerogenic epitopes that can be selected for are preferably those epitopes and/or antigens associated with autoimmune disease or allergic reactions.

A. Expression Cassettes and Vectors

An expression cassette of the invention includes a DNA sequence encoding a fusion immunoglobulin operably linked to transcriptional and translational control regions functional in a hemopoietic or lymphoid cell. The fusion immunoglobulin includes at least one heterologous tolerogenic epitope at the N-terminus variable region. The expression cassette is preferably incorporated into an vector that provides for stable maintenance and expression of the expression cassette in the host cell. If the host cell is a hemopoietic cell, the vector is preferably a vector that provides for integration of the vector into the chromosome of the hemopoietic cell. If the host cell is a lymphoid cell line, the vector can be a non-integrated vector such as a plasmid as long as it provides for stable maintenance and expression of the expression cassette over the lifetime of the cell. The expression cassettes and vectors of the invention are useful to provide fusion immunoglobulins to use as tolerogenic agents and/or to provide for maintenance of tolerance to an antigen and/or epitope.

A DNA sequence encoding a fusion immunoglobulin can be obtained and constructed using standard methods as described in Current Protocols in Molecular Biology, Chapter 3, J. Wiley/Greene Press (1992). DNA sequences encoding immunoglobulins can be obtained using known methods such as described by Hebell et al., Science, 254:102 (1991) and Huse et al., Science, 246:1275 (1989). Briefly, heavy and light chain sequences can be obtained by using reverse transcriptase polymerase chain reaction (RT-PCR) of messenger RNA (mRNA) isolated from spleen cells or, preferably, hybridomas producing an antibody of known specificity. The primers can be designed to amplify the variable light and heavy chain sequences including

BNSDOCID: <WO__9521926A1_I_>

the Fd fragment (V_H-CH1). Examples of such primers are disclosed in Huse et al., cited supra. and Ballard et al., PNAS, 83:9626 (1986). Typically such primers are designed to include restriction enzyme recognition sequences at both ends of the sequence to be amplified. The restriction endonuclease recognition sequences are known to those of skill in the art and can be selected to provide for ease of cloning into a vector at a specific location.

The DNA sequences encoding the immunoglobulin's light and heavy chains are preferably cDNA sequences so that any intervening sequence DNA has been removed and a fully functional immunoglobulin is encoded by the DNA sequence. The DNA sequence encoding the immunoglobulin molecule can encode a complete immunoglobulin having both heavy and light chains with the Fc fragment or it can encode portions of the immunoglobulin such as Fab fragment, $F(ab)_2$ fragment, or just the heavy chain. Modifications to the DNA sequence coding for the heavy chain can be made and still result in a fusion immunoglobulin molecule when the DNA sequence coding for the heavy chain is expressed in a cell of B cell lineage that can supply light chains to form the immunoglobulin. The DNA sequence can code for a secreted or membrane form of the immunoglobulin molecule.

Suitable examples of a DNA sequence coding for the heavy chain of an antibody specific for nitrophenyl are described by Hebell et al., cited supra. IgG1 or IgG2 (mouse) are preferred as carrier molecules for inducing tolerance. The DNA sequence preferably codes for the heavy chain of IgG1 or IgG2 types of immunoglobulin.

A DNA sequence coding for at least one tolerogenic epitope of an antigen can be obtained and prepared by standard methods. If the epitope is a small peptide of 15-20 amino acids, the nucleotide sequence encoding that epitope can be synthesized using automated DNA synthesis. If the DNA sequence codes for all or a portion of an antigen (i.e., codes for multiple epitopes), the DNA sequence coding for that antigen can be isolated and subcloned using published methods. The DNA sequences coding for all or a portion of some antigens can also be identified by searching in a database such as GenBank. Once the sequence is identified in such a database or by

BNSDOCID: <WO___9521926A1_I_>

reference to publications, the DNA sequence coding for all or a portion of an antigen can be obtained by automated synthesis or by polymerase chain reaction (PCR). For example, the DNA sequence coding for antigen E of ragweed pollen has been disclosed by Rafner et al., J. Biol. Chem., 266:1229 (1991); and Kuo et al., Molecular Immunol., 30:1077 (1993). Epitopes of antigen E have also been identified as described by Olson, J. Immunol., 136:2109 (1986); and Bond et al., J. Immunol., 146:3380 (1991). A DNA sequence encoding one or more of the epitopes of antigen E can be obtained by standard methods as described in Kuo et al., cited supra.

Suitable antigens are those that it would be desirable to induce and maintain immunological unresponsiveness to the epitope and/or antigen containing the epitope. Such antigens include pollen, ragweed, dustmites, and other known allergens. Suitable antigens also include autoantigens such as clotting factor VIII, acetylcholine receptors, collagen, myelin basic protein, thyroglobulin, and histocompatibility antigens. A suitable antigen also includes the epitopes from the λ-CI repressor protein. The amino acid sequences of many of these antigens as well as epitopes of these antigens are known to those of skill in the art. The preferred antigens include antigen E of ragweed and clotting factor VIII. The DNA sequences encoding suitable antigens can be obtained and prepared as described herein and in accord with published methods.

Before a DNA sequence coding for at least one tolerogenic epitope of an antigen is obtained and prepared, the epitope and/or antigen is selected. The epitope and/or antigen can be a single epitope or it can be all or a portion of an antigen containing many epitopes. The epitope can be one that interacts with T-cells, or one that interacts with B-cells, or one that interacts with both T- and/or B-cells.

The selection of the epitope and/or epitopes can be made based on the following criteria. Epitopes are first selected for the ability to induce tolerance to the peptide or an antigen containing the epitope, preferably an antigen associated with an allergic response or autoimmune response. Secondly, if tolerance is desired to a large and complex antigen, more than

BNSDOCID: <WO__9521926A1_I_>

one epitope can be selected to be combined into a fusion immunoglobulin. Preferably, the entire antigen may be included in the fusion immunoglobulin. Thirdly, epitopes may be selected if B- and/or T-cell tolerance is desired. Certain epitopes are known to those of skill in the art to be recognized by T-cells and not B-cells and *vice versa*. Fourthly, epitopes can be selected on the basis of reactivity with immune serum or lymphocytes from individuals having an allergic or autoimmune response to an antigen. For example, an epitope known to be immunodominant or to stimulate a strong autoantibody response can be selected so that the portion of the antigen included in the fusion immunoglobulin includes that epitope. Fifthly, if there is little or no information known about epitopes on the antigen, it may be desireable to include the entire antigen in the fusion immunoglobulin.

The DNA sequence coding for an epitope can include an epitope of about 5-6 amino acids or an antigen having a molecular weight of up to about 100,000 daltons. The preferred size range is about 9 amino acids to about 50,000 daltons. For example, epitopes recognized by T-cells have a consensus sequence including about 9 amino acids. It is believed that the minimal size of an epitope is about 5-6 amino acids. The maximum size of the antigen that can be presented in a fusion protein is the size that allows for the folding of both the antigen and the immunoglobulin carrier molecule. A preferred antigen is the A2 fragment of clotting factor VIII that has a molecular weight of about 40,000 daltons.

Once the epitope is selected and the DNA sequence encoding that epitope is obtained, the DNA sequence coding for the epitope is combined with the DNA sequence coding for the immunoglobulin to form a DNA sequence coding for a fusion immunoglobulin. The DNA sequence coding for the epitope is preferably combined with a DNA sequence for the immunoglobulin at the N-terminal variable region of the heavy chain in frame and in proper orientation. The location of the combination of the DNA sequence coding for the epitope can vary depending on the desired location of the epitope in the fusion immunoglobulin. If the epitope is the entire antigen or a large portion of the antigen (i.e., having a molecular weight of about

25,000 to about 100,000 daltons), the location of the epitope on the fusion immunoglobulin is such that it would allow folding of both the immunoglobulin carrier molecule as well as the antigen or the portion of the antigen. When the antigen and/or portion of the antigen is an epitope, it is preferably fused with the immunoglobulin at the amino terminus of the heavy chain at the amino acids at the N-terminus first framework region. Smaller epitopes (i.e., those containing about 5-50 amino acids) can be located at the first N-terminal framework region or within other regions on the variable portion of the immunoglobulin chain as long as the epitope remains exposed on the outer surface of the immunoglobulin molecule. Preferably, small epitopes can also be combined with the immunoglobulin at the amino acids of the first N-terminal framework region of the heavy chain.

Optionally, the DNA sequence coding for at least one heterologous tolerogenic epitope can include flanking DNA sequences on one or both ends of the DNA sequence. These flanking DNA sequences can include restriction endonuclease recognition sequences and/or can include a DNA sequence encoding a portion of the immunoglobulin sequence at the location where the two DNA sequences are to be combined. For example, a DNA sequence coding for an epitope that is combined at the first N-terminus framework region of a heavy chain of an immunoglobulin molecule can include a flanking DNA sequence encoding the first 5 amino acids of the first framework region on either or both ends of the DNA sequence coding for the epitope. The flanking DNA sequence can also include a recognition sequence for a restriction enzyme. The flanking DNA sequence is preferably about 3 to about 21 nucleotides long. When the flanking DNA sequence encodes a portion of the immunoglobulin amino acid sequence, that sequence is selected at the location of the point of combination of the epitopal DNA sequence with the immunoglobulin sequence. The flanking DNA sequence coding for a portion of the immunoglobulin amino acids can provide for amino acids in the fusion immunoglobulin that assist in the proper folding of both the epitope and/or antigen and the immunoglobulin at the point of fusion. The flanking DNA sequence can also insure that the DNA sequence coding for the epitope

BNSDOCID: <WO___9521926A1_1_>

are combined with the DNA sequence coding for the immunoglobulin in frame and proper orientation.

The DNA sequences coding for the immunoglobulin and the epitope are combined using standard subcloning methods. The combination of the two DNA sequences can be assisted by forming the DNA sequence encoding the epitope with flanking DNA sequences having certain restriction enzyme recognition sequences. These flanking sequences provide one of skill in the art with the ability to select the location at which the DNA sequence coding for the epitope will be combined with the DNA sequence coding for the fusion immunoglobulin and to insure the sequences are combined in frame and in proper orientation. When the DNA sequences coding for the immunoglobulin and the epitope are combined, they form a DNA sequence coding for a fusion immunoglobulin or a fusion heavy chain of an immunoglobulin molecule.

It should be understood that, due to the degeneracy of the genetic code, there are a number of DNA sequences that can code for an immunoglobulin and an epitope that have the same amino acid sequence. This set of sequences is a finite set and can be determined based on the amino acid sequence of the epitope and immunoglobulin. Alternative DNA sequences that code for an immunoglobulin molecule and an epitope with the same amino acid sequence are contemplated by and included within the scope of the invention.

The DNA sequence coding for a fusion immunoglobulin can then be combined with transcriptional and translational control regions functional in a hemopoietic or lymphoid cell. A control region that is important for expression of the DNA sequence coding for a fusion immunoglobulin includes a promoter. A suitable promoter is one that can function in a hemopoietic or lymphoid cell. The promoter preferably provides for constitutive expression of the DNA sequences coding for the fusion immunoglobulin. the promoter also preferably provides for an amount of the fusion immunoglobulin to induce and/or maintain tolerance. Suitable

examples of promoters include the β-actin promoter, the SV40 promoter, and the LTR Rous sarcoma virus promoter.

Other transcriptional and translational control regions include enhancer sequences and transcription termination and polyadenylation sequences. Enhancer sequences can be combined with and are usually found within or adjacent to promoter sequences. Certain enhancer sequences, such as those from SV40, are active in many mammalian cells and provide for stimulation of transcription up to 1,000-fold from the homologous or heterologous promoters. Polyadenylation sequences are found downstream from the coding sequence and provide for proper formation of mRNA. Polyadenylation sequences can be obtained from SV40. Transcription termination sequences are found downstream from the polyadenylation sequences within a few hundred nucleotides.

These transcriptional and translational control regions are available in commercially available vectors. A DNA sequence encoding a fusion immunoglobulin or fusion heavy chain can be combined with transcriptional and translational control regions in frame and proper orientation by subcloning into a vector having these control regions to form an expression cassette.

Vectors can be selected for the ability to provide for stable maintenance and/or gene expression in a hemopoietic or lymphoid cell. A vector is stably maintained in a cell if it can provide for expression of a fusion immunoglobulin over the lifetime of the cell. Stable maintenance can include maintenance and expression of a plasmid in a eukaryotic cell, preferably a cell such as a lymphoid cell. In that case, the plasmid including an expression cassette is not autonomously replicated or does not become integrated into the chromosome. The lifetime of a cell, such as a lymphoid cell, is about 14 to 60 days in the mouse or can be several years in humans. A plasmid vector containing an expression cassette can also be maintained in a lymphoid cell line such as the J558L cells without being replicated.

A vector can also be selected to provide for integration of the expression cassette into the chromosome of the host cell, such as a

BNSDOCID: <WO___9521926A1_I_>

213

hemopoietic cell. In a hemopoietic cell from the bone marrow of an animal, the vector is introduced into a mixed population of cells, some of which are dividing cells and some of which have not yet begun dividing. The vector can integrate into the chromosome and then be replicated along with the chromosome and transferred to progeny cells. The vector is stably integrated if gene expression can be detected in the cell population at about 1 to 12 weeks after infected cells are introduced into an animal or cultured in vitro.

Suitable vectors include the plasmids such as pSNR1, pEMBL, pBR322, pRSA101, pUC118, pUC119, pBluescript, and pComb (Barbas et al., PNAS, 88:7978 (1991)). Suitable vectors also include viral vectors such as baculovirus and retroviral vectors such as the MBAE vector (Chambers et al., PNAS, 89:1026 (1992)). The preferred vector for hemopoietic cells is the MBAE vector.

A bacterial strain containing a plasmid vector having a DNA sequence that codes for fusion heavy chain has ben designated E. coli DH5α (pQ3.EZ). The bacterial strain carries the plasmid pQ3.EZ which codes for fusion heavy chain that has a 12-26 amino acid epitope from λ-C1 repressor protein combined at the N-terminus first framework region of the heavy chain of an antibody specific for nitrophenyl. The bacterial strain has been deposited with the American Type Culture Collection at Rockville, MD on February 7, 1994 and given Accession No. 69555.

In a preferred version, a DNA sequence coding for an epitope such as the 12-26 epitope from the λ-C1 repressor protein is combined with the DNA sequence coding for an immunoglobulin variable region at the first N-terminal framework region of the heavy chain to form a DNA sequence coding for a fusion heavy chain. The DNA sequence coding for a fusion heavy chain is combined with a β-actin promoter in an MBAE retroviral vector. The vector is preferably used to transform bone marrow cells or other B-cell lineage cells that can produce light chains. The light chains combine with the fusion heavy chain to form a fusion immunoglobulin. Alternatively, a DNA sequence coding for a light chain could be included in the same vector

as that coding for the fusion heavy chain to provide for expression of a fusion immunoglobulin.

B. Transformed Cells

Vectors containing expression cassettes coding for a fusion immunoglobulin are used to transform cells. The transformed cells are used in methods of identifying novel tolerogenic epitopes and to produce a fusion immunoglobulin. Transformed cells can also be introduced into animals for induction and maintenance of tolerance to the heterologous epitope expressed by the transformed cells or to an antigen containing the heterologous epitope.

Suitable cells for transformation include hemopoietic cells, lymphoid cells, and lymphoid cell lines. The cells include bone marrow cells, lymphoid cells, and the J558L lymphoid cells. Host cells are preferably those that are capable of forming and secreting immunoglobulin molecules. The cell population transformed preferably includes cells of B-cell lineage and are those that synthesize light chains endogenously. Transformed cells that will be administered to animals are preferably syngeneic or share identical histocompatibility antigens to avoid rejection of the injected cells. For screening assays, bacterial host cells such as E. coli and the like can be suitable.

The vector can be introduced into cells using a variety of methods known to those of skill in the art such as calcium phosphate mediated transfection, polybrene mediated transfection, protoplast fusion, electroporation, and lipsomal mediated transfection.

Once the expression cassette is introduced into the cells, the transfected cells can be initially selected by detecting the presence of a selectable marker gene present on the vector. If the transfected cells are bone marrow cells or lymphoid cells, no selection may be employed. Transfected cells can then be screened for the presence and/or expression of the expression cassette coding for a fusion immunoglobulin. Transfected cells can be screened for the presence of an expression cassette using one or more techniques such as Southern blot, Northern blot, reverse transcriptase PCR,

BNSDOCID: <WO__9521926A1_I_>

Western blot, ELISA, and immunofluorescence. Detectably labelled DNA probes can be used in Southern and/or Northern blots. The probes are sufficiently complementary to nucleotide sequences coding for the epitope or a portion of an antigen so that the probe of about 50 to 100 nucleotides hybridizes under high stringency conditions. Primers for reverse transcriptase PCR can be designed as described previously to amplify cDNA sequences coding for the variable heavy and light chains of the immunoglobulin molecule.

Transfected cells in which the fusion immunoglobulin is being expressed can also be detected using a Western blot, ELISA, or immunofluorescence. Amounts of fusion immunoglobulins being expressed can be detected using a quantitative Western blot.

The amount of fusion immunoglobulin produced in a particular host cell type and with a particular promoter/enhancer sequence can be evaluated using a quantitative Western blot. The promoter/enhancer sequences providing for the most amount of constitutive expression of the fusion immunoglobulin can be determined by comparing the amount of fusion immunoglobulin produced in the same type of host cell over the same amount of time. A promoter/enhancer can be selected that would provide for a sufficient amount of fusion immunoglobulin to induce and/or maintain tolerance. The amount of a fusion immunoglobulin that will induce tolerance can vary in accordance with factors described herein and can be determined using standard methods.

C. Pharmaceutical Compositions

The invention also provides pharmaceutical compositions including a tolerogenic amount of a fusion immunoglobulin in a pharmaceutically acceptable excipient. The fusion immunoglobulin has at least one heterologous tolerogenic epitope on the N-terminal variable region of the immunoglobulin. Preferably, the heterologous tolerogenic epitope is combined with the immunoglobulin adjacent to the first N-terminal framework region of the heavy chain. The fusion immunoglobulin is combined with a

BNSDOCID: <WO__9521926A1_I_>

pharmaceutically acceptable excipient in amounts effective to induce tolerance to the tolerogenic epitope or to an antigen containing the epitope in an animal. The pharmaceutical composition can be administered to an animal to induce and/or maintain tolerance to the tolerogenic epitope. Induction of tolerance to the epitope or epitopes can minimize animal allergic reactions or the symptoms of autoimmune disease.

Fusion immunoglobulins can be isolated from transformed cells using standard methods. Fusion immunoglobulins can be isolated from cell supernatants by passage through protein A or other affinity columns in accord with standard methods.

Suitable tolerogenic epitopes are those epitopes associated with allergic or autoimmune responses. A tolerogenic epitope is one that can be administered in such a way as to result in immunological unresponsiveness to the epitope and/or an antigen containing the epitope. If the epitope is one that stimulates an immunodominant response, tolerance to that epitope can also result in tolerance to an antigen containing the epitope. Specific examples include antigen E or antigen K of ragweed pollen, dust mite antigens, heterologous histocompatibility antigens, clotting factor VIII, acetylcholine receptors, myelin basic protein, and thyroglobulin. The fusion immunoglobulin can contain a single tolerogenic epitope or a multiple tolerogenic epitopes. Preferably, the tolerogenic epitope is an epitope that is immunodominant in the allergic or autoimmune response.

The amount of the fusion immunoglobulin effective to induce tolerance in an animal depends on a factors but can be readily determined by one of skill in the art using standard dose response methods. The factors include the size of the animal to be treated, the number and type of epitopes, the type of tolerance, the age of the animal, the route and number of times of administration, and the duration of the tolerance desired.

The age of the animal can be an important factor in determining the effective tolerogenic amount of an epitope. A neonatal or infant animal may require about 100 to 1000-fold less of a single dose of a

BNSDOCID: <WO___9521926A1_I_>

217

fusion immunoglobulin administered intravenously than that required by an adult of the fusion immunoglobulin in order to induce tolerance to the epitope.

A tolerogenic amount of a fusion immunoglobulin also depends on the size of the animal and is typically about 10 to 100-fold higher (for B-cell tolerance) than the amount of the antigen and/or epitope given to the animal to elicit a protective immune response, except in the case of low does tolerance. A tolerogenic amount of an antigen per unit of mass is typically about 1 to 40 mg/kg of body weight to induce high dose tolerance for an epitope or antigen administered as a single dose intravenously to an animal. Low dose tolerance is also observed in some cases and can be obtained after multiple (>4) doses of submicrogram quantities in saline at weekly intervals intraperitoneally or intravenously.

Another factor that can vary the tolerogenic amount of a fusion immunoglobulin is whether the fusion immunoglobulin includes more than one epitope and whether those epitopes are immunodominant. If the fusion immunoglobulin has multiple epitopes, some of which are immunodominant, about a 10-fold lower dose of fusion immunoglobulin can induce tolerance when administered as a single dose to an animal intravenously.

The tolerogenic amount of a fusion immunoglobulin can also vary depending on whether a T-cell or B-cell tolerance is desired. Typically, T-cell tolerance requires a dose of antigen or epitope about 10 to 100-fold less than for B-cell tolerance to that same epitope or antigen.

Another factor is the persistence of the fusion immunoglobulin in the animal's circulation. A more slowly metabolized antigen provides for maintenance of tolerance for longer periods of time, typically about 2 to 10-fold greater time of maintenance of tolerance. The catabolic rate of epitopes or antigens depends on the half-life of isologous or the heterologous carrier immunoglobulin as well as the nature of the epitope or epitopes. The half-life rate of isologous or heterologous immunoglobulin is about 7 to 21 days (mouse). Epitopes having modified or unusual amino acids, such as D amino acids as well as complex antigens or epitopes, may not be degraded as rapidly as other types of epitopes.

BNSDOCID: <WO___9521926A1_I_>

Mode of administration can also influence the tolerogenic amount of the fusion immunoglobulin necessary. In the usual case, intravenous administration is the preferred route for inducing tolerance. The number of times the antigen is administered can also influence the amount of fusion immunoglobulin required per administration.

An effective tolerogenic amount for a particular heterologous tolerogenic epitope on a fusion immunoglobulin can be determined by conducting in vivo or in vitro dose response assays. The in vitro dose response assays can be conducted, for example, by using standard lymphocyte proliferation assays. For example, lymphocytes from an allergic or autoimmune animal can be combined with different doses of the fusion immunoglobulin and proliferation measured.

In vivo dose response can be determined by administering different doses of the fusion immunoglobulin in an excipient to an animal. The lack of immune responsiveness to the heterologous tolerogenic epitope can be determined by measuring the specific antibody response to the heterologous tolerogenic epitope or lymphocyte proliferation to a challenge dose of the fusion immunoglobulin.

Induction of tolerance is evaluated by measuring a decrease in immunological unresponsiveness. Methods of measuring immunological responsiveness can be conducted with in vivo or in vitro antigen presentation and challenge and are known to those of skill in the art. For example, the amount of antibody specific to the epitope and/or antigen can be measured as well as lymphocyte proliferation in response to a challenge with the epitope or fusion immunoglobulin. The decrease in immunological response that indicates tolerance has been induced can be about 2-fold to 100-fold, preferably about 20-fold to 100-fold reduction in antibody or lymphocyte responsiveness. The range of the decrease can vary depending on the sensitivity of the assay used to measure immunological responsiveness. For example, it is known that a decrease in the number of antibody-producing cells is more sensitive than a decrease in the amount of antibody. The range of the decrease can also vary if the epitope is an immunodominant epitope. A

BNSDOCID: <WO___9521926A1_I_>

2-fold change in responsiveness to an immunodominant epitope can result in significant levels of tolerance to the epitope and/or an antigen containing the epitope.

A single dose of a fusion immunoglobulin can induce tolerance. In some cases, the tolerance induced by a single dose in the mouse can last from about 2 months to about 6 months. However, for tolerance to be maintained in an animal, multiple doses are typically required. Maintenance of tolerance can be desired for at least that amount of time induced by a single dose of the fusion immunoglobulin to throughout the lifetime of the animal.

A tolerogenic amount of the fusion immunoglobulin is combined with a physiological excipient such as saline, buffered saline and incomplete Freund's adjuvant. The fusion immunoglobulin can be administered by a variety of routes such as intraperitoneally, orally, and intravenously but is preferably administered by the intravenous route. The animals that can be treated to induce tolerance to allergens or auto-antigens include mice, humans, rats, rabbits, and guinea pigs.

D. Methods of Identifying Epitopes That Can Serve as Tolerogens

The invention also provides methods of identifying epitopes that can serve as tolerizing epitopes. Identification of novel tolerogenic epitopes could be useful in diagnosis and treatment of autoimmune and allergic immune responses. One method includes the steps of providing a vector including a DNA sequence coding for a fusion immunoglobulin operably linked to transcriptional and translational control regions functional in a host cell. The fusion immunoglobulin has at least one heterologous epitope at the N-terminus variable region. The epitope can be one that is suspected of being able to induce tolerance. Cells are stably transformed with the vector as described previously. Transformed cells expressing the fusion immunoglobulin or the isolated fusion immunoglobulin are analyzed for the ability to immunoreact with immune serum or lymphocytes from allergic or autoimmune animals. Tolerance induction to a fusion immunoglobulin

BNSDOCID: <WO__9521926A1_I_>

identified by reactivity with immune serum or lymphocytes for autoimmune or allergic animals can be evaluated by in vitro or in vivo methods known to those of skill in the art. For example, fusion immunoglobulins that react with immune serum and/or stimulate lymphocyte proliferation can be administered to an animal and induction and maintenance of tolerance can be assessed as described herein.

In another method, the transformed hemopoietic or lymphoid cells can be introduced into an animal and induction and maintenance of tolerance to the heterologous epitope can be determined using assays for evaluating specific immunological responsiveness to the epitope as described previously.

Some epitopes and antigens are known to elicit immune responses. Some epitopes and antigens are known to elicit immunodominant immune responses associated with allergic or autoimmune immune responses. Those epitopes that elicit immune responses may or may not induce tolerance when presented in a fusion immunoglobulin. Epitopes of some antigens known to be associated with allergic or autoimmune immune responses have not been identified. The methods of the invention can be utilized to determine whether an epitope known to elicit an immune response can induce tolerance when presented in a fusion immunoglobulin or to identify novel tolerogenic epitopes of antigens.

In one method, a vector comprising a DNA sequence coding for a fusion immunoglobulin operably linked to transcriptional and translational control regions functional in the hemopoietic or lymphoid cell is transformed into a hemopoietic or lymphoid cell. The fusion immunoglobulin can include an epitope known to elicit an immune response or a novel tolerogenic epitope. The promoter/enhancer sequences preferably provide for expression of the fusion immunoglobulin in a hemopoietic or lymphoid cell at a level sufficient to induce tolerance to the epitope in vivo or in vitro. Such a promoter can be identified and screened for in an in vitro assay as described herein. The amount of fusion immunoglobulin that can induce tolerance in animals can be determined using standard dose response methodology.

BNSDOCID: <WO__9521926A1_I_>

The transformed cells are introduced into an animal. When transformed hemopoietic cells are introduced into an animal, preferably the animal has been irradiated before introduction of the transformed cells to destroy endogenous hemopoietic cells. The transformed cells are administered to an animal by intraperitoneal or intravenous injection. The animals are then analyzed for induction of tolerance to the epitope after about 2 to 20 days. Tolerance can be detected by measuring the specific antibody response or lymphocyte proliferation response to the heterologous tolerogenic epitope. A decrease in the specific antibody or lymphocyte proliferative response to the epitope of about 2 to 100-fold, preferably 10 to 100-fold, indicates tolerance to the epitope.

Preferably, the screening assays for identifying tolerogenic epitopes are conducted in mice. The transformed cells can be syngeneic mouse cells derived from another genetically identical mouse, or can be human hemopoietic or lymphoid cells. For example, screening assays can be done using human bone marrow tissue transformed with a vector. The human bone marrow tissue is then administered to immunodeficient mice such as the SCID-SCID mice according to the method described by Chambers et al., cited supra. Tolerance can be evaluated in the SCID-SCID mice by examining either the specific antibody response to the epitope or the lymphocyte proliferation response.

Another method of the invention provides for screening for novel tolerogens, preferably those associated with autoimmune or allergic immune responses. In this method, epitopes of antigens associated with allergic or autoimmune responses are screened for the ability to immunoreact with immune serum or to stimulate lymphocyte proliferation from animals having an allergic or autoimmune response. For example, different cDNA sequences coding for portions of a complex antigen such as clotting factor VIII can be combined with a DNA sequence coding for N-terminus variable region of an antibody to form a library of cDNA sequences coding for fusion immunoglobulins with different epitopes derived from clotting factor VIII. The DNA sequences coding for epitopes can be generated randomly, or can

222
BNSDOCID: <WO___9521926A1_I_>

be selected to encode overlapping linear amino acid sequence, or can be selected based upon the likelihood that the amino acids encoded by the DNA sequence are exposed (based on tertiary structure) on the surface of the clotting factor VIII molecule. The cDNA sequences coding for different portions of the antigen can be combined with cDNA sequences for the N-terminus variable region of an immunoglobulin, preferably at the first N-terminus framework region of the heavy chain as described previously.

A phagemid vector system such as pComb can be used to generate a cDNA library of heavy and light chains of antibodies having cDNA sequences coding for different portions of an antigen combined as described above. The phagemid vector can be constructed to carry these cDNA sequences using standard restriction enzyme digestion and ligation methods as described in Barbas et al., PNAS, 88:7978 (1991). The phagemid library can be screened for immunoreactivity with immune serum from allergic or autoimmune animals in a panning and/or filter Western blot assay similar to those described by Barbas et al., cited supra.

Briefly, the phagemid vectors carrying the Fab fragments with at least one heterologous epitope derived from an antigen are transformed into a E. coli strain. The E. coli strain is grown in the presence of antibiotics to select for those strains carrying the phagemid vector. Phage can be isolated and then screened for binding to cells coated with immune serum from an allergic or autoimmune animal as described by Barbas et al., cited supra. Adherent phage are eluted using elution buffer. Eluted phage can be transferred into E. coli cells and colonies can be examined for the presence of a phagemid carrying a Fab fragment with a heterologous epitope using a filter Western blot type assay with immune serum from an allergic or autoimmune animal.

Phagemid DNA from positive clones can be isolated and the DNA sequence coding for the fusion Fab can be subcloned into a vector that can be used to transform hemopoietic or lymphoid cells. The vector can contain additional DNA sequences so that a fusion immunoglobulin rather than Fab fragment is produced by the transformed cells. The fusion

BNSDOCID: <WO___9521926A1_I_>

immunoglobulin having a heterologous epitope that reacts with immune serum from allergic or autoimmune animals from a positive clone identified as described can be isolated and tested for the ability to induce tolerance in vitro or in vivo. Alternatively, transformed cells carrying such a vector can be introduced into an animal and induction of tolerance in vivo can be determined as described herein.

Once novel epitopes and/or fusion immunoglobulins that can induce tolerance are identified, they can be used in pharmaceutical compositions and methods for tolerizing animals to the epitopes. Alternatively, the identification of novel tolerogenic epitopes associated with autoimmune or allergic immune responses could be used in standard diagnostic assays to assess the presence of autoimmune or allergic immune responses or to monitor the effectiveness of treatment.

E. Methods of Tolerizing an Animal to an Epitope

The invention also provides methods for inducing and maintaining tolerance to an epitope in an animal. In one method, a pharmaceutical composition including a fusion immunoglobulin is administered to an animal as described previously. In another method, tolerance can be induced and maintained in an animal by introducing transformed hemopoietic or lymphoid cells producing the fusion immunoglobulin into the animal. Without limiting the invention in any way, it is believed that the persistent production of fusion immunoglobulin carrying the heterologous epitope by the transformed cells in vivo can allow for maintenance of tolerance as well or better than using a pharmaceutical composition of the fusion immunoglobulin.

In one method, a vector coding for a fusion immunoglobulin that can be stably maintained in a hemopoietic or lymphoid cell is provided. The fusion immunoglobulin has at least one heterologous tolerogenic epitope. Hemopoietic or lymphoid cells, such as peripheral blood cells, are transformed with a vector such as MBAE using polybrene. Transformed cells are not typically selected and the entire population of hemopoietic or lymphoid cells

BNSDOCID: <WO__9521926A1_I_>

are administered to the animal. Transformed cells can be evaluated for production of a fusion immunoglobulin in vivo or in vitro by detecting the presence of fusion immunoglobulin using antibodies or by detecting expression of fusion immunoglobulin mRNA using RT-PCR or Northern blots. Preferably, the transformed cell population is analyzed in vitro for production of fusion immunoglobulin at a level sufficient to induce and maintain tolerance to the heterologous epitope in an animal.

Transformed cell population prepared so that the fusion immunoglobulin is produced at a level sufficient to induce and/or maintain tolerance are introduced into an animal. The amount of cells introduced into the animal is that amount that provides for production of a fusion immunoglobulin at a level sufficient to induce tolerance, and preferably to maintain tolerance. The animal is monitored for induction and persistence of tolerance to the heterologous epitope using assays as described previously. In some cases, the animals are irradiated sufficiently to destroy endogenous hemopoietic or lymphoid cells before introduction of the transformed cell populations. An animal is considered tolerant to the epitope if about a 2 to 100-fold decrease in immunological responsiveness, such as lymphocyte proliferation or antibody response, is seen. Tolerance is considered to be maintained if the tolerant state is maintained at least as long as the tolerant state induced with a single intravenous injection of a tolerogenic pharmaceutical composition. In mice, a single injection of a tolerogenic amount of a fusion immunoglobulin can result in tolerance of about 2 to 20 days and as long as about 2 months to 6 months. Tolerance could be maintained throughout the lifetime of the animal.

Suitable transformed cells include bone marrow cells and lymphoid cells from mice or humans. Suitable animals include inbred strains of mice including immunodeficient mice such as the SCID-SCID mice. Induction and maintenance of tolerance to epitopes using human transformed cells can be evaluated by the development of tolerance to epitopes in human transformed cell populations administered to SCID-SCID mice. Other

transformed animal cells, such as bovine transformed cells, can also be evaluated for the induction of tolerance in SCID-SCID mice.

In another method, a tolerogenic amount of a fusion immunoglobulin can be used to induce tolerance and tolerance can be maintained by administration of transformed hemopoietic or lymphoid cells expressing the same fusion immunoglobulin. In the method, a tolerogenic amount of a fusion immunoglobulin can be administered as a single dose as described herein. After a state of immunological unresponsiveness is obtained, transformed hemopoietic or lymphoid cells expressing the fusion immunoglobulin can be administered to the animal. While not meant to limit the invention, it is believed that the transformed hemopoietic or lymphoid cells will result in the maintenance of tolerance to the epitope. The amount of fusion immunoglobulin that needs to be expressed when transformed cells are used to maintain rather than induce tolerance can be less than that required of cells that both induce and maintain tolerance. Typically, administration of about 10 to 100-fold less of the fusion immunoglobulin or antigen is required to maintain rather than induce tolerance.

EXAMPLE I

Preparation of

Fusion Immunoglobulin p12-26 Recombinant Constructs

Tolerance to the epitope comprising residues 12-26 of the bacteriophage λ cI protein was studied because this epitope can be recognized by both T- and B- cells, and it is the major immunodominant epitope of this protein in H-2^d mice. This epitope was expressed in a fusion protein of mouse IgG having the epitope at the N-terminus. Isologous IgG1 was chosen for the fusion protein because it is known to be a tolerogenic carrier. Isologous immunoglobulins (especially IgG's) are likely to make efficient tolerogenic carriers because of their ability to crosslink B-cell Fc receptors and to persist in the circulation, as well as their lack of "intrinsic immunogenicity", that is, the lack of the potential to elicit an immune response in a soluble form. DNA constructs coding for a fusion polypeptide

BNSDOCID: <WO___9521926A1_I_>

of immunoglobulin IgG containing the 12-26 epitope of λ cI repressor protein were obtained by modifying plasmid pSNR-1. (See Figure 1.)

The major immunodominant peptide of the λ cI repressor protein (residues 1-102) is found at residues 12-26, as described in Nature, 343:381 (1990). The DNA sequence coding for this peptide fragment was synthesized by standard automated methods. The synthetic oligonucleotide fragment coding for the 12-26 epitope has the following sequence (SEQ ID NO:1):

5' CTG GAG GAC GCG CGG CGG CTG AAG GCG ATA TAC GAG AAG AAG AAG 3'

3' GAC CTC CTG CGC GCC GCC GAC TTC CGC TAT ATG CTC TTC TTC CCT 5'

The corresponding amino acid sequence encoded by this fragment is:

Leu-Glu-Asp-Ala-Arg-Arg-Leu-Lys-Ala-Ile-Tyr-Glu-Lys-Lys-Lys (SEQ ID NO:2)

Plasmid pSNR-1 is a plasmid that includes a DNA sequence coding for the variable heavy chain domain (VH) and heavy chain constant regions 1-3 (CH1-3) from a murine immunoglobulin specific for 4-hydroxy-3-nitrophenyl. Plasmid pSNR-1 was constructed as described by Ballard et al., PNAS, 83:9626 (1986). The pSNR-1 plasmid was obtained from Douglas Fearon (Johns Hopkins, Baltimore, MD). To introduce the DNA sequence coding for the 12-26 epitope into the N-terminus of the variable heavy chain, the plasmid pSNR was manipulated as described below. A 1.3 kbp region of the pSNR-1 plasmid including the coding sequence for VH, 118 bp of DNA sequence 5' upstream promoter element to the VH coding sequence coding for a promoter element, and 3' downstream intron and IgH enhancer sequences was subcloned using standard methods. This sequence is defined between restriction enzyme sites BamHI and EcoRI, and was subcloned into the plasmid pBS (Stratagene) using BamHI and EcoRI restriction endonucleases. The pBS/VH was digested with PstI under conditions to isolate a single cut

PstI partial digest fragment, as described in Current Protocols in Molecular Biology, cited supra.

The 12-26 epitope was modified and then inserted into the VH region of the immunoglobulin at a location that provided for proper folding of that region. The DNA sequence coding for the 12-26 epitope was modified by adding the coding sequence for the first 5 amino acids of the framework region (FRI) of the VH coding sequence at the 3' end of the synthetic DNA sequence coding for the 12-26 epitope. This modification allowed for proper folding and was selected to result in minimal disruption in the tertiary structure of the immunoglobulin molecule. Regions of the Ig molecule that are likely to be sites where insertion of an epitope are not likely to disrupt the molecule can be determined by analyzing the amino acid sequence of the Ig molecule as well as the tertiary structure. The N-terminal and CDR regions on the Ig chain are preferable regions into which the epitopes can be inserted to result in minimal disruption of the tertiary structure. Insertion at the N-terminal region allows for insertion of larger polypeptide ≥ 10 kDa.

The modified 12-26 sequence including the sequence for the first five amino acids of the first framework region of the VH was obtained via polymerase chain reaction. A plasmid containing the 45 base pair nucleotide sequence coding for the 12-26 epitope was constructed by cloning the synthetic 45 base pair DNA sequence into the BamHI/ClaI site of a plasmid pPX1647 containing the H-ld flagellin gene (provided by Dr. P. Brey, Praxis-Lederle Corp.), a derivative plasmid of pUC119. The modified 12-26 sequence was amplified using PCR techniques and two primers.

The primers were designated OS-1 and OS-2. The primer OS-1 contains the coding sequence for the PstI site and the coding sequence for the first 5 amino acids of the 12-26 sequence. The sequence of OS-1 (SEQ ID NO:3) is:

5' TGATCTACTG CAGCTGGAGG ACGCGCGGCG G 3'.

The primer OS-2 was complementary to the coding sequence for the PstI site and to the coding sequence for the first 5 amino acids of the first framework

BNSDOCID: <WO__9521926A1_I_>

region of VH and the last 6 amino acids of the 12-26 sequence. The sequence for OS-2 (SEQ ID NO:4) is:

5' CGACCTCCTG CAGTTGGACC TGCTTCTTCT TCTCGTATAT 3'.

The 82 bp product of the PCR method, i.e., the modified 12-26 sequence, was isolated by high sieve agarose using standard methods.

The 82 base pair PCR fragment was digested with PstI to produce a 65 bp fragment coding for the modified 12-26 epitope including the first 5 amino acids of FR1. The 65 bp fragment was subcloned into the plasmid pBS at a pST1 site. The subcloning was done by digesting the modified 12-26 sequence with PstI. The selected plasmids containing the PstI fragment of modified 12-26 were sequenced to confirm the presence of that fragment in proper orientation. Plasmids containing the modified 12-26 sequence are referred to as pBS/12-26 and were sequenced to confirm structure.

The modified 12-26 fragment from pBS/12-26 was subcloned into pBS/VH. The subcloning was performed by initially doing a partial PstI digest of the pBS/VH to cut the VH region at a PstI site, which is located at the coding sequence for the first framework amino acids 4 and 5 of VH. The pBS/12-26 was fully digested with PstI. After ligation, plasmids containing the modified 12-26 sequence inserted after the coding sequence for the first 5 amino acids of the first framework region of the VH were selected by filter hybridization of bacterial colonies using a P^{32} labelled 12-26 oligonucleotide as probe. The resulting VH fusion sequence is as follows: L-FRI-12-26-FRI (L = leader sequence; FRI = the first 5 amino acids of the first framework region of VH). Double stranded sequencing was done to confirm proper site insertion as well as orientation. These plasmids are designated pBS/VH/12-26.

The presence of the VH/12-26 recombinant sequence in the plasmid was verified by DNA sequencing methodologies. The VH DNA sequence surrounding and including the modified 12-26 insert (SEQ ID NO:5) is as follows:

CAG GTC CAA CTG CAG CTG GAG GAC GCG CGG CGG CTG AAG GCG
L E D A R R L K A

ATA TAC GAG AAG AAG AAG CAG GTC CAA CTG CAG
I Y E K K K

The modified 12-26/VH recombinant from pBS/VH/12-26 was subcloned into a plasmid pSV2-neo at the BamH1/ECORI sites. The pSV2-neo plasmid is derived from pSNR (Dr. Al Bothwell, Yale University, New Haven, CT) and contains the V_H (NP-binding) inserted in an IgG_1 heavy chain. The 8.5 kbp EcoRI fragment from pSNR-1 and which contains the constant regions 1-3 (CH 1-3) of α_1 chain was also subcloned into the pSV2-neo. Deletion of a 8.5 kbp region between the EcoRI sites of plasmid pSNR-1, which includes the CH1-3 coding sequence, was carried out using standard techniques as disclosed in Current Protocols in Molecular Biology, Vol. 1: Supplement 3.1.3, John Wiley & Sons (1989). The complete plasmid contains the sequence coding for the variable heavy chain with the 65 base pair sequence coding for the 12-26 epitope inserted at the N-terminus first framework region of the variable heavy chain and the sequence coding for the (CH1-3) constant regions 1-3. The orientation of the modified variable region sequence and the constant regions were verified by Southern restriction analysis, as described in Current Protocols in Molecular Biology, cited supra. Successful recombinants were selected by ampicillin and a large scale plasmid preparation was grown using standard methods.

EXAMPLE II

Expression of

Fusion Immunoglobulin p12-26 Recombinant Constructs

The recombinant plasmids containing the coding sequence for both the VH/12-26 fusion and the CH1-3 of IgG1 were introduced into host cells and expression of the fusion protein was detected. Transformation and

BNSDOCID: <WO__9521926A1_I_>

detection of expression was carried out using standard methods as described in Current Protocols in Molecular Biology, cited supra.

The 12-26 IgG1 DNA construct (Q3) as well as the control pSNR construct (P6) were electroporated into J558L myeloma cells which synthesize only λ light chains. Stable integrants were selected for growth in the presence of the antibiotic G418. Transfectomas expressing the 12-26 IgG1 fusion protein were identified by analyzing cell culture supernatants by Western blot and ELISA.

Transfectomas were grown to high density in serum-free media (RPMI-1640 with 5% FCS) in roller bottles and in bulk culture. Purification from serum-free transfectoma supernatants was accomplished successfully via binding with protein-A sepharose at pH 8, with elution at pH 4, as well as with anti-mouse IgG affinity columns.

Purified supernatants from selected clones have been analyzed for expression of 12-26 epitopes by Western blotting and ELISA by standard methods. (See Figures 2 and 3.) For Western blotting, samples were electrophoresed on 10% SDS-PAGE. Gels were transferred to nitrocellulose and probed with anti-mouse IgG (left lanes) or anti-12-26 monoclonal antibody B3.11 (right lanes) plus alkaline phosphatase-conjugated antibodies as secondary reagents. The results are shown in Figure 2. Only those cell culture supernatants from transfectomas containing the 12-26 IgG1 construct (Q3) reacted with antibodies specific for mouse IgG (left lanes) and antibodies specific for the 12-26 epitope (right lanes).

For ELISA competitive inhibition assays, pre-titrated monoclonal antibody B3.11 was mixed with increasing amounts of 12-26 peptide, or the 12-26 peptide chemically coupled to rabbit gamma globulin (RGG/12-26), or 12-26 IgG1 (Q3). The ability of the mixtures to bind to immobilized 12-26 peptide was determined by standard methods. The results, shown in Figure 3, indicate that the 12-26 IgG fusion protein was able to effectively inhibit the binding of the monoclonal antibody to the 12-26 epitope compared with the 12-26 peptide in solution.

The competitive inhibition ELISA studies show that these fusion immunoglobulins can effectively compete with free synthetic peptide or 12-26 chemically-conjugated to rabbit IgG for binding to monoclonal antibody anti-12-26 B3.11. In addition, the 12-26-IgG is immunogenic for the 12-26 epitope when emulsified in CFA (data not shown). This suggests that the inserted peptide can be processed and presented in a physiologically relevant manner even in the context of a self-IgG molecule. Experiments also indicate that the 12-26 fusion immunoglobulins can stimulate IL-2 production (measured by CTLL assay) in an H-2^d restricted 12-26 specific T-cell hybridoma (9C127) (data not shown).

EXAMPLE III

Tolerance Induction in Mice with the 12-26 IgG1 Fusion Protein

A high dose pretreatment of animals with the 12-26 peptide injected intravenously or intraperitoneally in saline or emulsified in incomplete Freund's adjuvant (IFA) can induce T-helper cell tolerance upon subsequent immunization with peptide in complete Freund's adjuvant (CFA). Scherer et al., Symp. on Quant. Biol., Cold Spring Harbor, NY, 54:497 (1989). Tolerance induction to the 12-26 epitope has been confirmed in T-cell proliferation assays. However, animals treated with peptide are not tolerant at the B-cell level. That is, when challenged with 12-26 flagellin (providing "carrier epitopes"), the response was not diminished (see below). This indicates the reductions with peptide challenge were due to T- but not B-cell tolerance.

To determine whether the 12-26 IgG1 fusion protein can induce B-cell tolerance, the following experiment was conducted. Mouse spleen cells were cultured in vitro in RPMI-1640 + 5% FCS for 18 hours. The mouse spleen cells were then incubated with increasing concentrations of either free 12-26 peptide, a chemical conjugate of rabbit gamma globulin with 12-26 (RGG-12-26) or with 12-26-IgG1 (Q3). At 18 hours, these spleen cells were washed and then challenged with either lipopolysaccharide (a mitogenic

BNSDOCID: <WO__9521926A1_I_>

stimulus, not shown) or the A29 fusion protein of Salmonella flagellin that contains the 12-26 peptide. The Salmonella flagellin fusion protein containing the 12-26 epitope has been shown previously to be immunogenic both in vivo and in vitro (data not shown). As a control for induction of tolerance, spleen cells were treated with a rabbit anti-immunoglobulin previously shown to induce unresponsiveness in vitro. G. Warner et al., J. Immunol., 146:2185 (1991). The effect of anti-Ig is shown as an open circle on the right end of each graph. The responsiveness of the cells was measured by ELISA. The results are shown as Figure 4 (A29 fusion protein with 12-26 peptide challenge).

The results indicate that when spleen cells are challenged with the A29 fusion protein, the 12-26 IgG1 fusion protein (Q3.13), or the chemical conjugate (RGG-12-26) were both tolerogenic at microgram levels. In contrast, the free peptide does not inhibit B-cell responsiveness at any dose. Thus, these results indicate that the 12-26 IgG fusion proteins can induce tolerance in B-cells in vitro. Similar results were obtained in vivo as follows.

The 12-26-IgG fusion proteins were tested for induction of tolerance in vivo. CAF_1 mice were injected with 1 mg of the 12-26-IgG fusion protein, 12-26-IgG or free peptide in saline. Control mice received PBS in saline. Spleen cells from these mice were challenged 10 days later with the 12-26-flagellin fusion protein in vitro. Responsiveness to the 12-26 was measured by ELISA assays at 4 days after challenge as described for Figure 4. The results are shown in Figure 5.

The results indicate the 12-26-IgG fusion proteins as well as the chemical conjugate (RGG-12-26) can induce tolerance in vivo and in vitro. See Figures 4 and 5.

EXAMPLE IV

Construction of Retroviral Vector Containing a DNA Sequence Coding for the 12-26 IgG1 Fusion Protein

BNSDOCID: <WO___9521926A1_I_>

Several retroviral constructs have been prepared that are based on the murine Moloney leukemia retroviral vector MBAE, as described by Kang et al., Proc. Natl. Acad. Sci., 87:9803 (1990).

The retroviral vector MBAE can be obtained from Dr. Hozumi or prepared as described by Kang et al., cited supra. Briefly, the retroviral vector containing the Moloney murine leukemia long terminal repeats and the neo gene coding for G418 resistance was modified by insertion of the β-actin promoter and enhancer sequences. The β-actin promoter and enhancer sequences were cloned downstream from the neo gene. Heterologous genes can then be inserted downstream from the β-actin promoter by subcloning with HindIII and SalI.

DNA sequences subcloned into MBAE were derived from PCR-amplified reverse transcribed RNA from transfectoma Q3 which contains the 12-26-IgG H chain. The Q3 transfectoma was prepared as described in Example II. The RNA from the Q3 transfectoma was harvested and incubated with reverse transcriptase in a standard PCR reaction as described in Current Protocols in Molecular Biology, cited supra. to form cDNA molecules. The cDNA molecules were amplified using the following primers:

V_H 5' primer (SEQ ID NO:6):

5' TGG ACT AAG TCG ACA CCA TGG GAT GCA GC

pep 3' primer (SEQ ID NO:7):

5' GGC AAC AGA AGC TTT CAC TTC TTC TTC TCG TAT 3'

One such cDNA includes a DNA sequence coding for the leader sequence and the 12-26 epitope from the variable heavy chain gene followed by a stop codon. The stop codon was designed into the PCR primer at the end of the DNA sequence coding for the last amino acid of 12-26 (in primer) to construct a peptide minigene.

A DNA sequence coding for the leader sequence and the sequence coding for the 12-26 epitope followed by a stop codon was

BNSDOCID: <WO___9521926A1_I_>

subcloned into pBluescript and sequenced and then subcloned into the MBAE vector. Subcloning was performed using SalI and HindIII to insert the peptide minigene downstream from the β-actin promoter and enhancer sequences, as shown in Figure 7.

The recombinant MBAE vectors were transfected by lipofection into the ψ-2 cell line available from Dr. N. Hozumi (Toronto, Canada). The transfected cell lines were grown in RPMI 5% FCS in the presence of 0.8 mg/ml crude G418. G418 resistant clones were isolated by limiting dilution and viral titer was determined on NIH 3T3 cells in the presence of 0.8 mg/ml G418 (crude weight). For the peptide minigene construct, one transfected ψ-2 clone (MBAE pEP19) with a titer of 10^5-10^6 CFU/ml was chosen for subsequent gene transfer experiments. Presence of helper virus was assayed using standard methods ("horizontal spread of infection" method), as described by Current Protocols in Molecular Biology, cited supra, and was not detected. Virus producing lines were thawed out fresh for each individual experiment.

An A20.2J B-cell lymphoma cell, available from ATCC, infected with the viral vector expressed and secreted the peptide as detected by Western blot. See Figure 6A. After infection of A20.2J B-cell lymphoma cells, the cells were grown in G418 and 200 µl of supernatants were analyzed by Western blotting. Supernatants from four ψ-2/A20.2J clones infected with retroviral 12-26 minigene were slot blotted and reacted with monoclonal antibody B3.11 specific for the 12-26 epitope. As seen in Figure 6A, the peptide was expressed in the infected lymphoma cells.

The A20.2J infected cells not only produce the peptide but also present it to a 12-26 reactive T-cell hybridoma. Briefly, titrated volumes of supernatants from infected A20.2J cells were incubated with 12-26 reactive T-cell clones (T32) for 24-48 hours. The 12-26 reactive T-cell clones were obtained by Dr. Tom Briner and Dr. M. Gefter (Massachusetts Institute of Technology, Cambridge, MA). Responsiveness of the T-cell clone was measured by ^{3}H-thymidine incorporation and standard IL-2 assay. The results are shown in Figure 6B. The results indicate that A20 cells process this peptide so it can be presented to a 12-26 reactive T-cell clone. IL-2

BNSDOCID: <WO___9521926A1_I_>

production by these clones was also measured and the results show the 12-26 peptide is produced and secreted by the infected cells.

EXAMPLE V

Preparation of Mice Carrying Transfected Bone Marrow Cells

Mice carrying bone marrow cells transfected with the viral vector MBAE 12-26 coding for the 12-26 epitope (Figure 7) were prepared. Bone marrow progenitors from Balb/c mice were infected with the MBAE 12-26 vector as described by Chambers et al., Proc. Natl. Acad. Sci., 89:1026 (1992). Marrow donor Balb/c mice were pretreated intravenously with 150 mg/kg 5-fluorouracil for 3-4 days before marrow harvest. Fractionated marrow cells were kept on ice and then washed in complete RPM1 with 15% FC5 and 10 units/ml IL-3. The bone marrow cells were then cocultured with about an 80% confluent layer of irradiated (2000 rads) ψ-2 packaging lines. Co-culture with adherent ψ-2 virus producing line was done at 37°C for 48 hours as follows:

5×10^6 marrow cells per 6 wells in 10ml medium containing:

- 15% FCS
- 6 μg/ml polybrene
- 100 units/ml IL-6
- 200 units/ml IL-3

Nonadherent bone marrow cells were harvested after 48 hours, washed and resuspended in HEPES buffered Eaglis medium. Syngeneic recipient Balb/c mice were lethally irradiated with 900 rads and 4×10^6 cells in a volume of 400 μl were injected into the irradiated mice intravenously. Recipient mice were started on acidified water 1-2 weeks before transplantation to prevent gram negative infections and maintained in autoclaved microisolator cages with autoclaved food, bedding, and acidified water supplemented with antibiotics.

After two weeks, the lymphoid cells from the recipient mice were harvested from tail bleeds and examined for the presence of the 12-26

BNSDOCID: <WO___9521926A1_I_>

sequence by RT-PCR. Fragments of about 100 base pairs were detected in both infected lymphoid cells and the ψ-2 MBAE 12-26 containing cell line. See Figure 8.

Briefly, RNA from peripheral blood cells taken from the animals at 2 weeks or from infected [Ψ?]-2 packaging lines was reverse transcribed. DNA sequences coding for the 12-26 epitope were amplified using the V_H (SEQ ID NO:6) and pep (SEQ ID NO:7) primers. Amplified products were separated by agarose gel electrophoresis and products containing a DNA sequence coding for the 12-26 epitope were detected by Southern blot. The probe used to detect 12-26 coding sequences is as follows (SEQ ID NO:8):

5'- TGATCTACTG CAGCTGGAGG ACGCGCGGCG G - 3'

Hybridization was conducted under standard conditions as described in Current Protocols, cited supra. A fragment detected in peripheral blood cells by hybridization to 12-26 probe indicated expression of the 12-26 epitope was occurring in the cells 2 weeks after administration.

BNSDOCID: <WO__9521926A1_I_>

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: University of Rochester

(ii) TITLE OF INVENTION: Inducing Tolerance With Tolerogenic Fusion Proteins

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Schwegman, Lundberg & Woessner
(B) STREET: 3500 IDS Center
(C) CITY: Minneapolis
(D) STATE: Minnesota
(E) COUNTRY: United States of America
(F) ZIP: 55402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 10-FEB-1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Raasch, Kevin W.
(B) REGISTRATION NUMBER: 35,651
(C) REFERENCE/DOCKET NUMBER: 850.014WO1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 612-339-0331
(B) TELEFAX: 612-339-3061

BNSDOCID: <WO___9521926A1_I_>

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGGAGGACG CGCGGCGGCT GAAGGCGATA TACGAGAAGA AGAAG
45

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Leu Glu Asp Ala Arg Arg Leu Lys Ala Ile Tyr Glu Lys Lys Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

BNSDOCID: <WO___9521926A1_I_>

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGATCTACTG CAGCTGGAGG ACGCGCGGCG G 31

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGACCTCCTG CAGTTGGACC TGCTTCTTCT TCTCGTATAT
40

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 16..61

BNSDOCID: <WO___9521926A1_I_>

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAGGTCCAAC TGCAG CTG GAG GAC GCG CGG CGG CTG AAG GCG ATA TAC GAG 51
Leu Glu Asp Ala Arg Arg Leu Lys Ala Ile Tyr Glu
1 5 10

AAG AAG AAG C AGGTCCAACT GCAG 75
Lys Lys Lys
15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGGACTAAGT CGACACCATG GGATGCAGC 29

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGCAACAGAA GCTTTCACTT CTTCTTCTCG TAT 33

BNSDOCID: <WO___9521926A1_I_>

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGATCTACTG CAGCTGGAGG ACGCGCGGCG G 31

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 16, line s 30-32

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
February 8, 1994	69555

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☑ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
C. Williams	
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

WHAT IS CLAIMED IS:

1. An expression vector for persistently maintaining expression of an tolerogenic epitope in an animal comprising:

(a) a DNA sequence coding for a fusion immunoglobulin operably linked to transcriptional and translational control regions functional in a hemopoietic cell or lymphoid cell, wherein the fusion immunoglobulin has at least one heterologous tolerogenic epitope at the N-terminus variable region; and wherein said DNA sequence is operable linked to

(b) a vector that can provide for stable maintenance of the DNA sequence in the hemopoietic cell or lymphoid cell.

2. An expression vector according to claim 1, wherein the vector is a retroviral vector.

3. An expression vector according to claim 1, wherein the DNA sequence codes for a fusion IgG having a heterologous tolerogenic epitope inserted adjacent to the first framework region of the N-terminus variable region of the heavy chain.

4. An expression vector according to claim 3, wherein the DNA sequence encodes a fusion IgG including an epitope having the amino acid sequence of amino acids 12-26 of the λ CI repressor protein.

5. An expression vector according to claim 1, wherein the transcriptional and translational control regions provide for constitutive expression of the DNA sequence in the lymphoid cells.

6. A method for tolerizing an animal to an epitope comprising:

(a) providing a vector that can be stably maintained in a hemopoietic or lymphoid cell, wherein the vector comprises a DNA sequence that codes for a fusion immunoglobulin operably linked to

transcriptional and translational control regions functional in the hemopoietic or lymphoid cell, wherein the fusion immunoglobulin has at least one heterologous tolerogenic epitope at the N-terminus variable region;

(b) stably transforming a population of the hemopoietic or lymphoid cells from the animal with the vector to form a transformed population of hemopoietic or lymphoid cells expressing the fusion immunoglobulin; and

(c) introducing the transformed population of cells into an animal.

7. A method according to claim 6, wherein the fusion immunoglobulin has a tolerogenic epitope having the amino acid sequence of amino acids 12-26 of the λ CI repressor protein, wherein the tolerogenic epitope is inserted at the first framework region of the N-terminus of the variable heavy chain.

8. A method according to claim 7, wherein the vector is a retroviral vector.

9. A method according to claim 6, further comprising irradiating the animal sufficiently to destroy endogenous hemopoietic cells before introducing the transformed hemopoietic cells into the animal.

10. An expression cassette for expression of a DNA sequence in a hemopoietic or lymphoid cell comprising a DNA sequence coding for a fusion immunoglobulin wherein the fusion immunoglobulin has at least one heterologous tolerogenic epitope inserted adjacent to the first framework region at the N-terminus of the variable region of the immunoglobulin, operably linked to transcriptional and translational control regions functional in the hemopoietic or lymphoid cells.

11. An expression cassette according to claim 10, wherein the epitope has the amino acid sequence of amino acids 12-26 of the λ CI repressor protein.

12. An expression cassette according to claim 11, wherein the fusion immunoglobulin is an IgG.

13. A plasmid having the characteristics of ATCC No. 69555.

14. A pharmaceutical composition comprising:

(a) a tolerogenic amount of a fusion immunoglobulin, wherein the fusion immunoglobulin has at least one heterologous tolerogenic epitope adjacent to the first framework region of the N-terminus variable chain; and

(b) a pharmaceutically acceptable excipient.

15. A pharmaceutical composition according to claim 14, wherein the pharmaceutical immunoglobulin is an isologous IgG.

16. A pharmaceutical composition according to claim 15, wherein the fusion immunoglobulin has a heterologous tolerogenic epitope with an amino acid sequence of amino acids 12-26 of the λ CI repressor protein.

17. A pharmaceutical composition, wherein the excipient is selected from the group consisting of phosphate buffered saline, physiological saline and water.

18. A pharmaceutical composition, wherein the tolerogenic amount of the fusion immunoglobulin is about 4 to 40 mg/kg of body weight of the animal.

BNSDOCID: <WO__9521926A1_I_>

19. A transformed hemopoietic or lymphoid cell comprising an expression cassette stably maintained in the hemopoietic or lymphoid cell, wherein the expression cassette comprises a DNA sequence coding for a fusion immunoglobulin, wherein the fusion immunoglobulin has at least one heterologous tolerogenic epitope inserted adjacent to the first framework region at the N-terminal variable region, wherein said DNA sequence is operably linked to transcriptional and translational control regions functional in the hemopoietic or lymphoid cell.

20. A transformed cell according to claim 19, wherein the cell is a bone marrow cell.

21. A method for identifying tolerogenic epitopes comprising:

(a) providing a vector that can be stably maintained in a hemopoietic or lymphoid cell, wherein the vector comprises a DNA sequence that codes for a fusion immunoglobulin operably linked to transcriptional and translational control regions functional in the hemopoietic or lymphoid cell, wherein the fusion immunoglobulin has at least one heterologous epitope at the N-terminus variable region;

(b) stably transforming a population of hemopoietic or lymphoid cells from an animal with the vector to form a population of transformed cells;

(c) introducing the transformed cells into an animal and;

(d) identifying whether the heterologous epitope is a novel tolerogen by determining whether the animals are tolerant to the heterologous epitope.

22. A method of identifying tolerogenic epitopes comprising;

(a) providing a vector that can be stably maintained in a host cell, wherein the vector comprises a DNA sequence that codes for a fusion immunoglobulin operably linked to transcriptional

BNSDOCID: <WO___9521926A1_I_>

and translational control regions functional in the host cell, wherein the fusion immunoglobulin has at least one heterologous epitope at the N-terminus variable region of the immunoglobulin;

(b) stably transforming a population of host cells with the vector to form a population of transformed cells, producing the fusion immunoglobulins; and

(c) identifying whether the heterologous epitope on the fusion immunoglobulin is a tolerogenic epitope by determining whether the epitope is associated with an autoimmune or allergic immune response.

23. A method according to claim 22, wherein the host cell is E. coli.

24. A method according to claim 22, wherein the vector is a phagemid vector.

25. A method according to claim 22, wherein the host cell is a J558L cell.

26. A method according to claim 22, wherein the step of identifying whether the heterologous epitope on the fusion immunoglobulin is a tolerogen comprises determining whether the fusion immunoglobulin immunoreacts with immune serum from an autoimmune or allergic animal.

27. A method according to claim 22, wherein the step of identifying whether the heterologous epitope on the fusion immunoglobulin is a tolerogen comprises determining whether the fusion immunoglobulin stimulates proliferation of lymphocytes from an autoimmune or allergic animal.

28. A method according to claim 22 further comprising confirming that the heterologous epitope is a tolerogenic epitope by determining whether

BNSDOCID: <WO___9521926A1_I_>

the fusion immunoglobulin induces tolerance to the epitope in an animal.

29. A method of tolerizing an animal to an epitope comprising administering a fusion immunoglobulin having a heterologous tolerogenic epitope to an animal sufficiently to induce tolerance to the heterologous tolerogenic epitope, wherein the fusion immunoglobulin has the heterologous tolerogenic epitope at the first N-terminus framework region of the immunoglobulin.

30. A method of inducing and maintaining tolerance to an epitope in an animal comprising:

(a) administering a pharmaceutical composition according to claim 14 sufficiently to induce tolerance to an epitope; and

(b) administering transformed hemopoietic or lymphoid cells to the animal sufficiently to maintain tolerance to the epitope, wherein the transformed cell comprises a vector stably maintained in the transformed cell, wherein the vector comprises a DNA sequence coding for a fusion immunoglobulin operably linked to transcriptional and translational control regions functional in the cell, wherein the fusion immunoglobulin has at least one heterologous tolerogenic epitope at the N-terminus variable region of the immunoglobulin.

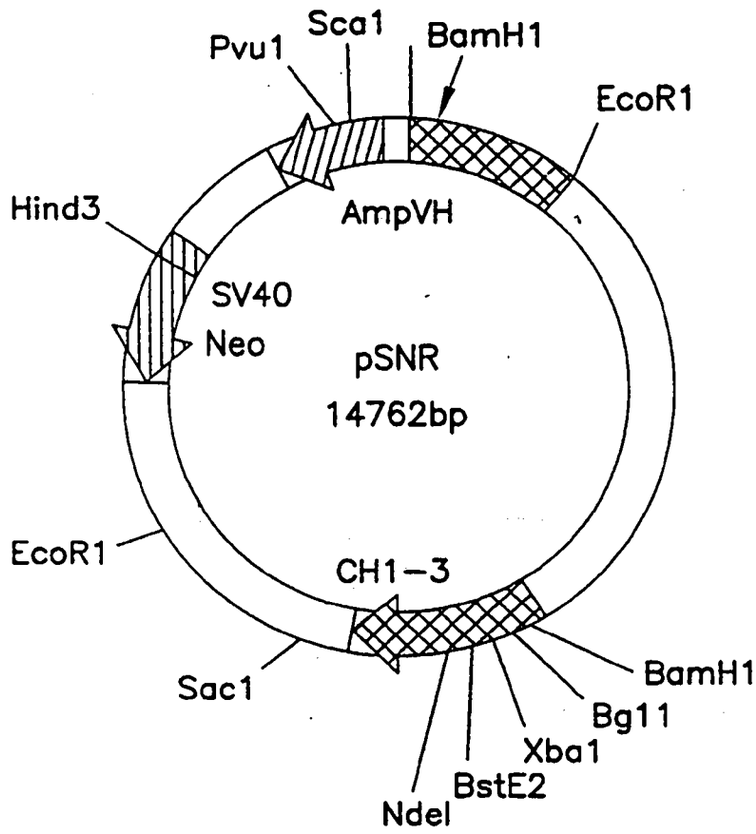


FIG. 1A

BNSDOCID: <WO___9521926A1_I_>

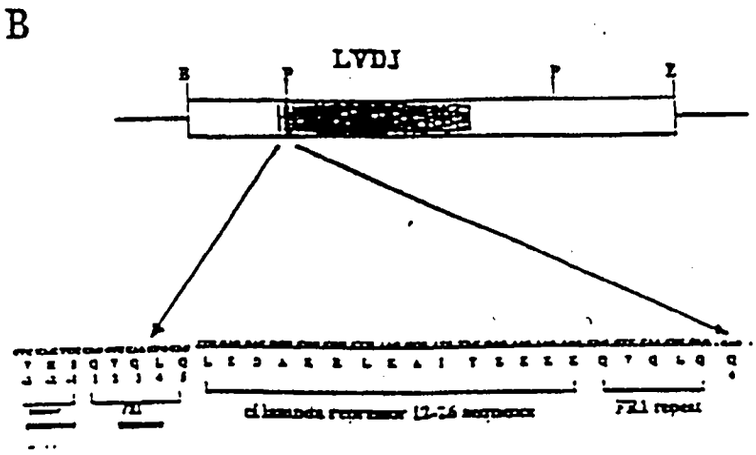


Figure 1

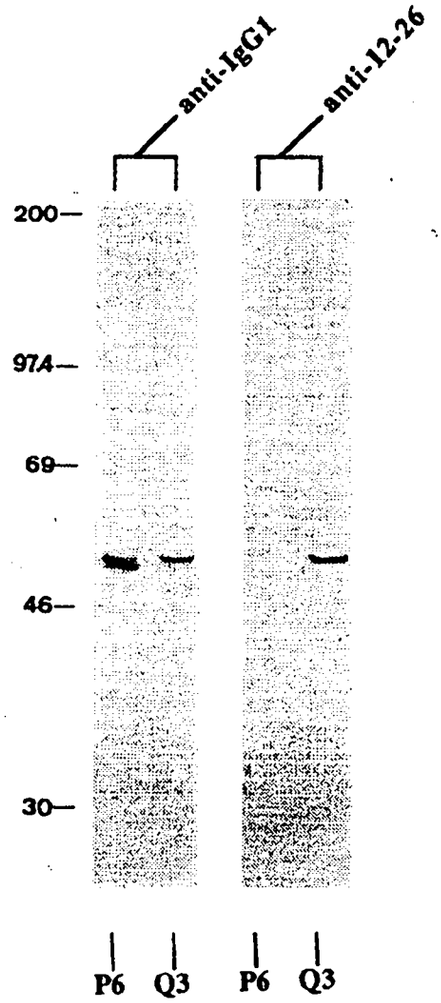


FIG. 2

BNSDOCID: <WO___9521926A1_I_>

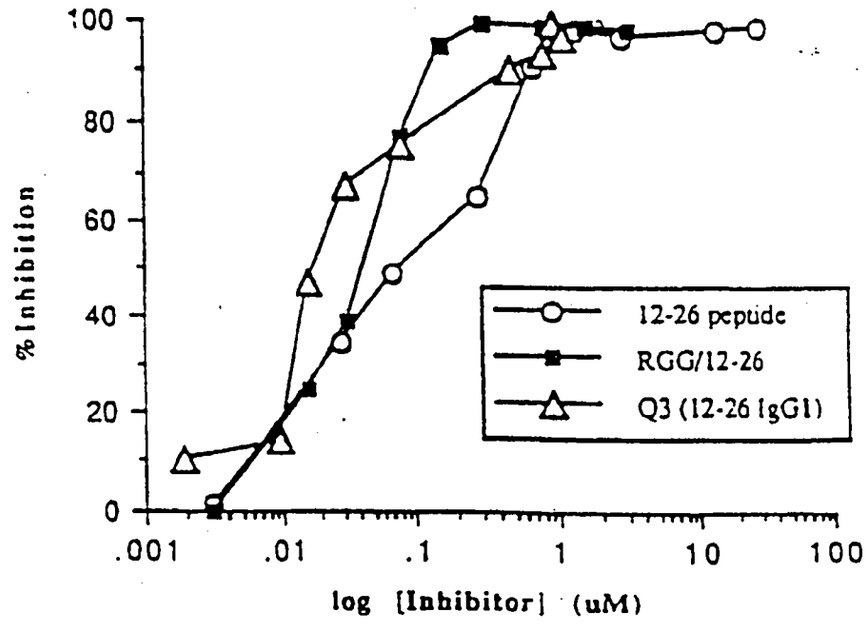

100
80
60
40
20
0
% Inhibition
.001
.01
.1
1
10
100
log [Inhibitor] (uM)
12-26 peptide
RGG/12-26
Q3 (12-26 IgG1)

FIG. 3

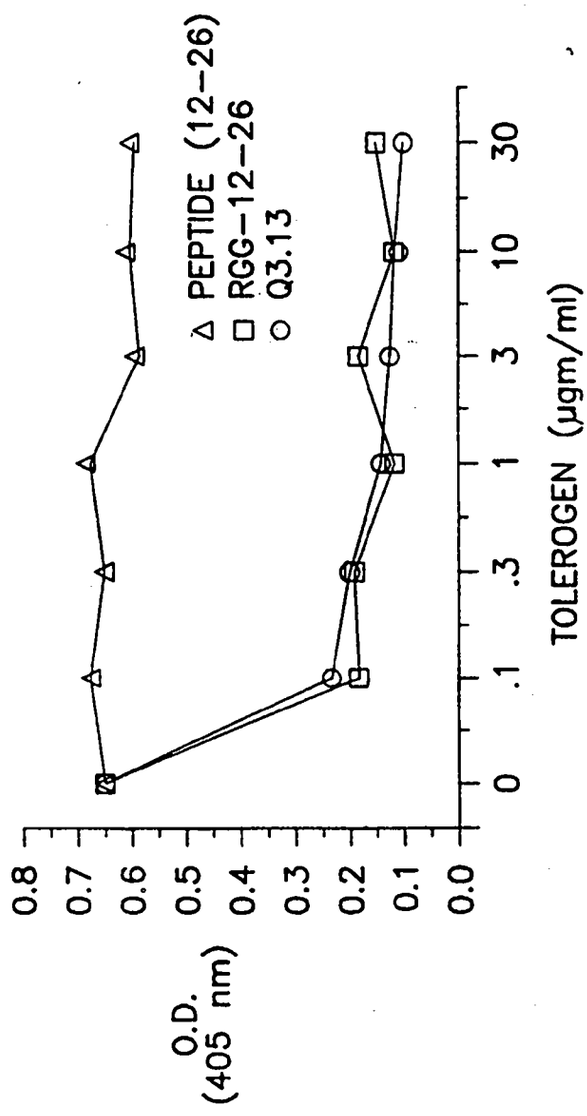


FIG. 4

BNSDOCID: <WO___9521926A1_I_>

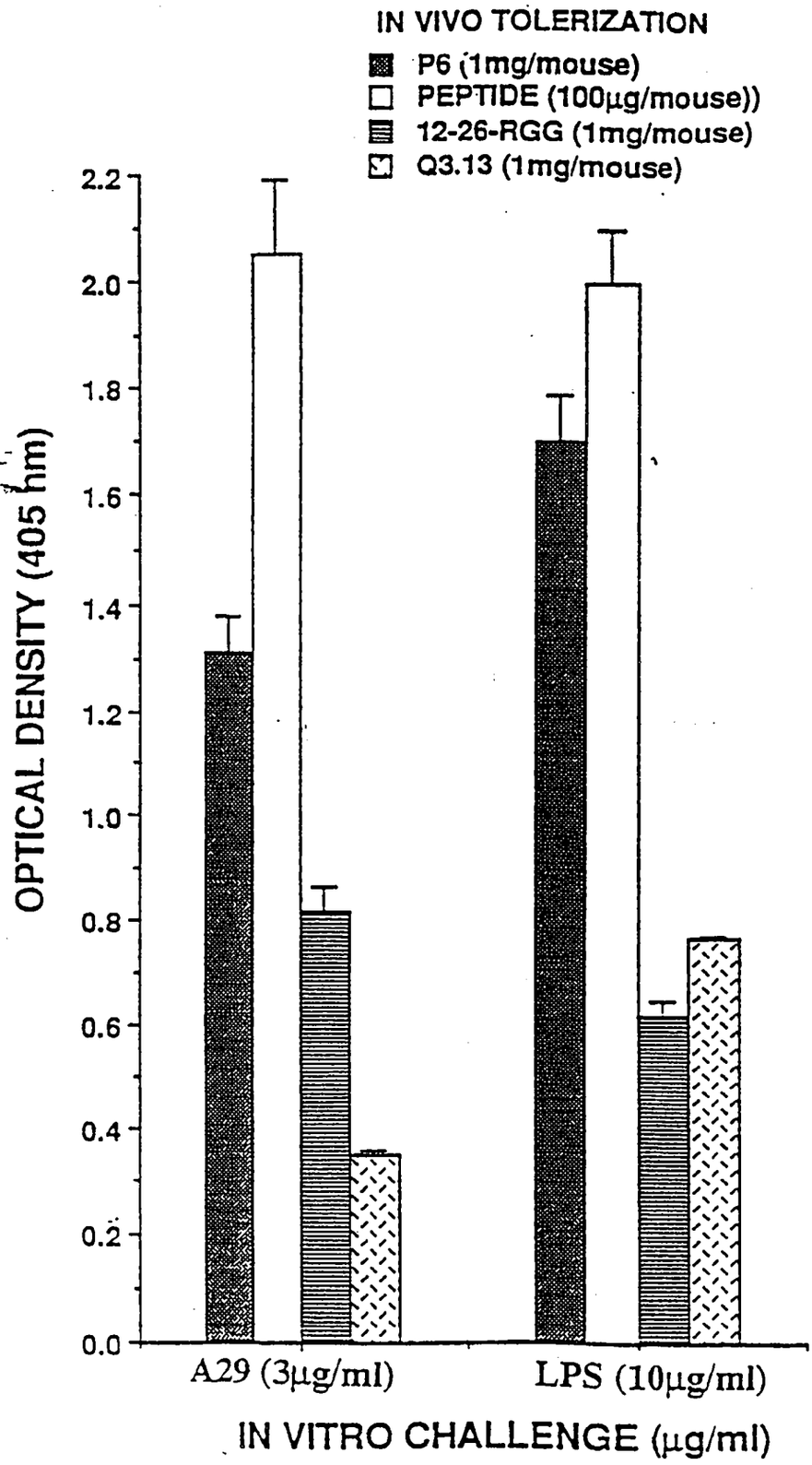

IN VIVO TOLERIZATION
P6 (1mg/mouse)
PEPTIDE (100µg/mouse))
12-26-RGG (1mg/mouse)
Q3.13 (1mg/mouse)
OPTICAL DENSITY (405 hm)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A29 (3µg/ml)
LPS (10µg/ml)
IN VITRO CHALLENGE (µg/ml)

Figure 5

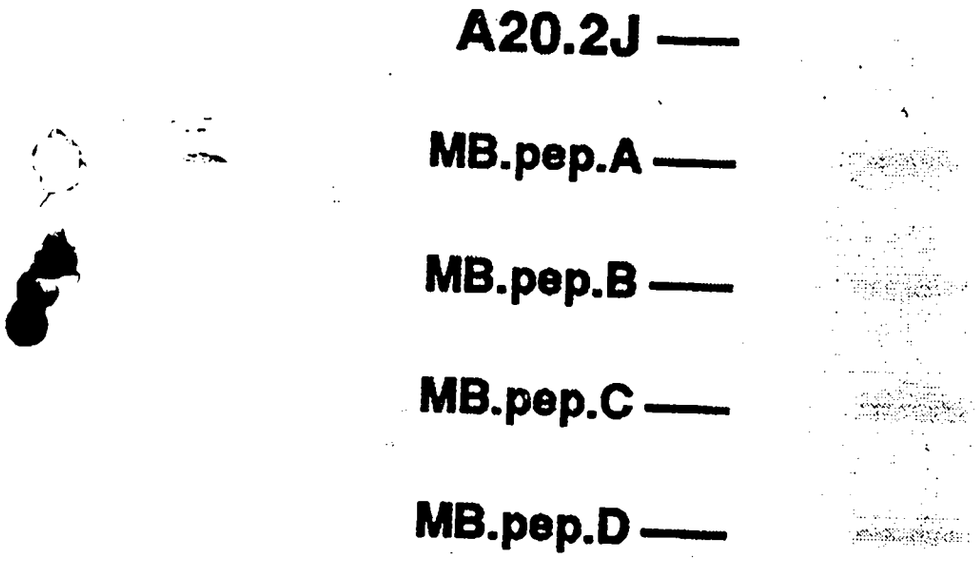
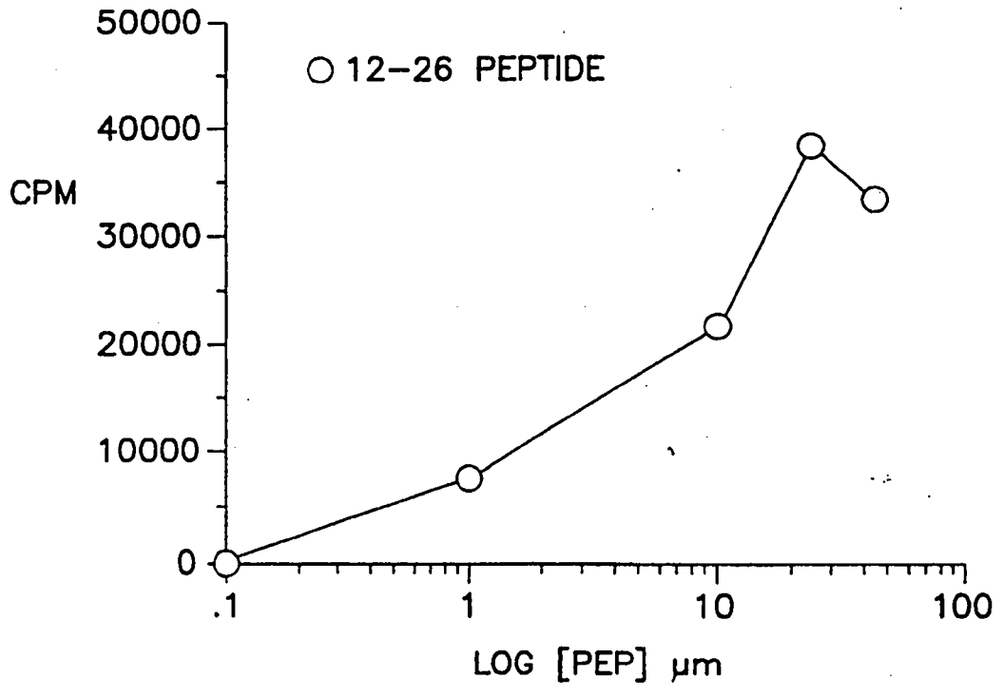


FIG. 6A

NSDOCID: <WO___9521926A1_I_>


50000
40000
30000
20000
10000
0
CPM
○ 12-26 PEPTIDE
.1
1
10
100
LOG [PEP] µm

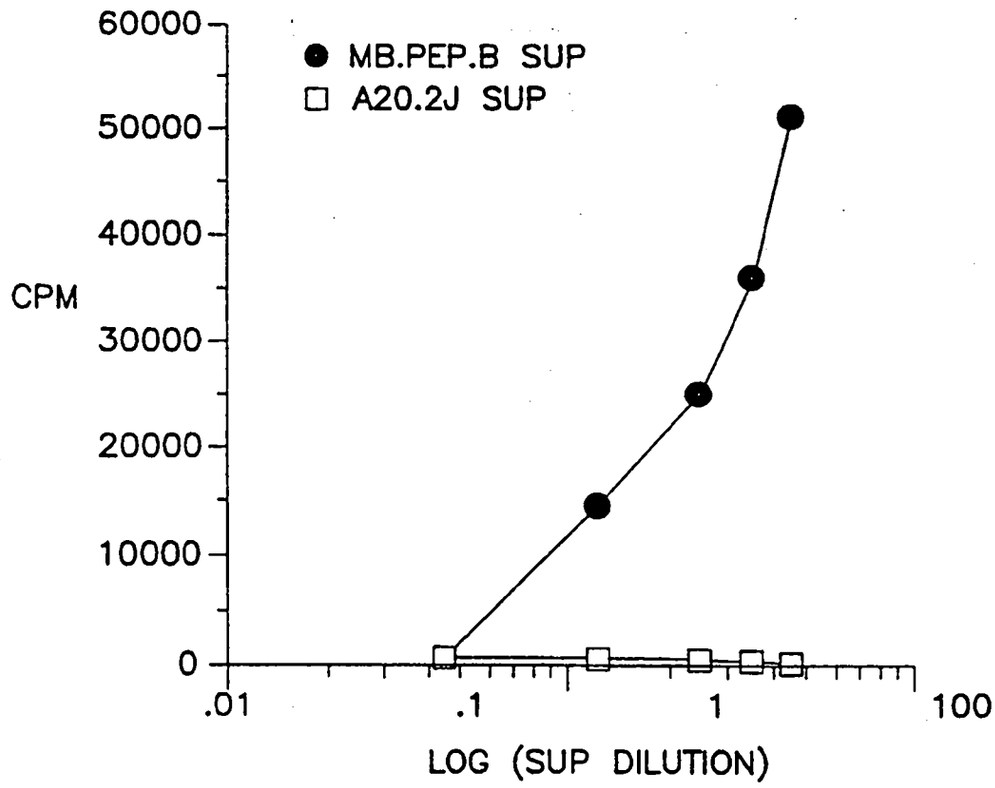

60000
50000
40000
30000
20000
10000
0
CPM
● MB.PEP.B SUP
□ A20.2J SUP
.01
.1
1
100
LOG (SUP DILUTION)

FIG. 6B

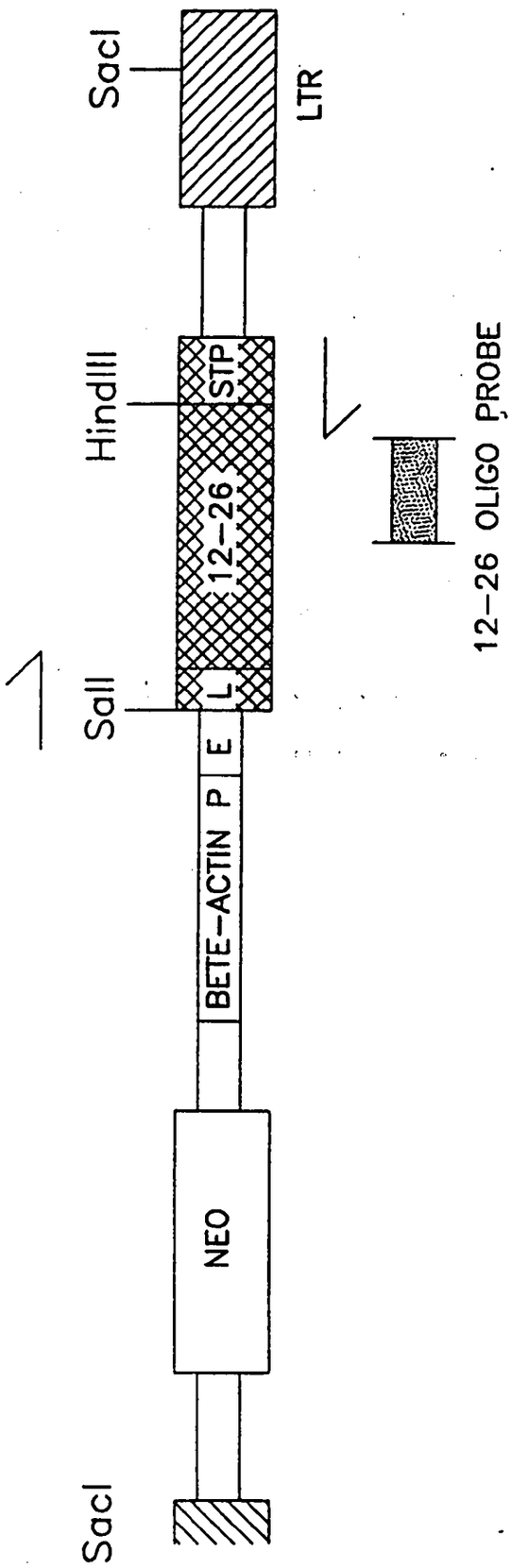


FIG. 7

BNSDOCID: <WO___9521926A1_I_>

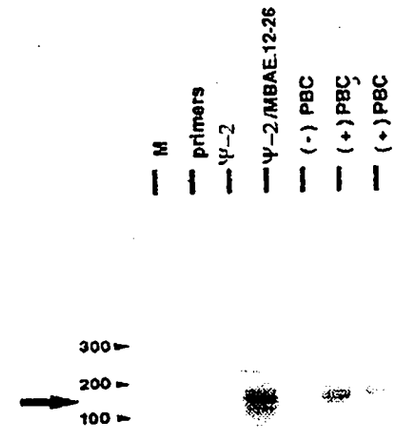


FIG. 8

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 95/01671

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/63 C12N15/86 C12N5/16 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,90 09804 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 7 September 1990 *see the whole patent* ---	1-30
X	Journal of Immunology, 1993, 8 Part 2, page 183A, abstract 1043. *see the whole abstract* ---	4,5,7, 11,16, 19,20,25
A	JOURNAL OF IMMUNOLOGY, vol. 119,no. 3, 1977 pages 1006-1009, VENKATARAMAN M. ET AL. 'Persistence of antigen-binding cells with surface tolerogen...' *see the whole article* -----	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 May 1995	21 -06- 1995

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Marie, A

Form PCT/ISA/210 (second sheet) (July 1992)

BNSDOCID: <WO___9521926A1_I_>

INTERNATIONAL SEARCH REPORT

information on patent family members

Int onal Application No
PCT/US 95/01671

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9009804	07-09-90	EP-A- 0460076	11-12-91
		JP-T- 4503605	02-07-92

Form PCT/ISA/210 (patent family annex) (July 1992)

275

Intestinal Immunology and Food Allergy,
edited by Alain L. de Weck and Hugh A. Sampson,
Nestlé Nutrition Workshop Series, Vol. 34. Nestec Ltd.,
Vevey/Raven Press. Ltd., New York © 1995.

XP-000981593

Induction of Systemic Tolerance by Tolerogens

PD 21-6-1995
P. 179-188 (11)

Yves Borel and Rodolphe Fritsché

Nestec Ltd., Nestlé Research Centre, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland

INTRODUCTION

Immunologic tolerance may be defined as a state of antigen-specific unresponsiveness induced by preexposure to an antigen. If the antigen is an allergen, the immune response is defined as allergy, an adverse reaction with an immunologic basis mediated by IgE immunoglobulin (1). The concept we wish to put forward is that by modifying the antigen presentation to the host, an allergic immune response to an allergen is prevented.

Oral tolerance is the normal immune response to the daily ingestion of food antigens, but there are rare instances where oral tolerance fails and food allergy occurs. When this happens, one can attempt to induce immunologic tolerance either orally or parenterally. These two strategic approaches are not mutually exclusive; both are beneficial but it remains to be seen whether they can be complementary. There are many differences between the two ways of inducing tolerance aside from the route of antigen administration. Oral and parenteral tolerance are both antigen specific. However, the specificity of tolerance induced by intravenous injection is only restricted to the antigen that induced it, while in oral tolerance, suppression may occur following administration of an antigen different from the disease-inducing antigen (bystander suppression) (2). The cellular mechanisms of both are still poorly understood. Regulatory T cells and soluble factors such as transforming growth factor-β2 (TGF-β2), as well as clonal anergy appear to play a central role.

It is known that several mechanisms play a role in eliciting food allergy, but the reasons why an antigen is allergenic are still poorly understood. Three factors appear to be critical: the physicochemical structure of the allergen, the genetic background of the host and environmental factors. A reduction in the allergenicity of food antigens poses a major challenge to the food industry. One of its main efforts has been in the reduction of allergenicity by physicochemical means such as, for instance, enzymatic digestion.

263

Here we consider an entirely different approach, that is, to change the allergen presentation to the host. We attempted to transform an allergen into a tolerogen, a compound that induces unresponsiveness. This idea is based on two main observations. First, haptens linked to isologous gamma globulin induce unresponsiveness to the same hapten linked to an immunogenic carrier molecule (3). Second, a method has been developed to covalently link either whole protein or peptide to gamma globulin and, under certain conditions, such protein gamma globulin conjugates were shown to diminish cellular and humoral immunity both *in vivo* and *in vitro* (4).

In this chapter, this principle is illustrated with an animal model, in which we prevented an allergic sensitization to a major cow's milk protein β-lactoglobulin (5). This is also shown for ovalbumin. The significance of these observations is discussed in the context of allergenicity versus tolerogenicity to prevent allergy. β-Lactoglobulin was selected for a number of reasons but primarily because cow's milk allergy is the most common allergy in children (6). Even though β-lactoglobulin constitutes only 10% of the total cow's milk protein, it is one of its most potent allergens (7). It resists acid digestion in the stomach and minute amounts of intact antigen can pass through the gut into the circulation. Furthermore, when secreted in human milk, it can sensitize breast-fed infants (8). Indeed, several cases of anaphylactic reactions to cow's milk protein have been reported (9). Thus β-lactoglobulin appears to be an ideal candidate to test the hypothesis that one can transform a protein antigen into a tolerogen by covalent linkage of the whole protein to isologous immunoglobulin G (IgG).

METHODS

Construction of Tolerogen

β-Lactoglobulin was linked to rat IgG as previously described (4) but with two minor modifications. First, we reduced by one third the amount of homobifunctional reagent [disuccinimidyl suberate (DSS)] used in the reaction. Second, before conjugation, we increased the initial molar ratio of β-lactoglobulin linked to IgG (from 1:1 to 10:1). The latter was done because an initial molar ratio of 1:1 failed to yield a tolerogenic conjugate. In contrast, a 10:1 molar ratio consistently produced a tolerogenic conjugate (5). In some experiments we conjugated ovalbumin to rat IgG instead of β-lactoglobulin to determine whether one can transform a different allergen into a tolerogen. An initial molar ratio of 10:1 was also the most effective for ovalbumin. The experimental protocol for both β-lactoblogulin–rat IgG and ovalbumin–rat IgG conjugates was the same. Only the former is described.

Experimental Design

Adult animals on a milk-free diet were injected intravenously with a single dose of 2 mg of either polymeric or monomeric conjugates made of β-lactoglobulin linked

to monomeric rat IgG. Controls consisted of rats similarly treated with the same dose of a mixture of β-lactoglobulin together with rat IgG but not covalently linked (or β-lactoglobulin or rat IgG linked to themselves), as well as untreated animals. Four days later, all rats were immunized with 100 μg of β-lactoglobulin and ovalbumin, an unrelated antigen, to test the specificity of the immune response. Two weeks later, the rats were bled and their sera were analyzed by enzyme-linked immuno-sorbent assay (ELISA) to determine the level of IgE, IgG, and IgA antibodies to both antigens. For β-lactoglobulin, the influence of tolerogen administration in rats immunized 4 days previously was also examined in both the primary and the secondary response. In addition, a [^{3}H]serotonin release assay was performed on peritoneal rat mastocytes either actively sensitized *in vivo* or passively sensitized *in vitro* (10).

In order to test the influence of the treatment on cellular immunity, a T cell proliferation assay was done *in vitro* with spleen lymphoid cells from either tolerized and immunized rats or controls (immunized animals). The cultured lymphoid cells were challenged *in vitro* with either β-lactoglobulin or β-lactoglobulin–rat IgG, or ovalbumin for specificity controls. Thus, in addition to one inflammatory manifestation of allergy, both cellular immunity and humoral immunity were examined.

RESULTS

Humoral Immunity

A single dose of tolerogen given 4 days before challenge suppresses the appearance of antibodies to β-lactoglobulin. This is the case for humoral antibodies in all isotypes, IgE, IgG, and IgA, as seen in Fig. 1. This was also observed in animals first immunized then tolerized in both the primary and the secondary response. Thus, it appears that one can prevent allergic sensitization in all classes of antibodies even if the tolerogen is given after immunization. For the conjugate to be tolerogenic, two conditions appear to be essential: the physical form of the conjugate (only monomeric conjugates containing unaggregated IgG are effective) and the molar ratio of allergen linked to rat IgG. This is also very well demonstrated in the case of ovalbumin linked to rat IgG. Here again only a 10:1 molar ratio seems to be effective and IgE antibody appears to be suppressed more than IgG antibody (Fig. 2). In all instances, the suppression of the antibody response was allergen specific since the immune response to the unrelated antigen was unaffected. Thus, suppression with β-lactoglobulin–IgG conjugate does not affect the response to ovalbumin and, reciprocally, suppression with ovalbumin–IgG conjugate failed to affect the immune response to β-lactoglobulin (Fig. 2).

Mastocyte Serotonin Release Assay

In the case of β-lactoglobulin, the effect of β-lactoglobulin–IgG conjugates on mast cell serotonin release was also determined. Initially, this was examined with normal

BNSDOCID: <XP___981593A_1_>

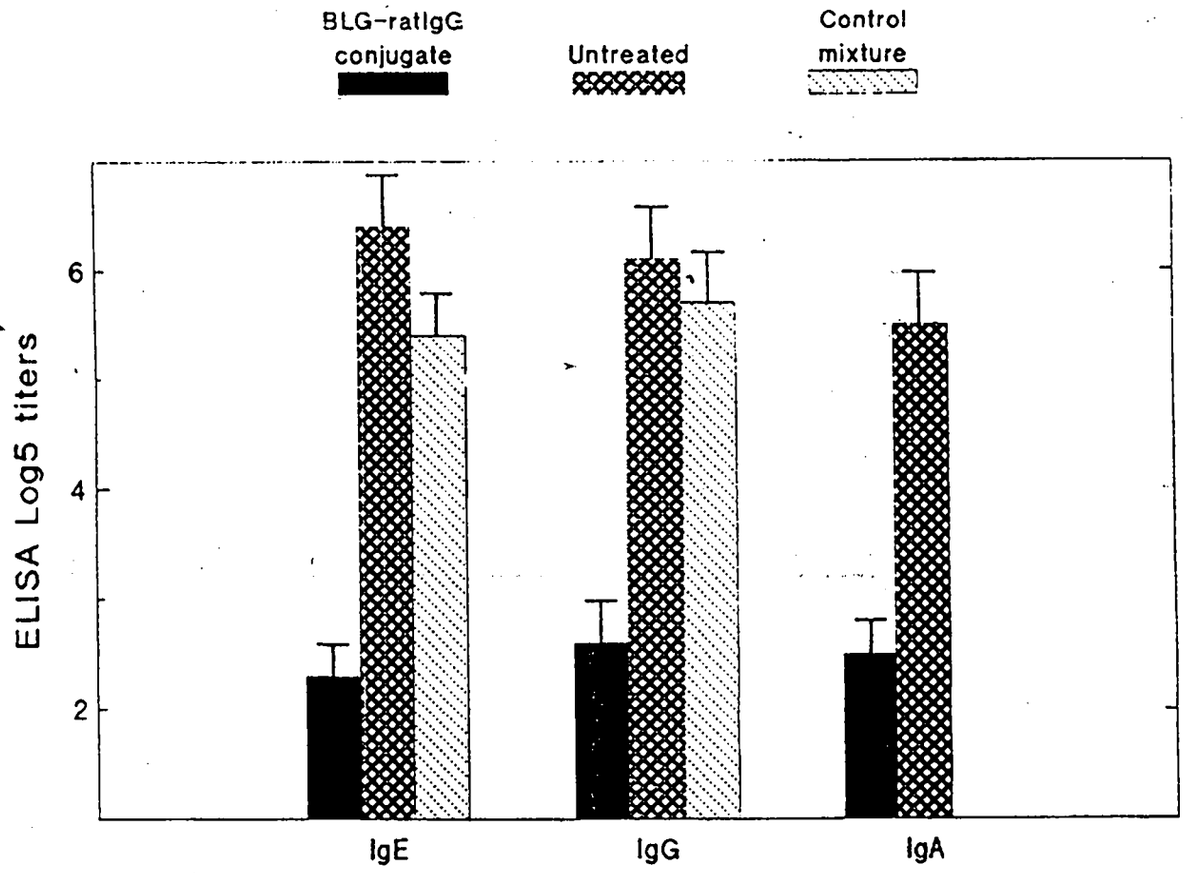


FIG. 1. Anti-β-lactoglobulin (BLG) – antibody suppression with tolerogen. ELISA titers of IgE, IgG, and IgA anti-β-lactoglobulin antibodies in sera from tolerized, control mixture injected, or untreated rats.

rat peritoneal mastocytes sensitized with rat anti-β-lactoglobulin reaginic antibodies. We found that sera containing high levels of IgE antibody, obtained from either untreated animals or controls treated with a mixture of β-lactoglobulin and rat IgG, mediate serotonin release following β-lactoglobulin triggering. In contrast, sera obtained from animals injected with a single dose of tolerogen failed to mediate serotonin release. Even though sera of animals successfully tolerized still contained low amounts of residual IgE antibody, the amounts were either qualitatively or quantitatively insufficient to sensitize mast cells for serotonin release. This was confirmed *in vivo* since mastocytes taken from rats injected with the tolerogen failed to release serotonin *in vitro* when compared to controls. Thus, it appears that a single dose of tolerogen suppresses this allergic inflammatory manifestation.

Because the serotonin release assay is a sensitive test to determine the allergenicity of a compound, it was of interest to determine the relative capacity of both antigen and tolerogen to trigger serotonin release. For a tolerogenic conjugate about 10% of the total protein is β-lactoglobulin (as determined by ELISA inhibition test). It represents 1 mole of β-lactoglobulin for 1 mole of IgG. The same amount of β-lactoglobulin

BNSDOCID: <XP____981593A_I_>

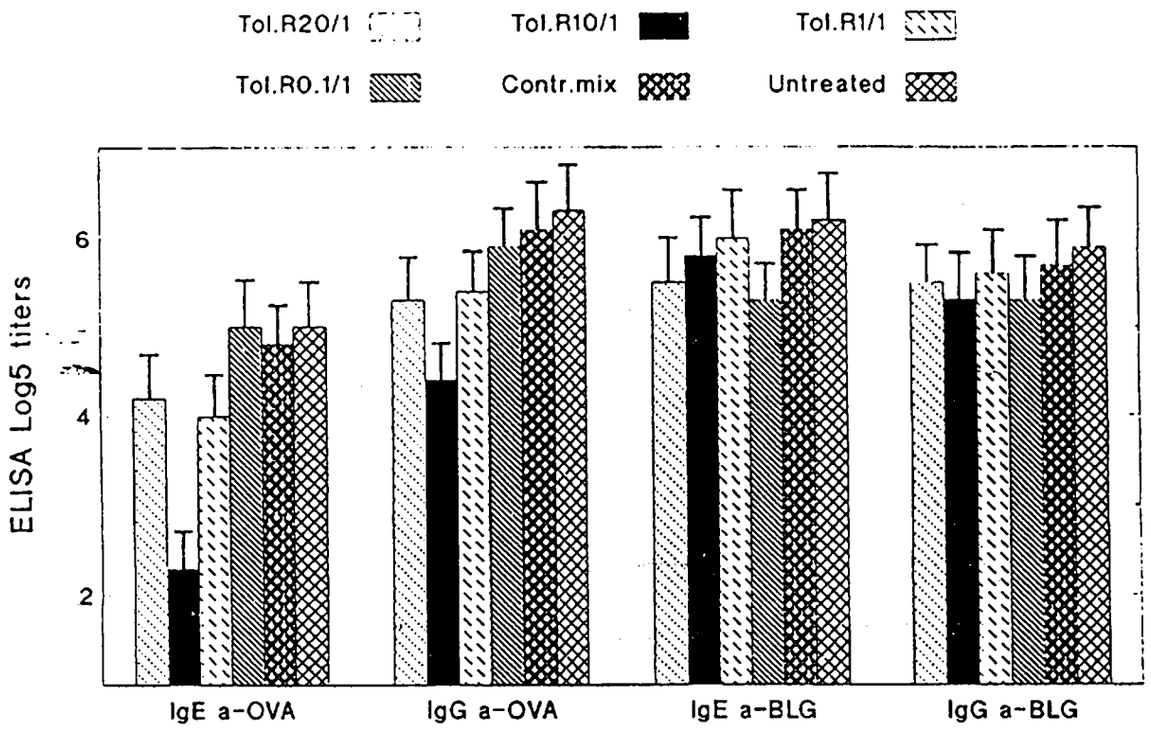


FIG. 2. Antibody suppression with tolerogens. ELISA titers of IgE and IgG anti-ovalbumin (OVA) or anti-β-lactoglobulin (BLG) antibodies in sera from tolerized (with different ovalbumin/rat IgG ratios), control mixture injected, or untreated rats.

either covalently linked to rat IgG or free was compared for its ability to trigger serotonin release. There is a tenfold difference between the two. Thus, one needs ten times more β-lactoglobulin linked to IgG compared to free β-lactoglobulin to produce about the same level of serotonin release (Fig. 3). This suggests that the tolerogen can still bind IgE antibody, but the spatial presentation of the allergenic epitope has been changed by covalent linkage of β-lactoglobulin to IgG.

Cellular Immunity

To test the influence of a single injection of protein conjugate on cellular immunity, a T cell proliferation assay was done with cultured spleen lymphoid cells. They were obtained either from control rats immunized with ovalbumin or β-lactoglobulin, or from rats tolerized with β-lactoglobulin–IgG and similarly immunized. They were challenged *in vitro* against β-lactoglobulin, β-lactoglobulin–IgG, and ovalbumin for specificity controls. The results are shown in Fig. 4. They show a reduction in T cell proliferation (as determined by [^{3}H]thymidine incorporation) to β-lactoglobulin in rats treated *in vivo* with β-lactoglobulin–IgG in contrast to the untreated controls. This was antigen specific since the response to ovalbumin was the same. In addition, it is interesting to note that in contrast to the allergen (β-lactoglobulin), the tolerogen

BNSDOCID: <XP___981593A_1_>

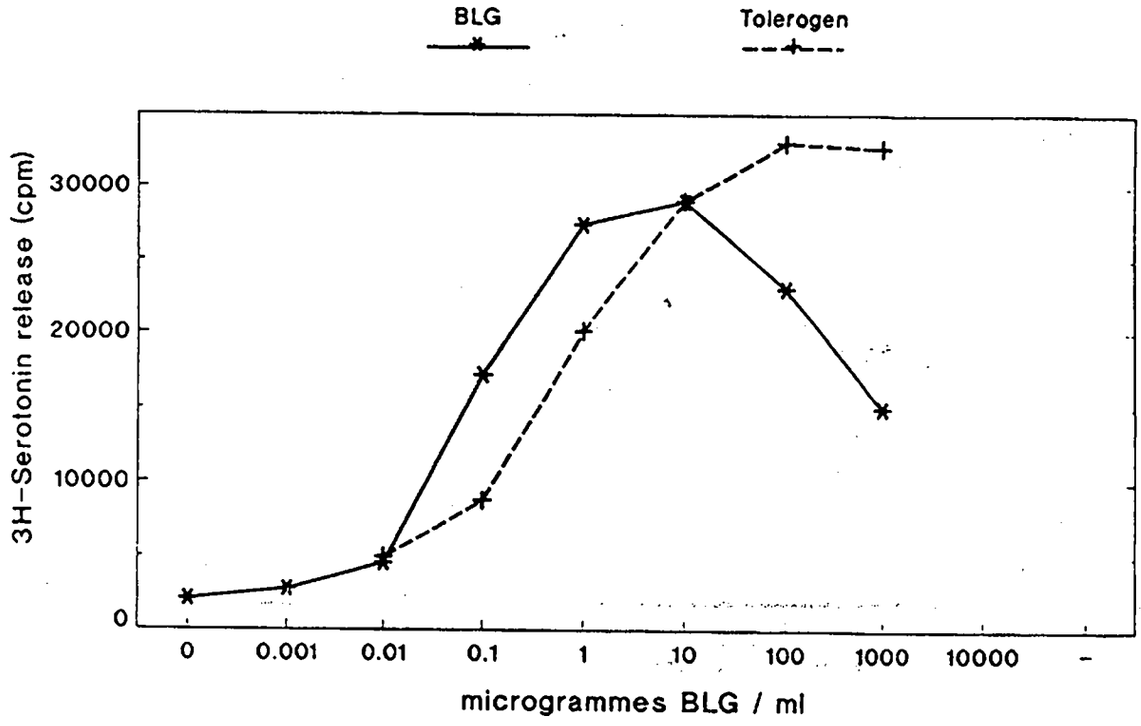


FIG. 3. [^{3}H]Serotonin release from normal rat mast cells passively sensitized *in vitro* with rat IgE anti-β-lactoglobulin (BLG). Triggering was done with β-lactoglobulin, either free or covalently linked to rat IgG (tolerogen).

(β-lactoglobulin–IgG) failed to stimulate T cell proliferation. This was the case in both controls and treated animals. It once again suggests that the antigenicity of β-lactoglobulin has been decreased by covalent binding to IgG.

DISCUSSION

We have shown that one can reduce an allergic response in rats to two allergens, β-lactoglobulin and ovalbumin, using the principle of carrier-induced tolerance (3). The allergen was covalently linked to isologous IgG, which by itself is a potent tolerogen already recognized by the host as self. For the conjugate to be tolerogenic, two conditions are essential: (a) the degree of substitution of the allergen linked to IgG and (b) the monomeric nature of the conjugate. Administration of tolerogen appears to reduce both T and B cell immune responses, suggesting that unresponsiveness instead of immunity is induced. The significance of these observations is twofold. First, it suggests an allergen-specific way to prevent allergy. Second, it provides a mean of analyzing allergenic versus tolerogenic epitopes for a single allergen. The data indicate that when β-lactoglobulin is covalently linked to IgG to form a tolerogen, the allergenicity of β-lactoglobulin is reduced. This is illustrated by two observations: there is a tenfold reduction in the capacity of conjugated β-lactoglobulin versus intact β-lactoglobulin to trigger serotonin release by rat mast cells. Second,

ANSDOCID: <XP 981593A 1 >

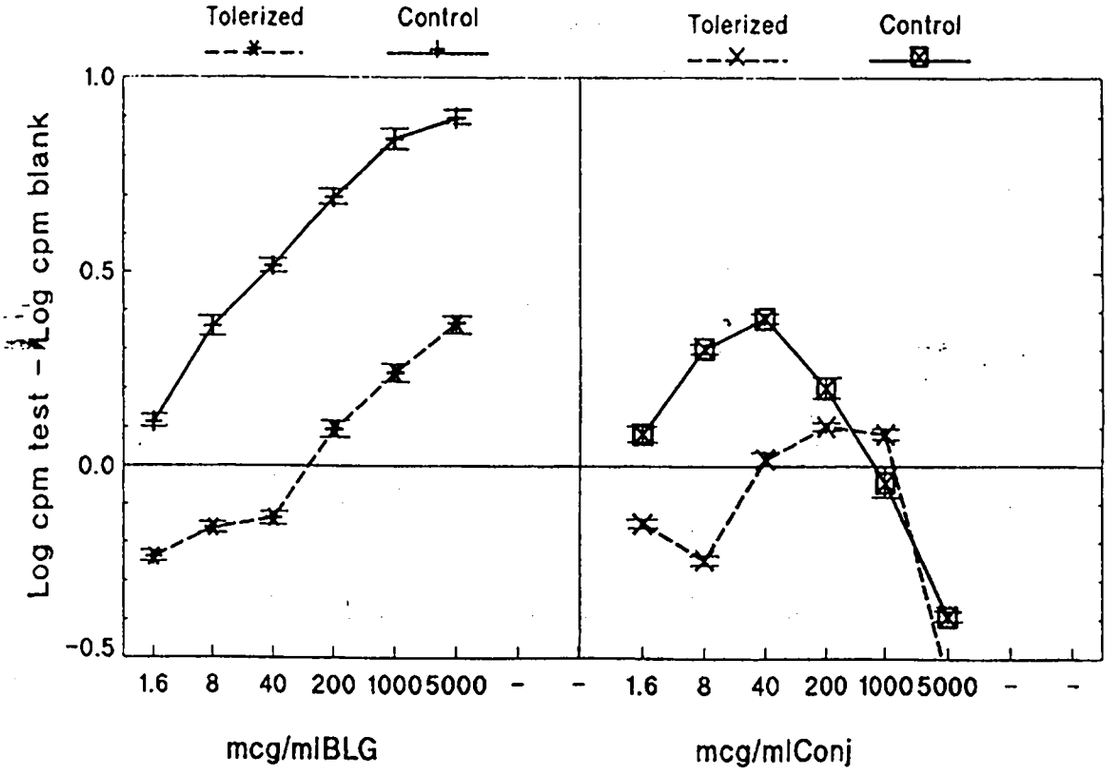


FIG. 4. Spleen lymphocyte stimulation ([^{3}H]thymidine incorporation assay) with cells from tolerized or control rats. *In vitro* stimulation was made with dilutions (1.6 to 5000 μg/ml) of either β-lactoglobulin (BLG), tolerogen, or ovalbumin (OVA). Results are expressed as the log of the ratio cpm test/cpm blank (log of stimulation index) and statistics made with Student's *t* test, ANOVA (variance), and ANCOVA (covariance) analyses.

β-lactoglobulin–IgG appears to be less immunogenic than β-lactoglobulin in eliciting T cell proliferation. This is the case for spleen lymphoid cells obtained from animals either tolerized or immunized *in vivo*. Thus the covalent binding of β-lactoglobulin to IgG decreases the immunogenicity of the allergen at both arms of the immune system, that is, the afferent level (T cell proliferation) and the efferent level (IgE binding on mast cells).

In order to analyze more precisely the nature of tolerogenic versus allergenic epitopes, individual peptides from a single allergen, for instance, ovalbumin, will have to be used. Increasing numbers of allergens are cloned and their molecular characteristics are known. Both conformational and sequential epitopes have been described. For example, the birch pollen allergen (Bet V1) has conformational epitopes present on the intact molecule even though sequential allergenic epitopes have also been characterized (11). For ovalbumin, several peptides have been characterized (12,13) but only a few are probably the major allergenic epitopes (peptides 323–339, peptides 105–122) (S. Elsayed, *personal communication*). Since peptides can be linked to IgG (4), this could provide a means to analyze whether tolerogenic and allergenic epitopes are the same. In addition, an important issue is to determine whether tolerization with

BNSDOCID: <XP___981593A_1_>

the major allergenic epitope of a single allergen can suppress the immune response to the whole molecule.

Allergy is one of the most common diseases since it affects about 10% of children and 3% to 4% of the adult population. Several strategies have been used to prevent it, including immunotherapy, chemical and enzymatic modification of the allergens (14), and administration of either allergen–antibody complexes (15) or peptides of the major allergenic epitopes (16). The multiplicity of these approaches shows both the complexity of the problem and the formidable challenge to downregulate an undesirable immune response. One possibility of doing so is to modulate the immune response following chemical modification of the allergen. This could alter the pattern of cytokine response, downregulating interleukin-4 (IL-4) and increasing interferon-γ (IFN-γ). These two cytokines have an opposite effect on IgE production. Consequently, IgE response is shifted to an IgG2a immune response (17). Thus, immunization but not allergy to the allergen occurs. Likewise, following immunotherapy, the so-called blocking antibody, usually IgG4, is raised while IgE antibody is downregulated. The balance between two populations of T helper cells, TH1 and TH2, appears to be the key for immunoregulation by cytokines (18). For example, IL-2 favors proliferation of TH1 cells, which secrete IFN-γ and IL-1, rather than TH2 cells, which secrete the IL-4 necessary for IgE production (19). It is unknown which cytokines are regulated by the administration of a protein–IgG conjugate.

In conclusion, we have shown using an animal model that one can specifically downregulate the immune responses to two potent food antigens (ovalbumin and β-lactoglobulin) by the administration of soluble protein–IgG conjugates. A single dose of tolerogen appears to suppress both T and B cell immune responses as well as mediator release from target mast cells. The method is simple and quite reproducible for a given protein allergen. These data have to be confirmed in other species and extended to airborne as well as food allergens. Our hope is that this novel approach will develop into an allergen-specific prevention therapy of allergy.

CONCLUSION

Allergy in general and food allergy in particular appear to be increasing. Various different strategies have been used to prevent this undesirable immune response, including immunotherapy, chemical modification of allergens, and administration of peptides (allergenic epitopes), to cite only a few. However, the challenge to develop an immunologically specific therapy for allergy is still open.

In this chapter, we have described how modifying the presentation of the allergen to the host can prevent allergic reactions. In essence, allergens are covalently linked to a "self-protein," such as isologous IgG, which is already tolerated by the host. Under certain conditions, the IgG conjugate has been shown in rats to prevent an allergic reaction to β-lactoglobulin or ovalbumin. The suppression is specific for the allergen. It affects both the T and B cell immune responses and stops mediator release from mast cells. β-Lactoglobulin linked to rat IgG not only induces unresponsiveness

BNSDOCID: <XP___981593A_I_>

in vivo but also appears to be less antigenic *in vitro* than intact β-lactoglobulin with respect to the efferent and/or afferent limbs of the immune system.

We suggest that a tolerogen made of allergens linked to isologous IgG may provide a novel strategy in the prevention of allergic reactions.

REFERENCES

1. Sampson HA. Differential diagnosis in adverse reactions to foods. *J Allergy Clin Immunol* 1986; 78: 212–9.
2. Miller A, Lider O, Weiner H. Antigen-driven bystander suppression after oral administration of antigens. *J Exp Med* 1991; 174: 791–8.
3. Borel Y. Haptens bound to self IgG induce immunologic tolerance, while when coupled to syngeneic spleen cells they induce immune suppression. *Immunol Rev* 1980; 50: 71–104.
4. Borel H, Borel Y. A novel technique to link either proteins or peptides to gammaglobulin to construct tolerogens. *J Immunol Methods* 1990; 126: 159–68.
5. Fritsché R, Borel Y. Prevention of allergic sensitization to β-lactoglobulin (BLG) with conjugates made of BLG coupled to isologous IgG. *J Allergy Clin Immunol* 1994; 93: 778.
6. Husby S, Høst A, Teisner B, Svehag SE. Infants and children with cow milk allergy/intolerance. Investigation of the uptake of cow milk protein and activation of the complement system. *Allergy* 1990; 45: 547–51.
7. Taylor SL. Immunologic and allergic properties of cow's milk proteins in humans. *J Food Protection* 1986; 49: 239–50.
8. Kilshaw PJ, Cant AJ. The passage of maternal dietary proteins into human breast milk. *Int Arch Allergy Appl Immunol* 1984; 75: 8–15.
9. Sampson HA, Mendelsohn L, Rosen JP. Fatal and near-fatal anaphylactic reactions to food in children and adolescents. *N Engl J Med* 1992; 327: 380–4.
10. Fritsché R, Bonzon M. Determination of cow milk formula allergenicity in the rat model by in vitro mast cell triggering and in vivo IgE induction. *Int Arch Allergy Appl Immunol* 1990; 93: 289–93.
11. Ebner C, Szepfahusi Z, Ferrerira F, *et al.* Identification of multiple T cell epitopes on Bet VI, the major birch pollen allergen, using specific T cell clones and overlapping peptides. *J Immunol* 1993; 150: 1047–54.
12. Elsayed S, Holen E, Hangstad MB. Antigenic and allergenic determinants of ovalbumin. *Scand J Immunol* 1988; 27: 587–91.
13. Johnsen G, Elsayed S. Antigenic and allergenic determinants of ovalbumin. III. MHC Ia-binding peptide (OA 323–339) interacts with human and rabbit specific antibodies. *Mol Immunol* 1990; 22: 821–7.
14. Hayglass KT, Stefura W. Isotype-selective abrogation of established IgE responses. *Clin Exp Immunol* 1990; 82: 429–34.
15. Machiels JJ, Somville MA, Lebrun PM, Lebecque SJ, Jacquemin MG, Saint-Remy JMR. Allergic bronchial asthma due to *Dermatophagoides pteronyssimus* hypersensitivity can be efficiently treated by inoculation of allergen–antibody complexes. *J Clin Invest* 1990; 85: 1024–35.
16. Pesce AS, Freisheim JM, Litwin A, Michael JG. Modulation of the immune response to allergens: phospholipase A degradation products suppress IgG and IgE response in mice. *Int Arch Allergy Appl Immunol* 1990; 92: 88–93.
17. Gieni RS, Yang X, Hayglass KT Allergen-specific modulation of cytokine synthesis patterns and IgE responses in vivo with chemically modified allergen. *J Immunol* 1993; 150: 302–10.
18. Romagnoni S. Induction of TH1 and TH2 responses: a key role for "natural" immune response? *Immunol Today* 1992; 13: 379–81.
19. Scott P. IL-12: initiation cytokine for cell-mediated immunity. *Science* 1993; 260: 496–7.

DISCUSSION

Dr. Schmitz: You have presented us with an intriguing finding. It is a very stimulating concept even for a single protein such as β-lactoglobulin. However, since there are several

271

different proteins in cow's milk that could render a baby sensitized, is it feasible to envisage that other milk proteins could be linked to IgG?

Dr. Borel: I don't think it would be feasible for any protein. Each protein is different, and it took us a great deal of time to find the conditions to transform β-lactoglobulin into a tolerogen by linking it to IgG. But we know now how to do it for β-lactoglobulin, and we know how to do it for ovalbumin. Whether it can be done for other proteins remains to be determined.

Dr. Aalberse: I missed one control. Do you really need IgG? What happens when you polymerize the β-lactoglobulin on its own?

Dr. Borel: It doesn't. I did not present the data but we did do this control; we definitely need IgG. In mice it seems to work best with Ig1 and Ig2A, so it is conceivable that certain isotypes work better than others. In rats, we use total IgG.

Dr. Aalberse: Did you check whether there is an interaction with Fc receptors?

Dr. Borel: I think the mechanism by which the conjugate induces nonresponsiveness needs to be worked out. We have measured the amount of β-lactoglobulin linked to IgG after we do the binding; we start with a molar ratio of 10:1 and we end up with a molar ratio of 1:1, so we have one molecule of β-lactoglobulin linked to one molecule of IgG. I presume that the Fc receptor of the IgG remains intact and can still function.

Dr. Sampson: Do you find a 1:1 molar ratio for ovalbumin as well?

Dr. Borel: We don't know yet, we haven't looked at the ratio after binding. What we do know is that we use the same ratio to start the reaction for both molecules but we haven't measured for the amount of ovalbumin linked to immunoglobulin.

Dr. Bienenstock: Do you think that because of the chemical alteration of the β-lactoglobulin molecule it is conceivable that the epitopes which are antigenic are in fact sufficiently spaced apart not to crosslink and act as a monomeric IgE?

Dr. Borel: We know very little about the allergenic epitope in β-lactoglobulin and it is unknown whether this epitope is still intact after the linkage, but the data I have presented suggest that there is a difference in the presentation of this epitope; whether it is the same epitope or a different one I do not know.

Dr. Bienenstock: Could you explain what you think is the mechanism whereby an antigen is crosslinked to IgG?

Dr. Borel: I think the mechanism affects T cells and B cells in different ways. On the B cell I think there is a linkage with the IgG through the Fc receptor as well as through the B cell antigen receptor, so there is a bridge between two receptors. We have shown that we can tolerize the B cell very easily *in vitro* by activating the link to IgG, so that is one mechanism. On the T cell, the mechanism seems to be different. I think the conjugate is probably presented by the B cell to the T cell, so the antigen-presenting cell might be the B cell which presents the tolerogen to the T cell.

Dr. Walker: I wonder if you have considered ways of delivering your β-lactoglobulin conjugate orally?

Dr. Borel: Yes, we tried and it seems to work but not as well as intravenously; probably the dose we have to give is much higher. I think if we want to consider the oral route in the human, I would choose IgA as the carrier protein because IgA is digested much less than IgG.

Dr. Walker: I was thinking more of the techniques that are now being used for vaccines, trying to facilitate transport across M cells by using a liposome or using other processes. Have you thought in such terms—taking the conjugate and linking it to something that might preferentially be taken up by M cells and direct

Dr. Borel: This is certainly a very interesting idea.

Dr. Bock: You mentioned that in one of your experiments you immunized the animals first. If you then inject that immunized animal with the β-lactoglobulin, do they have anaphylaxis?

Dr. Borel: It depends on the dose, but you can get anaphylaxis.

Dr. Cézard: Your model is very interesting but do you think that the way you immunize your animals could modify the tolerogen? For example, if you immunize them by oral immunization, do you think that could modify the mechanism?

Dr. Borel: It is a good question. We don't know whether animals that are sensitized from the gut could be suppressed by this procedure. This is one question that we would like very much to answer. We can immunize with complete Freund's adjuvant or with aluminum hydroxide, but whether oral immunization is suppressed by administration of tolerogen remains to be determined.

Dr. Marini: In humans, do you think that the high content of specific IgG and IgE in the colostrum may be protective for the neonate? The fetus will have received specific IgG from the mother via the placenta and the infant will receive β-lactoglobulin in the milk if the mother herself drinks milk. In this way, the child receives both antibodies and antigen.

Dr. Borel: There are people who believe that the IgG idiotype of a mother modulates the response of the child. What we have produced is in a way similar to an idiotype IgG molecule in which you have an antigen attached to IgG. Yes, it is a possibility. I think one of the issues is whether by treating the pregnant mother we can actually suppress the response in the fetus.

Dr. Sampson: When you talked about passively sensitizing the mast cells and then showing serotonin release, you said that even though there was some residual IgE in the tolerized animal you did not see a similar release. Did you dilute out your control animal sera so that the amount of IgE would be similar in the two groups, to try to sort out whether it is nonfunctional or whether it is just an issue of antibody concentration?

Dr. Borel: Perhaps Dr. Fritsché can answer this question because he did the experiment.

Dr. Fritsché: It is purely a dilution problem.

Dr. Turck: How could you use the tolerogen? Would you plan to feed it to all babies, even those not at risk for allergy, if you were able to produce an effective oral preparation?

Dr. Borel: Certainly the idea would be to prevent allergy to specific proteins. Whether we

(12) SOLICITUD INTERNACIONAL PUBLICADA SEGÚN EL TRATADO DE COOPERACION DE PATENTES (PCT)

(19) Organización Mundial de Propiedad Intelectual
Oficina Internacional

(43) Fecha de Publicación
28 de Marzo de 2002 (28.03.2002)

PCT

(10) Número de Publicación Internacional
WO 02/24883 A2

(51) Clasificación Internacional de Patentes[7]: C12N 9/52

(21) Número de Solicitud Internacional: PCT/EP01/10956

(22) Fecha de Registro Internacional: Septiembre 21 de 2001 (21.09.2001)

(25) Idioma de registro: Inglés

(26) Idioma de Publicación: Inglés

(30) Información de Prioridad:
00120867.7 Septiembre 25 de 2000 (25.09.2000) EP

(71) Solicitante: (para todos los Estados designados excepto Estados Unidos de América): **SOCIETE DES PRODUITS NESTLE S.A.** [CH/CH]; P.O. Box 353, CH-1800 Vevey (CH).

(72) Inventores; e

(75) Inventores/Solicitantes: (para Estados Unidos de América solamente): **GERMOND, Jacques, Edouard** [CH/CH]; 18, chemin des Cèdres, CH-1023 Crissier (CH). **CORTHESY, Blaise** [CH/CH]; 34C, Chemin du Polny, CH-1066 Epalinges (CH). **MOLLET, Beat** [CH/CH]; 9, avenue de l'Esplanade, CH-1012 Lausanne (CH). **FRITSCHE, Rodolphe** [CH/CH]; 48, chemin de la Chaumény, CH-1814 La Tour-de-Peilz (CH).

(74) Agente: **STRAUS, Alexander;** Becker, Kurig, Straus, Bavariastrasse 7, 80336 München (DE).

(81) Estados Designados (nacionales): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Estados Designados (regionales): Patente ARIPO (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), patente Eurasiatica (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM) Patente Europea (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), Patente OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Publicado:
- *sin reporte de búsqueda internacional y para ser publicada de nuevo en el caso de recibo de ese reporte*
- *totalmente en forma electrónica (excepto para esta página frontal) y disponible sobre la petición de la Oficina Internacional*

Para los códigos de dos letras y otras abreviaturas, refiérase a "Las Notas Guías sobre Códigos y Abreviaturas" que aparecen al comienzo de cada emisión regular de la Gaceta PCT.

(54) Título: BACTERIA DEL ACIDO LACTICO CAPAZ DE REDUCIR LA TENDENCIA DE UN INDIVIDUO A DESARROLLAR REACCIONES ALERGICAS

Peptide T1 (139-141) H-A-L-K-COOH

Peptide T2 (146-148) H-H-I-R-COOH

Peptide T3 (136-138) H-F-D-K-COOH

Peptide T4 (71-75) H-I-I-A-E-K-COOH

Peptide T5 (62-69) H-E-N-G-E-C-A-Q-K-COOH

Peptide T6 (84-91) H-I-D-A-L-N-E-N-K-COOH

Peptide T7 (9-14) H-G-L-D-I-Q-K-COOH

Peptide T8 (142-145) H-A-L-P-M-COOH

Peptide T9,T10, T11 (61-67,-68,-69) H-W-E-N-G-E-C-A,-Q,-K,-COOH

Peptide T12 (125-135) H-T-P-E-V-D-D-E-A-L-E-K-COOH

Peptide T13 (1-8) H-L-I-V-T-Q-T-M-K-COOH

Peptide T14 (142-148) = T3 (142-145) + T2 (146-148) H-A-L-P-M-H-I-R-COOH

Peptide T15 (78-82) H-I-P-A-V-F-K-COOH

Peptide T16 (15-20) H-V-A-G-T-W-Y-COOH

Peptide T17 (92-99,100) H-V-L-V-L-D-T-D-Y-K,-K-COOH

Peptide T18 (125-138) = T12 (125-135) + T3 (136-138) H-T-P-E-V-D-D-E-A-L-E-K-F-D-K-COOH

Peptide T19 (25-40) H-A-A-S-D-I-S-L-L-D-A-Q-S-A-P-L-R-COOH

Peptide T20, T21 (61, 62-69):S-S:(149-162)

H-W,-E-N-G-E-C-A-Q-K-COOH
|
H-L-S-F-N-P-T-Q-L-E-E-Q-C-H-I-COOH

Peptide T22 (21-40) H-S-L-A-M-A-A-S-D-I-S-L-L-D-A-Q-S-A-P-L-R-COOH

Peptide T23 (41-60) H-V-Y-V-E-E-L-K-P-T-P-E-G-D-L-E-I-L-L-Q-K-COOH

Peptide T24 (149-162) H-L-S-F-N-P-T-Q-L-E-E-Q-C-H-I-COOH

(57) Resumen: La presente invención tiene que ver con cepas novedosas de bacterias lácticas que son capaces de reducir la tendencia de un individuo a elevar reacciones alérgicas. En particular, la presente invención se refiere a la generación de cepas de bacterias lácticas que expresan proteínas que incluyen péptidos inductores de tolerancia y el uso de los mismos en la reducción de la tendencia de un individuo a desarrollar reacciones alérgicas. La invención también tiene que ver con composiciones alimenticias o farmacéuticas que contienen dichos microorganismos o fracciones activas de los mismos.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 March 2002 (28.03.2002)

PCT

(10) International Publication Number
WO 02/24883 A2

(51) International Patent Classification[7]: C12N 9/52

(21) International Application Number: PCT/EP01/10956

(22) International Filing Date:
21 September 2001 (21.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
00120867.7 25 September 2000 (25.09.2000) EP

(71) Applicant *(for all designated States except US)*: **SOCIETE DES PRODUITS NESTLE S.A.** [CH/CH]; P.O. Box 353, CH-1800 Vevey (CH).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GERMOND, Jacques, Edouard** [CH/CH]; 18, chemin des Cèdres, CH-1023 Crissier (CH). **CORTHESY, Blaise** [CH/CH]; 34C, Chemin du Polny, CH-1066 Epalinges (CH). **MOLLET, Beat** [CH/CH]; 9, avenue de l'Esplanade, CH-1012 Lausanne (CH). **FRITSCHE, Rodolphe** [CH/CH]; 48, chemin de la Chaumény, CH-1814 La Tour-de-Peilz (CH).

(74) Agent: **STRAUS, Alexander**; Becker, Kurig, Straus, Bavariastrasse 7, 80336 München (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian

[Continued on next page]

(54) Title: LACTIC ACID BACTERIA CAPABLE OF REDUCING AN INDIVIDUAL'S TENDENCY TO DEVELOP ALLERGIC REACTIONS

Peptide T1 H_2N-A-L-K-COOH (139-141)

Peptide T2 H_2N-H-I-R-COOH (146-148)

Peptide T3 H_2N-F-D-K-COOH (136-138)

Peptide T4 H_2N-I-I-A-E-K-COOH (71-75)

Peptide T5 H_2N-E-N-G-E-C-A-Q-K-COOH (62-69)

Peptide T6 H_2N-I-D-A-L-N-E-N-K-COOH (84-91)

Peptide T7 H_2N-G-L-D-I-Q-K-COOH (9-14)

Peptide T8 H_2N-A-L-P-M-COOH (142-145)

Peptide T9.T10, T11 H_2N-W-E-N-G-E-C-A,-Q,-K,-COOH (61-67,-68,-69)

Peptide T12 H_2N-T-P-E-V-D-D-E-A-L-E-K-COOH (125-135)

Peptide T13 H_2N-L-I-V-T-Q-T-M-K-COOH (1-8)

Peptide T14 H_2N-A-L-P-M-H-I-R-COOH (142-148) = T8 (142-145) + T2 (146-148)

Peptide T15 H_2N-I-P-A-V-F-K-COOH (78-82)

Peptide T16 H_2N-V-A-G-T-W-Y-COOH (15-20)

Peptide T17 H_2N-V-L-V-L-D-T-D-Y-K,-K-COOH (92-99,100)

Peptide T18 H_2N-T-P-E-V-D-D-E-A-L-E-K-F-D-K-COOH (125-138) = T12 (125-135) + T3 (136-138)

Peptide T19 H_2N-A-A-S-D-I-S-L-L-D-A-Q-S-A-P-L-R-COOH (25-40)

Peptide T20, T21 (61, 62-69):S-S:(149-162)

H_2N-W,-E-N-G-E-C-A-Q-K-COOH

H_2N-L-S-F-N-P-T-Q-L-E-E-Q-C-H-I-COOH

Peptide T22 H_2N-S-L-A-M-A-A-S-D-I-S-L-L-D-A-Q-S-A-P-L-R-COOH (21-40)

Peptide T23 H_2N-V-Y-V-E-E-L-K-P-T-P-E-G-D-L-E-I-L-L-Q-K-COOH (41-60)

Peptide T24 H_2N-L-S-F-N-P-T-Q-L-E-E-Q-C-H-I-COOH (149-162)

(57) Abstract: The present invention pertains to novel strains of lactic acid bacteria that are capable to reduce an individual's tendency to mount allergic reactions. In particular, the present invention relates to the generation of lactic acid bacteria strains that express proteins including tolerogenic peptides and the use thereof in reducing an individual's tendency to mount allergic reactions. The invention also pertains to food or pharmaceutical compositions containing said micro-organisms or active fractions thereof.

WO 02/24883 A2

patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

— *entirely in electronic form (except for this front page) and available upon request from the International Bureau*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

TRATADO DE COOPERACION DE PATENTES
PCT
REPORTE DE EXAMEN PRELIMINAR INTERNACIONAL

(PCT Artículo 36 y Regla 70)

Registro de referencia del solicitante o agente 80263 WO	**PARA FUTURAS ACCIONES**	Ver Notificación o Transmisión del Reporte de Examen Preliminar Internacional (Formato PCT/IPEA/416)
Solicitud Internacional No. PCT/EP01/10956	Fecha de registro internacional (día/mes/año) 21/09/2001	Fecha de prioridad (día/mes/año) 25/09/2000
Clasificación Internacional de Patentes (IPC) o clasificación nacional e IPC C12N 9/52		
Solicitante **SOCIETE DES PRODUITS NESTLE S.A. et al.**		

1. Este informe de examen preliminar internacional ha sido preparado por esta Autoridad Examinadora Preliminar Internacional y se transmite al solicitante de conformidad con el Artículo 36.

2. Este REPORTE consta de un total de 5 páginas, incluyendo la carátula.

☒ Este reporte también está acompañado de ANEXOS, es decir, páginas de descripción, reivindicaciones y/o dibujos que han sido modificados y son la base de este informe y/o páginas que contienen rectificaciones hechas ante esta Autoridad (ver Regla 70.16 y la Sección 607 de las Instrucciones Administrativas de conformidad con el PCT).

Estos anexos constan de un total de 2 páginas.

3. Este informe contiene indicaciones relacionadas con los siguientes ítems:

- I ☒ Bases del Reporte
- II ☐ Prioridad
- III ☐ No establecimiento de opinión respecto a novedad, nivel inventivo y aplicabilidad Industrial.
- IV ☐ Falta de unidad de invención
- V ☒ Afirmación razonada de conformidad con el Artículo 35(2) con respecto a novedad, nivel inventivo o aplicabilidad industrial; citaciones y explicaciones que sustentan dicha afirmación .
- VI ☐ Ciertos documentos citados
- VII ☐ Ciertos defectos en la solicitud internacional
- VIII ☐ Ciertas observaciones sobre la solicitud internacional

Fecha de presentación de la demanda 16/04/2002	Fecha de terminación de este reporte 27.01.2003
Nombre y dirección de correo de la autoridad de examen preliminar internacional: Oficina de Patentes Europea D-80298 Munich Tel. +49 89 2399-0 Tx: 523656 epmu d Fax: +49 89 2399-4465	Funcionario autorizado Scheffzyk-Sonnauer, Teléfono No. +49 89 2399 8602

I. Bases del reporte

1. Respecto a los **elementos** de la solicitud internacional: (Carátulas de remplazo que han sido suministradas a la oficina receptora en respuesta a una invitación bajo el artículo 14 están referenciados en este reporte como "presentados originalmente" y no están anexos a este reporte dado que no contienen modificaciones (Reglas 70.16 y 70.17):

Descripción, páginas:

1-17 como se presentaron originalmente

Reivindicaciones, No.:

1-13 como se recibieron en 07/01/2003 con carta de 07/01/2003

Parte del listado de secuencias de la descripción, páginas:

1-6, presentadas con la carta de 22.07.02

2. Respecto al **idioma,** todos los elementos marcados anteriormente estuvieron disponibles o fueron suministrados a esta Autoridad en el idioma en el cual la solicitud internacional fue presentada, a menos que se indique lo contrario bajo este ítem.

Estos elementos estuvieron disponibles o fueron suministrados a esta Autoridad en el idioma: , el cual es:

☐ el idioma de una traducción suministrada para una búsqueda internacional (según la Regla 23.1 (b)).
☐ el idioma de una publicación de la solicitud internacional (según la Regla 48.3 (b)).
☐ el idioma de la traducción suministrada para el examen preliminar internacional (según las Reglas 55.2 y/o 55.3).

3. Respecto al **nucleótido y/o secuencia amino ácida** revelado en la solicitud internacional, se llevó a cabo el examen preliminar internacional según el listado de secuencia:

☐ contenido en la solicitud internacional en forma escrita.
☐ registrado junto con la solicitud internacional en forma legible por computador.
☒ proporcionado subsiguientemente a esta Autoridad en forma escrita.
☒ proporcionado subsiguientemente a esta Autoridad en forma legible por computador.
☒ Se ha proporcionado la afirmación respecto a la cual el listado de secuencia escrito proporcionado subsiguientemente no va más allá de la revelación en la solicitud internacional según se presentó.
☒ Se ha proporcionado la afirmación respecto a la cual la información presentada en forma legible por computador es idéntica al listado de secuencia escrita.

4. Las modificaciones han resultado en la cancelación de:

☐ la descripción, páginas:
☐ las reivindicaciones, Nos.:
☐ los dibujos, hojas:

5. ☒ Este reporte ha sido establecido como si (algunas) de las modificaciones no se hubiesen hecho, ya que se ha considerado que van más allá de la revelación según se presentó, como se indicó en Casilla Suplementaria (Regla 70.2 (c)):

(Cualquier hoja de remplazo que contenga tales modificaciones debe ser referida bajo el ítem 1 y anexada a este reporte)
ver hoja separada

6. Observaciones adicionales, si son necesarias:

V. Afirmación razonada bajo el Articulo 35(2) respecto a novedad, paso inventivo o aplicabilidad industrial; citaciones y explicaciones que sustentan dicha afirmación

1. Afirmación

Novedad (N)	Sí:	Reivindicaciones	
	No:	Reivindicaciones	1-14
Nivel inventivo (IS)	Sí:	Reivindicaciones	
	No:	Reivindicaciones	1-14
Aplicabilidad industrial (IA)	Sí:	Reivindicaciones	1-14
	No:	Reivindicaciones	

2. Citaciones y explicaciones
ver hoja separada

SECCION I------

Las reivindicaciones nuevamente presentadas no cumplen los requerimientos del Art. 34(2)(b) del PCT dado que la combinación específica "proteasa anclada a la superficie de la célula de Lactobacillus Bulgaricus" introducida en la reivindicación nuevamente presentada únicamente es mencionada en la solicitud como fue presentada en combinación con péptidos inductores de tolerancia, especialmente T6 y T17 pero no en combinación con ningún péptido inductor de tolerancia. En consecuencia, no existe base en la solicitud como fue presentada para la generalización hecha en la reivindicación 1 nuevamente presentada. Reconocidamente, en la página 7 de la solicitud las proteínas ancladas a la superficie de la célula están mencionadas generalmente, sin embargo, no en combinación con Lactobacillus bulgaricus. Por lo tanto, en cuanto a las reivindicaciones nuevamente presentadas no cumplen los requerimientos del Art. 34(2)(b) del PCT las reivindicaciones nuevamente presentadas no son consideradas en este IPER. Siendo así, este IPER se basa sobre las reivindicaciones como fueron presentadas originalmente.

SECCION V----------

Se presenta una objeción según el Art. 6 del PCT dado que la expresión "péptido inductor de tolerancia" únicamente puede ser visto como un resultado a ser logrado, esto es, un péptido que exhibe actividades inductoras de tolerancia, pero no como una característica técnica. Correspondientemente, una persona versada en la materia que lea la reivindicación no está en la posición de asegurar sí o no una cepa bacterial dada, una composición alimenticia dada etc. esta protegida por el alcance de la presente invención. Por lo tanto, las presentes reivindicaciones no cumplen los requerimientos del Artículo 6 del PCT. Adicionalmente, debido al oscuro alcance de dicho término también se presenta una objeción por novedad dado que a este momento no se puede descartar que las cepas bacteriales enseñadas en la técnica que producen proteínas de fusión estén abarcadas por el alcance de las reivindicaciones presentes (ver por **e.j.** Vaccine 17 (1999), 2117-2128, Maassen C. et al. (1), International Journal of Food Microbiology, 41, 1998, 155-167, Pouwels P. et al. (2), WO 96/32486 (3) y WO 95/21926 (4)). Relacionado a esto, esta Autoridad también le gustaría llamar la atención del solicitante a la definición amplia del término "inductor de tolerancia" dado en la presente solicitud (ver página 6).

Además, las presentes reivindicaciones suministradas claramente son novedosas sobre lo revelado en la técnica anterior, el presente juego de reivindicaciones se objetaría según el Art. 33(3) del PCT dado que los péptidos inductores de tolerancia T6 y T17 especificados en la presente solicitud y el uso de los mismos para preparar productos alimenticios hipoalergénicos es ya enseñado en WO 00/42863 (5)(ver por ejemplo página 3). Adicionalmente, en vista del estado de la técnica el uso de bacteria de ácido láctico como vehículo para dichos péptidos no puede ser considerado a ser inventivo sino únicamente como una materia de una alternativa obvia para una persona versada en la materia (ver por ejemplo (3) o WO 97/0078 (6)).

2). Con el fin de cumplir los requerimientos del Art. 5 del PCT una invención debe ser practicable **sobre el área completa reivindicada** sin la necesidad de actividad inventiva y carga excesiva. Esta autoridad toma la opinión que estos requerimientos no son cumplidos por el presente caso dado que la presente solicitud falla en enseñar un concepto adecuado para generalización; esto es, las propiedades inductoras de tolerancia de los péptidos aparentemente no pueden ser predecibles sino que cada péptido tiene que ser verificado separadamente. Por lo tanto, en la ausencia de una guía general en la solicitud como fue presentada la cual proporcionaría a un lector versado con información concerniente a la "creación" de péptidos que exhiban propiedades inductoras de tolerancia esta Autoridad toma la opinión de que la producción de tales péptidos y la subsecuente evaluación de los mismos con respecto a su comportamiento inductor de tolerancia tiene que ser considerado como una carga excesiva y por lo tanto, se presenta una objeción según el Art. 5 del PCT en cuanto a la materia objeto considerada la cual va más allá de los ejemplos específicos dados en la solicitud. (Los ejemplos específicos, sin embargo, carecen de actividad inventiva - ver arriba). En orden a la complementación, se anota que en el caso el solicitante intenta discutir que la provisión de péptidos inductores de tolerancia es únicamente una materia de trabajo rutinario para una persona versada en la materia y que por ende los requerimientos del Art. 5 del PCT se cumplen, surgiría una objeción según el Art. 33(3) del PCT para el alcance total reivindicado por las razones dadas anteriormente, esto es, en vista de (5), (3) y (6).

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

STRAUS, Alexander
Becker, Kurig, Straus
Bavariastrasse 7
D-80336 München
ALLEMAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	27.01.2003
Applicant's or agent's file reference	IMPORTANT NOTIFICATION
80263 WO	

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP01/10956	21/09/2001	25/09/2000

Applicant
SOCIETE DES PRODUITS NESTLE S.A. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

For the purpose of deciding whether the claimed invention is patentable or not, the elected Offices may apply criteria additional to or different from the criteria on which the international preliminary examination report is based (see Articles 27(5), 33(5)). Additional criteria may include e.g. exemptions from patentability and the requirements of enabling disclosure and of clarity and support of claims.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Hingel, W Tel.+49 89 2399-8717

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
80263 WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP01/10956	21/09/2001	25/09/2000
International Patent Classification (IPC) or national classification and IPC		
C12N9/52		
Applicant		
SOCIETE DES PRODUITS NESTLE S.A. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
16/04/2002	27.01.2003
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Scheffzyk-Sonnauer, Telephone No. +49 89 2399 8602

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP01/10956

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, pages:

1-17 as originally filed

Claims, No.:

1-13 as received on 07/01/2003 with letter of 07/01/2003

Sequence listing part of the description, pages:

1-6, filed with the letter of 22.07.02

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
see separate sheet

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-14
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-14
Industrial applicability (IA)	Yes:	Claims	1-14
	No:	Claims	

2. Citations and explanations
see separate sheet

SECTION I-------

Newly-filed claims do not meet the requirements of Art. 34(2)(b) PCT since the specific combination "cell surface anchored protease of Lactobacillus Bulgaricus" introduced in newly-filed claim only is mentioned in the application as filed in combination with specific tolerogenic peptides, namely T6 and T17 but not in combination with any tolerogenic peptide. Hence, there is no basis in the application as filed for the generalization made in newly-filed claim 1. Admittedly, on page 7 of the application cell surface anchored proteins are generally mentioned, however, not in combination with Lactobacillus bulgaricus! Hence, in so far as newly-filed claims do not meet the requirements of Art. 34(2)(b) PCT newly-filed claims are not considered in this IPER. Thus, this IPER is based on the claims as originally filed.

SECTION V----------

An objection under Art. 6 PCT arises since the expression "tolerogenic peptide" only can be seen as a result to be achieved, i.e. a peptide exhibiting tolerogenic activities, but not as a technical feature. Correspondingly, a person skilled in the art reading the claim is not in the position to assess whether or not a given bacterial strain, a given food composition etc is covered by the scope of present claims. Therefore, present claims do not meet the requirements of Art. 6 PCT. In addition, due to the obscure scope of said term an objection under novelty also arises since at present one cannot rule out that the bacterial strains taught in the art producing fusion proteins are encompassed by the scope of present claims (see **e.g.** Vaccine 17 (1999), 2117-2128, Maassen C. et al. (1), International Journal of Food Microbiology, 41, 1998, 155-167, Pouwels P. et al. (2). WO 96/32486 (3) and WO 95/21926 (4)). Relating to this this Authority also would like to draw Applicant's attention to the broad definition of the term "tolerogenic" given in present application (see page 6).

Moreover, rendering present claims clearly novel over the disclosures of

the prior art present set of claims would be objected to under Art. 33(3) PCT since the tolerogenic peptides T6 and T17 specified in present application and the use thereof to prepare hypoallergenic food products are already taught in WO 00/42863 (5) (see e.g. page 3). In addition, in view of the state of the art the use of lactic acid bacteria as vehicles for said peptides cannot be considered to be inventive but only as a matter of an obvious alternative to a person skilled in the art (see e.g. (3) or WO 97/0078 (6)).

2). In order to meet the requirements of Art. 5 PCT an invention must be workable **over the whole area claimed** without the need of inventive activity and undue burden. This Authority takes the view that these requirements are not met by present case since present application fails to teach a concept fit for greneralisation; i.e. the tolerogenic properties of peptides apparently cannot be predicted but each peptide has to be checked separately. Hence, in the absence of a general guidance in the application as filed which would provide a skilled reader with information concerning the "creation" of peptides exhibiting tolerogenic properties this Authority takes the view that the production of such peptides and subsequent assessment thereof with respect to their tolerogenic behaviour has to be considered as an undue burden and thus an objection under Art. 5 PCT arises in so far as subject-matter is concerned which goes beyond the specific examples given in the application. (The specific examples, however, lack inventive activity- see above). For the sake of completeness, it is noted that in case the Applicant intends to argue that the provision of tolerogenic peptides is only a matter of routine work to a person skilled in the art and hence the requirements of Art. 5 PCT are met an objection under Art. 33(3) PCT for the whole scope claimed would arise for the reasons given above , i.e. in view of (5), (3) and (6).

Claims

1. A bacterial strain of the lactic acid bacterium group, expressing the cell surface anchored protease of *Lactobacillus bulgaricus*, said cell surface anchored protease of *Lactobacillus bulgaricus* containing a tolerogenic peptide heterologous to said cell surface anchored protease of *Lactobacillus bulgaricus*, such that the peptide is stabilized towards degradation by stomach/duodenal enzymes, and retains its tolerogenic properties and is displayed at the periphery of the cell surface anchored protease of *Lactobacillus bulgaricus*.

2. The bacterial strain according to claim 1, which is selected from Lactobacillus or Bifidobacterium.

3. The bacterial strain according to any of the preceding claims wherein the tolerogenic peptide is derived from a food material.

4. A food composition containing a bacterial strain according to any of the preceding claims or a supernatant thereof.

5. The food composition according to claim 4, which is selected from the group consisting of milk, yoghurt, curd, cheese, fermented milks, milk based fermented products, ice cream, fermented cereal based products, milk based powders, infant formulae or pet food.

6. The food material according to any of the claims 4 or 5, wherein the bacterial strain is contained therein in an amount ranging from 10^7 to 10^{11} cfu / dosage form.

7. A pharmaceutical composition containing a bacterial strain according to any of the claims 1 to 3 or a supernatant thereof.

8. The pharmaceutical composition according to claim 7, wherein the bacterial strain is

300

contained therein in an amount ranging from 10^{10} to 10^{12} cfu / dosage form.

9. Use of a bacterial strain according to any of the claims 1 to 3 or a supernatant thereof for the preparation of an ingestable carrier for the treatment of allergy.

10. Use of a food or pharmaceutical composition according to any of the claims 1 to 8 for the preparation of an ingestable carrier for the treatment of allergy.

11. The use according to claim 10, wherein the composition is administered the oral or the nasal route.

12. A vaccine comprising a bacterial strain according to any of the claims 1 to 3, or parts thereof.

13. The vaccine according to claim 12, wherein the cell membrane fraction of lysed bacteria are utilized.

TRATADO DE COOPERACION DE PATENTES

De la:
AUTORIDAD DE EXAMEN PRELIMINAR INTERNACIONAL

Para:
STRAUS, Alexander
Becker, Kurig, Straus
Bavariastrasse 7
D-80336 München
ALEMANIA

PCT

OPINION ESCRITA

(PCT Regla 66)

Fecha de envío
(día/mes/año) 04.10.2002

Registro de referencia del solicitante o agente
80263 WO

RESPUESTA VENCE
Dentro de 3 mes(es) contados a partir de la fecha anterior de envío

Solicitud Internacional No.	Fecha de registro internacional (día/mes/año)	Fecha de prioridad (día/mes/año)
PCT/EP01/10956	21/09/2001	25/09/2000

Clasificación Internacional de Patentes (IPC) o clasificación nacional e IPC
C12N9/52

Solicitante
SOCIETE DES PRODUITS NESTLE S.A. et al.

1. Esta opinión escrita la redacta **primero** la Autoridad Examinadora Preliminar Internacional.

2. Esta opinión contiene indicaciones relacionadas con los siguientes ítems:

I	☒	Bases de opinión
II	☐	Prioridad
III	☐	No establecimiento de opinión respecto a novedad, nivel inventivo y aplicabilidad Industrial.
IV	☐	Falta de unidad de invención
V	☒	declaración razonada de según la regla 66.2(a)(ii) respecto a novedad, nivel inventivo o aplicabilidad industrial; citaciones y explicaciones que sustentan dicha afirmación.
VI	☐	Ciertos documentos citados
VII	☐	Ciertos defectos en la solicitud internacional
VIII	☐	Ciertas observaciones sobre la solicitud internacional

3. El solicitante por el presente **está invitado a contestar** a esta opinión.

¿Cuándo? Ver el limite de tiempo indicado anteriormente. El solicitante puede, antes de la expiración del límite de tiempo, solicitar a esta Autoridad que otorgue una extensión, ver Regla 66.2(d).

¿Cómo? Presentando una respuesta escrita, acompañada de, cuando sea apropiado, de enmiendas, según Regla 66.3.

Para el formulario y el idioma de las enmiendas, ver las Reglas 66.8 y 66.9

También: Para una oportunidad adicional de presentar enmiendas, ver Regla 66.4.

Para la obligación del examinador de considerar las enmiendas y/o argumentos, ver Norma 66.4bis.

Para una comunicación informal con el examinador, ver Regla 66.6.

Si no se registra una respuesta, el informe del examen preliminar internacional se establecerá según esta opinión.

4. La fecha final por medio de la cual se debe establecer el informe del examen preliminar internacional según la Regla 69.2 es: 25/01/2003.

Nombre y dirección de correo de la autoridad encargada del examen preliminar internacional	Funcionario autorizado/Examinador
Oficina de Patentes Europea D-80298 Munich Tel. +49 89 2399-0 Tx: 523656 epmu d Fax: +49 89 2399-4465	SCHEFFZYK, I Agente de formalidades (incluyendo prolongación de limites de tiempo) Hingel, W No. Teléfono +49 89 2399 8717

I. Bases de la Opinión

1. Respecto a los **elementos** de la solicitud internacional: (Carátulas de remplazo que han sido suministradas a la oficina receptora en respuesta a una invitación bajo el artículo 14 están referenciados en esta opinión como "presentados originalmente"):

Descripción, páginas:

1-17 como se presentaron originalmente

Reivindicaciones, No.:

1-14 como se presentaron originalmente

Parte del listado de secuencias de la descripción, páginas:

1-6, presentadas con la carta de 22.07.02

2. Respecto al **idioma**, todos los elementos marcados anteriormente estuvieron disponibles o fueron suministrados a esta Autoridad en el idioma en el cual la solicitud internacional fue presentada, a menos que se indique lo contrario bajo este ítem.

Estos elementos estuvieron disponibles o fueron suministrados a esta Autoridad en el idioma, el cual es:

☐ el idioma de una traducción suministrada para una búsqueda internacional (según la Regla 23.1 (b)).
☐ el idioma de una publicación de la solicitud internacional (según la Regla 48.3 (b)).
☐ el idioma de la traducción suministrada para el examen preliminar internacional (según las Reglas 55.2 y/o 55.3).

3. Respecto al **nucleótido y/o secuencia amino ácida** revelado en la solicitud internacional, se llevó a cabo el examen preliminar internacional según el listado de secuencia:

☐ contenido en la solicitud internacional en forma escrita.
☐ registrado junto con la solicitud internacional en forma legible por computador.
☒ proporcionado subsiguientemente a esta Autoridad en forma escrita.
☒ proporcionado subsiguientemente a esta Autoridad en forma legible por computador.
☒ Se ha proporcionado la afirmación respecto a la cual el listado de secuencia escrito proporcionado subsiguientemente no va más allá de la revelación en la solicitud internacional según se presentó.
☒ Se ha proporcionado la afirmación respecto a la cual la información presentada en forma legible por computador es idéntica al listado de secuencia escrita.

4. Las modificaciones han resultado en la cancelación de:

☐ la descripción, páginas:
☐ las reivindicaciones, Nos:
☐ los dibujos, hojas:

5. ☐ Este reporte ha sido establecido como si (algunas) de las modificaciones no se hubiesen hecho, ya que se ha considerado que van más allá de la revelación según se presentó, como se indicó en Casilla Suplementaria (Regla 70.2 (c)):

(Cualquier hoja de remplazo que contenga tales modificaciones debe ser referida bajo el ítem 1 y anexada a este reporte.)

6. Observaciones adicionales, si son necesarias:

V. Declaración razonada según la Regla 66.2(a)(ii) respecto a la novedad, nivel inventivo o aplicabilidad industrial; citaciones y explicaciones que sustentan dicha declaración

1. Declaración

Novedad (N)	Reivindicaciones 1-14
Nivel inventivo (IS)	Reivindicaciones 1-14
Aplicabilidad industrial (IA)	Reivindicaciones

2. Citaciones y explicaciones

ver hoja separada

1). Se presenta una objeción según el Art. 6 del PCT ya que la expresión "péptido inductor de tolerancia" únicamente puede ser visto como un resultado a ser alcanzado, esto es, un péptido que exhibe actividades inductoras de tolerancia, pero no como una característica técnica. Correspondientemente, una persona versada en la materia que lea la reivindicación no está en la posición de asegurar sí o no una cepa bacterial dada, una composición alimenticia dada etc. es protegida por el alcance de la presente invención. Por lo tanto, las presentes reivindicaciones no cumplen los requerimientos del Artículo 6 del PCT. Adicionalmente, debido al oscuro alcance de dicho término también se presenta una objeción por novedad dado que en este momento no se puede descartar que las cepas bacteriales enseñadas en la técnica que producen proteínas de fusión estén abarcadas por el alcance de las presentes reivindicaciones (ver por **ej.** Vaccine 17 (1999), 2117-2128, Maassen C. et al. (1), International Journal of Food Microbiology, 41, 1998, 155-167, Pouwels P. Et al. (2), WO 96/32486 (3) y WO 95/21926 (4)). Relacionado a esto, esta Autoridad también le gustaría llamar la atención del solicitante a la definición amplia del término "inductor de tolerancia" dado en la presente solicitud (ver página 6).

Además, las presentes reivindicaciones suministradas claramente son novedosas sobre lo revelado en la técnica anterior, el juego de reivindicaciones se objetaría según el Art. 33(3) del PCT dado que los péptidos inductores de tolerancia T6 y T17 especificados en la presente solicitud y el uso de los mismos para preparar productos alimenticios hipoalergénicos es ya enseñado en WO 00/42863 (5)(ver por ejemplo página 3). Adicionalmente, en vista del estado de la técnica el uso de bacteria de ácido láctico como vehículo para dichos péptidos no puede ser considerado a ser inventivo sino únicamente como una materia de una alternativa obvia para una persona versada en la materia (ver por ejemplo (3) o WO 97/0078 (6)).

2). con el fin de cumplir los requerimientos del Art. 5 del PCT una invención debe ser practicable **sobre el área completa reivindicada** sin la necesidad de actividad inventiva y carga excesiva. Esta autoridad toma la opinión que estos requerimientos no son cumplidos por el presente caso ya que como es mencionado en la solicitud "es difícil obtener un péptido inductor de tolerancia el cual ha sido producido por cada lote de novo" (ver página 4). Esta declaración también se soporta por los ejemplos dados en la presente solicitud mostrando que las propiedades inductoras de tolerancia aparentemente no pueden ser predecibles sino que cada péptido ha sido verificado separadamente.

Por lo tanto, en la ausencia de una guía general en la solicitud como fue presentada la cual proporcionaría a un lector versado con información concerniente a la "creación" de péptidos que exhiban propiedades inductoras de tolerancia esta Autoridad toma la opinión de que la producción de tales péptidos y la subsecuente evaluación de los mismos con respecto a su comportamiento inductor de tolerancia tiene que ser considerado como una carga excesiva y por lo tanto, se presenta una objeción según el Art. 5 del PCT en cuanto a la materia objeto considerada la cual va más allá de los ejemplos específicos dados en la solicitud. (Los ejemplos específicos, sin embargo, carecen de actividad inventiva-ver arriba). En orden a la complementación, se anota que en el caso el solicitante intenta discutir que la provisión de péptidos inductores de tolerancia son únicamente una materia de trabajo rutinario para una persona versada en la materia y por lo tanto los requerimientos del Art. 5 del PCT son cumplidos, una objeción según el Art. 33(3) del PCT para el alcance total reivindicado se presentaría por las razones dadas anteriormente, esto es, en vista de (5), (3) y (6).

3). En caso que el solicitante intente presentar reivindicaciones modificadas, se le pide mostrar una base para todas las modificaciones hechas en las reivindicaciones.

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

STRAUS, Alexander
Becker, Kurig, Straus
Bavariastrasse 7
D-80336 München
ALLEMAGNE

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	04.10.2002
Applicant's or agent's file reference: 80263 WO	**REPLY DUE** **within 3 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP01/10956	21/09/2001	25/09/2000

International Patent Classification (IPC) or both national classification and IPC
C12N9/52

Applicant
SOCIETE DES PRODUITS NESTLE S.A. et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☐	Certain defects in the international application
VIII	☐	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4. For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis. For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 25/01/2003.

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer / Examiner
SCHEFFZYK, I

Formalities officer (incl. extension of time limits)
Hingel, W
Telephone No. +49 89 2399 8717

WRITTEN OPINION

International application No. PCT/EP01/10956

I. Basis of the opinion

1. With regard to the **elements** of the international application (Replacement *sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed"*):

Description, pages:

1-17 as originally filed

Claims, No.:

1-14 as originally filed

Sequence listing part of the description, pages:

1-6, filed with the letter of 22.07.02

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

WRITTEN OPINION

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-14
Inventive step (IS)	Claims	1-14
Industrial applicability (IA)	Claims	

2. Citations and explanations
see separate sheet

1). An objection under Art. 6 PCT arises since the expression "tolerogenic peptide" only can be seen as a result to be achieved, i.e. a peptide exhibiting tolerogenic activities, but not as a technical feature. Correspondingly, a person skilled in the art reading the claim is not in the position to assess whether or not a given bacterial strain, a given food composition etc is covered by the scope of present claims. Therefore, present claims do not meet the requirements of Art. 6 PCT. In addition, due to the obscure scope of said term an objection under novelty also arises since at present one cannot rule out that the bacterial strains taught in the art producing fusion proteins are encompassed by the scope of present claims (see **e.g.** Vaccine 17 (1999), 2117-2128, Maassen C. et al. (1), International Journal of Food Microbiology, 41, 1998, 155-167, Pouwels P. et al. (2), WO 96/32486 (3) and WO 95/21926 (4)). Relating to this this Authority also would like to draw Applicant's attention to the broad definition of the term "tolerogenic" given in present application (see page 6).

Moreover, rendering present claims clearly novel over the disclosures of the prior art present set of claims would be objected to under Art. 33(3) PCT since the tolerogenic peptides T6 and T17 specified in present application and the use thereof to prepare hypoallergenic food products is already taught in WO 00/42863 (5) (see e.g. page 3). In addition, in view of the state of the art the use of lactic acid bacteria as vehicles for said peptides cannot be considered to be inventive but only as a matter of an obvious alternative to a person skilled in the art (see e.g. (3) or WO 97/0078 (6)).

2). In order to meet the requirements of Art. 5 PCT an invention must be workable **over the whole area claimed** without the need of inventive activity and undue burden. This Authority takes the view that these requirements are not met by present case since as it is mentioned in the application "it is difficult to obtain a tolerogenic peptide which has to be produced for each batch de novo" (see page 4). This statement also is supported by the examples given in present application showing that tolerogenic properties apparently cannot be predicted but that each peptide has to be checked separately. Hence, in the absence of a general guidance in the application as filed which would provide a skilled reader with

information concerning the "creation" of peptides exhibiting tolerogenic properties this Authority takes the view that the production of such peptides and subsequent asessment thereof with respect to their tolerogenic behaviour has to be considered as an undue burden and thus an objection under Art. 5 PCT arises in so far as subject-matter is concerned which goes beyond the specific examples given in the application. (The specific examples, however, lack inventive activity- see above). For the sake of completeness, it is noted that in case the Applicant intends to argue that the provision of tolerogenic peptides is only a matter of routine work to a person skilled in the art and hence the requirements of Art. 5 PCT are met an objection under Art. 33(3) PCT for the whole scope claimed would arise for the reasons given above , i.e. in view of (5), (3) and (6).

3). In case the Applicant intends to submit amended claims he is asked to show a basis for all amendments made in the claims.

BACTERIA DEL ACIDO LACTICO CAPAZ DE REDUCIR LA TENDENCIA DE UN INDIVIDUO A DESARROLLAR REACCIONES ALERGICAS

RESUMEN-OBJETO Y FINALIDAD DE LA INVENCION

La presente invención tiene que ver con cepas novedosas de bacterias lácticas que son capaces de reducir la tendencia de un individuo a presentar reacciones alérgicas. En particular, la presente invención se refiere a la generación de cepas de bacterias lácticas que expresan a proteínas que incluyen a péptidos inductores de tolerancia y al uso de los mismos en la reducción de la tendencia de un individuo a presentar reacciones alérgicas. La invención también tiene que ver con composiciones alimenticias o farmacéuticas que contienen dichos microorganismos o fracciones activas de los mismos.

BACTERIA DEL ACIDO LACTICO CAPAZ DE REDUCIR LA TENDENCIA DE UN INDIVIDUO A DESARROLLAR REACCIONES ALERGICAS

La presente invención tiene que ver con cepas novedosas de bacterias lácticas que son capaces de reducir la tendencia de un individuo a desarrollar reacciones alérgicas. En particular, la presente invención se refiere a cepas novedosas de bacterias lácticas que expresan a polipéptidos que incluyen a péptidos inductores de tolerancia y al uso de los mismos en la reducción de la tendencia de un individuo a desarrollar reacciones alérgicas. La invención también tiene que ver con composiciones alimenticias o farmacéuticas que contienen dichos microorganismos o fracciones activas de los mismos.

Las alergias son reacciones inapropiadas del sistema inmune frente a una variedad de sustancias (alergenos). Generalmente, los individuos no generan una reacción inmune sustancial contra sustancias que se encuentran regularmente en el ambiente, tales como polen o materiales alimenticios, una no-reactividad que se atribuye principalmente a un mecanismo de supresión del propio sistema inmune. Sin embargo, en presencia de una condición de deterioro, el sistema inmune no cumple con dicha actividad de supresión (denominada tolerancia), dando lugar a una reacción inmune específica contra el alérgeno: la reacción alérgica. Las

reacciones alérgicas involucran la liberación de compuestos biológicamente activos, tales como los mediadores de la inflamación histamina, leucotrienos y enzimas, por parte de células particulares, principalmente mastocitos y granulocitos basófilos, al tejido circundante y las estructuras vasculares. Los mastocitos se dispersan a través de los tejidos del individuo mientras que los basófilos circulan dentro del sistema vascular. Tras una secuencia de eventos que involucran a anticuerpos de IgE los compuestos/mediadores se liberan de mastocitos objetivos, finalizando en reacciones farmacológicas.

Últimamente, el número de individuos que padecen de alergia ha aumentado, lo que se atribuye con frecuencia a una polución atmosférica siempre creciente provocada, por ejemplo, por los gases de escape. También se considera que el consumo extendido de materias proteicas contribuye a dicho desarrollo, en particular a la incidencia creciente de alergias alimentarias. Además, el déficit de infecciones microbianas hallado en los países desarrollados se ha sugerido como otra posible causa de aumento de las enfermedades atópicas.

Las alergias alimentarias y aún la intolerancia a los alimentos de algún tipo se han tornado muy comunes. Se ha encontrado que la mayoría de los individuos afectados exhiben una intolerancia a ciertos constituyentes de los alimentos, con aproximadamente un 5 por ciento de la población que inclusive desarrolla reacciones frente a sustancias ingeridas que son lo suficientemente graves como para requerir atención médica.

En años recientes se ha instalado la evidencia de que la principal causa de intolerancia a los alimentos reside en la absorción de sustancias

por parte del organismo que normalmente se excluyen, las cuales son, sin embargo, absorbidas por el tracto gastrointestinal en individuos susceptibles. Esta mala absorción se considera que deriva de defectos en el tejido que recubre el tracto digestivo (Peters y col., (1988) Can. J. of Gastroenterol. **2**, 127; Olaison y col., (1990) Scand. J. of Gastroenterol. **25**, 321; Hollander y col., (1986) Ann. of Int.Med. **105**, 883). La capacidad, recientemente identificada, de las células epiteliales que recubren el tracto gastrointestinal (GI) para procesar y presentar antígenos también podría explicar el desencadenamiento de reacciones inmunes inapropiadas que conducen a un epílogo inflamatorio y alérgico (Campbell y col., (1999) Immunol. Rev. **172**, 315).

La manifestación de una alergia alimentaria puede involucrar sustancialmente a cualquier tejido del organismo. Debido a su gran área de absorción, el tracto gastrointestinal es probablemente el mayor sitio de absorción de sustancias ofensivas. Muchos de los síntomas de las alergias alimentarias se ponen de manifiesto en el propio tracto digestivo, pero podrían afectar también a otros tejidos, incluyendo la piel y vías aéreas.

En el arte se proponen distintos enfoques para tratar la alergia, en particular la alergia alimentaria.

Uno de tales enfoques apunta a modificar la propia fuente del material alergénico de modo que se reduzca el potencial alérgico. Esto puede lograrse limitando o prohibiendo el alimento o los componentes del mismo, respectivamente, que serían la causa de dichos problemas. Un problema involucrado a menudo reside en el hecho de que la sustancia antigénica específica (alergeno) en el respectivo material alimenticio con

frecuencia se desconoce, de manera que en la mayoría de los casos no está claro qué componente debe eliminarse selectivamente.

En la Patente de los Estados Unidos 5.480.660 se informa que el inicio de la alergia en pacientes alérgicos al trigo puede aliviarse eliminando o reduciendo, en la harina, las proteínas que tengan pesos moleculares de no más de 30.000 Da y las proteínas que tengan un peso molecular entre 50.000 y 70.000 Da.

En la Patente Japonesa 11 04 67 21 se describe un método para reducir la alergenicidad de un material alimenticio que contiene proteínas, en el cual el material alimenticio se mezcla con harina de trigo y la mezcla se hornea durante más de 3 minutos a 180°C.

En la Patente de los Estados Unidos 4.293.571 se describe la preparación de una composición hipoalergénica en la cual se somete un material proteico a tratamiento hidrolítico, y las proteínas remanentes no hidrolizadas se someten a coagulación por tratamiento térmico seguido de un paso de ultrafiltración para eliminar el material coagulado y los macropéptidos que podrían constituir alergenos.

En la Patente de los Estados Unidos 5.039.532 se describe otro proceso para la preparación de un material alimenticio hipoalergénico en el cual se somete un producto a base de suero de leche a hidrólisis enzimática.

No obstante, todos estos métodos de tratamiento del material alimenticio o aun su exclusión de la dieta diaria demuestran a la larga ser dificultosos en la práctica ya que requieren revisión de la dieta, involucrando habitualmente medidas restrictivas estrictas, y pueden hasta afectar la calidad de vida y/o inhibir el crecimiento esperado del individuo.

Un enfoque diferente de tratamiento de la alergia alimentaria y de la intolerancia alimentaria se dirige a restaurar y mantener la integridad del intestino de manera tal que los alergenos alimentarios esencialmente no lo puedan atravesar. Al respecto la Patente de los Estados Unidos 5.192.750 describe el uso de la N-acetil glucosamina para permitir que la mucosa forme la necesaria barrera contra la transmisión de los alergenos alimentarios y para mantener un funcionamiento normal.

De acuerdo a otro enfoque de tratamiento de la alergia se sugiere la vacunación de los individuos contra las moléculas de IgE, que inhibe el desencadenamiento de los mastocitos y los basófilos. Con este fin, la Patente WO 97/31948 propone péptidos específicos para vacunación que se asemejan en su conformación tridimensional a partes de la molécula de IgE, inmunoglobulina involucrada en la regulación de las reacciones alérgicas e inflamatorias. Se considera que el propio sistema inmune del individuo formará a la larga anticuerpos dirigidos a dichas moléculas de IgE de tal manera que dichas inmunoglobulinas IgE sean barridas. Sin embargo, dicho método abriga la desventaja de que los anticuerpos formados pueden exhibir reactividad cruzada con otras clases de inmunoglobulinas, de manera que los mecanismos naturales de defensa del individuo pueden verse negativamente influídos.

En la Patente Europea 99200130.5, una solicitud de patente propiedad del presente solicitante, que no ha sido publicada aún, se describe otro enfoque más. Se propone una composición hipoalergénica que contiene un material proteico extensamente hidrolizado no alergénico y/o una base de aminoácidos libres y por lo menos un péptido inductor de

tolerancia de la respectiva proteína alergénica. Aunque esta composición suministra muchas ventajas por sobre el arte previo aún es difícil obtener el péptido inductor de tolerancia, que se debe producir para cada lote de novo.

Por lo tanto, existe la necesidad en el arte de proveer un mejor medio para el tratamiento de la alergia.

Un objetivo de la presente invención reside, por lo tanto, en proveer dicho medio.

El objetivo mencionado se ha resuelto proporcionando cepas novedosas de bacterias lácticas que son capaces de reducir la tendencia de un individuo a desarrollar reacciones alérgicas. Las cepas novedosas de bacterias lácticas expresan un polipéptido que alberga un péptido inductor de tolerancia de tal manera que se retienen la secuencia específica y posiblemente la configuración del péptido inductor de tolerancia, se preserva la estabilidad y así puede ser procesado y reconocido por el sistema inmune del individuo.

En las figuras,

la Fig. 1 muestra la secuencia primaria de péptidos contenidos en las respectivas fracciones del hidrolizado de β-lactoglobulina; las barras verticales representan enlaces disulfuro;

la Fig. 2 muestra los resultados obtenidos con péptidos inductores de tolerancia/fracciones de péptidos en diversos experimentos.

Se ha demostrado que las bacterias lácticas, es particular los Lactobacilos y las Bifidobacterias, modulan el sistema inmune sin abandonar su hábitat luminal produciendo mensajeros (citoquinas) involucrados en la regulación del sistema inmune. Las bacterias lácticas no

desencadenan la producción de citoquinas pro-inflamatorias, tales como IL-8, TNF-α, MCP-1 y GM-CSF, sino que más bien promueven la producción de citoquinas inhibidoras de la inflamación, tales como TGF-β (Blum y col., (1999) Antonie van Leewenhoek **76**, 199), con importantes implicancias concernientes al mantenimiento tanto de la homeostasis inmune intestinal como de la integridad de la barrera epitelial (Planchon y col., (1999) J. Cell Physiol. **181**, 55). Consistente con lo dicho, la administración oral de homogenatos de lactobacilos demostró ejercer un efecto supresor sobre la proliferación mitógeno-inducida de células mononucleadas. (Pessi y col., (1999) Appl. Environ. Microbiol. **65**, 4725).

Las bacterias lácticas (LAB) pueden utilizarse para la (super)producción heteróloga de proteínas y péptidos con sistemas que permitan la expresión de un gen extraño basada en la integración cromosómica o en elementos de un plásmido episomal (ver, por ej., Kuipers y col., (1997) Tibtech **15**, 140). Además, las bacterias lácticas tienen un potencial para colonizar superficies de mucosas y exhiben un carácter intrínseco de auxiliares asociadas con péptidos murámicos resultantes de la degradación de peptidoglicanos.

Asimismo, se ha demostrado que ciertas cepas de Lactobacilos promueven respuestas inmunes antígeno-específicas, particularmente en la clase de las IgA (Majamaa y col., (1995) J. Pediatr. Gastroenterol. Nutr. **20**, 333). La incapacidad de la IgA humana complejada con antígenos para activar un complemento puede tener importancia en el mantenimiento de la integridad de las superficies de las mucosas. Esto prevendría las reacciones inflamatorias locales, incluyendo el influjo de leucocitos y la

liberación de efectores inmunológicos que aumentan la permeabilidad de la membrana mucosal debido a deterioro de los tejidos. Además, a través de este mecanismo de exclusión inmune, la IgA podría limitar la posterior absorción de antígenos vecinos y de este modo anular las reacciones anafilácticas mediadas por anticuerpos de isotipos de IgE e IgG.

Más aún, se ha demostrado que los lactobacilos potencian la producción de IFN-gamma por parte de células T (Halpern y col, (1991) Int. J. Immunother. 7, 205). Recientemente se demostró que la IFN-gamma inhibe l liberación de TNF-alfa de los mastocitos de la mucosa intestinal (Bissonnette y col., (1995) Int. Arch. Allergy Immunol. **107**, 156). La secreción aumentada de IFN-gamma podría prevenir los efectos perjudiciales de la TNF-alfa (Hernández-Pando y col., (1994) Immunology **82**, 591), y en consecuencia contrarrestar el trastorno de permeabilidad asociado a la alergia alimentaria. Por ejemplo, el deterioro de la barrera contra la captación de antígenos en el eczema atópico puede ser la clave determinante en las respuestas inmunes exageradas a los alergenos ambientales y dietarios comunes.

Durante los extensos estudios que condujeron a la presente invención, los inventores diseñaron por lo tanto un medio para combinar las ventajosas propiedades de las bacterias lácticas con el potencial de los péptidos inductores de tolerancia para generar un poderoso agente que reduzca o aun minimice la tendencia de un individuo a las reacciones alérgicas.

La bacteria láctica a utilizar en la presente invención pertenece preferentemente al grupo de los Lactobacillus o al grupo de las

Bifidobacterium o al grupo de las Lactococcus, y deriva más preferentemente de los grupos de *L. Acidophilus, L. Johnsonii, L. Gasseri, L. Casei, L. Paracasei* o *L. Reuteri*, todas de origen humano o animal, respectivamente.

El péptido inductor de tolerancia, que es un péptido derivado de una dada proteína que provoca alergia en un individuo, generalmente tiene un tamaño de aproximadamente 200 a 6000 Da y es un péptido capaz de inducir tolerancia por lo menos hacia la proteína de la que deriva. Además, la tolerancia será inducida también hacia cualquier material que tenga el mismo determinante antigénico que el representado por el péptido inductor de tolerancia específico. De acuerdo con la presente invención, este péptido se inserta por un medio recombinante en un polipéptido sintetizado en la bacteria láctica, polipéptido que puede ser endógeno o exógeno con respecto a la bacteria. El péptido inductor de tolerancia se inserta de tal manera que se exponga sobre la periferia del polipéptido recombinante resultante, de modo que pueda ser reconocido por el sistema inmune.

El polipéptido puede expresarse en la bacteria láctica de acuerdo a métodos bien conocidos en el arte. Por ejemplo, los vectores disponibles comercialmente pNZ124 (Platteuw y col, (1994) Appl. Env. Microbiol. 60, 587), pGK12 (Walke y col., (1996) FEMS Microbiol. 138, 233) o pG+host9 (Magín y col., (1996) J. Bacteriol. 178, 931) pueden utilizarse para la expresión episomal. Sin embargo, teniendo en mente la estabilidad superior de la integración cromosómica, esta manera de hacer las cosas podría preferirse para que el gen recombinante codifique el respectivo polipéptido. Para la integración al cromosoma puede aplicarse la

recombinación homóloga utilizando por ejemplo un gen recombinante de bacterias lácticas que contenga el péptido inductor de tolerancia y reemplazando el gen endógeno. No obstante, existen métodos bien al alcance de los expertos en el arte para introducir genes recombinantes en un cromosoma de un huésped.

El polipéptido puede ser una proteína normalmente expresada para residir dentro de la célula, pero bien puede ser un polipéptido secretado por la bacteria láctica o que esté inserto en la pared celular de la misma, con la parte que contiene el péptido inductor de tolerancia localizada en la sección extracelular. Ejemplos de polipéptidos a utilizar en la presente invención son el pepN, pepX, la lactato deshidrogenasa o β-galactosidasa, o un miembro de la clase de las bacteriosinas o la proteína de la capa-S o una proteasa anclada en la pared celular.

La naturaleza del polipéptido no es crucial, con la salvedad de que el péptido inductor de tolerancia pueda estar inserto de modo tal que sea accesible para el sistema inmune. Sin embargo, una vez que un péptido inductor de tolerancia adecuado se ha identificado está dentro del alcance de los expertos en el arte acomodar el péptido inductor de tolerancia en semejante ubicación. Con este fin, el experto en el arte consultará modelos tridimensionales de los polipéptidos en los cuales se ha de insertar el péptido inductor de tolerancia y en base a dicha información diseñará una proteína recombinante correspondiente.

En una realización preferida los polipéptidos recombinantes se secretan o se acomodan en la pared celular de la bacteria de tal manera que el péptido inductor de tolerancia se exponga constantemente hacia el

ambiente.

El péptido inductor de tolerancia deriva de materiales alimenticios que causan reacción alérgica. No obstante no es esencial que su secuencia aminoacídica corresponda a la hallada en la proteína nativa, pero cualquier secuencia de péptido que posea esencialmente la misma secuencia aminoacídica y posiblemente la misma conformación tridimensional que el péptido de partida o un polipéptido derivado del mismo que exhiba esencialmente los mismos resultados, es decir que induzca una reducción de la respuesta alérgica, será apropiada para el fin buscado.

Como fuentes preferidas de péptidos inductores de tolerancia pueden mencionarse la leche, soja, maní, crustáceos, pescado, carne vacuna, sésamo o suero lácteo, tales como, por ejemplo, la β-lactoglobulina, albúmina de suero bovino o caseína.

Con el fin de identificar a los péptidos inductores de tolerancia, el material alergénico de interés se somete primeramente a una hidrólisis enzimática en una extensión de aproximadamente el 10 al 50% y la actividad enzimática residual de la mezcla proteica se inactiva, por ejemplo, por tratamiento térmico. La solución del hidrolizado proteico se clarifica y se purifica adicionalmente si se desea. Los péptidos así obtenidos se separan, por ejemplo, sometiendo la solución a un tratamiento de precipitación o pasándola a través de una columna cromatográfica.

Posteriormente, las fracciones que contienen diferentes péptidos se ensayan en busca de sus propiedades inductoras de tolerancia ensayándolas en un modelo animal. Con este fin, se alimentan animales, tales como ratones, con las respectivas fracciones de péptidos, y posteriormente se

inmunizan con el alérgeno y se mide la respuesta frente al mismo, como por ejemplo a través de un cambio en el aspecto exterior o midiendo específicamente los niveles de IgE en los animales. Como resultado, la fracción del péptido inductor de tolerancia reducirá la tendencia del animal a reaccionar frente al alérgeno, de modo que se puede determinar el respectivo péptido (la fracción).

Un vez que se han verificado el péptido inductor de tolerancia y su secuencia aminoacídica, se inserta la secuencia del ADN del polipéptido a ser expresado en la bacteria láctica por medio de ingeniería genética en el gen que codifica la proteína transportadora de la bacteria. Se describen métodos y técnicas para manipular las secuencias de ADN y para expresar proteínas en microorganismos en Sambrook y col., A Laboratory Manual Cold Spring Harbor (1992).

La presente invención también se refiere a una composición alimenticia y farmacéutica que contiene al menos una de las mencionadas bacterias lácticas.

La cepa bacteriana puede estar incluida en la composición en una proporción que oscila entre 105 y 1012 ufc (unidades formadoras de colonias) por g de material. De igual manera puede incluirse en la composición un sobrenadante de un cultivo de la bacteria láctica o una fracción activa del mismo.

La composición alimenticia puede ser leche, yogur, cuajada, queso, leches fermentadas, productos fermentados a base de leche, helado, productos fermentados a base de cereales, polvos a base de leche, fórmulas para bebés o, en el caso de animales, alimentos para mascotas.

La composición farmacéutica puede estar en forma de comprimidos, suspensiones bacterianas líquidas o preparaciones que comprenden partes de las bacterias, tales como meramente la fracción de la pared celular obtenida por lisado de las células bacterianas y recolección del componente de la pared celular, suplementos orales desecados, suplementos orales húmedos, alimentación por tubo seco o alimentación por tubo húmedo.

La vía de administración para la preparación farmacéutica es oral, pero también puede ser nasal. → Modo de uso ?

La presente invención también abarca vacunas, comprendiendo las bacterias de la presente invención o partes de las mismas. Las bacterias pueden estar vivas o pueden ser atenuadas, es decir debilitadas en su potencial de desarrollo, si se lo desea. Como partes de las bacterias, se aplicará primeramente la pared celular que alberga el antígeno de interés. La pared celular puede obtenerse fácilmente por lisado de las células y separación de los diferentes componentes de las mismas.

Los siguientes ejemplo ilustran adicionalmente la invención sin limitarla a los mismos.

Ejemplo 1

Aislamiento de péptidos inductores de tolerancia

a) Aislamiento de péptidos

se disolvieron 220 g de β-lactoglobulina (Sigma) en agua bidestilada a una concentración del 5% (p/p). Se agregó a la solución resultante tripsina tratada con TPCK utilizando una relación enzima/sustrato (E/S) de 1/100 (p/p) y la solución se incubó a 40°C y pH 7,6 bajo agitación constante. Después de 1 hora se agregó la misma cantidad de enzima para

dar una relación final (E/S) de 2/100. La incubación se continuó por otras 4 horas y la enzima se inactivó por tratamiento térmico (85°C, 5 minutos) para detener la reacción. El hidrolizado tríptico completo se liofilizó posteriormente y los productos peptídicos resultantes se separaron por cromatografía preparativa sobre una resina catiónica.

Se obtuvieron 15 fracciones peptídicas diferentes oscilando entre los 2 y los 23 aminoácidos (entre aproximadamente 240 y 2720 Da). Las fracciones individuales se nanofiltraron, diafiltraron, dializaron, se liofilizaron nuevamente y se conservaron secas a temperatura ambiente hasta su uso. Las fracciones también se caracterizaron por su contenido en péptido utilizando Cromatografía Líquida de Alta Resolución (HPLC) de fase revertida. En total se recogieron 15 facciones (F1 – F15) conteniendo como componentes principales los péptidos (T) indicados.

Fracciones	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15
Masas (g)	52	13,9	11	11,8	2,57	1,94	4,37	13,1	5,01	6,53	1,05	3,46	1,9	2,67	28,8
Péptido enriquecido	--	T6 T17 T18	T23	T23	–	T7 T9	T20 T21 T24	T9 T12 T24	T12 T21	T10 T21	T11	T10	T10 T11	T10 T11	--

Los péptidos se aislaron y se secuenciaron. Las secuencias de los mismos se indican en la Fig. 1.

Varios péptidos demostraron ser inductores de tolerancia en diversos experimentos que se describen en detalle en Pecquet, R., Bovetto L., Maynard F., Fritsché R. Los péptidos obtenidos por hidrólisis con tripsina de la B-lactoglobulina bovina inducen la tolerancia oral específica en

ratones, J. Allergy Clin. Immunol. (2000) 105:514-21, cuya referencia se incorpora a la presente a modo de referencia.

b) Síntesis de péptidos inductores de tolerancia

Tres péptidos inductores de tolerancia de la β-lactoglobulina (BLG), T6, T17 y T6/T17, identificados en la referencia anterior, así como también un péptido de control T13, se sintetizaron químicamente con residuos flanqueadores provenientes de la secuencia de la BLG, como así también un residuo extra de Cys, como se detalla a continuación:

T6	C F K **I D A L N E N K** V S R	
	84 91	
T17	C N K **V L V L D T D Y K** K Y S	
	92 100	
T6/T17	C F K **I D A L N E N K V L V L D T D Y K** K Y S	
	84 100	
T13	**L I V T Q T M K** S R C	
	1 8	

c) Ensayos en animales

Para evaluar las propiedades inductoras de tolerancia de los diversos péptidos se utilizaron ratones hembra Balb/c, obtenidos de IFFA-Crédo (L´Abresle, Francia). Los animales se criaron y desarrollaron en base a una dieta exenta de leche. Al comienzo de los experimentos los animales tenían 3 semanas de edad. Recibieron por cebadura β-lactoglobulina nativa (5 mg/g de peso corporal), diversas cantidades de β-lactoglobulina digerida y diversas cantidades de los diferentes péptidos obtenidos según el ítem a)

anterior. Los ratones de control fueron alimentados con solución salina. Después de 5 días mantenidos con dicha dieta todos los ratones fueron inmunizados con β-lactoglobulina y ovoalbúmina (grado V, Sigma) como antígeno no relacionado con el objeto de evaluar la especificidad de la respuesta inmune. Se realizó una evaluación de la hipersensibilidad de tipo demorado (DTH) 21 días después de la provocación sistémica comparando el espesor de la planta de la pata izquierda trasera, antes y después de la inmunización. 24 horas después de la inmunización se midieron los espesores de las plantas de las patas de los animales (expresados como Δ de espesor (mm)). Posteriormente, se extrajo sangre de todos los ratones, seguido de la extirpación de los bazos, que se unificaron de acuerdo al tratamiento de cada grupo. Se realizaron los ensayos de proliferación específica de los esplenocitos para cada grupo. Se recogieron individualmente los contenidos intestinales. Se congelaron individualmente muestras de suero y de intestino a –80°C hasta la realización de los respectivos ensayos. Se determinaron los niveles de IgE anti-β-lactoglobulina y de IgE anti-ovoalbúmina tanto en las muestras de suero como en las de intestino.

d) Ensayos de anticuerpos de IgE

Los fluidos séricos e intestinales se diluyeron y se ensayaron por duplicado para determinar anticuerpos de IgE anti-β-lactoglobulina y anti-ovoalbúmina, usando el test ELISA. Las muestras unificadas de 20 ratones no inmunizados se emplearon como controles negativos. Los títulos se determinaron calculando la dilución de la muestra que daba el doble de absorbancia que el control negativo. Los títulos se expresaron como log10

de la inversa de la dilución.

e) Cultivo de células

Se homogeneizaron soluciones de células de bazo en PBS y se purificaron. Las células se co-cultivaron en presencia de β-lactoglobulina o fitohemaglutinina A. Se agregó timidina tritilada ([^{3}H]-Thy (Amersham, Zürich) durante las últimas 6 horas de cultivo y las placas se cosecharon y analizaron por recuento por escintilación. Los índices de estimulación se calcularon como la razón entre el ensayo descontado del blanco y los niveles de control, expresada como los cpm promedio de la [^{3}H]-Thy incorporada por cultivos por triplicado.

f) Tolerancia inducida por la administración oral de péptidos sintéticos

Los péptidos sintéticos se analizaron de acuerdo a su capacidad inductora de tolerancia en el modelo parenteral en ratones. Se obtuvieron los siguientes resultados:

	Título de IgE (log) (ensayo/control)	SI de linfocitos del bazo (ensayo/control)	SI de linfocitos del MLN (ensayo/control)
T – 6	3,24/3,39	0,53	0,29
T – 17	2,67/3,39	0,31	0,41
T – 6/T – 17	3,40/3,39	0,92	0,81
T – 13	3,20/3,39	1,70	0,87

SI: Indice de Estimulación MLN: Nódulo Linfático Mesentérico

Puede verse que el péptido sintético T-17 redujo tanto la producción de IgE como la proliferación de células T mientras que el péptido T-6 suprimió sólo la respuesta de las células T. En contraste, el péptido T-13, el péptido de control, no indujo la tolerancia oral a la BLG.

Ejemplo 2

Construcción de un polipéptido recombinante

Los péptidos T6 y T17, identificados en el ejemplo 1 como poseedores de propiedades inductoras de tolerancia, se fusionaron con una proteasa de superficie celular para exponerse en la superficie de una bacteria. Como control negativo se usó el péptido 13, constituyendo parte del extremo N-terminal de la β-lactoglobulina.

La proteasa anclada en la superficie celular (anterior) fue la del *Lactobacillus bulgaricus*, cuya secuencia se publicó en Gilbert y col., (1996) J. Bacteriol., **178**, 3059-3065. esta proteína se caracterizó como una proteína de 2000 aminoácidos, compuesta por un péptido líder de 33 aminoácidos (pre-región) responsable de la exportación celular de la enzima, seguido de una serie de 154 aminoácidos (pro-región) que es responsable, al fragmentarse, de la activación de la actividad proteolítica de la enzima, y 700-800 aminoácidos para el sitio activo. La región posterior (alrededor de 1000 aminoácidos) se ha sugerido que juega un papel en la especificidad de ruptura y transporte en la célula de los péptidos generados y también de extender la pared celular. La proteasa está anclada a la pared celular por su extremo carboxilo, siendo los últimos 200 aminoácidos responsables de la unión covalente específica a la estructura de peptidoglicano de la pared celular.

El gen de la proteasa se amplificó primeramente con su promotor utilizando los dos cebadores siguientes:

5′-TTTTGTGGATCCTTAACTTCATAGCACG-3′

(hacia arriba del promotor del gen, portando un sitio BamHI)

5'-ATATTATCTAGAATTGAATAGATTGCC-3'

(hacia abajo del finalizador del gen rho-independiente, portando un sitio Xbal).

El producto de amplificación sufrió ruptura con BamHI y Xbal y se clonó en el vector pNZ124 de la bacteria láctica, que se había digerido con las mismas enzimas de restricción, y a la larga introducido por electroporación en el *Lactococcus lactis* libre de plásmidos (beta-galactosidasa y proteasa negativo).

La región del sitio activo de la proteasa clonada se reemplazó por el péptido/polipéptido de interés. Para lograr esto, la proteasa clonada se sometió a ruptura con NheI, que está ubicado 50 pares de bases hacia abajo en la secuencia del sitio de ruptura del péptido líder y con PvuI, 800 pares de bases más abajo en la secuencia. Se insertó una secuencia de ADN que codifica los péptidos de interés (hasta 10 aminoácidos) entre los dos sitios de restricción como dos oligonucleótidos, que se diseñaron de manera de generar los dos sitios de restricción en sus extremos una vez que se hibridicen. El diseño de los oligonucleótidos tiene en cuenta que al producirse la ligazón al gen de la proteasa, el armazón de lectura de la proteína recombinante permanece abierto.

La inserción de polipéptidos más grandes se efectuó por amplificación de ADN utilizando cebadores que contienen los dos sitios de restricción. El producto de la amplificación sufrió ruptura con las enzimas de restricción. En ambos casos los fragmentos de ADN se ligaron al gen de la proteasa y se introdujeron por electroporación en *Lactococcus lactis y Lactobacillus johnsonii.*

Los constructos de los plásmidos recombinantes que comprenden los péptidos T6 y T17, respectivamente, fueron depositados el 22 de Septiembre de 2000 bajo el Tratado de Budapest con el Instituto Pasteur y recibieron el no. de depósito CNCM I-2563 y CNCM; I-2564, respectivamente.

Ejemplo 3

Transformación de *Lactococcus lactis* y *Lactobacillus johnsonii*

Con el fin de lograr su transformación, se desarrollaron durante la noche una cepa de *Lactococcus lactis* (MG1363, libre de plásmidos) y la cepa La1 de *Lactobacillus johnsonii* (disponible del Instituto Pasteur bajo el acceso no. CNCM I-1225) en caldo MRS a 37°C en un sistema anaeróbico GasPak. Se utilizó una alícuota de este cultivo para inocular otro caldo de cultivo (MRS) que contenía sacarosa 0,5 M. Después de una re-inoculación adicional al 2% en 200 ml de MRS + sacarosa 0,5 M, el cultivo se desarrolló hasta una OD_{595} de 0,6. las células se recogieron por centrifugación a 5000 rpm a 4°C durante 10 minutos, el pellet se lavó una vez con la mitad de su volumen de una solución que contenía sacarosa 1 M y $CaCl_2$ 2,5 mM, una vez con un cuarto de su volumen de una solución que contenía sacarosa 1 M y $CaCl_2$ 2,5 mM y el pellet obtenido después de la centrifugación se resuspendió en 3,5 ml de una solución de sacarosa 1 M, $CaCl_2$ 2,5 mM + 0,459 ml de glicerol 87 % (10% de concentración final). Las células se utilizarían directamente para su transformación o se congelarían a –80°C.

Para la electroporación se mezclaron 40 µl de células con 10 – 100 ng de ADN (en <5 µl de volumen) y se transfirieron a una cubeta de

electroporación de 0,2 cm enfriada con hielo. Se aplicaron a la cubeta de electroporación de 0,2 cm enfriada con hielo pulsos a 200 Ω, 25 µF, 2,5 kV. Se agregaron a la cubeta 1 ml de MRS + $MgCl_2$ 20 mM, $CaCl_2$ 2 mM y la suspensión se incubó por 2-3 horas a la temperatura apropiada (a 30°C para *Lactococcus lactis* y a 37°C para *Lactobacillus johnsonii*. Cuando se emplean plásmidos ts (pG+host9) la incubación es a 30°C). Se colocaron alícuotas de 10 µl y 100 µl, respectivamente, en placas de agar MRS que contenían el antibiótico apropiado para lograr la transformación del ADN plasmídico y 100 µl y 500 µl, respectivamente, para lograr la transformación con la mezcla de ligazón. Las placas se incubaron anaeróbicamente durante 24-48 horas a la misma temperatura mencionada anteriormente.

Como medio de selección se utilizó MRS con eritromicina (2,5 µg/ml) o MRS con kanamicina (5 µg/ml).

Ejemplo 4

Generación de antisueros de rata y de anticuerpos monoclonales contra péptidos inductores de tolerancia a la BLG

Los péptidos de BLG T6, T17, T6/T17 y T13 (destacados en el ejemplo 1) se acoplaron a KLH activada con maleimida y se usaron junto con adyuvante de Titermax para inmunizar a ratas alimentadas en base a una dieta exenta de proteínas lácteas. Se comprobó por medio de ELISA que las muestras de sangre tomadas a intervalos de 2 semanas hasta 115 días después de la primera inmunización contenían anticuerpos específicos de los péptidos de BLG.

Los antisueros anti-T6 y anti-T13 también reaccionaron tanto con la

forma nativa (ELISA) como la desnaturalizada (SDS-PAGE + Western blot) de la BLG. Se comprobó que los antisueros anti-T6 y anti-T17 reconocían específicamente a los péptidos de BLG expresados en la superficie del L. Lactis recombinante. Los resultados se muestran en la Tabla I.

Tabla I

Reactividad de los antisueros anti péptidos de β-lactoglobulina

Antisuero específico para	T6			T17			T6/17			T13		
Número de rata	A1	A2	A3	B4	B5	B6	C7	C8	C9	D10	D11	D12
T6 (ELISA)	+++	+/-	+	-	NE	NE	-	NE	NE	-	NE	NE
T17 (ELISA)	-	NE	NE	+	-	-	+++	NE	NE	-	NE	NE
T6/17(ELISA)	+++	NE	NE	+	NE	NE	+++	+	-	-	NE	NE
T13 (ELISA)	-	NE	NE	-	NE	NE	-	NE	NE	+	+	+/-
BLG nativa (ELISA)	+++	+/-	+++	+/-	-	-	-	-	-	+	+	+/-
BLG desnat. (SDS-PAGE + WB)	+++	NE	++	-	NE	NE	-	NE	NE	+/-	+++	++
L1/T6 (ELISA)	+/-	-	+++	-	NE	NE	-	-	-	-	NE	NE
L1/T17 (ELISA)	-	NE	NE	+	-	-	-	-	-	-	NE	NE
L1/T13 (ELISA)	-	NE	NE	-	NE	NE	-	NE	NE	-	-	-

ELISA / OD490 nm (15'/37°C)

+++: >0,3

++: 0,2-0,3

+: 0,1-0,2

+/-: 0,05-0,1

-: <0,1

NE: no ensayado

BLG nativa: BLG cristalizada 3x (Sigma) disuelta en PBS

BLG desnaturalizada: BLG cristalizada 3x (Sigma) diluida y hervida en buffer de muestra SDS

LI/T: *Lactococcus lactis* recombinante portador de proteinasa de *Lactobacillus bulgaricus* con inserto de péptidos de BLG

Para producir hibridomas que segregaran anticuerpos monoclonales de péptidos anti-BLG se recuperaron los bazos de las ratas inmunizadas. Las células de bazo se fusionaron con células de mieloma de acuerdo con técnicas estándar y una primera ronda de screeening ya permitió la identificación de poblaciones celulares productoras tanto de anticuerpos anti-T6 como anti-T17. La clonación de estas células se logró usando un clasificador de células para garantizar la presencia de una sola célula por cavidad.

Ejemplo 5

Detección de péptidos inductores de tolerancia por anticuerpos

Con el objeto de determinar si las bacterias lácticas expresan los péptidos inductores de tolerancia de una manera tal que sean accesibles para y reconocidos por los anticuerpos, se desarrollaron bacterias que contenían el gen recombinante en un cultivo de 50 ml y se recogieron las células por centrifugación. Las células se lavaron posteriormente dos veces con 50 ml de TBS y a la larga las células se suspendieron en 6 ml de TBS.

Se transfirieron 75 µl de la suspensión a una cavidad (media placa de ELISA de cavidades de fondo plano) y las placas se dejaron destapadas durante 24 horas a 37°C de modo que las cavidades se secaran. Las placas se lavaron 3 veces con TBS/PBS hasta que las bacterias no unidas se lavaron y las cavidades se sometieron posteriormente a tratamiento de bloqueo con TBS-caseína (1,5 g/l) durante 2 horas a 37°C. Se agregó a las cavidades un antisuero de rata que contenía anticuerpos dirigidos a los péptidos específicos. Las cavidades se incubaron toda la noche a temperatura ambiente, usando una dilución en TBS-caseína que contenía 0,2% de Tween 20. Posteriormente, las cavidades se lavaron tres veces con TBS y se agregaron a las cavidades anticuerpos de desarrollo (IgG anti-rata de cabra conjugada con HRP) seguido de una incubación a 37°C diluida en solución de TBS-caseína que contenía 0,2% de Tween 20. las cavidades se lavaron tres veces con TBS y se desarrollaron con OPD/H_2O_2 y se leyeron a 490 nm.

Pudo observarse que ambas cepas de *Lactococcus lactis* y *Lactobacillus johnsonii* mostraron una reacción positiva, indicando que el péptido inductor de tolerancia es reconocido por los anticuerpos.

Ejemplo 6

Preparación de un antisuero de conejo dirigido a los residuos de aminoácidos 472-659 de la proteinasa prtB de *L bulgaricus*.

La secuencia del objetivo prtB (pares de bases 1414-1977) que codifica a los residuos de aminoácidos 472-659 (el aminoácido número 1 es la metionina de iniciación) se amplificó mediante PCR usando pMD114 como plantilla y el cebador ascendente (prtB5´-1414) de la secuencia

5′-GCGGATCCGGCTTGGGCGGTGCAGATG-3′ (conteniendo un sitio 5′-BamHI), y

5′-CGCAAGCTTGTGCGAAGTGTTCATGGC-3′ (conteniendo un sitio 5′-HindIII).

El producto específico de PCR se obtuvo utilizando una ADN polimerasa Taq (Perkin-Elmer), usando 3 ciclos consistentes en un paso de desnaturalización a 95°C durante 30 segundos, un paso de templado de cebador a 56°C durante 30 segundos y un paso de extensión a 72°C durante 45 segundos, seguido de 27 ciclos con un paso de templado llevado a 60°C.

Después de la desalinización y eliminación de los cebadores y de los dNTPs (High Pure PCR Product Purification Kit, de Roche), la muestra se digirió con BamHI y Hindi, luego se sometió a electroforesis en gel de agarosa y las bandas de ADN de interés se recuperaron del gel (E.Z.N.A. Gel Extraction Kit, Peqlab).

El producto de amplificación purificado se ligó entre los sitios BamHI y Hindi del vector de expresión pQE-9 de *E.coli* (Qiagen), en sentido descendente a una secuencia que codifica una marcación de 6-histidina.

La cepa M15 de *E. coli* [pREP4] se transformó con este vector y se obtuvo la expresión de la proteína heteróloga por inducción del cultivo mediada por IPTG 1mM.

Las células de un cultivo de 1 litro se peletizaron y se lisaron bajo condiciones desnaturalizantes (urea 8M). El lisado clarificado se obtuvo por centrifugación y se cargó en glóbulos de agarosa con Ni^{2+}-NTA (Qiagen), equilibrados a pH 8,0, permitiendo la unión de la proteína

marcada con 6-histidina. Luego de un paso de lavado a pH 6,3, se eluyó la proteína recombinante a pH 4,5.

El material se neutralizó con Tris-ClH (pH 7,5), se cuantificó por medio del ensayo de proteínas del ácido bicinconínico (Pierce) y se ajustó su concentración a 1 mg/ml usando el mismo buffer.

El antígeno se emulsionó en adyuvante completo de Freund y se administró por inyección intradérmica múltiple a dos conejos. Las inyecciones se realizaron los días 0, 14, 28 y 56. El desangrado final ocurrió el día 80.

La reactividad del antisuero, que fue sorprendentemente fuerte, se confirmó tanto por ELISA, sobre el Lc recombinante que expresa la proteinasa prtB de *L. Bulgaricus* en su superficie celular, como por inmunoblotting, sobre el sobrenadante del cultivo del Lc recombinante que expresa una forma secretada de la proteinasa.

REIVINDICACIONES

1. Una cepa bacteriana del grupo de las bacterias lácticas **caracterizada** porque expresa un polipéptido que contiene un péptido inductor de tolerancia heterólogo hacia dicho polipéptido, tal que el péptido se estabiliza frente a la degradación por las enzimas estomacales/duodenales y retiene sus propiedades inductoras de tolerancia y se expone en la periferia del polipéptido.

2. La cepa bacteriana de acuerdo a la reivindicación 1, la cual se selecciona entre Lactobacillus o Bifidobacterium.

3. La cepa bacteriana de acuerdo a la reivindicación 1 o la reivindicación 2, **caracterizada** porque el polipéptido se selecciona entre una proteína superficial, una proteína intracelular o una proteína secretada por la bacteria.

4. La cepa bacteriana de acuerdo a cualquiera de las reivindicaciones precedentes, **caracterizada** porque el péptido inductor de tolerancia deriva de un material alimenticio.

5. Una composición alimenticia **caracterizada** porque contiene una cepa bacteriana de acuerdo a cualquiera de las reivindicaciones precedentes o un sobrenadante de la misma.

6. La composición alimenticia de acuerdo a la reivindicación 5, **caracterizada** porque se selecciona del grupo integrado por leche, yogur, cuajada, queso, leches fermentadas, productos fermentados a base de leche, helado, productos fermentados a base de cereales, polvos a base de leche, fórmulas para bebés o alimentos para mascotas.

7. El material alimenticio de acuerdo a cualquiera de las

reivindicaciones 5 ó 6, **caracterizada** porque la cepa bacteriana está contenida en una proporción que oscila entre 10^7 y 10^{11} ufc/forma de dosificación.

8. Una composición farmacéutica caracterizada porque contiene una cepa bacteriana de acuerdo a cualquiera de las reivindicaciones 1 a 4 o un sobrenadante de la misma.

9. La composición farmacéutica de acuerdo a la reivindicación 8, **caracterizada** porque la cepa bacteriana está contenida en una proporción que oscila entre 10^{10} y 10^{12} ufc/forma de dosificación.

10. Uso de una cepa bacteriana de acuerdo a cualquiera de las reivindicaciones 1 a 4 o un sobrenadante de la misma para la preparación de un vehículo ingerible para el tratamiento de la alergia.

11. Uso de una composición alimenticia o farmacéutica de acuerdo a cualquiera de las reivindicaciones 1 a 9 para la preparación de un vehículo ingerible para el tratamiento de la alergia.

12. El uso de acuerdo a la reivindicación 11, **caracterizada** porque la composición se administra por vía oral o nasal.

13. Una vacuna **caracterizada** porque comprende una cepa bacteriana de acuerdo a cualquiera de las reivindicaciones 1 a 4, o partes de la misma.

14. La vacuna de acuerdo a la reivindicación 13, **caracterizada** porque se utiliza la fracción de membrana celular de la bacteria lisada.

Peptido T1 (139-141) H_2N-A-L-K-COOH

Peptido T2 (146-148) H_2N-H-I-R-COOH

Peptido T3 (136-138) H_2N-F-D-K-COOH

Peptido T4 (71-75) H_2N-I-I-A-E-K-COOH

Peptido T5 (62-69) H_2N-E-N-G-E-C-A-Q-K-COOH

Peptido T6 (84-91) H_2N-I-D-A-L-N-E-N-K-COOH

Peptido T7 (9-14) H_2N-G-L-D-I-Q-K-COOH

Peptido T8 (142-145) H_2N-A-L-P-M-COOH

Peptido T9, T10, T11 (61-67,-68,-69) H_2N-W-E-N-G-E-C-A,-Q,-K,-COOH

Peptido T12 (125-135) H_2N-T-P-E-V-D-D-E-A-L-E-K-COOH

Peptido T13 (1-8) H_2N-L-I-V-T-Q-T-M-K-COOH

Peptido T14 (142-148) = T8 (142-145) + T2 (146-148) H_2N-A-L-P-M-H-I-R-COOH

Peptido T15 (78-82) H_2N-I-P-A-V-F-K-COOH

Peptido T16 (15-20) H_2N-V-A-G-T-W-Y-COOH

Peptido T17 (92-99,100) H_2N-V-L-V-L-D-T-D-Y-K,-K-COOH

Peptido T18 (125-138) = T12 (125-135) + T3 (136-138) H_2N-T-P-E-V-D-D-E-A-L-E-K-F-D-K-COOH

Peptido T19 (25-40) H_2N-A-A-S-D-I-S-L-L-D-A-Q-S-A-P-L-R-COOH

Peptido T20, T21 (61, 62-69):S-S:(149-162)

H_2N-W,-E-N-G-E-C-A-Q-K-COOH

H_2N-L-S-F-N-P-T-Q-L-E-E-Q-C-H-I-COOH

Peptido T22 (21-40) H_2N-S-L-A-M-A-A-S-D-I-S-L-L-D-A-Q-S-A-P-L-R-COOH

Peptido T23 (41-60) H_2N-V-Y-V-E-E-L-K-P-T-P-E-G-D-L-E-I-L-L-Q-K-COOH

Peptido T24 (149-162) H_2N-L-S-F-N-P-T-Q-L-E-E-Q-C-H-I-COOH

Fig. 1

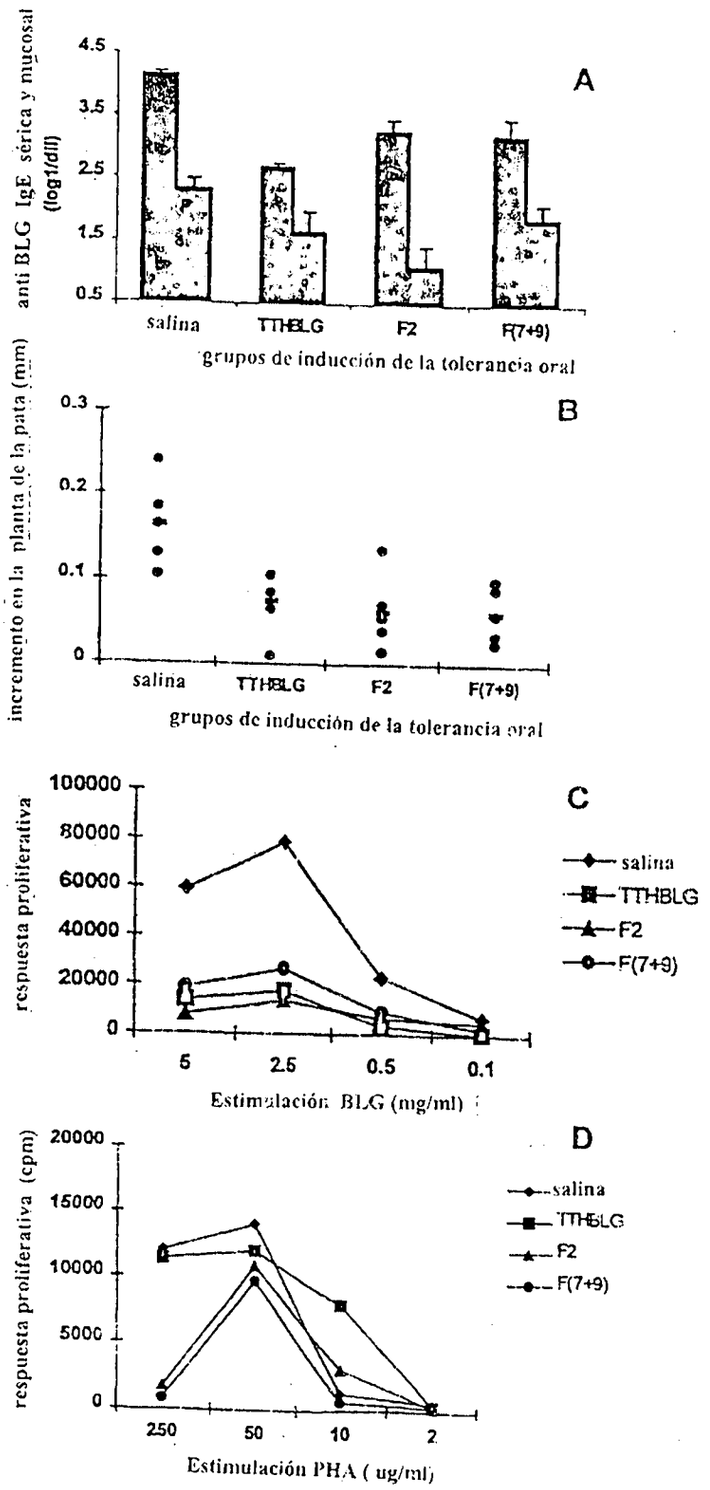

A
anti BLG IgE sérica y mucosal (log1/dil)
4.5
3.5
2.5
1.5
0.5
salina
TTHBLG
F2
F(7+9)
grupos de inducción de la tolerancia oral
B
incremento en la planta de la pata (mm)
0.3
0.2
0.1
0
salina
TTHBLG
F2
F(7+9)
grupos de inducción de la tolerancia oral
C
respuesta proliferativa
100000
80000
60000
40000
20000
0
5
2.5
0.5
0.1
Estimulación BLG (mg/ml)
salina
TTHBLG
F2
F(7+9)
D
respuesta proliferativa (cpm)
20000
15000
10000
5000
0
250
50
10
2
Estimulación PHA (ug/ml)
salina
TTHBLG
F2
F(7+9)

Fig. 2

LISTADO DE SECUENCIAS

<110> Société Des Produits Nestlé S. A.

<120> Bacteria del ácido lactico capaz de reducir la tendencia de un individuo a desarrollar reacciones alérgicas.

<130> 80263

<140> PCT/EP01/10956
<141> 2001-09-21

<150> EP 00120867.7
<151> 2000-09-25

<160> 29

<170> PatentIn Ver. 2.1

<210> 1
<211> 5
<212> PRT
<213> bovino

<400> 1
Ile Ile Ala Glu Lys
1 5

<210> 2
<211> 8
<212> PRT
<213> bovino

<400> 2
Glu Asn Gly Glu Cys Ala Gln Lys
1 5

<210> 3
<211> 8
<212> PRT
<213> bovino

<400> 3
Ile Asp Ala Leu Asn Glu Asn Lys
1 5

<210> 4
<211> 6
<212> PRT
<213> bovino

<400> 4
Gly Leu Asp Ile Gln Lys
1 5

<210> 5
<211> 4
<212> PRT
<213> bovino

<400> 5
Ala Leu Pro Met
1

<210> 6
<211> 7
<212> PRT
<213> bovino

<400> 6
Trp Glu Asn Gly Glu Cys Ala
1 5

<210> 7
<211> 8
<212> PRT
<213> bovino

<400> 7
Trp Glu Asn Gly Glu Cys Ala Gln
1 5

<210> 8
<211> 9
<212> PRT
<213> bovino

<400> 8
Trp Glu Asn Gly Glu Cys Ala Gln Lys
1 5

<210> 9
<211> 11
<212> PRT
<213> bovino

<400> 9
Thr Pro Glu Val Asp Asp Glu Ala Leu Glu Lys
1 5 10

<210> 10
<211> 8
<212> PRT
<213> bovino

<400> 10
Leu Ile Val Thr Gln Thr Met Lys
1 5

<210> 11
<211> 7
<212> PRT
<213> bovino

<400> 11
Ala Leu Pro Met His Ile Arg

1 5

<210> 12
<211> 6
<212> PRT
<213> bovino

<400> 12
Ile Pro Ala Val Phe Lys
1 5

<210> 13
<211> 6
<212> PRT
<213> bovino

<400> 13
Val Ala Gly Thr Trp Tyr
1 5

<210> 14
<211> 10
<212> PRT
<213> bovino

<400> 14
Val Leu Val Leu Asp Thr Asp Tyr Lys Lys
1 5 10

<210> 15
<211> 9
<212> PRT
<213> bovino

<400> 15
Val Leu Val Leu Asp Thr Asp Tyr Lys
1 5

<210> 16
<211> 14
<212> PRT
<213> bovino

<400> 16
Thr Pro Glu Val Asp Asp Glu Ala Leu Glu Lys Phe Asp Lys
1 5 10

<210> 17
<211> 16
<212> PRT
<213> bovino

<400> 17
Ala Ala Ser Asp Ile Ser Leu Leu Asp Ala Gln Ser Ala Pro Leu Arg
1 5 10 15

<210> 18
<211> 14
<212> PRT
<213> bovino

<400> 18
Leu Ser Phe Asn Pro Thr Gln Leu Glu Glu Gln Cys His Ile
1 5 10

<210> 19
<211> 20
<212> PRT
<213> bovino

<400> 19
Ser Leu Ala Met Ala Ala Ser Asp Ile Ser Leu Leu Asp Ala Gln Ser
1 5 10 15

Ala Pro Leu Arg
20

<210> 20
<211> 20
<212> PRT
<213> bovino

<400> 20
Val Tyr Val Glu Glu Leu Lys Pro Thr Pro Glu Gly Asp Leu Glu Ile
1 5 10 15

Leu Leu Gln Lys
20

<210> 21
<211> 14
<212> PRT
<213> bovino

<400> 21
Leu Ser Phe Asn Pro Thr Gln Leu Glu Glu Gln Cys His Ile
1 5 10

<210> 22
<211> 14
<212> PRT
<213> Secuencia Artificial

<220>
<223> Descripción de secuencia artificial : péptido sintetico

<400> 22
Cys Phe Lys Ile Asp Ala Leu Asn Glu Asn Lys Val Ser Arg
1 5 10

<210> 23
<211> 15
<212> PRT

<213> Secuencia Artificial

<220>
<223> Descripción de secuencia artificial : péptido sintetico

<400> 23
Cys Asn Lys Val Leu Val Leu Asp Thr Asp Tyr Lys Lys Tyr Ser
1 5 10 15

<210> 24
<211> 23
<212> PRT
<213> Secuencia Artificial

<220>
<223> Descripción de secuencia artificial: péptido sintetico

<400> 24
Cys Phe Lys Ile Asp Ala Leu Asn Glu Asn Lys Val Leu Val Leu Asp
1 5 10 15
Thr Asp Tyr Lys Lys Tyr Ser
20

<210> 25
<211> 11
<212> PRT
<213> Secuencia Artificial

<220>
<223> Descripción de secuencia artificial : péptido sintetico

<400> 25
Leu Ile Val Thr Gln Thr Met Lys Ser Arg Cys
1 5 10

<210> 26
<211> 28
<212> DNA
<213> Lactobacillus bulgaricus

<400> 26
ttttgtggat ccttaacttc atagcacg 28

<210> 27
<211> 27
<212> DNA
<213> Lactobacillus bulgaricus

<400> 27
atattatcta gaattgaata gattgcc 27

<210> 28
<211> 27
<212> DNA

<213> Secuencia Artificial

<220>
<223> Descripción de secuencia artificial : plantilla

<400> 28
gcggatccgg cttgggcggt gcagatg 27

<210> 29
<211> 27
<212> DNA
<213> Secuencia Artificial

<220>
<223> Descripción de secuencia artificial : plantilla

<400> 29
cgcaagcttg tgcgaagtgt tcatggc 27

348

SUPERINDUSTRIA Y COMERCIO
Radicacion : 03023841 00000000 Folios: 347
Fecha (AMD): 2003-03-20 16:39:36
Tramite : 011 PCT-PATENT 379 FASE NACI 411 PRESENTA
Dependencia: 2020 DIVISION DE NUEVAS CREACIONES

Industria y Comercio
SUPERINTENDENCIA

REQUISITOS MINIMOS PARA ADMISIÓN A TRAMITE

PCT

PATENTE DE INVENCIÓN [X] MODELO DE UTILIDAD [ ]

- Indicación de que se solicita la concesión de una patente [X] 1
- Datos de identificación del solicitante o de la persona que presenta la solicitud [X] 1
- Descripción de la invención [X] 311
- Dibujos de ser estos pertinentes [X] 340
- Comprobante de Pago de las tasas establecidas [X] 2,3

COMPLETA [X] INCOMPLETA [ ]

DISEÑO INDUSTRIAL [ ]

- Indicación de que se solicita el registro de Diseño Industrial. [ ]
- Datos de identificación del solicitante o de la persona que presenta la solicitud. [ ]
- Representación gráfica y fotográfica del Diseño Industrial o muestra del material que incorpora el diseño . [ ]
- Comprobante de pago de las tasas establecidas. [ ]

COMPLETA [ ] INCOMPLETA [ ]

ESQUEMA DE TRAZADO [ ]

- Indicación de que se solicita el registro de un Esquema de trazado. [ ]
- Datos de identificación del solicitante o de la persona que presenta la solicitud. [ ]
- Representación gráfica del esquema de trazado [ ]
- Comprobante de pago de las tasas establecidas. [ ]

COMPLETA [ ] INCOMPLETA [ ]

_____
Firma Responsable

Fecha: _____

Carrera 13 No. 27-00 Piso 5 y 10
Conmutador: 334 12 21-2-3-4
Fax: 281 31 25 e-mail: info@sic.gov.co
Santa Fe de Bogotá D.C. - Colombia