



DELEGATURA PROPIEDAD INDUSTRIAL
División de Nuevas Creaciones

PCT

SOLICITUD
PATENTE DE INVENCION

ENTRADA EN VIGENCIA NACIONAL

08-99559

21. EXPEDIENTE No.

54. TITULO

VACUNA DE SALMONELA CON VIRUS VIVOS ATENUADA

51. CLASIFICACION INTERNACIONAL

71. SOLICITANTE

VRIJE UNIVERSITEIT BRUSSEL

DOMICILIO

BRUSELAS, BELGICA

74. APODERADO

JUAN PABLO CADENA SARMIENTO

22. SANTAFE DE BOGOTÀ, D.C.

PETITORIO

AS: 108.467



SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 08-099559- -00000-0000

Fecha: 2008-09-19 16:24:47 Dep. 2020 NUECREACION
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 151

FORMULARIO ÚNICO DE SOLICITUD DE PATENTE PCT ENTRADA EN FASE NACIONAL

20 oct. 2008

1

SOLICITUD DE:



Patente de Invención.



Patente de Modelo de Utilidad.



Capítulo I.



Capítulo II.

2

SOLICITANTE (71)

Nombre: **VRIJE UNIVERSITEIT BRUSSEL**

Dirección: Pleinlaan 2, B-1050 Bruselas, BE
Nacionalidad o Domicilio: Bélgica
Lugar de Constitución: Bélgica

Teléfono:

Fax:

E-mail

IDENTIFICACIÓN

C.C.



NIT



C.E.



Otro



Cual _____

Número _____

3

REPRESENTANTE O APODERADO

Nombre: **JUAN PABLO CADENA SARMIENTO**

Dirección: Cra. 7 No. 71-21 Torre B – Piso 4,
Bogotá, Colombia.

Teléfono: 744-2200

Fax: 742-2200

E-mail:

IDENTIFICACIÓN

C.C.



NIT



C.E.



Otro



Cual _____

Número 80.410.337 TP 57492

4

INVENTOR (ES) (72)

Nombre: Henri Marcel Jozef DE GREVE, Connie Theresia ADRIAENSEN, Jean-Pierre Ernest Clément HERNALSTEENS.

Dirección: Jef Lambeauxlaan 11, B-1060 SINT-GILLIS, Bélgica; Leiekaai 33, B-9000 GHENT, Bélgica; François Desmedtstraat 156, B-1150 SINT-PIETERS WOLUWE, Bélgica (Respectivamente).

Nacionalidad o Domicilio: Bélgica (Respectivamente).

5 PCT (86)

Solicitud Internacional No. PCT/BE2006/000020

Fecha 20-03-2006

Publicación Internacional No WO 2007/112518 A1

Fecha 11-10-2007

6 Título (54):

VACUNA DE SALMONELA CON VIRUS VIVOS ATENUADA

7 Clasificación Internacional (51):

8 Prioridad

SI ☐

NO ☒

(33) País de Origen

(32) Fecha

(31) Número de Solicitud

9 Comprobante de pago No.

08 - 66, 152

Fecha

18-09-2008

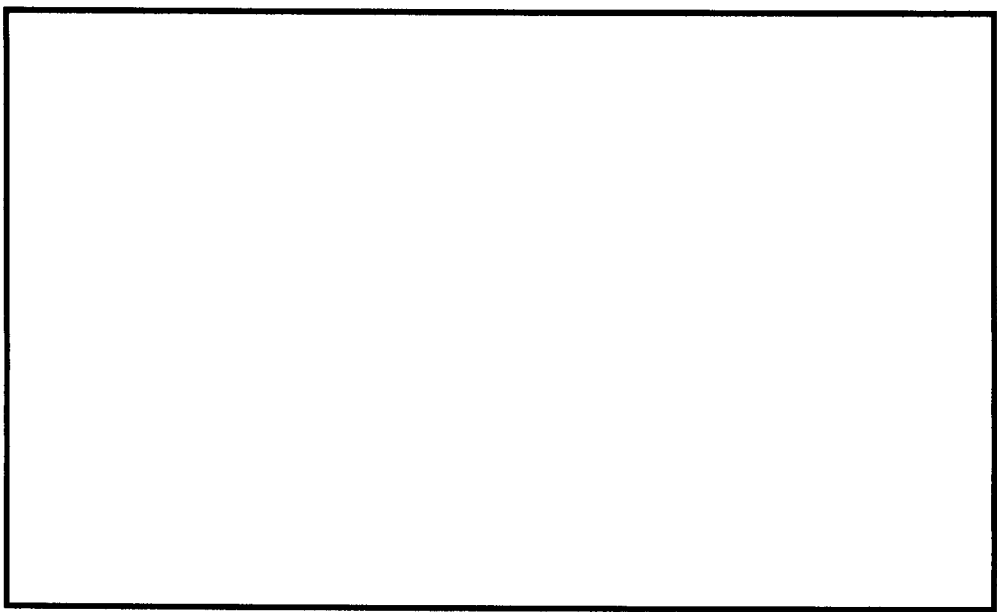
Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.

10

ANEXOS

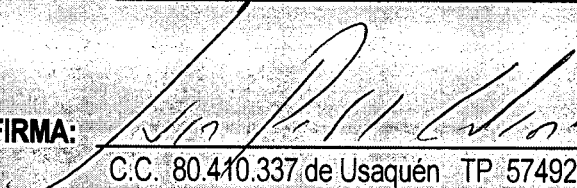
- ☒ Comprobante de pago de la tasa de presentación de la solicitud. \$ 501.000
- ☐ Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica.
- ☐ Poderes, si fuere el caso. **Fotocopia autenticada**
- ☐ Documento de cesión del inventor al solicitante o a su causante.
- ☐ Declaraciones.
- ☒ Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos)
- ☒ Traducción de la solicitud internacional al castellano. (Resumen, descripción, reivindicaciones, dibujos)
- ☐ Copia del examen preliminar efectuado por la IPEA.
- ☐ Traducción del examen preliminar 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 de las comunidades indígenas.
- ☐ De ser el caso, certificado de depósito del material biológico.
- ☐ 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, relacionadas parcial o totalmente con la invención de esta solicitud.
- ☒ Otros: Tarjeta de Propietarios

11 FIGURA CARACTERÍSTICA:



12 Solicito la concesión de la patente,

NOMBRE: JUAN PABLO CADENA SARMIENTO

FIRMA: 
C.C. 80.410.337 de Usaquén TP 57492 del C.S.J.

3

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 08 - 66,152
FECHA : AGOSTO 20 DE 2008

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 08-099559- -00000-0000

Fecha: 2008-09-19 16:24:47

Tra. 11 PATENTEIYII

Act. 411 PRESENTACION

Dep. 2020 NUECREACIONE

Eve: 378 FASENACIONALI

Folios: 151

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

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
BRIGARD & CASTRO LTDA	CONSIGNACION	BANCO DE BOGOTA	062754387	3257295	19/08/2008	61,283,000.00

***** 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	501,000.00
	TOTAL :	\$ 501,000.00

SON: QUINIENTOS UN MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

15:108467

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
11 October 2007 (11.10.2007)

PCT

(10) International Publication Number
WO 2007/112518 A1

(51) International Patent Classification:

C12N 1/20 (2006.01) A61K 39/112 (2006.01)
G01N 33/53 (2006.01) A61P 31/04 (2006.01)
A61K 39/108 (2006.01)

(74) Agents: VAN MALDEREN, Joëlle et al.; pronovern-OF-
FICE VAN MALDEREN, Avenue Josse Goffin 158, 1082
Brussels (BE).

(21) International Application Number:

PCT/BE2006/000020

(22) International Filing Date: 20 March 2006 (20.03.2006)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicant (for all designated States except US): VRIJE
UNIVERSITEIT BRUSSEL [BE/BE]; Pleinlaan 2,
B-1050 Brussels (BE).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV,
LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI,
NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG,
SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US,
UZ, VC, VN, YU, ZA, ZM, ZW.

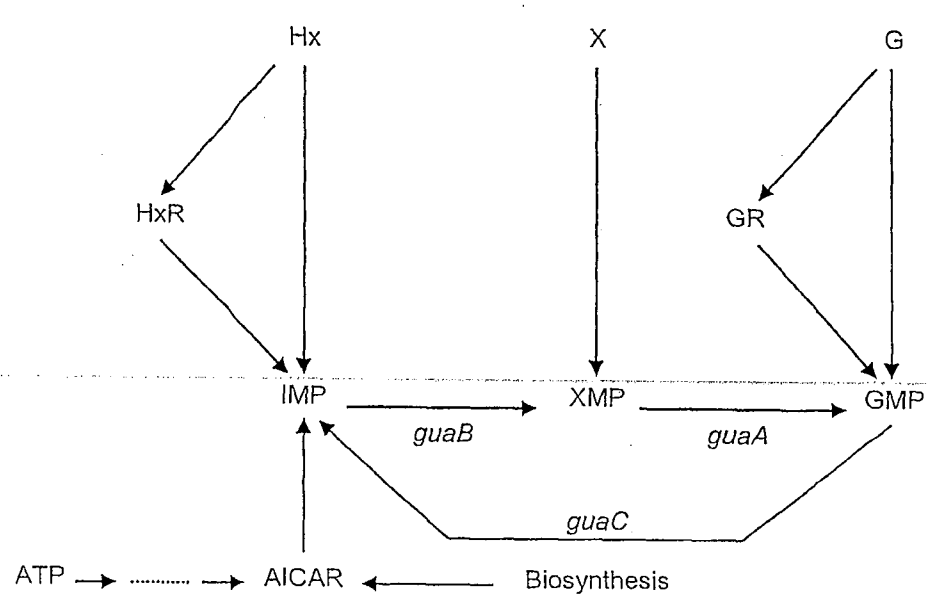
(72) Inventors; and

(75) Inventors/Applicants (for US only): DE GREVE, Henri
Marcel Jozef [BE/BE]; Jef Lambeauxlaan 11, B-1060
SINT-GILLIS (BE). ADRIAENSEN, Connie Theresia
[BE/BE]; Leiekaai 33, B-9000 GHENT (BE). HER-
NALSTEENS, Jean-Pierre Ernest Clément [BE/BE];
François Desmedtstraat 156, B-1150 SINT-PIETERS
WOLUWE (BE).

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT,
RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA,
GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: LIVE ATTENUATED SALMONELLA VACCINE



(57) Abstract: The present invention is related to double and triple attenuated mutant strains of a bacterium infecting veterinary species such as Salmonella enterica and/or (a pathogenic) Escherichia coli. The mutants of the invention contain at least one first genetic modification and at least one second genetic modification, said first modification in one or more motility genes, and said second modification in one or more genes involved in the survival or the proliferation of the pathogen in the host. The present invention further relates to live attenuated vaccines based on such mutants for preventing amongst others Salmonellosis and/or an infection by an E. coli pathogen in a veterinary species.

WO 2007/112518 A1

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
11 October 2007 (11.10.2007)

PCT

(10) International Publication Number
WO 2007/112518 A1

(51) International Patent Classification:

C12N 1/20 (2006.01) A61K 39/112 (2006.01)
G01N 33/53 (2006.01) A61P 31/04 (2006.01)
A61K 39/108 (2006.01)

(21) International Application Number:

PCT/BE2006/000020

(22) International Filing Date: 30 March 2006 (30.03.2006)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicant (for all designated States except US): **VRIJE UNIVERSITEIT BRUSSEL** [BE/BE]; Pleinlaan 2, B-1050 Brussels (BE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **DE GREVE, Henri** Marcel Jozef [BE/BE]; Jef Lambeauxlaan 11, B-1060 SINT-GILLIS (BE). **ADRIAENSEN, Connie** Theresia [BE/BE]; Leiekaai 33, B-9000 GHENT (BE). **HERNALSTEENS, Jean-Pierre** Ernest Clément [BE/BE]; François Desmedtstraat 156, B-1150 SINT-PIETERS WOLUWE (BE).

(74) Agents: **VAN MALDEREN, Joëlle** et al.; pronovem-OFFICE VAN MALDEREN, Avenue Josse Goffin 158, 1082 Brussels (BE).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

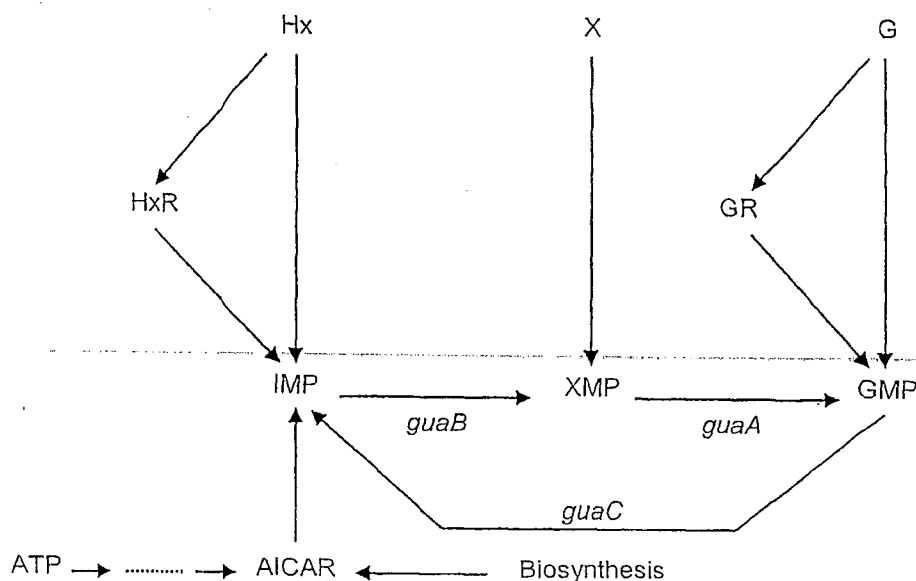
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

[Continued on next page]

(54) Title: **LIVE ATTENUATED SALMONELLA VACCINE**



(57) Abstract: The present invention is related to double and triple attenuated mutant strains of a bacterium infecting veterinary species such as *Salmonella enterica* and/or (a pathogenic) *Escherichia coli*. The mutants of the invention contain at least one first genetic modification and at least one second genetic modification, said first modification in one or more motility genes, and said second modification in one or more genes involved in the survival or the proliferation of the pathogen in the host. The present invention further relates to live attenuated vaccines based on such mutants for preventing amongst others *Salmonellosis* and/or an infection by an *E. coli* pathogen in a veterinary species.

7

WO 2007/112518 A1



Published:

- with international search report
- with sequence listing part of description published separately in electronic form and available upon request from the International Bureau

(48) Date of publication of this corrected version:

6 March 2008

(15) Information about Correction:

see Notice of 6 March 2008

LIVE ATTENUATED SALMONELLA VACCINE

Field of the invention

[0001] The present invention relates to attenuated bacterial mutants, in particular attenuated *Salmonella enterica* mutants, and to a live attenuated vaccine comprising same. The double, triple, multiple mutants of the invention advantageously allow a serological distinction between vaccinated animals and (non-vaccinated) animals that have been exposed to a wild-type field such as wild-type field *S. enterica*.

State of the art

[0002] *Salmonellae* are Gram-negative, facultative anaerobic, motile, non-lactose fermenting rods belonging to the family *Enterobacteriaceae*. *Salmonella* are usually transmitted to humans by the consumption of contaminated foods and cause Salmonellosis. *E. coli* is another member of the family *Enterobacteriaceae*.

[0003] *Salmonellae* have been isolated from many animal species including, cows, chickens, turkeys, sheep, pigs, dogs, cats, horses, donkeys, seals, lizards and snakes.

[0004] 95% of the important *Salmonella* pathogens belong to *S. enterica*, with *S. enterica* serovar Typhimurium (*S. Typhimurium*) and *S. enterica* serovar Enteritidis (*S. Enteritidis*) the most common forms.

[0005] *Salmonella* infections are a serious medical and veterinary problem world-wide and cause concern in the food industry. Contaminated food cannot be readily identified.

[0006] Control of Salmonellosis is important, to avoid potentially lethal human infections and considerable economic losses for the animal husbandry industry.

[0007] The ubiquitous presence of *Salmonella* in nature
5 complicates the control of the disease just by detection and eradication of infected animals.

[0008] Several control strategies based on the principles of competitive exclusion and vaccination have been tested to control the infection of e.g. poultry.

10 [0009] Vaccination of farm animals is often considered as the most effective way to prevent zoonoses caused by e.g. *Salmonella*.

[0010] Whole-cell killed vaccines and subunit vaccines are used with variable results in the prevention of
15 *Salmonella* infections in animals and in humans. Inactivated vaccines in general provide poor protection against Salmonellosis.

[0011] Live attenuated *Salmonella* vaccines are potentially superior to inactivated preparations owing to:
20 (i) their ability to induce cell-mediated immunity in addition to antibody responses; (ii) oral delivery with no risk of needle contamination; (iii) effectiveness after single-dose administration; (iv) induction of immune responses at multiple mucosal sites; (v) low production
25 cost; and (vi) their possible use as carriers for the delivery of recombinant antigens to the immune system.

[0012] The following attenuated mutant strains have been tested on their efficiency to induce a protective immune response in treated animals: (1) strains carrying mutations
30 in the *aro* genes (Alderton et al., 1991, *Avian diseases* 35:435-442; Schiemann and Montgomery, 1991, *Veterinary Microbiology* 27: 295-308), (2) strains carrying deletions in the *cya* (adenylate cyclase) and/or *crp* (cyclic AMP receptor) genes (US 5,389,368; US 5,855,879; US 5,855,880;

Hassan and Curtiss, 1997, *Avian Diseases* 41:783-791; Porter et al. 1993, *Avian Diseases* 37:265-273) and (3) strains that carry mutations in the genes of the *guaBA* operon of *S. typhi* (Wang et al., 2001, *Infection and Immunity* 69:4734-4741; WO99/58146 and US Patent 6,190,669).

[0013] So far only the vaccine strain Megan[®] Vac1, that carries deletions in the *cya* and *crp* genes, has been effective, at least in part. This strain does not provide full protection (<http://www.meganhealth.com/meganvac.html>).

[0014] McFarland and Stocker (1987, *Microbial pathogenesis* 3:129-141) reported on the virulence of *guaA* and *guaB* Tn10 insertion mutants of *S. Typhimurium* and *S. dublin* in BALB/c mice. At high dosage (2.5×10^7 CFU), these authors reported a significant lethality of animals, resulting from the multiplication of the auxotrophic strain.

[0015] Also the Δ *guaBA* mutant of *S. typhi* proved no suitable candidate for sound and safe protection against typhoid fever. It showed a significant residual virulence in mice (Wang et al., 2001).

[0016] There is thus still a need for improved live attenuated *Salmonella* vaccine strains, as well as for improved live attenuated vaccine strains of bacteria infecting veterinary species in general.

[0017] Vaccinated animals often produce antibodies against different antigens of the pathogen. Problem is that vaccinated animals as such can no longer be distinguished from animals that have been in contact with a wild-type field strain such as a *Salmonella* field strain, and are possibly infected therewith.

[0018] There is thus also a need for improved live attenuated strains like live attenuated *Salmonella* vaccine strains that would make such distinction possible.

Aims of the invention

[0019] An object of the present invention is to provide attenuated *Salmonella enterica* strains with a double or a triple mutation.

5 [0020] Another object of the present invention is to provide a live attenuated vaccine against Salmonellosis and methods of treatment based thereon.

[0021] Yet another object of the present invention is to provide attenuated *Salmonella* strains which are useful as
10 live vector and as DNA-mediated vaccines expressing foreign antigens. Such strains are thus highly suitable for the development of vaccines including polyvalent vaccines.

[0022] Still another object of this invention is to provide a method to achieve *S. enterica* deletion mutants of
15 the invention.

[0023] Still a further object of this invention is to provide an attenuated *Salmonella* strain that allows a serological distinction between vaccinated and non-vaccinated yet possibly infected animals.

20 [0024] Yet a further object of this invention is to provide the same materials and methods for the preparation of attenuated strains of a bacterium infecting veterinary species in general, more in particular poultry.

[0025] The general aim is to improve food safety and
25 animal health.

Summary of the invention

[0026] Some Δ *guaB* auxotrophic *Salmonella enterica* mutants with a deletion mutation in the *guaB* gene showed
30 residual virulence. It was found that further modifications (preferably deletions) in one or more genes involved in motility reduced the remaining virulence without affecting the immunogenic capacities of the strain.

[0027] A first aspect of the invention therefore relates

to an attenuated mutant strain of a bacterium infecting veterinary species, in particular an attenuated *S. enterica* mutant strain, wherein said mutant strain contains at least one first genetic modification and at least one second
5 genetic modification, said first modification in one or more (at least one) motility genes, and said second modification in one or more (at least one) genes involved in the survival or the proliferation of the bacterium or pathogen (e.g. *S. enterica*) in the host. The term
10 "bacterium infecting veterinary species" in the context of the invention refers in particular to bacteria that are pathogenic to veterinary species, and which can be attenuated by the above genetic modifications. The bacterium infecting veterinary species may be a Gram-
15 negative bacterium. Preferred are Gram-negative bacteria for poultry such as *Salmonella*, *Pasteurella*, *Escherichia coli*, etc. Most preferred are *Salmonella enterica* and (pathogenic) *E. coli*. By "pathogenic to" is meant that the bacterium, if not attenuated, is capable of causing an
20 infectious disease in the veterinary species.

[0028] The genetic modifications of the invention advantageously lead to a null-function, in other words impair or affect the gene function. The modification in the present context is also referred to as an "impairing
25 modification". The modification is said to inactivate the gene in question. Advantageously, said inactivation results in attenuation, at least to a degree that the mutant strain is suitable for use in a live attenuated vaccine.

[0029] The genetic modification may be an insertion, a
30 deletion, and/or a substitution of one or more nucleotides in said genes. Mutant strains according to the invention by such modification are affected in a motility gene function and in a gene function needed for the survival or the proliferation of the pathogen, leading to a null-function

(no functional gene product formed) of the affected genes.

[0030] Deletion mutants are preferred, as an insertion mutant may revert, thereby restoring the pathogenicity of the strain.

5 [0031] The first modification is in one or more (1, 2, 3, ...) motility genes. Examples of a gene involved in motility are the genes encoding flagellin. The mutant of the invention may have a (impairing) modification in the *fliC* and/or the *fljB* or the *fljBA* genes respectively (*fliC*;
10 *fljB*; *fljBA*; *fliC* and *fljB*; *fliC* and *fljBA*; ...). Advantageously mutants, in which all genes encoding flagellin are deleted, are incapable of swarming out on LB medium containing 0.4% agar and can thereby easily be distinguished from wild-type motile strains.

15 [0032] The second genetic modification is in one or more (1, 2, 3, ...) genes involved in the survival or the proliferation of the pathogen in its host. Such gene may be a house-keeping gene or a virulence gene. An example of a housekeeping gene that can lead to attenuated strains when
20 the gene function is affected, is the *guaB* gene encoding the enzyme IMP dehydrogenase. Such mutant is incapable of forming de novo guanine nucleotides. Also possible are impairing modifications in the *guaBA* operon, advantageously leading to a null-function of the gene(s) encoding for or
25 regulating proper IMP dehydrogenase activity.

[0033] Advantageously, the attenuated mutant strains of the invention are immunogenic.

[0034] The present invention in particular aims to provide attenuated *S. Enteritidis* and *S. Typhimurium*
30 strains.

[0035] Preferably the genetic modifications of the invention are introduced into parent strain *S. Enteritidis* phage type 4 strain 76Sa88 or into parent strain *S. Typhimurium* 1491S96. The 76Sa88 strain is a clinical

isolate from a turkey, obtained from the Veterinary and Agrochemical Research Centre, Groeselenberg 99, B-1180 Ukkel, Belgium, harboring the temperature sensitive replication plasmid pKD46, encoding the bacteriophage Lambda Red recombinase system. The 1491S96 strain is a clinical isolate from a chicken.

[0036] One of the attenuated *S. enterica* strains obtained according to the invention is *S. Enteritidis* strain SM73 having the deposit number LMG P-21642. Another example is the attenuated *S. Typhimurium* strain SM89 having the deposit number LMG P-21643.

[0037] A preferred mutant of the invention carries or comprises a genetic modification in the *guaB* gene and a genetic modification in the *fliC* gene.

[0038] Another preferred mutant of the invention carries or comprises a genetic modification in a *guaB* gene and a genetic modification in the *fljBA* genes.

[0039] Yet another preferred mutant of the invention carries or comprises a genetic modification in the *guaB* gene, a genetic modification in the *fliC* gene, and a genetic modification in the *fljBA* genes.

[0040] The attenuated strains of the invention are highly suitable for use in a live attenuated vaccine. The mutant strains of the invention may encode and express a foreign antigen.

[0041] Another aspect of the invention relates to a vaccine for immunizing a veterinary species against a bacterial infection, comprising:

- a pharmaceutically effective or an immunizing amount of an attenuated mutant strain according to the invention; and
- a pharmaceutically acceptable carrier or diluent. The present invention in particular relates to vaccines comprising attenuated mutant strains of *S. enterica* and/or

E. coli.

[0042] In general about 10^2 cfu to about 10^{10} cfu, preferably about 10^5 cfu to about 10^{10} cfu is administered (examples of a pharmaceutically effective or an immunizing amount). An immunizing dose varies according to the route of administration. Those skilled in the art may find that the effective dose for a vaccine administered parenterally may be smaller than a similar vaccine which is administered via drinking water, and the like.

10 [0043] The attenuated strains of the invention and pharmaceutical compositions or vaccines comprising same are highly suitable for immunizing animals such as veterinary species, livestock, and more specifically poultry. For instance, the attenuated *Salmonella* strains of the invention, and pharmaceutical compositions or vaccines comprising same, are highly suitable for immunizing veterinary species and in particular poultry such as chicken against Salmonellosis and possibly other diseases (e.g. in the case of a multivalent vaccine). The attenuated strains of the invention are particularly suited to protect the animal/veterinary species in question against an attack by the pathogen (the bacterium infecting veterinary species) in question.

[0044] A further aspect of the invention therefore concerns a method of immunizing animals, preferably veterinary species, more preferably poultry such as chicken against a disease caused by a bacterium infecting veterinary species, said method comprising the step of: administering to the animal or veterinary species in need thereof an immunizing amount of an attenuated mutant strain of the invention and/or of a vaccine comprising same, whereby a protective immune response is then invoked in the animal or veterinary species. The present invention in particular relates to methods of immunizing veterinary

species against Salmonellosis or against an infection by a pathogenic *E. coli*.

[0045] Examples of veterinary species to be immunized against Salmonellosis: poultry, small or heavy livestock
5 such as chicken, turkey, ducks, quails, guinea fowl, pigs, sheep, young calves, cattle etc. An immunizing amount is administered to these animals, preferably via the oral, nasal or parenteral route.

[0046] A further aspect of the invention relates to a
10 mutant strain of the invention for use as a medicament (e.g. for use in a vaccine). Yet another aspect of the invention relates to the use of an attenuated mutant strain of the invention for the preparation of a medicament, such as a vaccine, for the prevention (and/or treatment) of a
15 disease caused by a pathogen (the bacterium infecting veterinary species) such as Salmonellosis. Examples of animals or veterinary species to be treated and recommended doses are given above.

[0047] Yet another aspect of the invention concerns the
20 use of mutants of the invention, and in particular flagellin mutants, as serological markers to distinguish between vaccinated animals and animals that are naturally infected, *id est* have been into contact and became infected by a wild-type strain.

25 [0048] The invention for instance relates to a method for a serological distinction between vaccinated animals and animals infected by a wild-type strain, wherein the vaccinated animals have been immunized with a mutant strain wherein a flagellin gene is inactivated, said method
30 comprising the steps of:

- Assaying animals for the presence of antibodies raised against flagellin,
- Distinguishing infected animals from vaccinated

animals based on the presence or absence of said antibodies.

[0049] The method of the invention advantageously is an *in vitro* method. Advantageously animals infected by *Salmonellae* are as such distinguished from animals that have been immunized with an attenuated live vaccine according to the invention.

[0050] Livestock, such as poultry and in particular chicken are known to generate antibodies against flagellin gene products and in particular the *FliC* gene product. The antibodies in question will thus be detected in an animal infected by a wild-type strain (that generates such antibodies), yet not in an animal that has been vaccinated with a mutant strain wherein a flagellin gene(s) is/are inactivated. The latter do not generate antibodies against e.g. *FliC* and/or *FljB*.

[0051] The presence of said antibodies is indicative for the presence of wild-type strains and thus infection. The method of the invention thus advantageously allows detection or diagnosis of a *Salmonella* infection in animals vaccinated by a mutant strain wherein a flagellin gene(s) is/are inactivated. Such mutant strain may be one of the strains of the invention hereinabove described.

[0052] Inactivation of flagellin genes such as *fliC*, thus allows the use of serological tests, e.g. based on the detection of the *FliC* protein, for the diagnosis of the presence of wild-type strains, such as wild-type *S. enterica*, in (vaccinated) animals. In the method of the invention animals are preferably assayed for antibodies against *FliC*.

[0053] The method of the invention is in particular applicable to poultry, more preferably chickens.

Detailed description of the invention

[0054] It was surprisingly found that the combination of a flagellin mutation (an impairing modification in a gene coding for flagellin; also referred to as a flagellar mutation) and an auxotrophic mutation can lead to highly immunogenic attenuated *S. enterica* strains. The mutant strains according to the invention carry or comprise a (at least one) modification(s) in a motility gene(s) and a (at least one) modification(s) in a gene(s) involved in the survival or the proliferation of the pathogen in its host.

10 [0055] A gene involved in the survival or proliferation may be a house-keeping gene and/or a virulence gene. Examples of such housekeeping genes and virulence genes can be found in (Mastroeni et al., 2000, *The Veterinary Journal* 161:132-164, incorporated by reference herein). A preferred example of a house-keeping gene is the *guaB* gene, yet a modification in an *aro*, *pur*, *dap*, *pab*, *sipC*, *phoP*, *phoQ*, *pagC*, *cya*, and/or *crp* gene may also be envisaged.

[0056] The term "gene" as used herein refers to the coding sequence and its regulatory sequences such as promoter and termination signals.

[0057] Such inactivation may be obtained via a deletion by which the gene function is impaired. A person skilled in the art knows how to obtain such mutants and a simple test can tell whether the gene function is impaired. For instance, the mutant strain which fails to express a functional *guaB* gene product cannot grow on Minimal A medium, unless this medium is supplemented with (e.g. 0.3mM) guanine, xanthine, guanosine or xanthosine.

[0058] Another simple test can tell whether a motility gene function such as a flagellin gene function is impaired. These mutants do not swarm on LB medium containing 0.4% agar.

[0059] The modifications in the genes in question should result in attenuation of the mutant strains, preferably at

least to a degree that they are suitable for use in a live vaccine.

[0060] The Examples below show that additional mutations in a (at least one; one or more) motility gene(s) (*fliC*,
5 *fljB* and/or *fljBA*) advantageously alleviated the residual pathogenicity of a *guaB* deletion mutant, and improved the protection of the immunized animals against challenge with a lethal dose of wild-type *S. enterica*.

[0061] The flagellar filament of all members of the
10 genus *Salmonella* is a multimer of a single protein, the flagellin protein (van Asten et al., 1995, *Journal of Bacteriology* 177:1610-1613).

[0062] *FliC* is the phase 1 filament subunit protein of flagellin (Ciacchi-Woolwine et al., 1998, *Infection and*
15 *Immunity* 66:1127-1134).

[0063] *S. Typhimurium* has two flagellin genes (*fliC* and *fljB*) that are located at different sites of the chromosome and that show phase variation. The promoter of *fljB* forms part of a chromosomal fragment that can be inverted by
20 site-specific recombination. Depending on the orientation, either *fljB* is expressed together with *fljA*, the latter encoding a repressor of the *fliC* gene; or *fliC* is brought to expression. *E. coli*, another member of the *Enterobacteriaceae*, can also possess two flagellin genes
25 that respectively share homology with the *fliC* and *fljB* genes of *S. enterica* (Tominaga, 2004, *Genes Genet. Syst.* 79:1-8).

[0064] Inactivation of the *fliC* gene in *S. Enteritidis* (encoding the major flagellar protein) increased both the
30 safety and effectiveness of a vaccine administered to the inbred mouse line BALB/c. This line is very sensitive to systemic salmonellosis.

[0065] This proves amongst others that flagellin is not an essential antigen for the induction of a protective

immune response against *Salmonella* in BALB/c mice despite many indications therefore in literature.

[0066] *S. Enteritidis* flagellin is immunogenic in chickens and carries the H:g,m antigenic determinants (van Asten et al., 1995; Wyant et al., 1999, *Infection and Immunity* 67:1338-1346; Ogushi et al., 2001, *The Journal of Biological Chemistry* 276:30521-30526).

[0067] There is evidence that flagellae from various species of Gram-negative bacteria (e.g. those of *S. Enteritidis* and *S. typhi*) activate monocytes to produce proinflammatory cytokines (e.g. the tumor necrosis factor alpha) and mediate activation of interleukin-1 receptor-associated kinase (IRAK).

[0068] It is thus thought that Gram-negative flagellin plays an important and previously unrecognized role in the innate immune response to Gram-negative bacteria. *FliC* may be of particular importance during the course of infections in the gastrointestinal tract (Ciacci-Woolwine et al., 1998; Wyant et al., 1999; Moors et al., 2001, *Infection and Immunity* 69:4424-4429).

[0069] There is a lot of ambiguity in literature as to the degree to which flagella contributes to virulence in poultry and/or humans.

[0070] Van Asten et al. (2000, *FEMS Microbiol Lett.* 185:175-9) have shown that inactivation of the flagellin gene of *S. Enteritidis* strongly reduces (50-fold) invasion into Caco-2 cells (human colon carcinoma cell line), while the bacterial adherence was not really affected. Said report is limited to *in vitro* results.

[0071] Parker and Guard-Petter (2001, *FEMS Microbiology Letters* 204:287-291) on the other hand found that, upon oral challenge of chicks, a *fliC::Tn10* mutant was equally virulent to the wild-type. This indicates that the presence of flagellin was not necessary to achieve at least a

moderate level of invasion after oral challenge. When applied subcutaneously, flagellar mutants were significantly attenuated in comparison to the wild-type strain.

5 [0072] There are thus a lot of indications in the prior art that teach away from constructing e.g. *S. enterica* double (or triple) mutants according to the invention. Inactivation of one or more motility genes helps reduce the remaining virulence of for instance *guaB* deletion mutants
10 but there are other advantages as well.

[0073] The inactivation of e.g. the *fliC* gene advantageously allows the use of serological tests, based on the detection of antibodies directed against the *FliC* protein, for the diagnosis of the presence of wild-type *S.*
15 *enterica*, e.g. *S. Enteritidis*, in the (vaccinated) animals. Immunodetection is possible via ELISA, via RIA techniques and/or any other known immunological test or format.

[0074] IDEXX Laboratories has a test on the market (FlockChek[®] *Salmonella* Enteritidis Antibody Test Kit) to
20 reliably detect antibodies against H-antigenic determinants of the *FliC* flagellin of *S. Enteritidis* (H:g,m flagellar epitopes).

[0075] The above demonstrates that double and/or triple *S. enterica* mutants of the invention, bearing a (impairing)
25 genetic modification in a gene involved in survival or the proliferation of the pathogen in the host and in a gene(s) involved in motility have advantages over attenuated *S. enterica* strains that are in the art.

[0076] The mutant strains of the invention are highly
30 suitable for use in a live attenuated vaccine, as a live vector and/or a DNA-mediated vaccine. The term "vaccine" is meant to include prophylactic as well as therapeutic vaccines. Preferably the vaccine is prophylactic.

[0077] "Live vector" vaccines, also called "carrier

vaccines" and "live antigen delivery systems", comprise an exciting and versatile area of vaccinology (Levine et al, 1990, *Microecol. Ther.* 19:23-32). In this approach, a live viral or bacterial vaccine is modified so that it expresses
5 protective foreign antigens of another microorganism, and delivers those antigens to the immune system, thereby stimulating a protective immune response. Live bacterial vectors that are being promulgated include, among others, attenuated *Salmonella*.

10 [0078] An object of the invention is to provide attenuated mutant strains for use in a live vaccine, possibly a polyvalent live vaccine. By a "polyvalent vaccine" or "multivalent vaccine" is meant in particular a vaccine comprising antigenic determinants from a number of
15 different disease-causing organisms.

[0079] One of the objects of the invention is therefore to provide a vaccine against e.g. Salmonellosis comprising:

- a pharmaceutically effective or an immunizing amount of an attenuated mutant strain of the invention; and
- 20 - a pharmaceutically acceptable carrier or diluent.

[0080] Another object of the invention is to provide a live vector vaccine comprising:

- a pharmaceutically effective or an immunizing amount of an attenuated mutant strain of the invention, wherein
25 said mutant encodes and expresses a foreign antigen; and
- a pharmaceutically acceptable carrier or diluent.

[0081] The particular foreign antigen employed in the live vector is not critical to the present invention.

[0082] Still another object of the invention is to
30 provide a DNA-mediated vaccine comprising:

- a pharmaceutically effective amount or an immunizing amount of an attenuated mutant strain of the invention; wherein said mutant contains a plasmid which encodes and

expresses in a eukaryotic cell, a foreign antigen; and

- a pharmaceutically acceptable carrier or diluent.

[0083] Details as to the construction and use of DNA-mediated vaccines can be found in U.S. patent 5,877,159,

5 which is incorporated by reference herein in its entirety.

Again, the particular foreign antigen employed in the DNA-mediated vaccine is not critical to the present invention.

[0084] The decision whether to express the foreign antigen in the pathogen (using a prokaryotic promoter in a
10 live vector vaccine) or in the cells invaded by the pathogen (using an eukaryotic promoter in a DNA-mediated vaccine) may be based upon which vaccine construction for that particular antigen gives the best immune response in animal studies or in clinical trials, and/or, if the
15 glycosylation of an antigen is essential for its protective immunogenicity, and/or, if the correct tertiary conformation of an antigen is achieved better with one form of expression than the other (US Patent 5,783,196).

[0085] By a "pharmaceutically effective amount" is meant
20 an amount much greater than normal to overcome (prevent and/or treat) the disease in question, e.g. Salmonellosis. By an "immunizing amount" as used herein is in fact meant an amount that is able to induce a (protective) immune response in the animal that receives the pharmaceutical
25 composition/vaccine. The immune response invoked may be a humoral, mucosal, local and/or a cellular immune response.

As known in the art the necessary amounts may depend on age, sex, weight and many other factors.

[0086] The particular pharmaceutically acceptable
30 carriers or diluents employed are not critical to the present invention, and are conventional in the art. Examples of diluents include: buffer for buffering against gastric acid in the stomach, such as citrate buffer (pH 7.0) containing sucrose, bicarbonate buffer (pH 7.0) alone,

16
or bicarbonate buffer (pH 7.0) containing ascorbic acid, lactose, and optionally aspartame. Examples of carriers include: proteins, e.g., as found in skimmed milk; sugars; e.g. sucrose; or polyvinylpyrrolidone.

- 5 [0087] Deletion mutants according to the invention were created via standard homologous recombination techniques, whereby the entire gene(s) or at least part of the genes in question in a first step is replaced by a resistance gene and flanking FRT sites.
- 10 [0088] Preferably, in a second step, said resistance gene is removed by recombination between the two FRT sites. One FRT site and the priming sites P1 and P2 remain by the molecular mechanism of the recombination removing the antibiotics resistance gene according to Datsenko and
- 15 Wanner (2000) (see for instance Figure 4).

- [0089] The invention will be described in further details in the following examples and embodiments by reference to the enclosed drawings. Particular embodiments
- 20 and examples are not in any way intended to limit the scope of the invention as claimed. The rationale of the examples given here for *S. enterica* are equally well applicable to other (Gram-negative) bacteria infecting veterinary species, more in particular other (Gram-negative) bacteria
- 25 for poultry such as *Pasteurella*, (pathogenic) *E. coli*, etc.

Short description of the drawings

- [0090] Figure 1 gives a schematic overview of the biosynthetic pathway of guanosine monophosphate.
- 30 AICAR: 5'-phosphoribosyl-4-carboxamide-5-aminoimidazole; ATP: adenosine triphosphate; G: guanine; GMP: guanosine monophosphate; GR: guanosine; Hx: hypoxanthine; HxR: hypoxanthine riboside (inosine); IMP: Inosine monophosphate; X: Xanthine, XMP: Xanthosine monophosphate;

guaA: GMP synthetase, guaB: IMP dehydrogenase; guaC: GMP reductase.

[0091] Figure 2 represents contig 1294 of the *S. Enteritidis* genome (SEQ ID NO: 19). The ATG initiation
5 codon and TGA termination codon of the *guaB* gene are in bold. N can be A, C, T or G

[0092] Figure 3 represents the sequence of the Δ *guaB* fragment of *S. Enteritidis* cloned in pUC18 (SEQ ID NO: 20). The primers that were used are indicated by horizontal
10 arrows. The fragment generated with primers GuaB6-GuaB7 was cloned in pUC18. The ATG initiation and TGA termination codon of the *guaB* gene and the CCCGGG *Sma*I restriction site are indicated in bold.

[0093] Figure 4 represents the nucleotide sequence of
15 the *S. Enteritidis* PCR fragment, which includes the *guaB* deletion, obtained using primer GuaB10 (SEQ ID NO: 21). The PCR fragment was amplified with primers GuaB6-GuaB7, using total genomic DNA of the mutant SM20. The remaining FRT site is indicated in bold italic and the P1 and P2 primers
20 by arrows (Datsenko and Wanner, 2000, PNAS 97:6640-6645). The ATG initiation and TGA termination codon of the *guaB* gene are indicated in bold.

[0094] Figure 5 represents the *guaB* gene of *S. Typhimurium* LT2, section 117 of 220 of the complete genome
25 (SEQ ID NO: 22). The ATG initiation codon and TGA termination codon of the *guaB* gene are in bold.

[0095] Figure 6 shows the nucleotide sequence obtained after sequencing the PCR fragment amplified with primers FliC1-FliC2 on total DNA of the mutants SM73 and SM89,
30 using primer FliC1 and FliC2 (SEQ ID NO: 23). The remaining FRT site is indicated in bold italic, the ATG initiation and TAA stop codons in bold, and P1 and P2 are indicated with arrows.

[0096] Figure 7 shows the nucleotide sequence obtained

after sequencing the PCR fragment amplified with primers FljBA6-FljBA5 on total DNA of the *S. Typhimurium* mutant SM48, using primer FljBA6 (SEQ ID NO: 24). The remaining FRT site is indicated in bold, P1 and P2 are indicated with
5 arrows.

[0097] Figures 8-11 represent the deposit receipts for SM69, SM73, SM86 and SM89 respectively.

Examples

10 Example 1: auxotrophic mutation that affects the *guaB* gene

[0098] An auxotrophic insertion mutant of a wild type *S. Enteritidis* was obtained via insertion mutagenesis. Only when supplemented with 0.3M guanine, xanthine, guanosine or xanthosine could the mutant strain grow on Minimal A
15 medium.

[0099] These data strongly suggest that the auxotrophic mutation of the strain affects the *guaB* gene, encoding the enzyme IMP dehydrogenase (EC 1.1.1.205). This enzyme converts inosine-5'-monophosphate (IMP) into xanthosine
20 monophosphate (XMP) as indicated in Figure 1.

[0100] An insertion mutant can revert, thereby restoring the pathogenicity of the strain. This can limit its applicability in a live attenuated vaccine. In that aspect deletion mutants are preferred. *guaB* deletion
25 mutants of *S. Enteritidis* and *S. Typhimurium* were therefore created and tested. The *guaB* genes of both serovars are given in Figures 2 and 5.

Example 2: *guaB* deletion mutants

30 Construction of *guaB* deletion mutants

[0101] A method to generate deletion mutations in the genome of *E. coli* K12 that was previously published (Datsenko and Wanner, 2000, PNAS 97:6640-6645) was applied for this aim. This method relies on the homologous

recombination, mediated by the bacteriophage λ Red recombinase system, of a linear DNA fragment generated by PCR wherein the *guaB* sequence is substituted by an antibiotic resistance gene. This resistance gene is
5 surrounded by FRT sites and can be excised from the genome by site-specific recombination, mediated by the FLP recombinase.

[0102] Overlap PCR (Ho et al., 1989, Gene 77:51-59) was applied for the deletion of an internal segment of 861bp of
10 the *guaB* coding sequence. The principle relies on the use of two primer sets, GuaB3-GuaB4 (flanking the 5' end of the *guaB* gene) and GuaB5-GuaB2 (flanking the 3' end of the *guaB* gene). Both sets contain primers (GuaB4 and GuaB5) that are partially complementary and to which a *SmaI* restriction
15 site was added. After annealing of the resulting complementary sequences and chain elongation, PCR with the outward primers GuaB6 and GuaB7 generated a fragment with a 6 basepair *SmaI* site replacing an 861 basepair internal segment of the *guaB* coding sequence. This Δ *guaB* fragment
20 was cloned in the vector pUC18 (see Figure 3).

[0103] The chloramphenicol resistance gene (*cat*) with its flanking FRT sequences was amplified using the primers P1 and P2 (Datsenko and Wanner, 2000) and plasmid pKD3 DNA as a template. This PCR fragment was ligated in the *SmaI*
25 site of the cloned Δ *guaB* fragment. The desired fragment was generated using nested primers (GuaB6-GuaB7). The resulting PCR fragment was electroporated into *S. Enteritidis* 76Sa88 harbouring the temperature sensitive replication plasmid pKD46, encoding the bacteriophage Lambda Red recombinase
30 system. The chloramphenicol resistant transformants were tested on Minimal A medium and on Minimal A medium supplemented with 0.3 mM guanine. The Δ *guaB::catFRT* mutants were confirmed by PCR using the following primer combinations: GuaB6-GuaB7, GuaB6-P2, GuaB7-P1 and P1-P2.

- 78
- [0104] The *S. Enteritidis* Δ *guaB::catFRT* mutant (SM12) was electroporated with the temperature sensitive replication plasmid pCP20, encoding the FLP recombinase, to remove the *cat* gene. The resulting strain *S. Enteritidis* Δ *guaB* was named SM20. The PCR fragment in which the deletion is located was obtained using total genomic DNA of the mutant SM20 and the primer combination GuaB6-GuaB7. The Δ *guaB* mutation was confirmed by sequencing, using the primer GuaB10, of this fragment (see Figure 4).
- 10 [0105] The sequences of all above-mentioned primers are given in Table 1.
- [0106] To avoid the presence of possible additional mutations, caused by the expression of the Red recombinase system, an isogenic strain was constructed.
- 15 [0107] The Δ *guaB::catFRT* mutation of the mutant SM12 was transduced with bacteriophage P22 HT *int*⁻ (Davis, R.W., Botstein D. and Roth, J.R. (1980) *In Advanced Bacterial Genetics, A manual for genetic engineering*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) lysate of SM12
- 20 to wild type *S. Enteritidis* 76Sa88. The *cat* gene was removed using the plasmid pCP20. The resulting strain *S. Enteritidis* Δ *guaB* was called SM69 having deposit number LMG P-21641.
- [0108] A Δ *guaB* mutant of *S. Typhimurium* strain 1491S96
- 25 was constructed using the same procedure and the same primers. The resulting strain was named SM19. SM86 (having the deposit number LMG P-21646) is the isogenic strain obtained after transduction of Δ *guaB::catFRT* to *S. Typhimurium* strain 1491S96 using a bacteriophage P22 HT
- 30 *int*⁻ lysate of SM9, and after excision of the *cat* gene.
- [0109] The Δ *guaB* mutants SM19, SM20, SM69 and SM86 are sensitive to bacteriophage P22 HT *int*⁻. This proves the presence of intact lipopolysaccharides (LPS).

Virulence and protection tests with the *S. Enteritidis guaB* deletion mutant SM20 in mice.

[0110] The virulence of the mutant SM20 in mice was tested by oral infection of 6-8 week old female BALB/c mice (Pattery et al., 1999, Mol. Microbiol. 33(4):791-805) in two independent experiments. These were performed as described above. The wild type strain *S. Enteritidis* 76Sa88 was tested in parallel as a positive control. The *S. Enteritidis* 76Sa88 Δ aroA mutant SM50 was included in the experiment as a vaccine control. This mutant carries a precise deletion of the complete *aroA* coding sequence and was constructed by the method of Datsenko and Wanner (2000).

[0111] The complete data are given in Tables 2 and 3. These results demonstrate that the Δ guaB mutant SM20 is strongly attenuated in mice but still shows some residual pathogenicity when administered at this high dose. Oral immunization with this mutant induces protective immunity against infection by a high dose of the corresponding pathogenic wild type *S. Enteritidis* strain 76Sa88. The protection is at least equal to the protection conferred by the *S. Enteritidis* Δ aroA mutant SM50.

Virulence and protection tests with the isogenic *guaB* deletion mutants SM69 and SM86 in mice.

[0112] The virulence of the mutants SM69 and SM86 in mice was tested by oral infection of 6-8 week old female BALB/c mice. These were performed as described above. The wild type strains *S. Enteritidis* 76Sa88 and *S. Typhimurium* 1491S96 were tested in parallel as positive controls.

[0113] The complete data are given in Tables 6, 7, and 10-13. These results demonstrate that the Δ guaB mutants SM69 and SM86 are strongly attenuated in mice, yet still show some residual pathogenicity when administered at this

14

high dose. Oral immunization with the mutants induces protective immunity against infection by a high dose of the corresponding pathogenic wild type strain.

5

Example 3: Flagellin mutants of *S. Enteritidis* and *S. Typhimurium*

[0114] It was then tested whether an additional (a further) modification in a motility gene (e.g. a flagellin gene) could further reduce the residual pathogenicity that remained in single mutants like SM20 that carry a deletion mutation in the *guaB* gene.

[0115] *S. Enteritidis* strains that contain only one gene coding for flagellin, *fliC*, were used in preliminary experiments. Double mutants were constructed wherein the *guaB* and *fliC* genes of *S. Enteritidis* were inactivated. For *S. Typhimurium*, double (Δ *guaB* Δ *fliC*; Δ *guaB* Δ *fljBA*) and triple (Δ *guaB* Δ *fliC* Δ *fljBA*) mutants were constructed.

20 Construction of Δ *fliC* mutants (SM24, SM30)

[0116] PCR using the *FliCP1-FliCP2* primer combination on the template plasmid pKD3 (*catFRT*) or pKD4 (*kanFRT*) amplifies the recombinant fragment which contain the antibiotic resistance gene together with the FRT sites and priming sites P1 and P2, and extensions homologous to the initial 50 (1-50) and the terminal (1468-1518) 50 nucleotides of the *fliC* coding sequence. In this region, *S. Typhimurium* 1491S96 and *S. Enteritidis* 76Sa88 show respectively 100% and 98% sequence identity with the primers. The primer *FliCP1* contains an additional G at position 37 compared to SEQ ID NO 22. Therefore the Δ *fliC* mutant allele encodes a 16 amino acid peptide, of which the first 12 amino acids correspond to the amino terminus of

FliC. An internal segment of 1416 bp (51-1467) of the *fliC* coding sequence (1-1518) will be substituted.

[0117] The resulting PCR product (1 µg) was electroporated to *S. Typhimurium* 1491S96 (pKD46) and
5 *S. Enteritidis* 76Sa88 (pKD46), previously induced with 0.2% arabinose, encoding the Lambda recombinase system.

[0118] Antibiotic resistant candidate substitution mutants were confirmed by PCR, using primers FliC1 and FliC2 and total DNA of the mutant strains and the wild type
10 strain. Restriction analysis was carried out to distinguish between PCR fragments with approximately the same size. For the restriction of the wild type *S. Typhimurium* PCR fragment amplified with FliC1-FliC2, the enzyme *EcoRV* was used. Two fragments (470bp and 1021bp) were obtained. The
15 fragment amplified for the *fliC* substitution mutant doesn't contain an *EcoRV* restriction site. In case of *S. Enteritidis* the enzyme *ApoI* was used. This enzyme cuts the wild type *fliC* fragment of *S. Enteritidis* in 2 pieces (345bp and 1147bp). The fragment obtained for the *fliC*
20 substitution mutant doesn't contain an *ApoI* restriction site.

[0119] The motility of the mutants was tested on LB medium (Miller, 1992, *A short course in bacterial genetics, a laboratory manual and handbook for Escherichia coli and
25 related bacteria*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) with 0.4% agar. Wild type *S. Typhimurium* and wild type *S. Enteritidis* swarm on this medium. The *S. Typhimurium fliC* substitution mutant swarms out, as the *fljB* gene is still present in the mutant. The
30 *S. Enteritidis fliC* substitution mutant doesn't swarm anymore. These results were confirmed by microscopic observation.

[0120] Electrocompetent cells of the different mutants

were prepared and transformed by electroporation with plasmid pCP20 (extracted from *S. Typhimurium* χ 3730, R. Curtiss, III, S.M. Kelly, P.A. Gulig, C.R. Gentry-Weeks and J.E. Galán. Avirulent *Salmonella* expressing virulence antigens from other pathogens for use as orally-administered vaccines. In: J. Roth, Editor, *Virulence Mechanisms*, American Society for Microbiology, Washington DC (1988), p. 311) to remove the antibiotic resistance gene. The transformants were incubated at 43°C. This will eliminate the temperature sensitive pCP20 plasmid and should eliminate the antibiotic resistance gene. The loss of the antibiotic resistance gene in *S. Typhimurium* and *S. Enteritidis* Δ fliC::catFRT mutants was confirmed.

[0121] The deletion mutants, originating from the chloramphenicol resistant substitution mutants, were confirmed by PCR using the primer combination FliC1/FliC2. For both *S. Typhimurium* Δ fliC and *S. Enteritidis* Δ fliC a fragment of 185bp was amplified.

[0122] The deletion was confirmed by sequencing the amplified fragments using primer FliC3.

[0123] The obtained mutants were tested on LB medium containig 0,4% agar: wild type *S. Typhimurium* and wild type *S. Enteritidis* swarms out on this medium, also *S. Typhimurium* Δ fliC swarms out (*fljB* flagellar gene is still present). *S. Enteritidis* Δ fliC as expected doesn't swarm out.

Construction of Δ fljBA mutants (SM48)

[0124] *S. Typhimurium* contains a second flagellin gene, *fljB*. This gene is expressed together with *fljA*, that codes for the repressor of *fliC*. In the present case, both *fljA* and *fljB* were deleted. *FljB* is 1520bp long and codes for the protein flagellin. *FljA* is 539bp long and codes for the

repressor of *fliC*. The total length of the fragment that was deleted (*fljBA*): 2127bp.

[0125] Primers were designed which show 51 nucleotides homology with sequences of the *fljBA* gene and homology with sequences of the template plasmid, which flank the antibiotic resistance gene and FRT sites. Primer FljBAP1 shows homology with the sequence starting from the startcodon of *fljB* till 51bp downstream (1-51) and primer FljBAP2 shows homology with the sequence starting from the stop codon of *fljA* till 51bp upstream (2076-2127). Primers FljBAP1 and FljBAP2 show homology at their 3' ends with the priming sites P1 and P2 in the template plasmid flanking the resistance gene with the FRT sites.

[0126] PCR using primers FljBAP1 and FljBAP2 (sequences in table 1) and template DNA pKD3 (*catFRT*) or pKD4 (*kanFRT*) amplified fragments of the desired length.

[0127] 1 µg of the PCR product was electroporated to *S. Typhimurium*, transformed with pKD46 or pKD20. The selected kanamycine and chloramphenicol resistant transformants were confirmed by PCR.

[0128] The mutants were tested on LB medium containing 0,4% agar. Wild type *S. Typhimurium*, *S. Typhimurium* $\Delta fljBA::kanFRT$ and *S. Typhimurium* $\Delta fljBA::catFRT$ swarm out (*fliC* is still present). Motility of the three strains was confirmed by microscopic observation.

[0129] Electrocompetent cells of the different mutants were electroporated with the pCP20 plasmid (originated from *S. Typhimurium* LT2 restriction mutant $\chi 3730$) to remove the antibiotic resistance gene. After 2 hours of incubation at 28°C the culture was plated on LB medium with

carbenicilline. After incubation of the transformants at 43°C on LB, they were tested on the loss of the plasmid and the antibiotic resistance gene. The deletion mutations were confirmed by means of PCR and sequencing of the fragment.

5 [0130] PCR using primer combination FljBA6/FljBA5 (sequence in table 1) amplified a fragment of 2112bp for the wild type *S. Typhimurium* and a fragment of 185bp for the *S. Typhimurium* $\Delta fljBA$ mutant SM48.

[0131] The deletion in mutant SM48 was confirmed by
10 sequencing using primer FljBA6 on the PCR fragment obtained using primers FljBA6-FljBA5 (Figure 7).

Construction of the *S. Typhimurium* 1491S96 $\Delta fljBA \Delta fliC$ double mutant (SM23)

15 [0132] The strain *S. Typhimurium* $\Delta fljBA::kanFRT$ (pKD46) was used to construct the double mutant. Electrocompetent cells were prepared at a temperature of 28°C (temperature sensitive plasmid pKD46). The electrocompetent cells were electroporated with the recombinant *fliC* fragment, in which
20 the *fliC* gene is substituted with the chloramphenicol resistance gene (see earlier examples). To screen and confirm the candidate mutants the procedure used in the construction of the *fliC* mutant was followed. The desired genotype: *S. Typhimurium* $\Delta fljBA::kanFRT \Delta fliC::catFRT$. To
25 eliminate the antibiotic resistance gene the protocol previously described was followed. The deletions in *S. Typhimurium* $\Delta fljBA \Delta fliC$ (SM23) were confirmed by PCR.

[0133] The double mutant *S. Typhimurium* $\Delta fljBA \Delta fliC$ (SM23) was as expected not motile on LB medium with 0,4%
30 agar. The non-motility of the strain was confirmed by microscopic observation.

Combination of the auxotrophic and the flagellar mutations

[0134] P22-transduction (Davis, R.W., Botstein D. and Roth, J.R. (1980) *In Advanced Bacterial Genetics, A manual for genetic engineering*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) was used to combine the
5 mutations. P22-lysates of the substitution mutants were used to construct the combined deletion mutants. The transduction was confirmed by PCR (the same protocol and primers were used as in previous confirmations). For the elimination of the antibiotic resistance genes the
10 previously described protocol using the pCP20 helper plasmid was used. The deletions were confirmed by PCR. Mutants constructed this way are: *S. Typhimurium* $\Delta fliC$ $\Delta guaB$ (SM32), *S. Typhimurium* $\Delta fljBA$ $\Delta guaB$ (SM35), *S. Typhimurium* $\Delta fliC$ $\Delta fljBA$ $\Delta guaB$ (SM27) and *S. Enteritidis*
15 $\Delta fliC$ $\Delta guaB$ (SM21).

Construction of isogenic deletion mutants

[0135] To exclude the possibility that additional unknown mutations (which can have an effect on the
20 attenuation of the strains) are present in the candidate vaccine strains, dedicated to the use of the method described by Datsenko and Wanner (2000) for the construction of the deletion mutants, isogenic deletion mutants were constructed. The mutations were transduced to
25 a wild type background, by means of P22 phage transduction (Davis, R.W., Botstein D. and Roth, J.R. (1980) *In Advanced Bacterial Genetics, A manual for genetic engineering*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). The antibiotic resistant substitution mutants were used as
30 donor strain. For the elimination of the antibiotic resistance genes and confirmation of the deletions, the same protocols were used as in the previous experiments.

[0136] Constructed mutants: *S. Enteritidis* $\Delta guaB$

(SM69, having the deposit number LMG P-21641); *S. Enteritidis* $\Delta fliC$ (SM71); *S. Typhimurium* $\Delta guaB$ (SM86, having the deposit number LMG P-21646); *S. Typhimurium* $\Delta fliC$ (SM91); *S. Typhimurium* $\Delta fljBA$ (SM90), *S. Enteritidis* $\Delta guaB \Delta fliC$ (SM73, having the deposit number LMG P-21642); *S. Typhimurium* $\Delta guaB \Delta fliC$ (SM104); *S. Typhimurium* $\Delta guaB \Delta fljBA$ (SM87); *S. Typhimurium* $\Delta fljBA \Delta fliC$ (SM83); *S. Typhimurium* $\Delta guaB \Delta fljBA \Delta fliC$ (SM89, having the deposit number LMG P-21643).

10

Example 4: virulence and protection experiments with *S. Enteritidis* vaccine strains

Effect of the inactivation of the *fliC* gene on the virulence of a *S. Enteritidis* vaccine strain.

15 [0137] To study the effect of the inactivation of the *fliC* gene on the immunogenicity of a *S. Enteritidis* vaccine strain, two independent virulence and protection tests were carried out in 7 weeks old female BALB/c mice with both mutant SM20 ($\Delta guaB$) and SM 21 ($\Delta guaB \Delta fliC$) (Tables 4 and
20 5).

[0138] For the virulence assay, the mice were orally infected with a dose of about 10^8 CFU, which corresponds to approximately 10^5 times the LD₅₀ of the wild type strain (Pattery et al., 1999, *Molecular Microbiology* 33:791-805).
25 The mice were observed during 21 days. All mice inoculated with the wild type *S. Enteritidis* strain 76Sa88 died within 9 days after infection, while the non-infected control mice remained healthy during the observation period of 21 days. In the first experiment mice infected with the *S. Enteritidis* $\Delta guaB$ mutant SM20 showed typical disease
30 symptoms (reduced activity, untidy coat and curved back) and one out of ten died. In the second experiment no

disease symptoms were observed with SM20. The *S. Enteritidis* Δ *guaB* Δ *fliC* mutant SM21 was asymptomatic in both experiments.

5 Efficacy of the mutants SM20 and SM21 to confer protection:
protection tests

[0139] The efficacy of the mutants SM20 and SM21 to confer protection was tested three weeks after the initial immunization by oral challenge with about 10^5 LD50 of the
10 wild type *S. Enteritidis* strain 76Sa88 (LD50= 10^3 CFU). The mice were observed during 21 days. All non-immunized mice died after challenge. In the second experiment, one out of three mice vaccinated with SM20 died. All other vaccinated mice survived the challenge without observable disease
15 symptoms. These data show that both mutants are attenuated and confer protection against challenge with the corresponding wild type strain.

[0140] To ascertain that no additional unknown mutations were present, which could contribute to the attenuation of
20 the candidate vaccine strains, the mutations containing the selectable resistance genes were transferred to a wild type background by P22 transduction (Davis, R.W., Botstein D. and Roth, J.R. (1980) In *Advanced Bacterial Genetics*, A manual for genetic engineering. Cold Spring Harbor
25 Laboratory, Cold Spring Harbor, N.Y.).

Efficacy of the inactivation of the *fliC* gene on the virulence of *S. Enteritidis*: virulence and protection tests with isogenic strains.

30 [0141] The virulence and protection tests in BALB/c mice were repeated with the isogenic strains SM69 (Δ *guaB*), SM71 (Δ *fliC*) and SM73 (Δ *guaB* Δ *fliC*) after confirmation by PCR and phenotypic characterization as earlier described. In this virulence assay, a Δ *fliC* mutant was included, to study

the effect of the inactivation of the *fliC* gene on the virulence of *S. Enteritidis*. Similar conditions as described above were used for the virulence and challenge experiments. Data obtained for the transductants SM69 and
5 SM73 (Tables 6 and 7) confirmed the observations made in the earlier experiments.

[0142] Comparison between both *guaB* deletion mutants indicates that the *S. Enteritidis* Δ *guaB* Δ *fliC* double mutant SM73 is more attenuated and confers better protection
10 against challenge with high doses of the wild type strain than the *S. Enteritidis* Δ *guaB* single mutant SM69. The virulence assay performed with the *S. Enteritidis* Δ *fliC* mutant SM71 showed that this mutant remained as virulent as the wild type strain under the applied conditions.

15

Immunological responses and antibody production

[0143] Fifty-four days following the initial immunization in the first experiment, blood samples were collected from the tail artery of the mice. Anti-
20 lipopolysaccharide (LPS) IgG titers were determined by means of enzyme-linked immunosorbent assay (ELISA) using *S. Enteritidis* LPS (Sigma) for coating. Comparison between sera of mice immunized with SM20 and SM21 showed that in both cases anti-LPS serum IgG responses were elicited and
25 that no significant differences in titer were measured. Oral immunization with a second and third dose, 66 and 95 days after the initial immunization did not enhance the anti-LPS IgG levels in serum (data not shown).

30 Example 5: virulence and protection experiments with *S. Typhimurium* transductants

[0144] Virulence and protection experiments were carried out with the transductants in 7 weeks old female BALB/c mice. The mice were orally inoculated with approximately

10^8 cells. The mice were observed daily for a period of 21 days. After this period, the mice were challenged with 10^8 cells of the wild type pathogenic strain, and the mice were observed over a period of 21 days.

5 [0145] Symptoms of disease and the survival rate are noted in Tables 10-13. The single and double flagellar mutants remained highly virulent when orally administered, all mice died. The mice inoculated with the *guaB* mutant showed mild symptoms of diseases (mice had been fighting,
10 only two remained alive). The combined Δ *guaB* Δ *fljBA*, Δ *guaB* Δ *fliC* and Δ *guaB* Δ *fliC* Δ *fljBA* mutants were highly attenuated. No symptoms of disease were observed during the 21 days observation period after vaccination. These mutants conferred good protection after challenge with a high dose
15 of the wild type strain. Only a reduced activity of the mice after challenge can be observed.

[0146] These results also show that the flagellar mutations do not affect the immunogenic capacities of the strains when administered to BALB/c mice. The flagellar
20 mutations can be useful as a serological marker to distinguish between the vaccine strain and the wild type strain. Combination of the auxotrophic mutation with the flagellar mutation(s) gives the best results concerning the reduced virulence of the mutants in mice and the protection
25 against the corresponding wild type strain.

[0147] The attenuation of the *S. Typhimurium* Δ *guaB* Δ *fliC* Δ *fljBA* triple deletion mutant and the *S. Typhimurium* double deletion mutants, Δ *guaB* Δ *fljBA* and Δ *guaB* Δ *fliC*, were comparable.

30

Example 6: Safety evaluation of *S. enteritidis* vaccine strains

Safety evaluation SM69 in chicks inoculated at the age of one day by the intratracheal or oral gavage route

[0148] The objective of this study was to evaluate the safety of *S. Enteritidis* Δ guaB mutant strain SM69 master seed in one-day-old chickens. Mortality was used as a primary parameter for the determination of safety.

5 [0149] Chicks at one day of age were leg-banded and randomly placed in each of the four treatment groups (Group 1: SM69-IT, group 2: SM69-OG, group 3: PBS-IT and Group 4: PBS-OG). After the master seed inoculation, the birds from groups 1 and 2 were placed in one isolator and those of
10 groups 3 and 4 in another isolator.

[0150] Chickens in groups 1 and 2 were inoculated with the SM69 master seed by the intratracheal (IT) route or oral gavage (OG) route, respectively, with an actual titer of 1.3×10^8 CFU/0.2 ml per bird. Chickens in groups 3 and
15 4 were administered with 0.2 ml PBS (phosphate buffered saline) per bird by the intratracheal or oral gavage, respectively.

[0151] Following the inoculation of SM69 or PBS, chick mortality was observed daily until 38 days post
20 inoculation. Table 8 summarizes the results of mortality for all 4 groups. In group 1, one bird died during the inoculation due to inoculation trauma. Two birds died at 2 days post inoculation (DPI). Three birds died from day 3 to day 13 (at 3, 5 and 13 DPI respectively). A total of 6
25 birds thus died in group 1. In group 2, two birds died in total. One died due to inoculation trauma and one died at day 5 post inoculation. No birds died in the PBS treated groups either by the intratracheal or oral gavage route.

[0152] This study indicates that the *S. Enteritidis*
30 Δ guaB mutant strain SM69 is not safe when administered at 1.3×10^8 CFU per bird at one day of age by the intratracheal or oral gavage route.

Safety evaluation of SM69 in chicks inoculated at the age

of 2 weeks by the intratracheal or oral gavage route

[0153] Safety of the *S. Enteritidis* Δ guaB mutant strain SM69 was then evaluated in 2 week-old SPF chickens by the intratracheal and oral gavage routes. Mortality was used as
5 a primary criterion and body weight as a secondary criterion for the determination of safety.

[0154] Birds at 2 weeks of age were leg-banded and randomly placed in each of the four treatment groups: SM69-IT, SM69-OG, Poulvac ST-IT and PBS-IT. Ten birds in group 1
10 were inoculated with SM69 by the intratracheal route; ten birds in group 2 were inoculated with SM69 by oral gavage; ten birds in group 3 were inoculated with a *Salmonella* Typhimurium AroA⁻ vaccine (Poulvac[®] ST) by the intratracheal route; and five birds in group 4 were
15 inoculated with PBS by the intratracheal route.

[0155] Chickens in groups 1 and 2 were inoculated with SM69 master seed by the intratracheal or oral gavage route, respectively, with the actual titer of 2.3×10^8 CFU/0.2 ml per bird. Chickens in group 3 were administered with
20 Poulvac[®] ST by the intratracheal route with 2.2×10^8 CFU/0.2 ml per bird. Chickens in group 4 were administered by the intratracheal route with 0.2 ml PBS per bird.

[0156] After inoculation, the birds from treatment groups 1 and 2 were placed in one isolator and those from
25 groups 3 and 4 in another isolator.

[0157] Following inoculations, mortality was observed daily until 21 days post-inoculation. Body weight of all birds was also recorded at the end of the study period (21 days). Poulvac[®] ST and PBS were used as intratracheal
30 procedure controls.

[0158] During the 21-day observation period, one bird in the SM69 intratracheal treatment group (group 1) died from an infected yolk sac. No mortality was associated with SM69 inoculation, indicating that the SM69 strain is safe at the

titer tested, 2.3×10^8 CFU per bird by the intratracheal and oral gavage routes. As expected, no death was observed either in the Poulvac® ST treated birds at the titer of 2.2×10^8 CFU per bird or in the PBS treated birds, indicating that the study was valid (Table 9).

[0159] Body weight was compared amongst groups in an analysis of variance (ANOVA) model with body weight as the dependent variable and treatment included as an independent variable. Group comparisons were made using Tukey's test for multiple comparisons. The level of significance was set at $p < 0.05$. The study was considered valid because the control chickens (PBS control group) remained healthy and free of clinical signs of diseases or mortality throughout the study.

[0160] There were no significant differences in the final body weight in chickens administered with SM69 by the intratracheal or oral gavage inoculation, Poulvac® ST, or PBS (Table 9). Even though no baseline was established of the birds in each group at one day of age, it was unlikely that there was a significant difference in the initial body weight amongst the four groups since the birds were randomly placed into each of the 4 treatment groups.

[0161] It can be concluded from the present experiment that SM69 is safe when administered at the tested titer of 2.3×10^8 CFU per bird at 2 weeks of age by either the intratracheal or oral gavage route.

Safety evaluation of SM73 in chicks inoculated at the age of one day by the intratracheal or oral gavage route

[0162] Safety of the *S. Enteritidis* deletion mutant strain SM73 (Δ guaB Δ fliC) was evaluated in chickens by the intratracheal and oral gavage routes. Mortality was used as a primary criterion and body weight as a secondary criterion for the determination of safety.

[0163] All birds were leg-banded and randomly assigned to one of the four groups of birds included in this study (Group 1: SM73-IT, group 2: SM73-OG, group 3: Poulvac ST-IT and Group 4: PBS-IT). Ten birds in group 1 were inoculated
5 with SM73 by the intratracheal route; ten birds in group 2 were inoculated with SM73 by oral gavage; ten birds in group 3 were inoculated with a *S. Typhimurium* AroA⁻ vaccine (Poulvac® ST) by the intratracheal route; and five birds in group 4 were inoculated with PBS by the intratracheal
10 route. A total of 4 isolators was used (one for each group) in which the chickens were housed for the duration of the study.

[0164] Chickens were inoculated at one day of age. Chickens in groups 1 and 2 were inoculated with SM73 master
15 seed by the intratracheal or oral gavage route, respectively, with an actual titer of 2.5×10^7 CFU/0.2 ml per bird. Chickens in group 3 were administered with Poulvac® ST by the intratracheal route with 2.1×10^7 CFU/0.2 ml per bird. Chickens in group 4 were administered
20 by the intratracheal route with 0.2 ml PBS per bird.

[0165] Mortality was observed and body weight recorded as described above for SM69. Poulvac® ST and PBS were once again used as intratracheal procedure controls.

[0166] During the 21-day observation period, no
25 mortality was recorded for any bird at all. There was further no difference in the final body weight of PBS-IT, Poulvac ST-IT and SM73-OG inoculated birds. The average body weight of the SM73-IT groups was significantly lower in comparison to the SM73-OG group ($p = 0.0009$) but this is
30 most probably due to an experimental error.

[0167] It can be concluded from the present experiment that SM73 is safe when administered at the tested titer of a 2.5×10^7 CFU per bird at one day of age by the intratracheal or oral gavage route.

74

[0168] A deposit has been made according to the Budapest Treaty at the BCCM/LMG Culture Collection, Laboratorium voor Microbiologie, K.L. Ledeganckstraat 35, B-9000 Gent
5 (Belgium) for the following micro-organisms: *Salmonella* Enteritidis SM69 under deposit number LMG P-21641 (deposit date: 9 August, 2002); *S. Enteritidis* SM73 under deposit number LMG P-21642 (deposit date: 9 August, 2002), *S. Typhimurium* SM86 under deposit number LMG P-21646 (deposit
10 date: 28 August, 2002) and *S. Typhimurium* SM89 under deposit number LMG P-21643 (deposit date: 9 August, 2002). The deposits have been made in the name of Prof. J.-P. Hernalsteens, previous address: Vrije Universiteit Brussel, Laboratorium Genetische Virologie, Paardenstraat 65, B-1640
15 Sint-Genesius-Rhode, current address: Vrije Universiteit Brussel, Onderzoeksgroep Genetische Virologie, Pleinlaan 2, B-1050 Brussels, Belgium.

Table 1 : Primer sequences

SEQ ID NO	Primer	Sequence
1	GuaB2	5' CGTTCAGCG CAACAGGCG TTGT 3'
2	GuaB3	5' GGCTGGGATT GGCAGGTAG TA 3'
3	GuaB4	5' GGATGATCCG GGCCTCAAC GTCAGGGCTT CTTTA 3'
4	GuaB5	5' TTGACGCCG GGATCACCA AGAGTCCCG AACTA 3'
5	GuaB6	5' GCAACACTC CTGCTGGTTA 3'
6	GuaB7	5' AGACCGAGGA TCACTTTATC 3'
7	GuaB10	5' AGGAAGTTG AGAGGATAA 3'
8	P1	5' GTGTAGGCTG GAGCTGCTTC 3'
9	P2	5' CATATGAATA TCCTCCTTAG 3'
10	FliCP1	5' ATGGCACAAG TCATTAAATAC AACAGCCCTG TCGCTGGTTG ACCAGAATA ATGTGTAGGC TGGAGCTGCT TC 3'
11	FliCP2	5' CGCATTAAACG CAGTAAAGAG AGGACGTTT GCGGAACCTG GTMGCCCTGC GCCACATATG AATATCCTCC TTAG 3'
12	FliC1	5' ATGGCACAAG TCATTAAATAC AACAG 3'
13	FliC2	5' CGCATTAAACG CAGTAAAGAG AGGAC 3'
14	FliC3	5' TATCGCAAT CTGGAGGCAA 3'
15	FljBAP1	5' ATGGCACAAG TAATCAACAC TAACAGTCTG TCGCTGCTGA CCCAGAATAA CTGTGTAGGC TGGAGCTGCT TC 3'
16	FljBAP2	5' TTATTACAGC TAGTCCGAAG ACGTGATCCT GCTCAACCAG TCAACACATAA CCATATGAAT ATCCTCCTTA G 3'
17	FljBA5	5' CAGCGTAGTC CGAAGACGTC ATC 3'
18	FljBA6	5' AACTAACAG TCTGTGCTG CT 3'

Table 2: Virulence test in BALB/c mice of the *S. Enteritidis* Δ guaB mutant SM20

Infection		Survival	Day of death	State of the mice
Strain	Dose			
First Experiment				
Negative control: milk	/	11/11	/	No disease symptoms
Positive control : <i>S. Enteritidis</i> 76Sa88	2.1×10 ⁸	0/5	7, 7, 8, 8, 9	
Vaccine control: <i>S. Enteritidis</i> ΔaroA SM50	2.5×10 ⁸	10/10	/	No disease symptoms
<i>S. Enteritidis</i> AguaB SM20	5.1×10 ⁸	9/10	13	Disease symptoms between the 7 th and the 14 th day after infection
Second Experiment				
Negative control: milk	/	4/4	/	No disease symptoms
Positive control : <i>S. Enteritidis</i> 76Sa88	1.4×10 ⁸	0/3	8, 9, 9	
Vaccine control: <i>S. Enteritidis</i> ΔaroA SM50	2.1×10 ⁸	3/3	/	No disease symptoms
<i>S. Enteritidis</i> AguaB SM20	1.9×10 ⁸	3/3	/	No disease symptoms

Table 3: Challenge of mice vaccinated with the *S. Enteritidis* guaB mutant SM20

Vaccination		Challenge		Survival	Day of death	State of the mice
Strain	Dose	Strain	Dose			
First Experiment						
Negative control: milk	/	negative control: milk	/	6/6	/	No disease symptoms
Negative control: milk	/	S. Enteritidis 76Sa88	1.5x10 ⁸	0/5	7, 8, 8, 9	Disease symptoms starting on the 5 th day after challenge
Vaccine control: S. Enteritidis ΔaroA SM50	2.5x10 ⁸	S. Enteritidis 76Sa88	1.5x10 ⁸	3/5	9, 13	Disease symptoms between the 7 th and the 14 th day after challenge
S. Enteritidis ΔguaB SM20	5.1x10 ⁸	S. Enteritidis 76Sa88	1.5x10 ⁸	5/5	/	Mice are less active between the 11 th and the 14 th day after challenge.

Table 3 continued:

Vaccination		Challenge		Survival	Day of death	State of the mice
Strain	Dose	Strain	Dose			
Second Experiment						
Negative control: milk	/	negative control: milk	/	2/2	/	No disease symptoms
Negative control: milk	/	S. Enteritidis 76Sa88	1.5x10 ⁸	0/2	9,18	
Vaccine control: S. Enteritidis ΔaroA SM50	2.1x10 ⁸	S. Enteritidis 76Sa88	1.5x10 ⁸	1/3	9,21	Disease symptoms between the 7 th and the 21 st day after challenge.
S. Enteritidis AguaB SM20	1.9x10 ⁸	S. Enteritidis 76Sa88	1.5x10 ⁸	2/3	10	Disease symptoms starting on the 9 th day after infection.

Table 4: Virulence test in BALB/c mice of the *S. Enteritidis* mutants SM20 and SM21

Infection		Survival	Day of death	State of the mice
Strain	Dose			
First Experiment				
Negative control : not infected	/	11/11	/	Asymptomatic
Positive control : <i>S. Enteritidis</i> 76Sa88	2.1x10 ⁸	0/5	7,7,8,8,9	Severe symptoms from day 5 onwards
<i>S. Enteritidis</i> ΔguaB SM20	5.1x10 ⁸	9/10	13	Mild to severe symptoms from day 7 till day 17
<i>S. Enteritidis</i> ΔguaB ΔfliC SM21	4.3x10 ⁸	10/10	/	Asymptomatic
Second Experiment				
Negative control : not infected	/	4/4	/	Asymptomatic
Positive control : <i>S. Enteritidis</i> 76Sa88	1.4x10 ⁸	0/3	8,9,9	Severe symptoms from day 6 onwards
<i>S. Enteritidis</i> ΔguaB SM20	1.9x10 ⁸	3/3	/	Asymptomatic
<i>S. Enteritidis</i> ΔguaB ΔfliC SM21	3.2x10 ⁸	3/3	/	Asymptomatic

22

Table 5: Challenge of mice vaccinated with the *S. Enteritidis* mutants SM20 and SM21

Vaccination		Challenge		Survival	Day of death	State of the mice
Strain	Dose	Strain	Dose			
First Experiment						
Negative control: milk	/	S. Enteritidis wild type strain 76Sa88	1.6x10 ⁸	0/5	7, 8, 8, 8, 9	Severe symptoms onwards from day 6
S. Enteritidis wild type strain 76Sa88	2.1x10 ⁸	S. Enteritidis wild type strain 76Sa88	1.6x10 ⁸	/	/	/
S. Enteritidis ΔguaB SM20	5.1x10 ⁸	S. Enteritidis wild type strain 76Sa88	1.6x10 ⁸	4/4	/	Asymptomatic
S. Enteritidis ΔguaB ΔfliC SM21	4.3x10 ⁸	S. Enteritidis wild type strain 76Sa88	1.6x10 ⁸	5/5	/	Asymptomatic

Table 5: Continuation

Vaccination		Challenge		Survival	Day of death	State of the mice
Strain	Dose	Strain	Dose			
Second Experiment						
Negative control: milk	/	S. Enteritidis wild type strain 76Sa88	1.5x10 ⁸	0/2	9, 18	Severe symptoms onwards from day 6
S. Enteritidis wild type strain 76Sa88	1.4x10 ⁸	S. Enteritidis wild type strain 76Sa88	1.5x10 ⁸	/	/	/
S. Enteritidis Δ guaB SM20	1.9x10 ⁸	S. Enteritidis wild type strain 76Sa88	1.5x10 ⁸	2/3	10	No symptoms, except one with severe symptoms
S. Enteritidis Δ fliC SM21	3.2x10 ⁸	S. Enteritidis wild type strain 76Sa88	1.5x10 ⁸	3/3	/	Asymptomatic

Table 6: Virulence test in BALB/c mice of the *S. Enteritidis* mutants SM71, SM73 and SM69

Infection		Survival	Day of death	State of the mice
Strain	Dose			
Negative control : not infected	/	4/4	/	Asymptomatic
Positive control : <i>S. Enteritidis</i> 76Sa88	3.7×10^8	0/3	7, 8, 9	Severe symptoms onwards from day 5
<i>S. Enteritidis</i> Δ fliC SM71	1.4×10^8	0/3	6, 8, 8	Severe symptoms onwards from day 4
<i>S. Enteritidis</i> Δ guaB SM69	7.6×10^8	5/5	/	Mild symptoms, from day 11 till day 18
<i>S. Enteritidis</i> Δ guaB Δ fliC SM73	1.2×10^8	5/5	/	Reduced activity, from day 11 till day 13

Table 7: Challenge of mice vaccinated with the *S. Enteritidis* mutants SM71, SM73 and SM69

Vaccination		Challenge		Survival	Day of death	State of the mice
Strain	Dose	Strain	Dose			
Negative control: milk	/	<i>S. Enteritidis</i> wild type strain 76Sa88	3.1×10^8	0/4	8, 8, 8, 9	Severe symptoms onwards from day 5
<i>S. Enteritidis</i> wild type strain 76Sa88	3.7×10^8	<i>S. Enteritidis</i> wild type strain 76Sa88	3.1×10^8	/	/	/
<i>S. Enteritidis</i> Δ fliC SM71	1.4×10^8	<i>S. Enteritidis</i> wild type strain 76Sa88	3.1×10^8	/	/	/
<i>S. Enteritidis</i> AquaB SM69	7.6×10^8	<i>S. Enteritidis</i> wild type strain 76Sa88	3.1×10^8	2/5	8, 8, 19	Severe symptoms onwards from day 5
<i>S. Enteritidis</i> Δ fliC SM73	1.2×10^8			5/5	/	Asymptomatic

36

Table 8: Safety evaluation of the *Salmonella* mutant SM69 in one-day-old chickens

Infection	Group	N	Titer	Route	Survival	Day of death (DPI)
Strain						
S. Enteritidis SM69	1: SM69-IT	10	1.3x10 ⁸ CFU/0.2 ml	intratracheal	4/10*	0, 2, 3, 5, 13
S. Enteritidis SM69	2: SM69-OG	10	1.3x10 ⁸ CFU/0.2 ml	oral gavage	8/10**	0, 5
Negative control: PBS	3: PBS-IT	10	PBS - 0.2 ml	intratracheal	10/10	-
Negative control: PBS	4: PBS-OG	10	PBS - 0.2 ml	oral gavage	10/10	-

IT Intratracheal

OG Oral gavage

DPI Days post inoculation

5 * In group 1, 1 bird died during inoculation; 1 bird died at 3, 5 and 13 DPI; and 2 birds at 2 DPI respectively

** In group 2, 1 bird died during inoculation; and 1 bird died at 5 days DPI

Table 9: Safety evaluation of the Salmonella mutant SM69 in 2-week-old chickens

Infection	Group	N	Titer	Route	Survival	Day of death (DPI)	Mean weight (kg)	Std weight
Strain								
S. Enteritidis SM69	1:SM69-IT	10	2.3x10 ⁸ CFU/0.2 ml	IT	9/10*	13	0.429	0.064
S. Enteritidis SM69	2:SM69-OG	10	2.3x10 ⁸ CFU/0.2 ml	OG	10/10	-	0.420	0.044
Vaccine control: Poulvac® ST*	3:Poulvac-IT	10	2.2x10 ⁸ CFU/0.2 ml	IT	10/10	-	0.423	0.046
Negative control: PBS	4:PBS-IT	5	PBS ~ 0.2 mL	OG	5/5	-	0.388	0.019

IT Intratracheal
OG Oral gavage
DPI Days post inoculation
* Death due to yolk sac infection
** A live S. Typhimurium AraA⁻ vaccine against S. Typhimurium

Table 10: Virulence experiments with *S. Typhimurium* mutant strains in BALB/c mice. Oral inoculation with approximately 10^8 cells of the *S. Typhimurium* strain 1491S96

Infection	Survival (day of death)	State of the mice
Strain (<i>S. Typhimurium</i> 1491S96)		
Wild type SM2	0/3 (9, 9, 10)	Disease symptoms from day 4 onwards
Δ fliC SM91	0/5 (9, 10, 11, 15, 15)	Disease symptoms from day 5 onwards
Δ fliJBA SM90	0/5 (8, 10, 10, 11, 34)	Severe disease symptoms from day 7 onwards
Δ fliJBA Δ fliC SM83	0/5 (8, 9, 12, 14, 15)	Severe disease symptoms from day 7 onwards
Δ guaB SM86	2/5* (2, 2, 2)	Mild disease symptoms from day 9 until challenge
Δ guaB Δ fliJBA SM87	5/5	No symptoms
Δ guaB Δ fliC Δ fliJBA SM89	5/5	No symptoms
Control (not infected)	4/4	No symptoms

* died after a fight

Table 11: Challenge experiments with *S. Typhimurium* mutant strains in BALB/c mice. Oral vaccination with approximately 10⁸ cells of the *S. Typhimurium* strain 1491S96

Vaccination Strain	Challenge Strain	Dose (CFU)	Survival (day of death)	State of the mice
(<i>S. Typhimurium</i> 1491S96)	(<i>S. Typhimurium</i> 1491S96)			
ΔguaB SM86	<i>S. Typhimurium</i> 1491S96	1.3 x 10 ⁷	2/2	Mild symptoms until days 14, afterwards one mouse showed clear symptoms, the other was healthy again
ΔguaB ΔfljBA SM87	<i>S. Typhimurium</i> 1491S96	1.3 x 10 ⁷	5/5	Reduced activity between day 8 and day 10
ΔguaB ΔfliC ΔfljBA SM89	<i>S. Typhimurium</i> 1491S96	1.3 x 10 ⁷	5/5	Reduced activity between day 8 and day 10
Control (not infected)	<i>S. Typhimurium</i> 1491S96	1.3 x 10 ⁷	0/4	Severe symptoms from day 6 onwards

33

Table 12: Virulence experiments with *S. Typhimurium* 1491S96 mutant strains in BALB/c mice.

Infection			
Strain	Dose	Survival (day of death)	State of the mice
(<i>S. Typhimurium</i> 1491S96)			
Wild type SM2	0.8 x 10 ⁸	1/4 (11, 13, 14)	Disease symptoms from day 6 onwards
ΔguaB SM86	0.8 x 10 ⁸	5/5	Weak symptoms on day 13 and 14
ΔguaB ΔfliC SM91	2.5 x 10 ⁸	5/5	No symptoms
ΔguaB ΔfljBA SM87	1.5x 10 ⁸	5/5	No symptoms
ΔguaB ΔfliC ΔfljBA SM89	1.7x 10 ⁸	5/5	No symptoms
Control (not infected)	-	5/5	No symptoms

Table 13: Protection experiments with *S. Typhimurium* 1491S96 mutant strains in BALB/c mice.

Vaccination		Challenge				
Strain	Dose	Strain (<i>S. Typhimurium</i> 1491S96)	Dose	Survival (day of death)	State of the mice	
<i>ΔguaB</i> SM86	0.8 x 10 ⁸	<i>S. Typhimurium</i> 1491S96	2.7 x 10 ⁸	5/5	Reduced activity from day 6 till day 16	
<i>ΔguaB ΔfliC</i> SM91	2.5 x 10 ⁸	<i>S. Typhimurium</i> 1491S96	2.7 x 10 ⁸	5/5	Reduced activity onwards from day 7; weak symptoms from day 8 untill day 14	
<i>ΔguaB ΔfliJBA</i> SM87	1.5x 10 ⁸	<i>S. Typhimurium</i> 1491S96	2.7 x 10 ⁸	3/5 (10,28)	Weak symptoms between day 6 and day 16	
<i>ΔguaB ΔfliC ΔfliJBA</i> SM89	1.7x 10 ⁸	<i>S. Typhimurium</i> 1491S96	2.7 x 10 ⁸	4/5 (19)	Reduced activity between day 8 and day 16	
Control (not infected)	-	<i>S. Typhimurium</i> 1491S96	2.7 x 10 ⁸	0/5 (8,9,10,11,16)	Severe symptoms from day 6 onwards	

34

CLAIMS

1. An attenuated mutant strain of a bacterium infecting veterinary species, wherein said mutant contains
5 at least one first genetic modification and at least one second genetic modification, said first modification in one or more motility genes, and said second modification in one or more genes involved in the survival or proliferation of the bacterium in the host.
- 10 2. The mutant strain of any of the preceding claims, wherein the veterinary species is poultry.
3. The mutant strain of any of the preceding claims, which is a *Salmonella enterica* or *Escherichia coli* strain.
- 15 4. The mutant strain of any of the preceding claims, wherein the motility gene is a gene encoding flagellin.
5. The mutant strain of claim 4, with a mutation in the *fliC* and/or *fljB* or *fljBA* genes.
- 20 6. The mutant strain of claim 4 or 5, which is incapable of swarming out on LB medium containing 0.4% agar.
7. The mutant strain of any of the preceding claims, wherein the gene involved in the survival is a
25 house-keeping gene or a virulence gene.
8. The mutant strain of claim 7, wherein the housekeeping gene that is inactivated is the *guaB* gene.
9. The mutant strain of claim 8, wherein the mutant strain contains a deletion mutation that impairs the
30 *guaB* gene function.
10. The mutant of claim 8 or 9, which is incapable of forming *de novo* guanine nucleotides.
11. The mutant strain of any of the preceding claims, wherein said mutant strain encodes and expresses a

foreign antigen.

12. The mutant strain of any of the preceding claims, which is an attenuated *S. Enteritidis* or a *S. Typhimurium* strain.

5 13. The mutant strain of any of the preceding claims, wherein the genetic modifications are introduced into parent strain *S. Enteritidis* phage type 4 strain 76Sa88.

10 14. The mutant strain of claim 13, which is the attenuated *S. Enteritidis* strain SM73 having the deposit number LMG P-21642.

15 15. The mutant strain of any of claims 1 to 12, wherein the genetic modifications are introduced into parent strain *S. Typhimurium* 1491S96.

16 16. The mutant strain of claim 15, which is the attenuated *S. Typhimurium* strain SM89 having the deposit number LMG P-21643.

20 17. The mutant of any of the preceding claims, containing a genetic modification in the *guaB* gene and a genetic modification in the *fliC* gene.

18. The mutant of any of the preceding claims, containing a genetic modification in a *guaB* gene and a genetic modification in the *fljBA* genes.

25 19. The mutant of any of the preceding claims, containing a genetic modification in the *guaB* gene, a genetic modification in the *fliC* gene, and a genetic modification in the *fljBA* genes.

30 20. The mutant strain of any of the preceding claims, wherein the modification is selected from the group consisting of an insertion, a deletion, and/or a substitution of one or more nucleotides in said genes.

21. A vaccine for immunizing a veterinary species against a bacterial infection, said vaccine comprising: a pharmaceutically effective or an immunizing amount of a

31

mutant strain according to any of the preceding claims; and a pharmaceutically acceptable carrier or diluent.

22. The vaccine of claim 21, wherein said mutant strain encodes and expresses a foreign antigen.

5 23. The vaccine of claim 21 or 22, wherein said mutant strain comprises a plasmid, which encodes and expresses, in a eukaryotic cell, a foreign gene.

24. A method of immunizing a veterinary species against a bacterial infection, said method comprising the
10 step of:

administering to a veterinary species in need thereof an immunizing amount of a mutant strain according to any of the claims 1 to 20 and/or a vaccine according to any of the claims 21 to 23.

15 25. The method of claim 24 wherein the veterinary species is poultry.

26. The method of any of claims 24 or 25 wherein the mutant strain is an attenuated strain of *S. enterica* or *E. coli*.

20 27. The method of any of claims 24 to 26 wherein the mutant strain and/or the vaccine is administered via oral, nasal or parenteral routes.

28. The use of a mutant strain according to any of the claims 1 to 20 for the preparation of a vaccine for
25 the prevention and/or the treatment of infections in veterinary species, preferably poultry.

29. A method for a serological distinction between vaccinated animals and animals infected by a wild-type strain, wherein the vaccinated animals have been
30 immunized with a mutant strain wherein a flagellin gene is inactivated, said method comprising the steps of:

- Assaying animals for the presence of antibodies raised against flagellin,

- Distinguishing infected animals from vaccinated animals based on the presence or absence of said antibodies.

30. The method of claim 29 wherein said
5 antibodies are generated by an animal infected by a wild-type strain, yet not by an animal that has been vaccinated with a mutant strain wherein a flagellin gene is inactivated, such as a mutant according to any of claims 4-20.

10 31. The method of any of claims 29-30 wherein the presence of said antibodies indicates the presence of wild-type strains and thus infection.

32. The method of any of claims 29-31, wherein
15 animals infected by *Salmonellae* are distinguished from animals that have been immunized with an attenuated live vaccine according to any of claims 21-23.

33. The method of any of claims 29-32, whereby the animals are assayed for antibodies raised against FliC.

20 34. The method of any of claims 29-32, wherein the animal is a veterinary species, preferably poultry, more preferably a chicken.

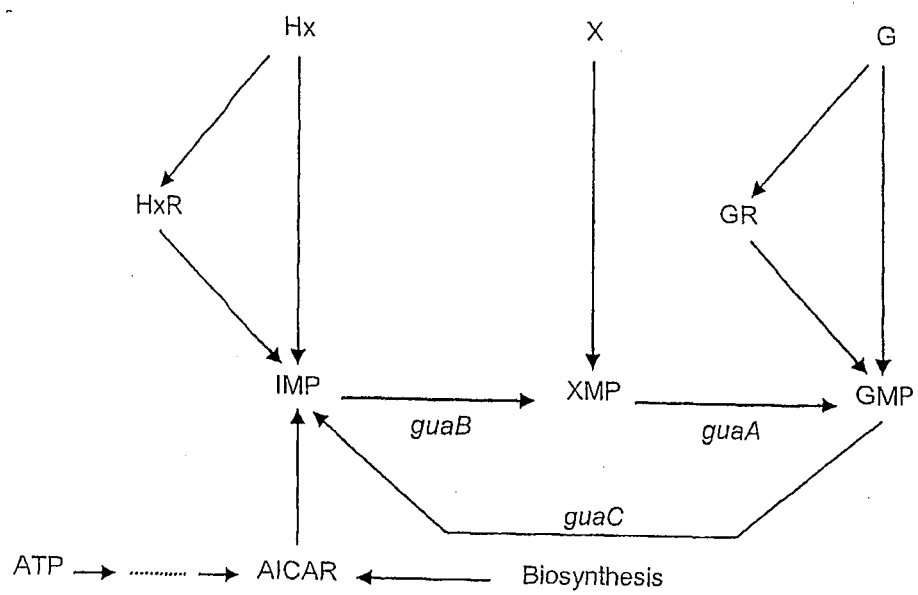


Fig. 1

CTGATCGAACAAAGCCTTCGGCCTGCAGTTTGGCTTTTAGCTGCTCATATTTCTGCTGCAA - 60
CAAGCCTTCGCCCCGCGGGCTGCATACTTTCGGCGATGATTTGATAATCGCCGCGCGGCTC - 120
GTACAGCGTAATGTTGGCGCGTACCAGCACCTGCTGCCCCGTGCTGCGGGCGGAACGTCAC - 180
CCGGCGGATTGCTGTTACGGAACATCGCACAGCGCACCTGAGCGGTATCGTCTTTGAGCGT - 240
AAAGTACCAGTGGCCCGACGCAGGCTGCGTGAAATTAGAAATCTCGCCGCTGATCCATAC - 300
CTGTCCCATCTCCTGTTCTAACAGCAGACGAACCGTCTGGTTAAGGCGGCTTACGGTAAA - 360
AATTGAGGAAGTTTGAGAGGATAACATGTGAGCGGGATCAAATTCTAAATCAGCAGGTTA - 420
TTCAATCGATAGTAACCTGCTCACGGGGGATCGCAAGCACTATTTGCAAAAAAATGTAGA - 480
TGCAACCGATTACGTTCTGTATAATGCCGCGGGAATATTTATTAACCTCCAGCGGAGAT - 540
ATTGCCCATGCTACGTATCGCTAAAGAAGCTCTGACGTTTGACGACGTCCTCCTTGTTC - 600
CGCTCACTCCACCGTTTTCGCGAATACTGCCGATCTCAGCAGCGAGTTGACGAAAACTAT - 660
TCGTCTGAATATTCTATGCTTTCTGCGCGGATGGACACCGTGACGGAAGCGCGCTGGC - 720
AATTGCCCTGGCCCGAAGGCGGCATTGGTTTTATCCACAAAAACATGTCTATTGAGCG - 780
CCAGGCGGAAGAAGTTTCGCGCGTGAAGAAACACGAGTCCGGCGTAGTGACCGACCCGCA - 840
GACCGTCTTGCCAACCACACGTTGTCATGAAGTGAAAGCCCTGACCGAGCGTAACGGTTT - 900
TGCGGGCTATCCGGTGGTGACTGAAGATAACGAGCTGGTGGGTATCATCACCGGTCGTGA - 960
CGTGCGTTTTTGTGACTGACCTGAACCGCGGTGAGTGTCTACATGACACCGAAAGAGCG - 1020
TCTGGTGACCGTTTCGTGAAGCGAAGCCCGTGAAGTCGTGCTGGCAAAAATGCACGAAA - 1080
ACGCGTAGAAAAAGCGCTGGTTCGTGATGATAACTTCCATCTGCTTGGCATGATTACCGT - 1140
AAAAGATTTCCAGAAAGCGGAACGTAAACCAAACCTCTGTAAAGATGAGCAGGGCCGTTT - 1200
ACGTGTTCGCGCGCGGCTCGGCGCAGGCGCGGGCAACGAAGAGCGCGTTGACGCGCTGGT - 1260
GGCGGCAGGCGCTGACGTACTGCTGATCGACTCCTCTCACGGTCACTCTGAAGGCGTGT - 1320
GCAACGTATCCGTGAGACGCGTGCTAAATATCCTGACCTGCAAATCATCGCGGGAACGT - 1380
TGCGAGCGGCGCAGGCGCTCGCGCACTGGCGGAAGCCGGTTGCAACCGCGTGAAAGTGGG - 1440
TATCGGCCCCGGTTCCATCTGTACGACTCGTATCGGGACTGGTGTGGGCGTTCCGCAGAT - 1500
CACCGCTGTTTCTGACGCGGTGGAAGCGCTGGAAGGCACCGGGATTCCGGTTATCGCTGA - 1560
CGGCGGTATCCGTTTCTCCGNNACATCGCCAAAGCCATCGCCGCGAGGCGCGAGCGCGGT - 1620
AATGGTGGGTTCTATGCTGGCCGGTACCGAAGAATCNCGGGCGAAATCGAACTCTACCA - 1680
GGGCGGTTCTTACAAATCTTATCGCGGTATGGGTTCTCTGGGCGCGATGTCCAAAGGTTT - 1740
CTCCGACCGTTACTTCCAGAGCGACAACGCGCCGACAAACTGGTGCCGGAAGGTATCGA - 1800
AGGCCGCGTAGCCTATAAAGGTCGCTGAAAGAGATCATTACACGAGATGGGCGGCCT - 1860
GCGCTCCTGTATGGGGCTGACCGGTTGTGCTACCATCGACGAACTGCGTACTAAAGCGGA - 1920
GTTTGTGCGTATCAGCGGTGCGGGTATCCAGGAAAGCCACGTTACGACGTGACCATCAC - 1980
CAAAGAGTCCCCGAACCTACCGTCTGGGCTCCTGATTTTCTTCGCCCCGACCTTCGCGTCGG - 2040
GCGATTTATTTAATCTGTTTCACTTGCCCTCGGAATAAGCGTCAATGACGGAACATTCA - 2100
TAAGCATCGCATCTCTCATTCTGGACTTCGGTTCTCAGTACACTCAACTGGTTGCGCGCGG - 2160
CGTGCGTGAGCTGGGTGTTTACTGTGAACGTGGGCGTGGGATGTGACAGAAGCACAAAT - 2220
TCGTGACTTCAACCCAAGCGGCATTATTTCTTCCGGCGGCCCCGAAAGCACCCGAAGA - 2280
AAACAGCCCCGCGCGCGCCGAGTATGTCTTTGAAGCAGGCGTGCCGGTATTTGGCGTTTG - 2340
CTATGGTATGCAGACCATGGCGATGCAGCTTGGCGGTCTATGTAGAAGGTTCTAATGAGCG - 2400
TGAATTTGGTTATGCGCAGGTGCAAGTGTGACCGACAGCGCGCTGGTTTCGCGGTATTGA - 2460
AGATTTCCCTGACCGCAGACGGCAAACCGTGTGACGCTGTGGATGAGCCACGGCGATAA - 2520
AGTGACGGCGATTCCGTCCGACTTCGTGACCGTCCGACGACCGAGAGCTGCCCGTTTCGC - 2580
CATTATGGCCAACGAAGAAAAACGCTTCTACGGCGTACAGTTCCACCCGGAAGTGACTCA - 2640
CACCCGCCAGGGTATGCGCATGCTGGAGCGTTTTGTACGCGATATCTGCCAGTGTGAAGC - 2700
GTTGTGAGACGCCGCGAAGATCATCGATGACGCCGTGGCGCGCATTCGCGAGCAGGTAGG - 2760
CGACGATAAAGTGATCTTCGGTCTCTCCGGCGGCGTGGATTCTTCCGTACCGCCATGCT - 2820
GCTGCACCGCGCGATCGGTAAAAATCTGACCTGTGTTTTCTCGTCAACCGCCTGTTGCG - 2880
CCTGAACGAAGCCGAGCAGGTGA - 2903 (SEQ ID NO: 19)

Fig. 2

29

quaB3
1 GGCTGCGATTGGCGAGGTAGTAGTCCATCGCCATGCCCAGACGCTGCTGGCGGANCTGGA

quaB6
61 TCTGACGCAACAACCTCCTGCTGGTTACGGCTGACAAATTTCTGCCGCTGCTGATGGCGTAG
121 CGCGCGCAGGTCGGCGACAAAATCAGCTATCGTGACGTCCGTTTCGTGACCGACGGCGCT
181 GACCACCGGAATGCGGCTGGCAAAAATCGCCCGCGCCACGCGTTCGTTCGTTAAAACTCCA
241 CAAATCTTCCAGCGAACC GCCCGCGCCGACGATCAGTACATCACATTGCGCCGCGCGC
301 GTTCGCCAGTTTCGATAGCACGAACGATCTGCCCCGGCGCGTTCGTGCGCTGGACTGCGGT
361 TGGATAGATAATAACGGGCAGGGATGGGTCACGACGCTTTAGCACGTGGAGAATATCGTG
421 CAGCGCCGCGCGGTTTTCGAAGTGATCACCCCAACGCAGTGGGCCGGGAGGGCAACGG
481 CTGTTTATGCTGCTGATCGAACAAGCCTTCGGCCTGCAGTTTGGCTTTTAGCTGCTCATA
541 TTTCTGCTGCAACAAGCCTTCGCCCGCGGGCTGCATACTTTCGGCGATGATTTGATAATC
601 GCCGCGCGGCTCGTACAGCGTAATGTTGGCGGTACCAGCACCTGCTGCCCGTGTGCGG
661 GCGGAACGTCAACCGGCGATTGCTGTTACGGAACATCGCACAGCGCACCTGAGCGGTATC
721 GTCTTTGAGCGTAAAGTACAGTGGCCCCGACGAGGCTGCGTGAAATTAGAAATCTCGCC
781 GCTGATCCATACCTGTCCCATCTCCTGTTCTAACAGCAGACGAACCGTCTGGTTAAGGCG

quaB10
841 GCTTACGGTAAAAATTGAGGAAGTTTGAGAGGATAACATGTGAGCGGGATCAAATTCCTAA
901 ATCAGCAGGTTATTCAATCGATAGTAACCTGCTCACGGGGGATCGCAAGCACTATTTGCA
961 AAAAAATGTAGATGCAACCGATTACGTTCTGTATAATGCCGCGGCAATATTTATTAACCT

quaB4
1021 CCCAGGTGAGATATTGCCCATGCTACGTATCGCTAAAGAAGCTCTGACGTTTGACGCCCG

quaB5
1081 GGATCACCAAAGAGTCCCGAACTACCGTCTGGGCTCCTGATTTTCTTCGCCCCGACCTTC
1141 GCGTCGGGCGATTTATTTAATCTGTTTCACTTGCTCGGAATAAGCGTCAATGACGGAAA
1201 ACATTATAAGCATCGCATCCTCATTCTGGACTTCGGTTCTCAGTACACTCAACTGGTTG
1261 CGCGCCGCGTGCCTGAGCTGGGTGTTTACTGTGAAGTGTGGGCGTGGGATGTGACAGAAG
1321 CACAAATTCGTGACTTCAACCCAAGCGCATTATTTCTTCCGGCGGCCCGGAAAGCACCA
1381 CCGAAGAAAACAGCCCGCGCGCGCCGAGTATGCTTTGAAGCAGGCGTGCCGGTATTTG
1441 GCGTTTGCTATGGTATGCAGACCATGCGGATGCAGCTTGGCGGTGATGTAGAAGGTTCTA
1501 ATGAGCGTGAATTTGGTTATGCGCAGGTGCAAGTGTGACCGACAGCGCGCTGGTTTCGCG
1561 GTATTGAAGATTCCCTGACCGCAGACGGCAAACCGCTGCTGGACGTGTGGATGAGCCACG
1621 GCGATAAAGTGACGGCGATTCCGTCCGACTTCGTGACCGTCGCCAGCACCGAGAGCTGCC
1681 CGTTCGCCATTATGGCCAACGAAGAAAAACGCTTCTACGGCGTACAGTTCCACCCGGAAG
1741 TGACTCACACCCGCCAGGGTATGCGCATGCTGGAGCGTTTTGTACGCGATATCTGCCAGT
1801 GTGAAGCGTTGTGGACGCCGGCGAAGATCATCGATGACGCCGTGGCGCGCATTGCGGAGC

quaB7
1861 AGGTAGGCGACGATAAAGTGATCCTCGGTCTCTCCGGCGGCGTGGATTCTTCCGTCACCG

quaB2
1921 CCATGCTGCTGCACCGCGCATCGGTAAAAATCTGACCTGTGTTTTCGTCGACAACGGCC
1981 TGTTCGCGCTGAACG

(SEQ ID NO: 20)

Fig. 3

1 CATGTGTGAGCGGGATCAAATCTAAATCAGCAGGTTATTCAATCGATAG
51 TAACCTGCTCACGGGGGATCGCAAGCACTATTTGCAAAAAAATGTAGAT
101 GCAACCGATTACGTTCTGTATAATGCCCGGCAATATTTATTAACCTCCCA
151 GGTGAGATATTGCGCATGTACGTATCGCTAAAGAAGCCCTGACGTTTGAC

P1
→

201 GCCCCTGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATA

←
P2

251 GGAAGTCCGGAATAGGAACTAAGGAGGATATTCATATGGGATCACCAAAG
301 ATCCCCGAACTACCGTCTGGGCTCTGATTTTCTTCGCCCCGACCTTCGCG

(SEQ ID NO: 21)

Fig. 4

1 ATTTGCAAAA AAATGTAGAT GCAACCGATT ACGTTCTGTA TAATGCCGCG GCAATATTTA
61 TTAACCTCCC AGGTGAGATA TTGCCCATGC TACGTATCGC TAAAGAAGCC CTGACGTTTG
121 ACGACGTCCT CTTGTTCCC GCTCACTCCA CCGTTTTGCC GAATACTGCC GATCTCAGCA
181 CGCAGTTGAC GAAAACTATT CGTCTGAATA TTCCTATGCT TTCTGCGGCG ATGGACACCG
241 TGACGGAAGC GCGCCTGGCA ATTGCCCTGG CCCAGGAAGG CGGCATTGGT TTTATCCACA
301 AAAACATGTC CATTGAGCGC CAGGCGGAAG AAGTTCGCG CGTGAAGAAA CACGAGTCCG
361 GCGTAGTGAC CGACCCGCG ACCGTCTGC CAACCACCAC GTTGCATGAA GTGAAAGCCC
421 TGACCGAGCG TAACGGTTTT GCGGGCTATC CCGTGGTGAC TGAAGATAAC GAGCTGGTGG
481 GTATCATCAC CGGTCGTGAC GTGCGTTTTG TGACTGACCT GAACCAGCCG GTTAGCGTTT
541 ACATGACGCC GAAAGAGCGT CTGGTGACCG TTCGTGAAG CGAAGCCCGT GAAGTCGTGC
601 TGGCAAAAAT GCACGAAAAA CCGGTAGAAA AAGCGCTGGT CGTTGATGAT AACTCCATC
661 TGCTTGCGCAT GATTACCGTA AAAGATTTCC AGAAAGCGGA ACGTAAACCA AACTCCTGCA
721 AAGATGAGCA GGGCCGTTTA CGTGTGCGCG CCGCGGTCCG CGCAGGCGCG GGCAACGAAG
781 AGCGCGTTGA CGCGCTGGTG GCGGCAGGCG TTGACGTACT GCTGATCGAC TCCTCTCACG
841 GTCATTCAGA AGGCGTGTG CAACGTATCC GTGAAACCCG TGCTAAATAT CCTGACCTGC
901 AAATCATCGG CGGCAACGTC GCGACAGGCG CAGGCGCTCG CGCACTGGCG GAAGCCGGTT
961 GCAGCGCGGT GAAAGTCGGT ATTGGCCCG GTTCCATCTG TACCACTCGT ATCGTGA CTG
1021 GCGTGGGCGT TCCGCAGATC ACCGCTGTTT CTGACGCAGT TGAAGCGCTG GAAGGTACCG
1081 GTATTCCGGT TATCGCTGAC GCGGATATCC GTTTCTCCGG CGACATAGCC AAAGCGATTG
1141 CCGCAGGTGC AAGCGCGGTA ATGGTGGGTT CCATGCTGGC GGGTACGGAA GAATCCCCGG
1201 GCGAAATCGA ACTCTACCAG GGCCGTTCTT ACAAATCTTA CCGCGGCATG GGCTCGCTGG
1261 GTGCGATGTC CAAAGGTTCC TCCGACCGTT ACTTCCAGAG CGACAACGCC GCTGACAAAC
1321 TGGTGCCGGA AGGTATCGAA GGTGCGGTAG CCTATAAAG TCGCCTGAAA GAGATCATTC
1381 ACCAGCAGAT GGGCGGCC TGCTCCTGTA TGGGGCTGAC CGGTTGTGCT ACCATCGACG
1441 AACTGCGTAC TAAAGCGGAG TTTGTGCGTA TCAGCGGTGC GGGCATTGAG GAAAGCCACG
1501 TTCACGACGT GACCATCACC AAAGAGTCCC CGAACTACCG TCTGGGCTCC TGA

(SEQ ID NO: 22)

Fig. 5

CTATGGCACAAGTCATTAATACAAACAGCCTGTCGCTGGTTGACCCAGAATAATGTGTAG
 P1 →
 GCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATAGGAAC
 TCGGAATAGGAACTAAGGAGGATATTCATATGTGGCGCAGGCGAACCAGGT
 P2 ←
 TCCGCAAAACGTCCTCTCTTTACTGGCGTTAATGCG
 (SEQ ID NO: 23)

Fig. 6

1 AGAATAACTGTGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGA
 P1 →
 51 GAATAGGAACTTCGGAATAGGAACTAAGGAGGATATTCATATGGTTATGT
 P2 ←
 101 TTGACTGGGTGAGCAGGATCACGTCTTCGGACTACGCTGT
 (SEQ ID NO: 24)

Fig. 7

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION 02-132
Page 1 of Form BCCM/LMG/BP/4/..... Receipt in the case of an original deposit

KOPIE

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the
International Depositary Authority BCCM/LMG identified at the bottom of next page

International Form BCCM/LMG/BP/4/02-132.....

To: Name of the depositor : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

1. Identification of the microorganism:

1.1 Identification reference given by the depositor:

SM69

1.2 Accession number given by the International Depositary Authority:

LMG P-21641

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION

Page 2 of Form BCCM/LMG/BP/4/.....02-132 Receipt in the case of an original deposit

KOPIE

II. Scientific description and/or proposed taxonomic designation

The microorganism identified under I above was accompanied by :

(mark with a cross the applicable box(es)):

☒ a scientific description

☒ a proposed taxonomic designation

III. Receipt and acceptance

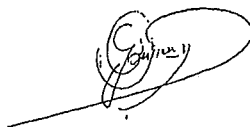
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

August 9, 2002

IV. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):



Dr. D. Janssens, curator IDA

Date : September 5, 2002

8/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 1 of Form BCCM/LMG/BP/9/...02-132...Viability statement

KOPIE

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Viability statement issued pursuant to Rule 10.2 by the International Depositary
Authority BCCM/LMG identified on the following page

International Form BCCM/LMG/BP/9/...02-132...

To: Party to whom the viability statement is issued:

Name : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Depositor:

I.1 Name : Prof. J.-P. Hernalsteens

I.2 Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

II. Identification of the microorganism

II.1 Accession number given by the International Depositary Authority:

LMG P-21641

II.2 Date of the original deposit (or where a new deposit or a transfer has been made, the most recent relevant date) :

August 9, 2002

III. Viability statement.

The viability of the microorganism identified under II above was tested on

: August 9, 2002

(Give date. In the cases referred to in Rule 10.2(a)(II) and (III), refer to the most recent viability test).

On that date, the said microorganism was: (mark the applicable box with a cross)

- ☒ viable
☐ no longer viable

40

WO 2007/112518

9/21

PCT/BE2006/000020

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 2 of Form BCCM/LMG/BP/9/.....02-132 Viability statement

KOPIE

IV. Conditions under which the viability test has been performed:

(Fill in if the information has been requested and if the results of the test were negative).

V. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) :



Dr. D. Janssens, curator IDA

Date : September 5, 2002

10/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION

Page 1 of Form BCCM/LMG/BP/4/...02-133, Receipt in the case of an original deposit

KOPIE

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the
International Depositary Authority BCCM/LMG identified at the bottom of next page

International Form BCCM/LMG/BP/4/...02-133...

To: Name of the depositor : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

1. Identification of the microorganism:

1.1 Identification reference given by the depositor:

SM73

1.2 Accession number given by the International Depositary Authority:

LMG P-21642

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 2 of Form BCCM/LMG/BP/4/.....⁰²⁻¹³³ Receipt in the case of an original deposit

KOPIE

II. Scientific description and/or proposed taxonomic designation

The microorganism identified under I above was accompanied by :

(mark with a cross the applicable box(es)):

- ☒ a scientific description
☒ a proposed taxonomic designation

III. Receipt and acceptance

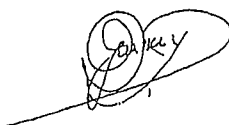
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

August 9, 2002

IV. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or, of authorized official(s):



Dr. D. Janssens, curator IDA

Date : September 5, 2002

12/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 1 of Form BCCM/LMG/BP/9/.....02-133 Viability statement

KOPIE

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Viability statement issued pursuant to Rule 10.2 by the International Depositary
Authority BCCM/LMG identified on the following page

International Form BCCM/LMG/BP/9/.....02-133

To: Party to whom the viability statement is issued:

Name : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Depositor:

I.1 Name : Prof. J.-P. Hernalsteens

I.2 Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

II. Identification of the microorganism

II.1 Accession number given by the International Depositary Authority:

LMG P-21642

II.2 Date of the original deposit (or where a new deposit or a transfer has been made, the most recent relevant date) :

August 9, 2002

III. Viability statement.

The viability of the microorganism identified under II above was tested on

: August 9, 2002

(Give date. In the cases referred to in Rule 10.2(a)(II) and (III), refer to the most recent viability test).

On that date, the said microorganism was: (mark the applicable box with a cross)

☒ viable

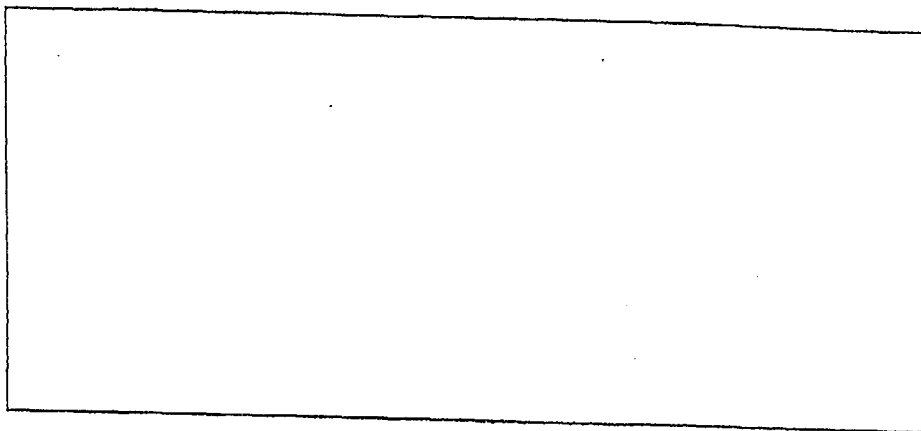
☐ no longer viable

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 2 of Form BCCM/LMG/BP/9/02-133 Viability statement

KOPIE

IV. Conditions under which the viability test has been performed:

(Fill in if the information has been requested and if the results of the test were negative).



V. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) :



Dr. D. Janssens, curator IDA

Date : September 5, 2002

Fig. 9

14/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 1 of Form BCCM/LMG/BP/4/.....02-134 Receipt in the case of an original deposit

KOPIE

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the
International Depositary Authority BCCM/LMG identified at the bottom of next page

International Form BCCM/LMG/BP/4/02-134.....

To: Name of the depositor : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Identification of the microorganism:

I.1 Identification reference given by the depositor:

SM89

I.2 Accession number given by the International Depositary Authority:

IMG P-21643

WO 2007/112518

15/21

PCT/BE2006/000020

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION

Page 2 of Form BCCM/LMG/BP/4/.....02-134
Receipt in the case of an original deposit

KOPIE

II. Scientific description and/or proposed taxonomic designation

The microorganism identified under I above was accompanied by :

(mark with a cross the applicable box(es));

- ☒ a scientific description
☒ a proposed taxonomic designation

III. Receipt and acceptance

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

August 9, 2002

IV. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):



Dr. D. Janssens, curator IDA

Date : September 5, 2002

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 1 of Form BCCM/LMG/BP/9/...02-134 Viability statement

KOPIE

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Viability statement issued pursuant to Rule 10.2 by the International Depositary
Authority BCCM/LMG identified on the following page

International Form BCCM/LMG/BP/9/...02-134...

To: Party to whom the viability statement is issued:

Name : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Depositor:

I.1 Name : Prof. J.-P. Hernalsteens

I.2 Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

II. Identification of the microorganism

II.1 Accession number given by the International Depositary Authority:

LMG P-21643

II.2 Date of the original deposit (or where a new deposit or a transfer has been made, the most recent relevant date) :

August 9, 2002

III. Viability statement.

The viability of the microorganism identified under II above was tested on

: August 9, 2002

(Give date. In the cases referred to in Rule 10.2(a)(II) and (III), refer to the most recent viability test).

On that date, the said microorganism was: (mark the applicable box with a cross)

☒ viable

☐ no longer viable

14

WO 2007/112518

17/21

PCT/BE2006/000020

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 2 of Form BCCM/LMG/BP/9/.....02-134 Viability statement

KOPIE

IV. Conditions under which the viability test has been performed:

(Fill in if the information has been requested and if the results of the test were negative).

V. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or
of authorized official(s) :



Dr. D. Janssens, curator IDA

Date : September 5, 2002

Fig. 10

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 1 of Form BCCM/LMG/BP/4/02-135

Receipt in the case of an original deposit

KOPIE

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the
International Depositary Authority BCCM/LMG identified at the bottom of next page

International Form BCCM/LMG/BP/4/02-135...

To: Name of the depositor : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Identification of the microorganism:

I.1 Identification reference given by the depositor:

SM86

I.2 Accession number given by the International Depositary Authority:

LMG P-21646

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
 LMG-COLLECTION
 Page 2 of Form BCCM/LMG/BP/4/...02-135... Receipt in the case of an original deposit

KOPIE

II. Scientific description and/or proposed taxonomic designation

The microorganism identified under I above was accompanied by :

(mark with a cross the applicable box(es)):

- ☒ a scientific description
☒ a proposed taxonomic designation

III. Receipt and acceptance

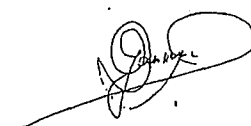
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

August 28, 2002

IV. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
 Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
 Universiteit Gent
 K.L. Ledeganckstraat 35
 B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):



Dr. D. Janssens, curator IDA

Date : September 5, 2002

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 1 of Form BCCM/LMG/BP/9/.....02-135 Viability statement

KOPIE

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Viability statement issued pursuant to Rule 10.2 by the International Depositary
Authority BCCM/LMG identified on the following page

International Form, BCCM/LMG/BP/9/.....02-135

To: Party to whom the viability statement is issued:

Name : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Depositor:

I.1 Name : Prof. J.-P. Hernalsteens

I.2 Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

II. Identification of the microorganism

II.1 Accession number given by the International Depositary Authority:

LMG P-21646

II.2 Date of the original deposit (or where a new deposit or a transfer has been made, the most recent relevant date) :

August 28, 2002

III. Viability statement.

The viability of the microorganism identified under II above was tested on

: August 28, 2002

(Give date. In the cases referred to in Rule 10.2(a)(II) and (III), refer to the most recent viability test).

On that date, the said microorganism was: (mark the applicable box with a cross)

☒ viable

☐ no longer viable

WO 2007/112518

21/21

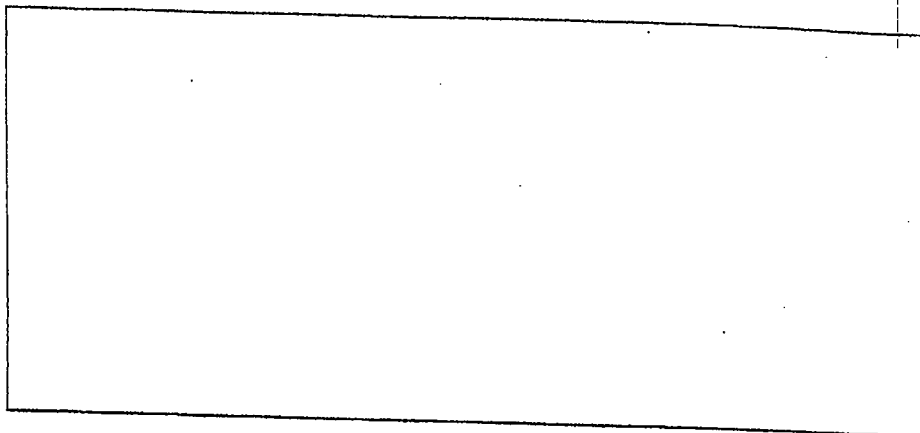
PCT/BE2006/000020

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 2 of Form BCCM/LMG/BP/9/.....02-135 Viability statement

KOPIE

IV. Conditions under which the viability test has been performed:

(Fill in if the information has been requested and if the results of the test were negative).



V. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or
of authorized official(s) :



Dr. D. Janssens, curator IDA

Date : September 5, 2002

Fig. 11

FDAH02AWO.ST25.txt
SEQUENCE LISTING

<110> Fort Dodge Animal Health

<120> LIVE ATTENUATED SALMONELLA VACCINE

<130> BP.FDAH.002A/EP

<160> 24

<170> PatentIn version 3.3

<210> 1

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> GuaB2 primer

<400> 1

cgttcaggcg caacaggccg ttgt

24

<210> 2

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> GuaB3 primer

<400> 2

ggctgcgatt ggcgaggtag ta

22

<210> 3

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> GuaB4 primer

<400> 3

ggatgatcccg ggcgtcaaac gtcagggctt cttta

35

<210> 4

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> GuaB5 primer

<400> 4

ttgacgcccg ggatcaccaa agagtccccg aacta

35

<210> 5

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> GuaB6 primer

41

WO 2007/112518

PCT/BE2006/000020

FDAH02AWO.ST25.txt

<400> 5
gcaacaactc ctgctggta 20

<210> 6
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> GuaB7 primer

<400> 6
agaccgagga tcactttatc 20

<210> 7
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> GuaB10 primer

<400> 7
aggaagtttg agaggataa 19

<210> 8
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> P1 primer

<400> 8
gtgtaggctg gagctgcttc 20

<210> 9
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> P2 primer

<400> 9
catatgaata tcctccttag 20

<210> 10
<211> 72
<212> DNA
<213> Artificial Sequence

<220>
<223> FlicP1 primer

<400> 10
atggcacaag tcattaatac aaacagcctg tcgctggttg acccagaata atgtgtaggc 60
tggagctgct tc 72

<210> 11
<211> 74

FDAH02AWO.ST25.txt

<212> DNA
<213> Artificial Sequence

<220>
<223> FlicP2 primer

<400> 11
cgcataacg cagtaaagag aggacgtttt gcggaacctg gttmgcctgc gccacatatg 60
aatatcctcc ttag 74

<210> 12
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Flic1 primer

<400> 12
atggcacaag tcattaatac aaacag 26

<210> 13
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Flic2 primer

<400> 13
cgcataacg cagtaaagag aggac 25

<210> 14
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Flic3 primer

<400> 14
tatcggaat ctggaggcaa 20

<210> 15
<211> 72
<212> DNA
<213> Artificial Sequence

<220>
<223> FljBAP1 primer

<400> 15
atggcacaag taatcaacac taacagtctg tcgctgctga cccagaataa ctgtgtaggc 60
tggagctgct tc 72

<210> 16
<211> 71
<212> DNA
<213> Artificial Sequence

<220>

Ux

WO 2007/112518

PCT/BE2006/000020

FDAH02AWO.ST25.txt

<223> FljBAP2 primer

<400> 16

ttattcagcg tagtccgaag acgtgacccct gctcaccag tcaaacataa ccatatgaat 60

atcctcctta g 71

<210> 17

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> FljBA5 primer

<400> 17

cagcgtagtc cgaagacgtg atc 23

<210> 18

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> FLjBA6 primer

<400> 18

acactaacag tctgtcgtg ct 22

<210> 19

<211> 2903

<212> DNA

<213> Salmonella Enteritidis

<220>

<221> misc_feature

<223> Contig 1294 of the S. Enteritidis genome

<220>

<221> misc_feature

<222> (1581)..(1583)

<223> n is a, c, g, or t

<220>

<221> misc_feature

<222> (1657)..(1657)

<223> n is a, c, g, or t

<400> 19

ctgatcgaac aagccttcgg cctgcagttt ggcttttagc tgctcatatt tctgctgcaa 60

caagccttcg cccgcgggct gcatactttc ggcgatgatt tgataatcgc cgcgcggctc 120

gtacagcgta atgttgggcg gtaccagcac ctgctgcccg tgctgcgggc ggaacgtcac 180

ccggcgattg ctgttacgga acatcgaca gcgcacctga gcggtatcgt ctttgagcgt 240

aaagtaccag tggcccgcag caggctgcgt gaaattagaa atctcgccgc tgatccatac 300

ctgtcccatc tcctgtttcta acagcagacg aaccgtctgg ttaaggcggc ttacggtaaa 360

aattgaggaa gtttgagagg ataacatgtg agcgggatca aattctaaat cagcaggtta 420

ttcaatcgat agtaacctgc tcacggggga tcgcaagcac tatttgcaaa aaaatgtaga 480

FDAH02AWO.ST25.txt

tgcaaccgat tacgttctgt ataatgccgc gggaatattt attaacctcc caggcgagat	540
attgcccattg ctacgtatcg ctaaagaagc tctgacgttt gacgacgtcc tccttggtcc	600
cgctcactcc accgttttgc cgaatactgc cgatctcagc acgcagttga cgaaaactat	660
tcgtctgaat attcctatgc tttctgcggc gatggacacc gtgacggaag cgcgcctggc	720
aattgccctg gccaggaag gcggcattgg ttttatccac aaaaacatgt ctattgagcg	780
ccaggcggaa gaagttcgcc gcgtgaagaa acacgagtcg ggcgtagtga ccgacccgca	840
gaccgtcctg ccaaccacca cgttgcatga agtgaaagcc ctgaccgagc gtaacgggtt	900
tgcggtctat ccggtggtga ctgaagataa cgagctggcg ggtatcatca ccggtcgtga	960
cgtgcgtttt gtgactgacc tgaaccagcc ggtgagtgct tacatgacac cgaaagagcg	1020
tctggtgacc gttcgtgaag gcgaagcccg tgaagtcgtg ctggcaaaaa tgcacgaaaa	1080
acgcgtagaa aaagcgctgg tcgttgatga taacttccat ctgcttggca tgattaccgt	1140
aaaagatttc cagaaagcgg aacgtaaacc aaactcctgt aaagatgagc agggccgttt	1200
acgtgtcggc gcggcggtcg gcgcaggcgc gggcaacgaa gagcgcgttg acgcgctggt	1260
ggcgccaggc gttgacgtac tgctgatcga ctctctcac ggtcactctg aaggcggtgt	1320
gcaacgtatc cgtgagacgc gtgctaaata tcctgacctg caaatcatcg gcgggaacgt	1380
tgcgacgggc gcaggcgctc gcgcactggc ggaagccggt tgcaacgcgg tgaaagtggg	1440
tatcggtccc ggttccatct gtacgactcg tatcggtact ggtgtgggcg ttccgcagat	1500
caccgctgtt tctgacgcgg tggaagcgct ggaaggcacc gggattccgg ttatcgtga	1560
cggcggtatc cgtttctccg nnnacatcgc caaagccatc gccgcaggcg cgagcgcggt	1620
aatggtgggt tctatgctgg ccggtaccga agaatcnccg ggcgaaatcg aactctacca	1680
gggcccgttc tacaaatctt atcgcggtat gggttctctg ggcgcgatgt ccaaaggttc	1740
ctccgacctg tacttccaga gcgacaacgc cgccgacaaa ctggtgccgg aaggtatcga	1800
aggccgcgta gcctataaag gtcgcctgaa agagatcatt caccagcaga tgggcggcct	1860
gcgctcctgt atggggctga ccggttggtg taccatcgac gaactgcgta ctaaagcgga	1920
gtttgtgcgt atcagcggtg ccggttatcca ggaaagccac gttcacgacg tgaccatcac	1980
caaagagtcc ccgaactacc gtctgggctc ctgattttct tcgcccgacc ttcgcgtcgg	2040
gcgatttatt taatctgttt cacttgccctc ggaataagcg tcaatgacgg aaaacattca	2100
taagcatcgc atctcattc tggacttcgg ttctcagtag actcaactgg ttgcgcgccc	2160
cgtgcgtgag ctgggtgttt actgtgaact gtgggcgtgg gatgtgacag aagcacaat	2220
tcgtgacttc aacccaagcg gcattattct ttccggcggc ccggaaagca ccaccgaaga	2280
aaacagcccc cgcgcgccgc agtatgtctt tgaagcaggc gtgccggtat ttggcggttg	2340
ctatggtatg cagaccatgg cgatgcagct tggcggtcat gtagaagggt ctaatgagcg	2400
tgaatttggt tatgcgcagg tcgaagtgtt gaccgacagc gcgctgggtc gcggtattga	2460
agattccctg accgcagacg gcaaaccgct gctggacgtg tggatgagcc acggcgataa	2520

629

FDAH02AWO.ST25.txt

agtgacggcg attccgtccg acttcgtgac cgtcgccagc accgagagct gcccgttcgc 2580
cattatggcc aacgaagaaa aacgcttcta cggcgtagcag ttccacccgg aagtgactca 2640
caccgcccag ggtatgcgca tgctggagcg ttttgtagcg gatattctgcc agtgtgaagc 2700
gttggtggacg ccggcgaaga tcatcgatga cgccgtggcg cgcattcgcg agcaggtagg 2760
cgacgataaa gtgacacctg gtctctcccg cggcgtaggatt tcttccgtca ccgccatgct 2820
gctgcaccgc gcgatcggta aaaatctgac ctgtgttttc gtcgacaacg gcctgttgcg 2880
cctgaacgaa gccgagcagg tga 2903

<210> 20
<211> 1995
<212> DNA
<213> Artificial sequence

<220>
<223> sequence of the delta-guaB fragment of S. Enteritidis cloned in pUC18

<220>
<221> misc_feature
<222> (55)..(55)
<223> n is a, c, g, or t

<400> 20
ggctgcgatt ggcgaggtag tagtccatcg ccatgcccag acgctgctgg cgganctgga 60
tctgacgcaa caactcctgc tggttacggc tgacaatttc tgccgctgct gatggcgtag 120
cgcgcgagg tcggcgacaa aatcagctat cgtgacgtcc gtttcgtgac cgacggcgct 180
gaccaccgga atgcggctgg caaaaatcgc ccgcgccacg cgttcgtcgt taaaactcca 240
caaatcttcc agcgaaccgc cgccgcgccc gacgatcagt acatcacatt cgccgcgcgc 300
gttcgccagt tcgatagcac gaacgatctg ccccggcgcg tcgtcgccct ggactgcggt 360
tggatagata ataacgggca gggatgggtc acgacgcttt agcacgtgga gaatatcgtg 420
cagcgccgcg ccggttttcg aagtgatcac cccaacgcag tgggcccggg agggcaacgg 480
ctgtttatgc tgctgatcga acaagccttc ggcctgcagt ttggctttta gctgctcata 540
tttctgctgc aacaagcctt cgccgcgggg ctgcatactt tcggcgatga tttgataatc 600
gccgcgcggc tcgtacagcg taatgttggc gcgtaccagc acctgctgcc cgtgctgcgg 660
gcggaacgtc acccggcgat tgctgttacg gaacatcgca cagcgcacct gagcgggtatc 720
gtctttgagc gtaaagtacc agtggcccga cgcaggctgc gtgaaattag aaatctcgcc 780
gctgatccat acctgtccca tctcctgttc taacagcaga cgaaccgtct ggtaagggcg 840
gcttacggta aaaattgagg aagtttgaga ggataacatg tgagcgggat caaattctaa 900
atcagcaggt tattcaatcg atagtaacct gctcacgggg gatcgcaagc actatttgca 960
aaaaaatgta gatgcaaccg attacgttct gtataatgcc gcggcaatat ttattaacct 1020
cccagggtgag atattgcccga tgctacgtat cgctaaagaa gctctgacgt ttgacgcccc 1080

FDAH02AWO.ST25.txt

```

ggatcaccaa agagtccccg aactaccgtc tgggtcctg attttcttcg cccgaccttc 1140
gcgtcgggcg atttatttaa tctgtttcac ttgcctcgga ataagcgtca atgacggaaa 1200
acattcataa gcatcgcata ctcatcttgg acttcgggtc tcagtacact caactgggtg 1260
cgcgccgcgt gcgtgagctg ggtgtttact gtgaactgtg ggcgtgggat gtgacagaag 1320
cacaaattcg tgacttcaac ccaagcggca ttattctttc cggcggcccc gaaagcacca 1380
ccgaagaaaa cagcccgcg cgcgccgagt atgtctttga agcaggcgtg ccggtatttg 1440
gcgttttgcta tgggtatgcag accatggcga tgcagcttgg cggtcagtga gaaggttcta 1500
atgagcgtga atttgggtat gcgcaggctg aagtgttgac cgacagcgcg ctggttcgcg 1560
gtattgaaga ttccctgacc gcagacggca aaccgctgct ggacgtgtgg atgagccacg 1620
gcgataaagt gacggcgatt ccgtccgact tcgtgaccgt cgccagcacc gagagctgcc 1680
cgttcgccat tatggccaac gaagaaaaac gcttctacgg cgtacagttc caccgggaag 1740
tgactcacac ccgccagggt atgcgcagtc tggagcggtt tgtacgcgat atctgccagt 1800
gtgaagcgtt gtggacgccg gcgaagatca tcgatgacgc cgtggcgcg attcgcgagc 1860
aggtaggcga cgataaagt atcctcggtc tctccggcgg cgtggattct tccgtcaccg 1920
ccatgctgct gcaccgcgcg atcggtaaaa atctgacctg tgttttcgtc gacaacggcc 1980
tgttgcgcct gaacg 1995

```

<210> 21
 <211> 349
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Nucleotide sequence of the S. Enteritidis PCR fragment, which includes the guaB deletion, obtained using primer GuaB10

```

<400> 21
catgtgtgag cgggatcaaa ttctaaatca gcaggttatt caatcgatag taacctgctc 60
acgggggatc gcaagcacta tttgcaaaaa aaatgtagat gcaaccgatt acgtttctgta 120
taatgcccgg caatatttat taacctccca ggtgagatat tcgccatgta cgtatcgcta 180
aagaagccct gacgtttgac gcccgtgtag gctggagctg cttcgaagtt cctatacttt 240
ctagagaata ggaactccgg aataggaact aaggaggata ttcatatggg atcaccaaag 300
atccccgaac tacctgtctg gctctgattt tcttcgcccg accttcgcg 349

```

<210> 22
 <211> 1553
 <212> DNA
 <213> Salmonella Typhimurium

<220>
 <221> misc_feature
 <223> guaB gene of S. Typhimurium LT2, section 117 of 220 of the complete genome

<400> 22

SD

WO 2007/112518

PCT/BE2006/000020

FDAH02AWD.ST25.txt
 atttgcaaaa aaatgtagat gcaaccgatt acgttctgta taatgccgcg gcaatattta 60
 ttaacctccc aggtgagata ttgcccattg tacgtatcgc taaagaagcc ctgacgtttg 120
 acgacgtcct ccttggtccc gctcactcca ccgttttgcc gaatactgcc gatctcagca 180
 cgcagttgac gaaaactatt cgtctgaata ttcctatgct ttctgcggcg atggacaccg 240
 tgacggaagc gcgcctggca attgccctgg cccaggaagg cggcattggg tttatccaca 300
 aaaacatgtc cattgagcgc caggcggaag aagttcgccg cgtgaagaaa cacgagtccg 360
 gcgtagtgac cgacccgcag accgtcctgc caaccaccac gttgcatgaa gtgaaagccc 420
 tgaccgagcg taacggtttt gcgggctatc cggtggtgac tgaagataac gagctggtgg 480
 gtatcatcac cggtcgtgac gtgcgttttg tgactgacct gaaccagccg gttagcgttt 540
 acatgacgcc gaaagagcgt ctggtgaccg ttcgtgaagg cgaagcccgt gaagtcgtgc 600
 tggcaaaaat gcacgaaaaa cgcgtagaaa aagcgtggt cgttgatgat aacttccatc 660
 tgcttgcat gattaccgta aaagatttcc agaaagcggg acgtaaacca aactcctgca 720
 aagatgagca gggccgttta cgtgtcggcg cggcggtcgg cgcaggcgcg ggcaacgaag 780
 agcgcgttga cgcgctggtg gcggcaggcg ttgacgtact gctgatcgac tcctctcacg 840
 gtcattcaga aggcgtggtg caacgtatcc gtgaaacccg tgctaaatat cctgacctgc 900
 aaatcatcgg cggcaacgtc gcgacaggcg caggcgctcg cgcactggcg gaagccggtt 960
 gcagcgcggt gaaagtcggt attggcccg gttccatctg taccactcgt atcgtgactg 1020
 gcgtgggcgt tccgcagatc accgctgttt ctgacgcagt tgaagcgtg gaaggtaccg 1080
 gtattccggt tatcgtgac ggcggtatcc gtttctccgg cgacatagcc aaagcgattg 1140
 ccgcagggtc aagcgcggtg atggtgggtt ccatgctggc gggtagcgaa gaatccccgg 1200
 gcgaaatcga actctaccag ggccgttctt acaaacttta ccgcggcatg ggctcgttg 1260
 gtgcgatgtc caaaggttcc tccgaccgtt acttccagag cgacaacgcc gctgacaaac 1320
 tgggtgccgga aggtatcgaa ggtcgcgtag cctataaagg tcgcctgaaa gagatcattc 1380
 accagcagat gggcggcctg cgctcctgta tggggctgac cggttggtg accatcgacg 1440
 aactgcgtac taaagcggag ttgtgctgta tcagcgggtg gggcattcag gaaagccacg 1500
 ttcacgacgt gaccatcacc aaagagtccc cgaactaccg tctgggctcc tga 1553

<210> 23
 <211> 193
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Nucleotide sequence obtained after sequencing the PCR fragment amplified with primers Flic1-Flic2 on total DNA of the mutants SM73 and SM89, using primers Flic1 and Flic2

<400> 23
 ctatggcaca agtcattaat acaaacagcc tgctcgtggt tgacccagaa taatgtgtag 60
 gctggagctg cttcgaagtt cctatacttt ctagagaata ggaacttcgg aataggaact 120

FDAH02AWO.ST25.txt

aaggaggata ttcatatgtg gcgcaggcga accagggtcc gcaaaacgtc ctctctttac 180
tggcgттаат gcg 193

<210> 24

<211> 140

<212> DNA

<213> Artificial sequence

<220>

<223> Nucleotide sequence obtained after sequencing the PCR fragment
amplified with primers FljBA6-FljBA5 on total DNA of the S.
Typhimurium mutant SM48, using primer FljBA6

<400> 24

agaataactg tgtaggctgg agctgcttcg aagttcctat actttctaga gaataggaac 60
ttcggaatag gaactaagga ggatattcat atggttatgt ttgactgggt gagcaggatc 120
acgtcttcgg actacgctgt 140

INTERNATIONAL SEARCH REPORT

International application No
PCT/BE2006/000020

CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/20 G01N33/53 A61K39/108 A61K39/112 A61P31/04

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)
C12N G01N A61K A61P

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, BIOSIS, EMBASE, MEDLINE, SCISEARCH, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KODAMA CHIE ET AL: "Salmonella flagellin is not a dominant protective antigen in oral immunization with attenuated live vaccine strains" INFECTION AND IMMUNITY, vol. 72, no. 4, April 2004 (2004-04), pages 2449-2451, XP002408910 ISSN: 0019-9567 page 2450 ----- -/-	1-7,12, 13,15, 20,21

☒ Further documents are listed in the continuation of Box C.

☒ 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" 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

23 November 2006

13/12/2006

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

Domingues, Helena

INTERNATIONAL SEARCH REPORT

International application No

PCT/BE2006/000020

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MATSUI HIDENORI ET AL: "Oral immunization with ATP-dependent protease-deficient mutants protects mice against subsequent oral challenge with virulent Salmonella enterica serovar Typhimurium." INFECTION AND IMMUNITY, vol. 71, no. 1, January 2003 (2003-01), pages 30-39, XP002408911 ISSN: 0019-9567 page 31	1-7, 12, 13, 15, 20, 21
X	MANN B J ET AL: "Protection in a gerbil model of amebiasis by oral immunization with Salmonella expressing the galactose/N-acetyl d-galactosamine inhibitable lectin of Entamoeba histolytica" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 15, no. 6-7, April 1997 (1997-04), pages 659-663, XP004064528 ISSN: 0264-410X page 659	1-7, 11-13, 15, 20-23
X	US 2004/120962 A1 (CURTISS ROY [US] ET AL) 24 June 2004 (2004-06-24) page 6; table 1 examples 3, 4	1-7, 11-13, 15, 20-23
Y	CURTISS R ET AL: "NONRECOMBINANT AND RECOMBINANT AVIRULENT SALMONELLA VACCINES FOR POULTRY" VETERINARY IMMUNOLOGY AND IMMUNOPATHOLOGY, AMSTERDAM, NL, vol. 54, no. 1/4, November 1996 (1996-11), pages 365-372, XP000876519 ISSN: 0165-2427 the whole document	1-34
Y	BARBEZANGE CYRIL ET AL: "Some safety aspects of Salmonella vaccines for poultry: In vivo study of the genetic stability of three Salmonella typhimurium live vaccines" FEMS MICROBIOLOGY LETTERS, vol. 192, no. 1, 1 November 2000 (2000-11-01), pages 101-106, XP002408912 ISSN: 0378-1097 the whole document	1-34
	-/-	

52

PCT/BE2006/000020

Category*

Citation of document, with indication, where appropriate, of the relevant passages

Relevant to claim No.

Y

MCFARLAND W C ET AL: "EFFECT OF DIFFERENT
 PURINE AUXOTROPHIC MUTATIONS ON
 MOUSE-VIRULENCE OF A VI-POSITIVE STRAIN OF
 SALMONELLA-DUBLIN AND OF TWO STRAINS OF
 SALMONELLA-TYPHIMURIUM"
 MICROBIAL PATHOGENESIS, ACADEMIC PRESS
 LIMITED, NEW YORK, NY, US,
 vol. 3, no. 2, 1987, pages 129-142,
 XP002376858
 ISSN: 0882-4010
 table 1
 page 135, paragraph 1; table 2
 page 138, paragraph 3

1-34

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/BE2006/000020

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2004120962 A1	24-06-2004	NONE	

(12) SOLICITUD INTERNACIONAL PUBLICADA SEGÚN EL TRATADO DE COOPERACIÓN INTERNACIONAL EN MATERIA DE PATENTES (PCT)

VERSIÓN CORREGIDA

(19) Organización Mundial de Propiedad Intelectual
Oficina Internacional



(43) Fecha de Publicación Internacional
11 de octubre de 2007 (11.10.2007)

PCT

(10) Número de Publicación Internacional
WO 2007/112518 A1

- (51) Clasificación Internacional de Patentes⁷:
C12N 1/20 (2006.01) A61K 39/112 (2006.01)
G01N 33/53 (2006.01) A61P 31/04 (2006.01)
A61K 39/108 (2006.01)
- (21) No. Solicitud Internacional: PCT/BE2006/000020
- (22) Fecha de Presentación Internacional:
20 de marzo de 2006 (20.03.2006)
- (25) Idioma de Presentación: Inglés
- (26) Idioma de Publicación: Inglés
- (71) Solicitante (para todos los países destinatarios con excepción de EU): **VRIJE UNIVERSITEIT BRUSSEL** [BE/BE]; Pleinlaan 2, B-1050 Brussels (BE).
- (72) Inventores, e
- (75) Inventores/Solicitantes (para US únicamente): **DE GREVE, Henri Marcel Jozef**; Jef Lambeauxlaan 11, B-1060 SINT-GILLIS (BE). **ADRIAENSEN, Connie Theresia**; Leiekaai 33, B-9000 GHENT (BE). **HERNALSTEENS, Jean-Pierre Ernest Clément**; François Desmedtstraat 156, B-1150 SINT-PIETERS WOLUWE (BE).
- (74) Agente: **VAN MALDEREN, Joëlle**; pronovem-OFFICE VAN MALDEREN, Avenue Josse Goffin 158, 1082 Brussels (BE).

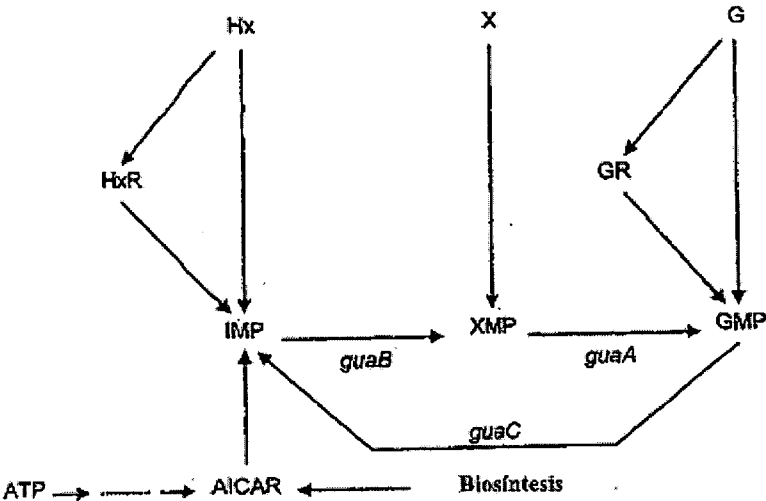
- (81) Países designados (a menos que se indique lo contrario para cada clase de protección disponible):
AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) Países designados (a menos que se indique lo contrario para cada clase de protección disponible):
ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasiática (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Europea (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Publicado:
— con informe internacional de búsqueda
— con el listado de secuencias parte de la descripción publicada separadamente en forma electrónica y disponible bajo petición de la Oficina Internacional.

(48) Fecha de publicación de esta versión corregida:
6 de marzo de 2008

(15) Información sobre la modificación
ver comunicación del 6 de marzo de 2008

(54) Título: VACUNA DE SALMONELA CON VIRUS VIVOS ATENUADA



(57) Extracto: La presente invención está relacionada con una cepa mutante atenuada doble y triple de una bacteria que infecta especies veterinarias tales como Salmonella enterica y/o (a patógenos) Escherichia coli. Los mutantes de la invención contienen por lo menos una primera modificación genética y por lo menos una segunda modificación genética, dicha primera modificación en uno o más genes con movilidad, y dicha segunda modificación en uno o más genes implicados en la supervivencia o en la proliferación del patógeno en el hospedador. La presente invención se refiere a vacunas vivas atenuadas basadas en esos mutantes para la prevención de entre otros Salmonelosis y/o una infección por un patógeno E. coli en una especie veterinaria.

24

- 1 -

VACUNA DE SALMONELA CON VIRUS VIVOS ATENUADA

DESCRIPCION

5

CAMPO DE LA INVENCION

10 La presente invención se refiere a mutantes bacterianos atenuados, en particular a mutantes atenuados de *Salmonella enterica* y a una vacuna con virus vivos atenuada que comprende los mismos. Los mutantes dobles, triples, múltiples de la invención permiten con ventaja una diferenciación serológica entre los animales vacunados y los animales (no vacunados) que se han expuesto a un campo natural tal como el campo natural de *S. enterica*.

15 Las salmonellas son bacilos Gram-negativos, anaerobios facultativos, móviles, que fermentan azúcares distintos de la lactosa, que pertenecen a la familia de las enterobacterias. Las salmonelas habitualmente se transmiten a los seres humanos por el consumo de alimentos contaminados y producen salmonelosis. *E. coli* es otro miembro de la familia de las *Enterobacteriaceae*.

20 Las salmonelas se han aislado de muchas especies animales incluyendo, las vacas, los pollos, los pavos, las ovejas, los cerdos, los perros, los gatos, los caballos, los monos, las focas, los lagartos y las serpientes.

25 El 95% de los patógenos importantes de *Salmonella* pertenecen a *S. enterica*, con la *S. enterica* serovariedad *Typhimurium* (*S. Typhimurium*) y *S. enterica* serovariedad *Enteritidis* (*S. Enteritidis*) las formas más frecuentes.

30 Las infecciones por *Salmonella* son un problema médico y veterinario grave en todo el mundo y producen preocupación en la industria alimentaria. El alimento contaminado no puede identificarse fácilmente. El control de la salmonelosis es importante, para impedir infecciones humanas potencialmente letales y considerables pérdidas económicas en la industria conservera de animales.

La presencia omnipresente de la *Salmonella* en la naturaleza complica el control de la enfermedad precisamente por la detección y erradicación de animales infectados.

Se han probado varias estrategias de control basadas en los principios de exclusión competitiva y vacunación para controlar la infección de por ejemplo, las aves de corral.

5 La vacunación de animales de granja se considera con frecuencia la vía más eficaz para prevenir las zoonosis producidas por la *Salmonella*.

Las vacunas atenuadas de células completas y las vacunas de subunidades se utilizan en la prevención de las infecciones por *Salmonella* en los animales y en los seres humanos con resultados variables. Las vacunas inactivadas en general proporcionan poca protección frente a la salmonelosis.

10 Las vacunas de *Salmonella* atenuadas con virus vivos son potencialmente superiores a las preparaciones inactivadas debido a: (i) su capacidad para producir inmunidad mediada por las células además de respuestas al anticuerpo; (ii) la administración oral sin riesgo de contaminación por la aguja; (iii) eficacia después de la administración de una sola dosis; (iv) producción de respuestas inmunitarias en
15 múltiples puntos de la mucosa; (v) bajo coste de producción; y (vi) su posible utilización como vehículos para la administración de antígenos recombinantes al sistema inmunitario.

Se ha probado la eficacia de las siguientes cepas mutantes atenuadas para provocar una respuesta inmunitaria protectora en los animales tratados: (1) cepas que
20 llevan mutaciones en los genes *aro* (Alderton et al., 1991, *Avian diseases* 35: 435-422; Schiemann y Montgomery, 1991, *Veterinary Microbiology* 27: 295-308), (2) cepas que llevan eliminaciones en los genes *cya* (adenilato ciclase) y/o *crp* (receptor de AMP cíclico) (patentes US. nº 5.389.368; US nº 5.855.879; US nº 5.855.880; Hassan y Curtiss, 1997; *Avian Diseases* 41:783-791; Porter et al. 1993, *Avian Diseases* 37:265-
25 273) y (3) cepas que llevan mutaciones en los genes del operón *guaBA* de *S. typhi* (Wang et al., 2001, *Infection and Immunity* 69:4734-4741; documento WO 99/58146 y patente US nº 6.190.669).

Hasta ahora solamente la cepa Megan® Vac1 de vacuna, que lleva consigo eliminaciones en los genes *cya* y *crp* ha sido eficaz, al menos en parte. Esta cepa no
30 proporciona protección completa (<http://www.meganhealth.com/meganvac.html>).

McFarland y Stocker (1987, *Microbial pathogenesis* 3:129-141) describieron la virulencia de los mutantes de la inserción de *guaA* y *guaB* Tn10 de *S. Typhimurim* y *S. dublin* en ratones BALB/c. A alta dosis ($2,5 \times 10^7$ CFU para *S. Typhimurium* y 10^4 cfu



para *S. dublin*), estos autores describieron una letalidad significativa de los animales, procedente de la multiplicación de la cepa auxótrofa.

Asimismo el mutante ΔguaB demostró no ser candidato adecuado para una protección firme y segura contra la fiebre tifoidea. Esté presentó una virulencia residual significativa en los ratones (Wang *et al.*, 2001).

Hay por lo tanto todavía necesidad de cepas de la vacuna de *Salmonella* atenuadas con virus vivos, así como de cepas de la vacuna de *Salmonella* atenuadas con virus vivos mejoradas de bacterias que infectan especies veterinarias en general.

Los animales vacunados con frecuencia producen anticuerpos contra diferentes antígenos del patógeno. El problema es que los animales vacunados como tales no pueden ya distinguirse de los animales que han estado en contacto con una cepa de ámbito natural tal como la cepa del ámbito de *Salmonella*, y son posiblemente infectadas con ésta.

Por lo tanto, existe también necesidad de cepas atenuadas con microbios vivos mejoradas similares a las cepas de la vacuna atenuada contra *Salmonella* que harían posible dicha diferenciación.

OBJETIVOS DE LA INVENCION

Un objetivo de la presente invención consiste en proporcionar cepas de *Salmonella enterica* atenuadas con una mutación doble o triple.

Otro objetivo de la presente invención consiste en proporcionar una vacuna con virus vivos atenuada contra la salmonelosis y procedimientos de prevención basados en la misma.

Todavía otro objetivo de la presente invención consiste en proporcionar cepas de *Salmonella* atenuadas que son útiles como vector con microbios vivos y como vacunas mediadas por ADN que expresan xenoantígenos. Dichas cepas son muy adecuadas para el desarrollo de vacunas incluyendo las vacunas polivalentes.

Todavía otro objetivo de la presente invención consiste en proporcionar un procedimiento para conseguir mutantes por eliminación de *S. enterica* de la invención.

Además otro objetivo de la presente invención consiste en proporcionar una cepa de *Salmonella* atenuadas que permite una diferenciación serológica entre animales vacunados y no vacunados incluso posiblemente de animales infectados.

Además otro objetivo de la presente invención consiste en proporcionar los mismos materiales y procedimientos para la preparación de cepas atenuadas de bacterias que infectan especies veterinarias en general, más en particular aves de corral.

- 5 El objetivo general consiste en mejorar la seguridad de los alimentos y la salud de los animales.

SUMARIO DE LA INVENCION

- 10 Algunos mutantes de *Salmonella enterica* auxótrofos de $\Delta guaB$ con una mutación por eliminación en el gen *guaB* presentan virulencia residual. Se descubrió que modificaciones adicionales (preferentemente eliminaciones) en uno o más genes implicados en la movilidad reducían la virulencia restante sin afectar las capacidades inmunógenas de la cepa.

- 15 Un primer aspecto de la invención por consiguiente se refiere a una cepa mutante atenuada de una bacteria que infecta especies veterinarias, en particular una cepa mutante atenuada de *S. enterica*, en la que dicha cepa mutante contiene por lo menos una primera modificación genética y por lo menos una segunda modificación genética, dicha primera modificación en uno o más (por lo menos uno) genes con
20 movilidad y dicha segunda modificación en uno o más (por lo menos uno) genes implicados en la supervivencia o en la proliferación de la bacteria o del patógeno (por ejemplo *S. enterica*) en el hospedador. La expresión "especie bacteriana que infecta la bacteria" en el contexto de la invención se refiere en particular a bacterias que son patógenas para especies veterinarias, y que pueden ser atenuadas mediante las
25 modificaciones genéticas anteriores. La especie veterinaria que infecta la bacteria puede ser una bacteria Gram-negativa. Para las aves de corral se prefieren las bacterias Gram-negativas tales como *Salmonella*, *Pasteurella*, *Escherichia coli*, etc. Las más preferidas son *Salmonella enterica* y *E. coli* (patógena). "Patógena para" significa que la bacteria, si no está atenuada, es capaz de producir una enfermedad
30 infecciosa en la especie veterinaria.

Las modificaciones genéticas de la invención conducen favorablemente a una función inválida, en otras palabras alteran o afectan la función génica. La modificación en el presente contexto se denomina también "modificación por alteración". La

modificación se dice que inactiva el gen en cuestión. Con ventaja, dicha inactivación produce atenuación, por lo menos en un grado que la cepa mutante es adecuada para su utilización en una vacuna con microbios vivos atenuada.

La modificación genética puede ser una inserción, una eliminación y/o una sustitución de uno o más nucleótidos en dichos genes. Las cepas mutantes según la invención por dicha modificación están afectadas en una función génica de movilidad y en una función génica necesaria para la supervivencia o la proliferación del patógeno, que conduce a una función inválida (no se forma ningún producto génico funcional) de los genes afectados.

Se prefieren mutantes por eliminación, ya que un mutante por inserción puede revertir, restaurando de este modo la patogenicidad de la cepa.

La primera modificación consiste en uno o más (1, 2, 3, ...) genes con movilidad. Ejemplo de un gen implicado en la movilidad son los genes que codifican la flagelina. El mutante de la invención puede tener una modificación (alteración) en los genes *fliC* y/o el *fljB* o el *fljBA* respectivamente (*fliC*; *fljB*; *fljBA*; *fliC* y *fljB*; *fliC* y *fljBA*; ...). Favorablemente los mutantes, en los que se han eliminado todos los genes que codifican la flagelina, son incapaces de congregarse en el medio LB que contiene agar-agar al 0,4% y pueden de este modo distinguirse fácilmente de las cepas móviles naturales.

La segunda modificación genética consiste en uno o más genes (1, 2, 3, ...) implicados en la supervivencia o en la proliferación del patógeno en su hospedador. Dicho gen puede ser un gen constitutivo o un gen con virulencia. Un ejemplo de un gen constitutivo que puede conducir a cepas atenuadas cuando la función génica está afectada, es el gen *guaB* que codifica la enzima IMP deshidrogenasa. Dicho mutante es incapaz de formar de nuevo nucleótidos de guanina. También son posibles modificaciones por alteración en el operón *guaBA*, que conducen favorablemente a una función inválida del gen o genes que codifica(n) o regula(n) la actividad propia de IMP deshidrogenasa.

Favorablemente, las cepas mutantes atenuadas de la invención son inmunógenas.

La presente invención en particular tiene por objeto proporcionar cepas atenuadas de *S. Enteritidis* y *S. Typhimurium*.

Preferentemente las modificaciones genéticas de la invención se introducen en una cepa progenitora 76Sa88 tipo 4 del fago *S. Enteritidis* o en una cepa precursora 1491S96 de *S. Typhimurium*. La cepa 76Sa88 es una cepa clínica procedente de un pavo, obtenida en el Veterinary and Agrochemical Research Centre, Groeselenberg 99, B-1180 Ukkel, Bélgica, que alberga el plásmido pKD46 de replicación sensible a la temperatura, que codifica al sistema de bacteriófago Lambda Red recombinasa. La cepa 1491S96 es una cepa clínica procedente de un pollo.

Una de las cepas atenuadas de *S. enterica* obtenida según la invención es la cepa SM73 de *S. Enteritidis* que tiene el número de depósito LMG P-21642. Otro ejemplo es la cepa SM89 atenuada de *S. Typhimurium* que tiene el número de depósito LMG P-21643.

Un mutante preferido de la invención lleva o comprende una modificación genética en el gen *guaB* y una modificación genética en el gen *fliC*.

Otro mutante preferido de la invención lleva o comprende una modificación genética en un gen *guaB* y una modificación genética en los genes *fljBA*.

Las cepas adecuadas de la invención son muy adecuadas para su utilización en una vacuna atenuada con virus vivos. Las cepas mutantes de la invención pueden codificar y expresar un xenoantígeno.

Otro aspecto de la invención se refiere a una vacuna para inmunizar una especie veterinaria contra una infección bacteriana que comprende:

- una cantidad farmacéuticamente eficaz o inmunizadora de una cepa mutante atenuada según la invención; y

- un vehículo o diluyente farmacéuticamente aceptable. La presente invención se refiere en particular a vacunas que comprenden cepas mutantes atenuadas de *S. entérica* o *E. coli*.

En general se administran aproximadamente de 10^2 cfu a aproximadamente 10^{10} cfu, preferentemente aproximadamente 10^5 cfu a aproximadamente 10^{10} cfu (ejemplos de una cantidad farmacéuticamente eficaz o inmunizadora). Una dosis inmunizadora oscila según la vía de administración. Los expertos en la materia pueden encontrar que la dosis eficaz para una vacuna administrada por vía parenteral puede

63

- 7 -

ser inferior a la de una vacuna similar que se administre mediante agua potable y similares.

5 Las cepas atenuadas de la invención y las composiciones farmacéuticas o las vacunas que comprenden las mismas son muy adecuadas para inmunizar animales tales como especies veterinarias, ganado y más específicamente aves de corral. Por ejemplo, las cepas atenuadas de *Salmonella* de la invención, y las composiciones farmacéuticas o las vacunas que comprenden las mismas, son muy adecuadas para inmunizar especies veterinarias y en particular aves de corral tales como pollos frente a la salmonelosis y posiblemente otras enfermedades (por ejemplo, en el caso de un
10 vacuna polivalente). Las cepas atenuadas de la invención son particularmente adecuadas para proteger la especie animal/veterinaria en cuestión contra un ataque por el patógeno (la bacteria que infecta especies veterinarias) en cuestión.

Un aspecto adicional de la invención se refiere por consiguiente a un procedimiento de inmunización de animales, preferentemente especies veterinarias,
15 más preferentemente aves de corral tales como pollos contra una enfermedad producida por una bacteria que infecta especies veterinarias, comprendiendo dicho método las etapas siguientes:

- administrar al animal o especie veterinaria que lo necesita un agente
20 inmunizador de una cepa mutante atenuada de la invención y/o de una vacuna que comprende la misma, con lo que a continuación se provoca una respuesta inmunitaria protectora en el animal o especie veterinaria. La presente invención en particular se refiere a los procedimientos de inmunización de especies veterinarias contra la salmonelosis o contra una infección por *E. coli* patógena.

25 Ejemplos de especies veterinarias que van a ser inmunizadas contra la salmonelosis: aves de corral, ganado pequeño o pesado tales como pollos, pavos, patos, codornices, gallinas de Guinea, cerdos, ovejas, terneras, vacas, etc. Se administra una cantidad inmunizadora a estos animales, preferentemente por vía oral, nasal o parenteral.
30

Un aspecto adicional de la invención se refiere a una cepa mutante de la invención para su utilización como medicamento (por ejemplo para su utilización en una vacuna). Otro aspecto todavía de la invención se refiere a la utilización de una

cepa mutante atenuada de la invención para la preparación de un medicamento, tal como una vacuna, para la prevención (y/o tratamiento) de una enfermedad producida por un patógeno (la bacteria que infecta especies veterinarias) tal como la salmonelosis. Los ejemplos de animales o especies veterinarias que se va a tratar y dosis recomendadas se proporcionan anteriormente.

Todavía otro aspecto de la invención se refiere a la utilización de mutantes de la invención, y en particular a mutantes de flagelina, como marcadores serológicos para distinguir entre los animales vacunados y los animales que están infectados de manera natural, es decir que se han puesto en contacto y llegan a infectarse por una cepa natural.

La invención, por ejemplo, se refiere a un procedimiento para una diferenciación serológica entre los animales vacunados y los animales infectados por una cepa natural, en el que los animales vacunados han sido inmunizados con una cepa mutante en la que un gen de flagelina está inactivado, comprendiendo dicho procedimiento las etapas siguientes:

- ensayar en animales la presencia de anticuerpos contruidos contra la flagelina,
- distinguir los animales infectados de los animales vacunados basándose en la presencia o ausencia de dichos anticuerpos.

El procedimiento de la invención convenientemente es un procedimiento *in vitro*. Convenientemente los animales infectados por salmonelas se distinguen como tales de los animales que han sido inmunizados con una vacuna atenuada con microbios vivos según la invención.

Los animales de granja, tales como las aves de corral y en particular los pollos se sabe que generan anticuerpos contra los productos del gen de la flagelina y en particular el producto del gen FliC. Los anticuerpos en cuestión serán detectados de este modo en un animal infectado por una cepa natural (que genera dichos anticuerpos), no obstante no en un animal que ha sido vacunado con una cepa mutante en la que un(os) gen(es) de flagelina está(n) inactivado(s). Estos últimos no generan anticuerpos contra por ejemplo, FliC y/o FljB.

La presencia de dichos anticuerpos es indicadora de la presencia de cepas naturales y de este modo de la infección. El procedimiento de la invención de este modo permite con ventaja la detección o el diagnóstico de una infección por *Salmonella* en los animales vacunados por una cepa mutante en los que un(os) gen(es) de flagelina está(n) inactivado(s). Dicha cepa mutante puede ser una de las cepas de la invención descritas anteriormente en la presente memoria.

La inactivación de los genes de flagelina tal como *fliC*, permite de este modo la utilización de pruebas serológicas, por ejemplo, basadas en la detección de la proteína FliC, para el diagnóstico de la presencia de cepas naturales, tal como de *S. enterica* natural, en animales (vacunados). En el procedimiento de la invención en los animales se ensayan preferentemente anticuerpos contra FliC.

El procedimiento de la invención es aplicable en particular a aves de corral, más preferentemente a pollos.

DESCRIPCIÓN DETALLADA DE LA INVENCION

Se descubrió sorprendentemente que la combinación de la mutación de flagelina (una modificación por alteración en un gen que codifica flagelina; denominada también mutación flagelada) y una mutación auxótrofa pueden conducir a cepas atenuadas muy inmunógenas de *S. enterica*. Las cepas mutantes según la invención llevan o comprenden una(s) (por lo menos una) modificación o modificaciones en un(os) gen(es) con movilidad y una(s) (por lo menos una) modificación o modificaciones en un(os) gen(es) implicado(s) en la supervivencia o la proliferación del patógeno en su hospedador.

Un gen implicado en la supervivencia o proliferación puede ser un gen constituido y/o un gen con virulencia. Ejemplos de dichos genes constituidos y de genes con virulencia pueden encontrarse en (Mastroeni et al., 2000, *The Veterinary Journal* 161:132-164, incorporada como referencia a la presente memoria). Un ejemplo preferido de un gen constitutivo es el gen *guaB*, incluso puede imaginarse también una modificación en un gen *aro*, *pur*, *dap*, *pab*, *sipC*, *phoP*, *phoQ*, *pagC*, *cya*, y/o *crp*.

El término "gen" tal como se utiliza en la presente memoria se refiere a la secuencia de codificación y a sus secuencias reguladoras tales como las señales de activación y terminación.

67

Dicha inactivación puede obtenerse mediante una eliminación mediante la cual la función del gen *guaB* se altera. Un experto en la materia conoce cómo obtener dichos mutantes y una prueba sencilla puede decir si la función del gen está alterada. Por ejemplo, la cepa mutante que no puede expresar un producto del gen *guaB* funcional en medio Mínimo A, a menos que este medio esté enriquecido con guanina, xantina, guanosina o xantosina.

Otro ejemplo sencillo puede decir si una función del gen con movilidad tal como si se altera una función del gen de flagelina. Estos mutantes no se congregan en un medio LB que contiene agar-agar al 0,4%.

Las modificaciones en los genes en cuestión producirían atenuación de las cepas mutantes, preferentemente por lo menos hasta un grado que sean adecuadas para su utilización en una vacuna con microbios vivos.

Los ejemplos que se proporcionan a continuación demuestran que las mutaciones adicionales en un(os) (por lo menos uno; uno o más) gen(es) con movilidad (*fliC*, *fljB* y/o *fljBA*) con ventaja alivió la patogenicidad residual de un mutante por eliminación de *guaB* y mejoró la protección de los animales inmunizados contra la prueba de provocación con una dosis letal de *S. enterica* natural.

El filamento flagelado de todos los miembros del género *Salmonella* es un polímero de una sola proteína, la proteína flagelina (*van Asten et al.*, 1995, *Journal of Bacteriology* **177**:1610-1613).

FliC es la proteína de la subunidad del filamento en fase 1 de la flagelina (*Ciacci-Woolwine et al.*, 1998, *Infection and Immunity* **66**:1127-1134).

S. Typhimurim tiene dos genes de flagelina (*fliC* y *fljB*) que están situados en diferentes puntos del cromosoma y que presentan variación de fase. El activador de *fljB* forma parte de un fragmento cromosómico que puede invertirse por recombinación específica en el punto. Dependiendo de la orientación, *fljB* se expresa junto con *fljA*, este último que codifica un represor del gen *fliC*; o *fliC* se lleva hasta expresión. *E. coli*, otro miembro de las *enterobacterias*, puede poseer también dos genes de flagelina que comparten respectivamente homología con los genes *fliC* y *fljB* de *S. enterica* (*Tominaga*, 2004, *Genes Genet. Syst.* **79**:1-8).

La inactivación del gen *fliC* en *S. Enteritidis* (que codifica la proteína flagelada mayor) aumentó tanto la seguridad como la eficacia de una vacuna administrada a la

64

estirpe BALB/c de ratón consanguíneo. Esta estirpe es muy sensible a la salmonelosis generalizada.

5 Esto demuestra entre otras cosas que la flagelina no es antígeno esencial para la provocación de una respuesta inmunitaria protectora contra la *Salmonella* en ratones BALB/c a pesar sin embargo de muchas indicaciones en la bibliografía.

La flagelina de *S. Enteritidis* es inmunógena en pollos y lleva los determinantes antigénicos H:g,m (van Asten et al., 1995; Wyant et al., 1999, *Infection and Immunity* 67:1338-1346; Ogushi et al., 2001, *The Journal of Biological Chemistry* 276:30521-30526).

10 Existen pruebas de que las flageladas de varias especies de bacterias Gram-negativas (por ejemplo las de *S. Enteritidis* y *S. typhi*) activan los monocitos para producir citocinas proinflamatorias (por ejemplo, el factor alfa de la necrosis tumoral) y median la activación de la cinasa asociada al receptor de interleucina-1 (IRAK).

15 Se cree por lo tanto que la flagelina Gram-negativa desempeña una función importante y previamente no reconocida en la respuesta inmunitaria innata a las bacterias Gram-negativas. FliC puede ser de particular importancia durante el transcurso de las infecciones en el aparato digestivo (Ciacci-Woolwine et al., 1998; Wyant et al., 1999; Moors et al., 2001, *Infection and Immunity* 69:4424-4429).

20 Existe mucha ambigüedad en la bibliografía en cuanto al grado en el que los flagelados contribuyen a la virulencia en aves de corral y/o seres humanos.

Van Asten et al. (2000, *FEMS Microbiol. Lett.* 185:175-9) han demostrado que la activación del gen de flagelina de *S. Enteritidis* reduce en gran medida (50 veces) la invasión en las células Caco-2 (estirpe celular de carcinoma de colon humano), mientras que la adherencia bacteriana no fue afectada realmente. Dicho informe se limita a resultados *in vitro*.

25 Parker y Guard-Petter (2001, *FEMS Microbiology Letters* 204:287-291) por otra parte descubrieron que, en la prueba de provocación oral de pollos, un mutante *fliC::Tn10* fue igualmente virulento para el tipo natural. Esto indica que la presencia de flagelina no fue necesaria para conseguir por lo menos un nivel moderado de invasión después de la prueba de provocación oral. Cuando se aplica por vía subcutánea, los mutantes flagelados se atenuaron de manera significativa en comparación con la cepa natural.

30

Existen por lo tanto muchas indicaciones en la técnica anterior que dan a conocer aparte de construcciones por ejemplo, mutantes dobles (o triples) de *S. enterica* según la invención. La inactivación de uno o más genes con movilidad ayuda a reducir la virulencia restante o por ejemplo los mutantes por eliminación de *guaB* pero existen también otras ventajas.

La inactivación por ejemplo, del gen *fliC* permite con ventaja la utilización de pruebas serológicas, basadas en la detección de anticuerpos dirigidos contra la proteína FliC, para el diagnóstico de la presencia de *S. enterica* natural, por ejemplo, *S. Enteritidis*, en los animales (vacunados). La inmunodetección es posible por ELISA, por las técnicas RIA y/o cualquier otra prueba o formato inmunológicamente conocido.

IDEXX Laboratories posee una prueba en el mercado (FlockChek® *Salmonella Enteritidis* Antibody Test Kit) para detectar de manera fiable anticuerpos contra determinantes H-antigénicos de la flagelina FliC de *S. Enteritidis* (epítomos flagelados H:g,m).

Lo que se ha comentado anteriormente demuestra que los mutantes dobles y/o triples de *S. enterica* de la invención, que llevan una modificación genética (alteración) en un gen implicado en la supervivencia o la proliferación del patógeno en el huésped y en un(os) gen(es) implicado(s) en la movilidad presenta ventajas sobre las cepas atenuadas de *S. enterica* que forman parte de la técnica.

Las cepas mutantes de la invención son muy adecuadas para su utilización en una vacuna atenuada con virus vivos, tal como un vector con microbios vivos y/o una vacuna mediada por ADN. El término "vacuna" significa que incluye vacunas profilácticas así como terapéuticas. Preferentemente la vacuna es profiláctica.

Las "Vacunas de vector con microbios vivos", denominadas también "vacunas vehículo" y "sistemas de administración de antígenos de microbios vivos", comprenden un área excitante y versátil de la vacunología (Levine et al., 1990, *Microecol. Ther.* 19:23-32). En este procedimiento, se modifica una vacuna vírica o bacteriana con microbios vivos de modo que expresa xenoantígenos protectores de otros microorganismos y administra los antígenos al sistema inmunitario, estimulando de este modo una respuesta inmunitaria protectora. Los vectores bacterianos vivos que se están publicando incluyen, entre otros, la *Salmonella* atenuada.

Un objetivo de la invención consiste en proporcionar cepas mutantes atenuadas para su utilización en una vacuna con microbios vivos, posiblemente una

vacuna con microbios vivos polivalente o multivalente. "Vacuna polivalente" o "vacuna multivalente" significa en particular una vacuna que comprende determinantes antigénicos de numerosos organismos diferentes causantes de enfermedades.

Uno de los objetivos de la invención consiste, por consiguiente, en proporcionar una vacuna contra por ejemplo la salmonelosis que comprende:

- una cantidad farmacéuticamente eficaz o inmunizadora de una cepa mutante atenuada de la invención; y
- un vehículo o diluyente farmacéuticamente aceptable.

Otro objetivo de la invención consiste en proporcionar una vacuna con virus vivos de vector que comprende:

- una cantidad farmacéuticamente eficaz o inmunizadora de un cepa mutante de la invención, en la que dicho mutante codifica y expresa un xenoantígeno; y
- un vehículo o diluyente farmacéuticamente aceptable.

El xenoantígeno específico empleado en el vector con microbios vivos no es crítico para la presente invención.

Aún otro objetivo de la invención consiste en proporcionar una vacuna mediada por ADN que comprende:

- una cantidad farmacéuticamente eficaz o una cantidad inmunizadora de una cepa mutante atenuada de la invención; en la que dicho mutante contiene un plásmido que codifica y se expresa en una célula eucariótica, un xenoantígeno; y
- un vehículo o diluyente farmacéuticamente aceptable.

Los detalles en cuanto a la construcción y utilización de vacunas mediadas por ADN pueden encontrarse en la patente US nº 5.877.159, que se incorpora a la presente memoria como referencia en su totalidad. De nuevo, el xenoantígeno específico empleado en la vacuna mediada por ADN no es crítico para la presente invención.

65

5 La decisión de si expresar el xenoantígeno en el patógeno (utilizando un activador procariótico en una vacuna de vector con microbios vivos) o en las células invadidas por el patógeno (utilizando un activador eucariótico en una vacuna mediada por ADN) puede basarse en qué construcción de vacuna para este antígeno específico proporciona la mejor respuesta inmunitaria en los estudios con animales o en las pruebas clínicas, y/o, si la glucosilación de un antígeno es esencial para su inmunogenicidad protectora, y/o, si la configuración terciaria correcta de un antígeno se consigue mejor con una forma de expresión que con la otra (patente US nº 5.783.196).

10 La expresión "Cantidad farmacéuticamente eficaz" significa una cantidad mucho mayor que la normal para superar (prevenir y/o tratar) la enfermedad en cuestión, por ejemplo la salmonelosis. "Cantidad inmunizadora" tal como se utiliza en la presente memoria significa de hecho una cantidad que es capaz de provocar una respuesta inmunitaria (protectora) en el animal que recibe la composición/vacuna
15 farmacéutica. La respuesta inmunitaria provocada puede ser una respuesta humoral, de las mucosas, local y/o celular. Como es sabido en la técnica las cantidades necesarias pueden depender de la edad, el sexo, el peso y de muchos otros factores.

Los vehículos o diluyentes específicos farmacéuticamente aceptables empleados no son críticos para la presente invención y son convencionales en la
20 técnica. Ejemplos de diluyentes incluyen: tampón para tamponar los ácidos gástricos en el estómago, tal como tampón de citrato (pH 7,0) que contiene sacarosa, tampón de bicarbonato (pH 7,0) solo o tampón bicarbonato (pH 7,0) que contiene ácido ascórbico, lactosa y opcionalmente aspartamo. Ejemplos de vehículos incluyen: proteínas, por ejemplo, como las que se encuentran en la leche desnatada; azúcares;
25 por ejemplo sacarosa; o polividona.

Los mutantes por eliminación según la invención se crearon por técnicas de recombinación homólogas convencionales, por lo que el/los gen(es) completo(s) o por lo menos parte de los genes en cuestión en una primera etapa se sustituye(n) por un gen con resistencia y las secuencias de FRT adyacentes.

30 Preferentemente, en una segunda etapa, dicho gen con resistencia se separa por recombinación entre las dos secuencias de FRT. Una secuencia de FRT y las secuencias P1 y P2 de cebado permanecen por el mecanismo molecular de la

recombinación que elimina el gen con resistencia a antibióticos según Datsenko y Wanner (2000) (véase por ejemplo la Figura 4).

La invención se describirá con más detalle en los ejemplos y formas de realización siguientes haciendo referencia a los dibujos adjuntos. Las formas de realización y ejemplos específicos no pretenden en modo alguno limitar el alcance de la invención reivindicada. El fundamento de los ejemplos proporcionados en la presente memoria para *S. enterica* son asimismo muy aplicables a otras bacterias (Gram-negativa) que infectan especies veterinarias, más en particular otras bacterias (Gram-negativas) para aves de corral tales como *Pasteurella*, *E. coli* (patógena), etc.

BREVE DESCRIPCIÓN DE LOS DIBUJOS

La Figura 1 proporciona una visión de conjunto esquemática de la serie de reacciones de la biosíntesis del monofosfato de guanosina.

AICAR: 5'-fosforribosil-4-carboxamida-5-aminoimidazol; ATP: trifosfato de adenosina; G: guanina; GMP: monofosfato de guanosina; GR: guanosina; Hx: hipoxantina; HxR: ribósido de hipoxantina (inosina); IMP: monofosfato de inosina; X: xantina, XMP: monofosfato de xantosina; *guaA*: GMP sintetasa, *guaB*: IMP deshidrogenasa; *guaC*: GMP reductasa.

La Figura 2 representa el cóntigo 1294 del genoma de *S. Enteritidis* (SEC. ID. nº: 19). El codón de iniciación **ATG** y el codón de terminación **TGA** del gen *guaB* están en negrita. N puede ser A, C, T o G.

La Figura 3 representa la secuencia del fragmento Δ *guaB* de *S. Enteritidis* clonado en pUC18 (SEC. ID. nº: 20). Los cebadores que se utilizaron son los indicados por flechas horizontales. El fragmento generado con los cebadores GuaB6-GuaB7 se clonó en pUC18. El codón de iniciación **ATG** y de terminación **TGA** del gen *guaB* de la enzima de restricción *SmaI* **CCCGGG** se indican en negrita.

La Figura 4 representa la secuencia nucleotídica del fragmento por PCR de *S. Enteritidis*, que incluye la eliminación de *guaB*, obtenida tras el secuenciado, utilizando el cebador GuaB10 (SEC. ID. nº: 21). El fragmento por PCR se amplió con los cebadores GuaB6-GuaB7, utilizando el ADN genómico completo del mutante SM20. La secuencia FRT restante está indicada en letra negrita en cursiva y los cebadores

P1 y P2 por flechas (Datsenko y Wanner, 2000, PNAS 97:6640-6645). Los codones de iniciación **ATG** y de terminación **TGA** del gen *guaB* están indicadas en negrita.

La Figura 5 representa el gen *guaB* de *S. Typhimurium* LT2, apartado 117 de 220 del genoma completo (SEC. ID. n°: 22). El codón de iniciación **ATG** y el codón de terminación **TGA** del gen *guaB* están en negrita.

La Figura 6 presenta la secuencia nucleotídica obtenida después del secuenciado del fragmento por PCR ampliado con cebadores FliC1-FliC2 en el ADN completo de los mutantes SM73 y SM89, utilizando los cebadores FliC1 y FliC2 (SEC. ID. n°: 23). La secuencia FRT restante se indica en letra negrita cursiva, los codones de iniciación **ATG** y de terminación **TAA** en negrita, y P1 y P2 están indicados con flechas.

La Figura 7 presenta la secuencia nucleotídica obtenida después del secuenciado del fragmento PCR ampliado con los cebadores FljBA6-FljBA5 en el ADN completo del mutante SM48 de *S. Typhimurium*, utilizando el cebador FljBA6 (SEC. ID. n°: 24). El punto FRT restante está indicado en negrita, P1 y P2 están indicados con flechas.

Las Figuras 8 a 11 representan las recetas del depósito para SM69, SM73, SM86 y SM89 respectivamente.

EJEMPLOS

Ejemplo 1: mutación auxótrofa que afecta al gen *guaB*

Un mutante auxótrofo de la inserción de una *S. Enteritidis* natural se obtuvo por mutagénesis de la inserción. Solamente cuando se enriquece con guanina, xantina, guanosina o xantosina 0,3 mM pudo desarrollarse la cepa mutante en medio Mínimo A.

Estos datos sugieren fuertemente que la mutación auxótrofa de la cepa afecta al gen *guaB*, que codifica la enzima IMP deshidrogenasa (EC 1.1.1.205). Esta enzima transforma la inosina-5'-monofosfato (IMP) en monofosfato de xantosina (XMP) como se indica en la Figura 1.

Un mutante de la inserción puede revertir, restaurando de este modo la patogenicidad de la cepa. Esto limita su aplicación en una cepa con virus vivos

atenuada. En este aspecto se prefieren mutantes por eliminación. Se crearon por consiguiente y se probaron los mutantes por eliminación de *guaB* de *S. Enteritidis* y *S. Typhimurium*. Los genes *guaB* de ambas serovariantes se proporcionan en las Figuras 2 y 5.

5

Ejemplo 2: mutantes por eliminación de *guaB*

Construcción de los mutantes por eliminación de *guaB*

10

Se aplicó con este objetivo un procedimiento para generar mutaciones por eliminación en el genoma K12 de *E. coli* (Datsenko y Wanner, 2000, PNAS 97:6640-6645). Este procedimiento se basa la recombinación homóloga, mediada por el sistema bacteriófago λ Red recombinasa, de un fragmento lineal de ADN generado por PCR en el que la secuencia *guaB* se sustituye por un gen con resistencia a

15

antibióticos. Este gen con resistencia es adyacente a las secuencias de FRT y puede escindirse del genoma por recombinación específica para el sitio, mediada por la FLP recombinasa.

20

Se aplicó PCR por solapamiento (Ho et al., 1989, Gene 77:51-59) para la eliminación de un segmento interno de 861 bp de la secuencia de codificación de *guaB*. El principio se basa en la utilización de dos series de cebador, GuaB3-GuaB4 (adyacente al extremo 5' del gen *guaB*). Ambas series contienen cebadores (GuaB4 y GuaB5) que son parcialmente complementarios y a los cuales se añade una secuencia de restricción *SmaI*. Tras la hibridación de las secuencias complementarias resultantes y la elongación de la cadena, la PCR con los cebadores externos GuaB6 y GuaB7 generó un fragmento con una secuencia *SmaI* de 6 pares de bases que sustituye a un

25

segmento interno de 861 pares de bases de la secuencia de codificación *guaB*. Este fragmento Δ *guaB* se clonó en el vector pUC18 (véase la Figura 3).

30

El gen con resistencia al cloranfenicol (*cat*) con sus secuencias FRT adyacentes se amplió utilizando los cebadores P1 y P2 (Datsenko y Wanner, 2000) y el ADN plásmido pKD3 (Datsenko y Wanner, 2000) como plantilla. Este fragmento de PCR se ligó en la secuencia *SmaI* del fragmento Δ *guaB* clonado. El fragmento deseado se generó utilizando cebadores anidados (GuaB6-GuaB7). El fragmento por PCR resultante se electroporó en 76Sa88 de *S. Enteritidis* que alberga el plásmido

pKD46 de replicación sensible a la temperatura, que codifica al sistema de bacteriófago Lambda Red recombinasa. Se probaron transformantes resistentes al cloranfenicol en medio Mínimo A y en medio Mínimo A enriquecido con guanina 0,3 mM. Los mutantes $\Delta\text{guaB}::\text{catFRT}$ se confirmaron por PCR utilizando las siguientes combinaciones de cebador: GuaB6-GuaB7, GuaB6-P2, GuaB7-P1 y P1-P2.

El mutante $\Delta\text{guaB}::\text{catFRT}$ de *S. Enteritidis* (SM12) se electroporó con el plásmido pCP20 de replicación sensible a la temperatura, que codifica la FLP recombinasa, para eliminar el gen *cat*. La cepa ΔguaB de *S. Enteritidis* resultante se denominó SM20. El fragmento por PCR en el que está situada la eliminación se obtuvo utilizando ADN genómico completo del mutante SM20 y la combinación de cebador GuaB6-GuaB7. La mutación ΔguaB se confirmó por secuenciado, utilizando el cebador GuaB10, de este fragmento (véase la Figura 4).

En la Tabla 1 se proporcionan las secuencias de todos los cebadores mencionados anteriormente.

Para evitar la presencia de posibles mutaciones adicionales, producidas por la expresión del sistema de la Red recombinasa, se construyó una cepa isógena.

La mutación $\Delta\text{guaB}::\text{catFRT}$ de la SM12 mutante fue transducida por el bacteriófago P22 HT *int* (Davis, R.W., Botstein D. y Roth, J.R. (1980) en *Advanced Bacterial Genetics, A manual for genetic engineering*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) lisado de SM12 para 76Sa88 de *S. Enteritidis* natural. Se eliminó el gen *cat* utilizando el plásmido pCP20. La cepa ΔguaB de *S. Enteritidis* resultante se denominó SM69 con el número de depósito LMG P-21641.

Se construyó un mutante ΔguaB de la cepa 1491S96 de *S. Typhimurium* utilizando el mismo procedimiento y los mismos cebadores. La cepa resultante se denominó SM19. SM86 (con el número de depósito LMG P-21646) es la cepa isógena obtenida de la misma después de la transducción de $\Delta\text{guaB}::\text{catFRT}$ a la cepa 1491S96 utilizando un lisado del bacteriófago P22 HT *int* de SM9 y después la escisión del gen *cat*.

Los mutantes SM19, SM20, SM69 y SM86 de ΔguaB son sensibles al bacteriófago P22 HT *int*. Esto prueba la presencia de lipopolisacáridos (LPS) intactos.

Pruebas de virulencia y protección con el mutante SM20 por eliminación de *guaB* de *S. Enteritidis* en ratones

5 Se probó la virulencia del mutante SM20 en ratones por infección oral de ratones BALB/c hembra de 6 a 8 semanas de vida (*Pattery et al., 1999, Molecular Microbiology* **33**:791-805) en dos experimentos independientes. Éstos se realizaron como se describió anteriormente. La cepa natural 76Sa88 de *S. Enteritidis* se probó en paralelo como referencia positiva. El mutante SM50 de 76Sa88 Δ aroA de *S. Enteritidis* se incluyó en el experimento como referencia de la vacuna. Este mutante lleva una
10 eliminación precisa de la secuencia de codificación *aroA* completa y se construyó por el procedimiento de Datsenko y Wanner (2000).

15 Los datos completos se dan en las Tablas 2 y 3. Estos resultados demuestran que el mutante SM20 de Δ *guaB* está muy atenuado en los ratones pero presenta todavía alguna patogenicidad residual cuando se administra a esta dosis elevada. La inmunización oral con el mutante produce inmunidad protectora contra la infección por una dosis alta de la correspondiente cepa 76Sa88 de *S. Enteritidis* natural patógena. La protección es por lo menos igual a la protección proporcionada por el mutante SM50 de Δ aroA de *S. Enteritidis*.

20 **Pruebas de virulencia y protección con los mutantes SM69 y SM86 por eliminación de *guaB* isógeno en ratones**

25 Se probó la virulencia de los mutantes SM69 y SM86 en ratones por infección oral de ratones BALB/c hembra de 6 a 8 semanas de vida. Las pruebas se realizaron como se describió anteriormente. Las cepas naturales *S. Enteritidis* 76Sa88 y *S. Typhimurium* 1491S96 se probaron en paralelo como controles positivos.

30 Los datos completos se dan en las Tablas 6 y 7, y 10 a 13. Estos resultados demuestran que los mutantes SM69 y SM86 de Δ *guaB* están muy atenuados en los ratones, aunque presentan todavía alguna patogenicidad residual cuando se administra a esta dosis elevada. La inmunización oral con el mutante produce inmunidad protectora contra la infección por una dosis alta de la correspondiente cepa natural patógena.

Ejemplo 3: mutantes de flagelina de *S. Enteritidis* y *S. Typhimurium*

Se probó a continuación si una modificación adicional (una más) del gen de movilidad (por ejemplo un gen de flagelina) podría reducir más la patogenicidad residual que queda en mutantes individuales como SM20 que lleva una mutación por eliminación en el gen *guaB*.

Se utilizaron en experimentos preliminares cepas de *S. Enteritidis* que contienen únicamente un gen que codifica la flagelina, *fliC*. Se construyeron mutantes dobles en los que los genes *guaB* y *fliC* de *S. Enteritidis* estaban inactivados. Para *S. Typhimurium*, se construyeron mutantes dobles ($\Delta\textit{guaB}\Delta\textit{fliC}$; $\Delta\textit{guaB}\Delta\textit{fljBA}$) y triples ($\Delta\textit{guaB}\Delta\textit{fliC}\Delta\textit{fljBA}$).

Construcción de mutantes $\Delta\textit{fliC}$ (SM24, SM30)

Utilizando la PCR la combinación del cebador FliCP1-FliCP2 en el plásmido pKD3 (*catFRT*) o pKD4 (*kanFRT*) de la plantilla se amplía el fragmento recombinante que contiene el gen con resistencia a antibióticos junto con las secuencias FRT y las secuencias P1 y P2 de cebado y prolongaciones homólogas para los 50 nucleótidos iniciales (1-50) y los 50 terminales (1468-1518) de la secuencia de codificación *fliC*. En esta zona, 1491S96 de *S. Typhimurium* y 76Sa88 de *S. Enteritidis* presentan respectivamente una identidad de secuencia del 100% y 98% con los cebadores. El cebador FliCP1 contiene una G adicional en la posición 37 en comparación con la SEC. ID. nº: 22. Por consiguiente, el alelo mutante $\Delta\textit{fliC}$ codifica un péptido de 16 aminoácidos, del que los primeros 12 aminoácidos corresponden al terminal amino de FliC. Un segmento interno de 1416 bp (51-1467) de la secuencia de codificación *fliC* (1-1518) será sustituido.

El producto resultante de la PCR (1 µg) se electroporó a 1491S96 de *S. Typhimurium* (pKD46) y 76Sa88 de *S. Enteritidis* (pKD46), previamente producido con arabinosa al 0,2%, que codifica el sistema de lambda recombinasa.

Los mutantes de la sustitución del candidato resistente a antibióticos fueron confirmados por PCR, utilizando los cebadores FliC1 y FliC2 y ADN completo de las cepas mutantes y la cepa natural. Se llevó a cabo análisis de restricción para distinguir entre los fragmentos de PCR con aproximadamente el mismo tamaño. Para la

restricción del fragmento de PCR de *S. Typhimurium* natural ampliado con FliC1-FliC2, se utilizó la enzima *EcoRV*. Se obtuvieron dos fragmentos (de 470 bp y 1021 bp). El fragmento ampliado para el mutante de la sustitución de *fliC* no contiene una secuencia de restricción. En el caso de *S. Enteritidis* se utilizó la enzima *ApoI*. Esta enzima corta el fragmento *fliC* natural de *S. Enteritidis* en 2 piezas (de 345 bp y 1147 bp). El fragmento obtenido para el mutante por sustitución *fliC* no contiene una secuencia de restricción *ApoI*.

Se probó la movilidad de los mutantes en el medio LB (Miller, 1992, *A short course in bacterial genetics, a laboratory manual and handbook for Escherichia coli and related bacteria*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, Nueva York) con agar-agar al 0,4%. *S. Typhimurium* natural y *S. Enteritidis* natural se congregan en este medio. El mutante por sustitución *fliC* de *S. Typhimurium* se aglomeran, ya que el gen *fljB* está todavía presente en el mutante. El mutante por sustitución *fliC* de *S. Enteritidis* no se aglomera más. Estos resultados fueron confirmados por observación al microscopio.

Las células electrocompetentes de diferentes mutantes se prepararon y transformaron por electroporación con el plásmido pCP20 (producido a partir de χ 3730 de *S. Typhimurium*, R. Curtiss, III, S.M. Kelly, P.A. Gulig, C.R. Gentry-Weeks and J. E. Galán. *A virulent Salmonella expressing virulence antigens from other pathogens for use as orally-administered vaccines*. En: J. Roth, Editor, *Virulence Mechanisms*, American Society for Microbiology, Washington DC (1988), pág. 311) para eliminar el gen con resistencia a antibióticos. Se incubaron transformantes a 43°C. Esto eliminará el plásmido pCP20 sensible a la temperatura y debería eliminar el gen con resistencia a antibióticos. Se confirmó la pérdida del gen con resistencia a antibióticos en los mutantes $\Delta fliC::catFRT$ de *S. Typhimurium* y *S. Enteritidis*.

Los mutantes por eliminación, que se originan a partir de los mutantes por sustitución del resistente al cloranfenicol, se confirmaron por PCR utilizando la combinación de cebador FliC1/FliC2. Tanto para $\Delta fliC$ de *S. Typhimurium* y $\Delta fliC$ de *S. Enteritidis* se amplió un fragmento de 185 bp.

La eliminación se confirmó por secuenciado de los fragmentos ampliados utilizando el cebador FliC3.

Los mutantes obtenidos se probaron en medio LB que contenía agar-agar 0,4%: *S. Typhimurium* natural y *S. Enteritidis* natural congregados en este medio,

73

asimismo $\Delta fliC$ de *S. Typhimurium* se aglomera (el gen flagelado *fljB* está todavía presente). Como cabía esperar no se aglomeró $\Delta fliC$ de *S. Enteritidis*.

Construcción de mutantes de $\Delta fljBA$ (SM48)

5

S. Typhimurium contiene un segundo gen de flagelina, *fljB*. Este gen se expresa junto con *fljA*, que codifica el represor de *fliC*. En el presente caso, se eliminaron tanto *fljA* como *fljB*. *fljB* tiene 1520 bp de longitud y codifica la proteína flagelina. *fljA* tiene 539 bp de longitud y codifica el represor de *fliC*. Longitud total del

10

fragmento que se eliminó (*fljBA*): 2.127 bp.

Se diseñaron cebadores que presentan homología de 51 nucleótidos con las secuencias del gen *fljBA* y homología con las secuencias del plásmido de la plantilla, que flanquean el gen con resistencia a antibióticos y las secuencias FRT. El cebador FljBAP1 presenta homología con la secuencia que parte del codón de iniciación de *fljB* hasta 51 bp corriente abajo (1-51) y el cebador FljBAP2 presenta homología con la

15

secuencia de iniciación del codón de terminación de *fljA* hasta 51 bp corriente arriba (2076-2127). Los cebadores FljBAP1 y FljBAP2 presentan homología en sus extremos 3' con las secuencias de cebado P1 y P2 en el plásmido de la plantilla que flanquea el gen con resistencia con las secuencias FRT.

20

La PCR que utiliza los cebadores FljBAP1 y FljBAP2 (secuencias en la tabla 1) y el ADN pKD3 de la plantilla (*catFRT*) o pKD4 (*kanFRT*) ampliaron los fragmentos de longitud deseada.

25

Se electroporó 1 μ g del producto de PCR para *S. Typhimurium*, transformado con pKD46 o pKD20. Se confirmaron por PCR los transformantes resistentes a kanamicina y cloranfenicol seleccionados.

30

Se probaron los mutantes en medio LB que contenía agar-agar al 0,4%. *S. Typhimurium*, $\Delta fljBA::kanFRT$ de *S. Typhimurium* y $\Delta fljBA::catFRT$ de *S. Typhimurium* congregado (*fliC* está todavía presente). La movilidad de estas cepas se confirmó por observación al microscopio.

Las células electrocompetentes de diferentes mutantes se prepararon y transformaron por electroporación con el plásmido pCP20 (producido a partir de χ 3730 mutante de restricción de LT2 de *S. Typhimurium*) para eliminar el gen con resistencia a antibióticos. Después de 2 horas de incubación a 28°C se colocó el cultivo en placas

7f

en medio LB con carbenicilina. Tras la incubación de los transformantes a 43°C en LB se probaron en la pérdida de plásmido y del gen con resistencia a antibióticos. Se confirmaron las mutaciones por eliminación mediante la PROCARIÓTIC y el secuenciado del fragmento.

5 La PCR que utiliza la combinación de cebadores FljBA6/FljBA5 (secuencia en la tabla 1) amplió un fragmento de 2112 bp para *S. Typhimurium* natural y un fragmento de 185 bp para el mutante SM48 de $\Delta fljBA$ de *S. Typhimurium*.

10 Se confirmó la eliminación en el mutante SM48 por secuenciado utilizando el cebador FljBA6 en el fragmento por PCR obtenido utilizando los cebadores FljBA6-FljBA5 (Figura 7).

Construcción del mutante doble 1491S96 $\Delta fljBA\Delta fliC$ de *S. Typhimurium* (SM23)

15 Se utilizó la cepa $\Delta fljBA::kanFRT$ (pKD46) de *S. Typhimurium* para construir el mutante doble. Se prepararon células electrocompetentes a una temperatura de 28°C (plásmido pKD46 sensible a la temperatura). Las células electrocompetentes se electroporaron con el fragmento *fliC* recombinante, en el que el gen *fliC* está sustituido por el gen con resistencia al cloranfenicol (véase los ejemplos al principio). Para identificar y confirmar los mutantes experimentales se siguió el procedimiento utilizado en la construcción del mutante *fliC*. Genotipo deseado: $\Delta fljBA::kanFRT \Delta fliC::catFRT$ de *S. Typhimurium*. Para eliminar el gen con resistencia al antibiótico se siguió el protocolo descrito anteriormente. Se confirmaron por PCR las eliminaciones en $\Delta fljBA \Delta fliC$ (SM23) de *S. Typhimurium*.

20 El mutante doble $\Delta fljBA \Delta fliC$ de *S. Typhimurium* (SM23) cabe esperar que no sea móvil en el medio LB con agar-agar al 0,4%. La inmovilidad de la cepa se confirmó por observación al microscopio.

Combinación de las mutaciones auxótrofas y flageladas

30 Se utilizó la transducción de P22 (Davis, R.W., Botstein D. y Roth, J.R. (1980) en *Advanced Bacterial Genetics, A manual for genetic engineering*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) para combinar las mutaciones. Los lisados de P22 de los mutantes por sustitución se utilizaron para construir los mutantes por

75

5 eliminación combinados. Se confirmó la transducción por PCR (se utilizaron el mismo protocolo y cebadores que en las confirmaciones anteriores). Para la eliminación de los genes con resistencia al antibiótico se utilizó el protocolo descrito anteriormente que utiliza el plásmido cooperador pCP20. Se confirmaron las eliminaciones por PCR. Los mutantes contruidos de esta manera son: $\Delta fliC \Delta guaB$ de *S. Typhimurium* (SM32), $\Delta fljBA \Delta guaB$ de *S. Typhimurium* (SM35), $\Delta fliC \Delta fljBA \Delta guaB$ de *S. Typhimurium* (SM27) y $\Delta fliC \Delta guaB$ de *S. Enteritidis* (SM21).

10 Construcción de mutantes isógenos por eliminación

15 Para excluir la posibilidad de que mutaciones desconocidas adicionales (que pueden tener efecto sobre la atenuación de las cepas) estén presentes en las cepas de vacuna experimentales, dedicadas a la utilización del método descrito por Datsenko y Wanner (2000) para la construcción de los mutantes por eliminación, se construyeron mutantes isógenos por eliminación. Las mutaciones se transdujeron al fondo natural, por medio de transducción del fago P22 (Davis, R.W., Botstein D. y Roth, J.R. (1980) en *Advanced Bacterial Genetics, A manual for genetic engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.*). Se utilizaron mutantes por sustitución resistentes al antibiótico como cepa donante. Para la eliminación de los genes con resistencia al antibiótico y confirmación de las deleciones, se utilizaron los mismos protocolos que en los experimentos anteriores.

20 Mutantes contruidos: $\Delta guaB$ de *S. Enteritidis* (SM69, con el número de depósito LMG P-21641); $\Delta fliC$ de *S. Enteritidis* (SM71); $\Delta guaB$ de *S. Typhimurium* (SM86, con el número de depósito LMG P-21646); $\Delta fliC$ de *S. Typhimurium* (SM91); $\Delta fljBA$ de *S. Typhimurium* (SM90), $\Delta guaB \Delta fliC$ de *S. Enteritidis* (SM73, con el número de depósito LMG P-21642); $\Delta guaB \Delta fliC$ de *S. Typhimurium* (SM104); $\Delta guaB \Delta fljBA$ de *S. Typhimurium* (SM87); $\Delta fljBA \Delta fliC$ de *S. Typhimurium* (SM83); $\Delta guaB \Delta fljBA \Delta fliC$ de *S. Typhimurium* (SM89, con el número de depósito LMG P-21643).

Ejemplo 4: experimentos de virulencia y protección con las cepas de la vacuna con *S. Enteritidis*

Efecto de la inactivación del gen *fliC* sobre la virulencia de una cepa de la vacuna de *S. Enteritidis*

Para estudiar el efecto de la inactivación del gen *fliC* sobre la inmunogenicidad de la cepa de la vacuna con *S. Enteritidis*, se realizaron dos pruebas independientes de virulencia y protección en ratones BALB/c hembra de 7 semanas de vida tanto con el mutante SM20 (ΔguaB) y SM 21 ($\Delta\text{guaB } \Delta\text{fliC}$) (Tablas 4 y 5).

Para el ensayo de virulencia, se infectaron los ratones por vía oral con una dosis de aproximadamente 10^8 CFU, lo que corresponde a aproximadamente 10^5 veces el LD_{50} de la cepa natural (Pattery et al., 1999, *Molecular Microbiology* 33:791-805). Se observaron los ratones durante 21 días. Todos los ratones inoculados con la cepa 76Sa88 de *S. Enteritidis* natural murieron en 9 días después de la infección, mientras que los ratones de referencia no infectados permanecieron sanos durante el periodo de observación de 21 días. El primer ratón del experimento infectado con el mutante SM20 ΔguaB de *S. Enteritidis* presentaba síntomas típicos de la enfermedad (actividad reducida, pelo desordenado y dorso curvado) y uno de cada diez murieron. En el segundo experimento no se observaron síntomas de la enfermedad con SM20. El mutante SM21 de $\Delta\text{guaB } \Delta\text{fliC}$ de *S. Enteritidis* fue asintomático en ambos experimentos.

Eficacia de los mutantes SM20 y SM21 para proporcionar protección: pruebas de protección

La eficacia de los mutantes SM20 y SM21 para proporcionar protección se probó tres semanas después de la inmunización inicial mediante prueba de provocación oral con aproximadamente 10^5 LD_{50} de la cepa 76Sa88 de *S. Enteritidis* natural ($\text{LD}_{50}=10^3$ CFU). Se observaron los ratones durante 21 días. Todos los ratones no inmunizados murieron después de la prueba de provocación. En el segundo experimento, murió uno de cada tres ratones vacunados con SM20. Todos los demás ratones vacunados sobrevivieron a la prueba de provocación sin síntomas de

enfermedad observables. Estos datos demuestran que ambos mutantes están atenuados y proporcionan protección contra la prueba de provocación con la cepa natural correspondiente.

Para determinar qué mutaciones desconocidas no adicionales estaban presentes, que podrían contribuir a la atenuación de las cepas de vacuna experimentales, se transfirieron las mutaciones que contenían los genes con resistencia seleccionables a un fondo natural por transducción de P22 (Davis, R.W., Botstein D. y Roth, J.R. (1980) en *Advanced Bacterial Genetics, A manual for genetic engineering*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Eficacia de la inactivación del gen *fliC* sobre la virulencia de *S. Enteritidis*: pruebas de virulencia y protección con cepas isógenas

Las pruebas de virulencia y protección en ratones BALB/c se repitieron con las cepas SM69 isógenas (Δ *guaB*), SM71 (Δ *fliC*) y SM73 (Δ *guaB* Δ *fliC*) tras la confirmación por PCR y caracterización fenotípica como se describió al principio. En este ensayo de virulencia, se incluyó un mutante de Δ *fliC*, para estudiar el efecto de la inactivación del gen *fliC* sobre la virulencia de *S. Enteritidis*. Similares condiciones a las descritas anteriormente se utilizaron para los experimentos de virulencia y prueba de provocación. Los datos obtenidos para los transductantes SM69 y SM73 (Tablas 6 y 7) confirmaron las observaciones hechas en los experimentos iniciales.

La comparación entre ambos mutantes por eliminación *guaB* indica que el doble mutante SM73 Δ *guaB* Δ *fliC* de *S. Enteritidis* está más atenuada y proporciona mejor protección frente a la prueba de provocación con grandes dosis de la cepa natural que el mutante individual SM69 Δ *guaB* de *S. Enteritidis*. El ensayo de virulencia realizado con el mutante SM71 Δ *fliC* de *S. Enteritidis* demostró que este mutante permanecía virulento como cepa natural a las condiciones aplicadas.

Respuestas inmunológicas y producción de anticuerpos

Cuarenta y cuatro días después de la inmunización inicial en el primer experimento, se recogieron muestras de sangre de la arteria de la cola de los ratones. Se determinaron los títulos de IgG del antilipopolisacárido (LPS) por medio del ensayo

inmunosorbente con enzima ligada (ELISA) utilizando LPS (Sigma) de *S. Enteritidis* para recubrimiento. La comparación entre los sueros de los ratones inmunizados con SM20 y SM21 demostró que en ambos casos se produjeron respuestas de IgG del suero anti-LPS y que no se midieron diferencias significativas en el valor. La inmunización oral con una segunda y tercera dosis, 66 y 95 días después de la inmunización inicial no aumentó las concentraciones de IgG anti-LPS en el suero (datos no mostrados).

Ejemplo 5: experimentos de virulencia y protección con transductantes de *S. Typhimurium*

Se realizaron experimentos de virulencia y protección con los transductantes en ratones BALB/c hembra de 7 semanas de vida. Se inocularon por vía oral a los ratones aproximadamente 10^8 células. Se observaron los ratones diariamente durante un periodo de 21 días. Después de este periodo, los ratones se sometieron a la prueba de provocación con 10^8 células de la cadena patógena natural, y los ratones se observaron durante un periodo de 21 días.

En las Tablas 10 a 13 se apuntan los síntomas de la enfermedad y la tasa de supervivencia. Los mutantes flagelados individuales y dobles permanecieron muy virulentos cuando se administró por vía oral a todos los ratones muertos. Los ratones inoculados con el mutante *guaB* presentaban síntomas leves de enfermedades (los ratones habían luchado, solamente dos quedaban vivos). Los mutantes $\Delta\textit{guaB}$ $\Delta\textit{fliJBA}$, $\Delta\textit{guaB}$ $\Delta\textit{fliC}$ y $\Delta\textit{guB}$ $\Delta\textit{fliC}$ $\Delta\textit{fliJBA}$ combinados estaban muy atenuados. No se observaron síntomas de enfermedad durante el periodo de 21 días de observación tras la vacunación. Estos mutantes proporcionaron buena protección después de la prueba de provocación con una dosis elevada de la cepa natural. Solamente pudo observarse una actividad reducida de los ratones después de la prueba de provocación.

Estos resultados también demuestran que las mutaciones flageladas no afectan las capacidades inmunógenas de las cepas cuando se administran a ratones BALB/c. Las mutaciones flageladas pueden ser útiles como marcador serológico para distinguir entre la cepa de la vacuna y la cepa natural. La combinación de la mutación auxótrofa con la(s) mutación o mutaciones flagelada(s) da los mejores resultados en lo

que se refiere a la virulencia reducida de los mutantes en ratones y a la protección frente a la correspondiente cepa natural.

La atenuación de los mutantes por eliminación triple $\Delta\text{guaB } \Delta\text{fliC } \Delta\text{fliBA}$ de *S. Typhimurium* y los mutantes por eliminación doble de *S. Typhimurium*, $\Delta\text{guaB } \Delta\text{fliBA}$ y $\Delta\text{guaB } \Delta\text{fliC}$, eran comparables.

Ejemplo 6: Evaluación de la seguridad de las cepas de vacuna de *S. enteritidis*

Evaluación de seguridad del mutante SM69 en pollos de un día de vida inoculados por las vías intratraqueal y de alimentación forzada por vía oral

El objetivo de este estudio fue evaluar la seguridad de la cepa patrón SM69 mutante de ΔguaB de *S. Enteritidis* sembrada en pollos de un día de vida. Se utilizó la mortalidad como parámetro principal para la determinación de la seguridad.

Los pollos con un día de vida se agruparon por las patas y se colocaron al azar en cada uno de los cuatro grupos de tratamiento (Grupo 1: SM69-IT, grupo 2: SM69-OG, grupo 3: PBS-IT y Grupo 4: PBS-OG). Después de la inoculación por siembra del patrón, las aves de los grupos 1 y 2 se colocaron en un separador y las de los grupos 3 y 4 en otro separador.

Los pollos de los grupos 1 y 2 se inocularon con el patrón SM69 sembrado por vía intratraqueal (IT) o por vía de alimentación forzada por vía oral (OG), respectivamente, con un valor existente de $1,3 \times 10^8$ cfu/0,2 ml por ave. A los pollos en los grupos 3 y 4 se les administraron 0,2 ml de PBS (solución salina tamponada con fosfato) por ave por vía intratraqueal o alimentación forzada por vía oral, respectivamente.

Después de la inoculación de SM69 o de PBS, se observó la mortalidad de los pollos diariamente hasta 38 días después de la inoculación. La Tabla 8 resume los resultados de la mortalidad para los 4 grupos. En el grupo 1, un ave murió durante la inoculación debido a traumatismo por inoculación. Dos aves murieron 2 días después de la inoculación (DPI). Tres aves murieron desde el día 3 al día 13 (a 3, 5 y 13 DPI respectivamente). Un total de 6 aves por lo tanto murieron en el grupo 1. En el grupo 2, murieron dos aves en total. Una murió durante la inoculación debido al traumatismo por inoculación y otra murió al 5° día después de la inoculación. No murió ningún ave

en los grupos tratados con PBS ya sea por vía intratraqueal o por alimentación forzada por vía oral.

Este estudio indica que la cepa SM69 mutante de $\Delta guaB$ de *S. Enteritidis* no es segura cuando se administra a razón de $1,3 \times 10^8$ cfu por ave con un día de vida por vía intratraqueal o por alimentación forzada por vía oral.

Evaluación de la seguridad de SM69 en pollos de 2 semanas de vida inoculados por las vías intratraqueal y de alimentación forzada por vía oral

Se evaluó a continuación la seguridad de la cepa SM69 mutante de $\Delta guaB$ de *S. Enteritidis* en pollos SPF de 2 semanas de vida por las vías intratraqueal y de alimentación forzada por vía oral. Se utilizó la mortalidad como criterio principal y el peso corporal como criterio secundario para la determinación de la seguridad.

Las aves a las 2 semanas de vida se agruparon por las patas y se colocaron al azar en cada uno de los cuatro grupos de tratamiento: SM69-IT, SM69-OG, Poulvac ST-IT y PBS-IT. Diez aves del grupo 1 se inocularon con SM69 por vía intratraqueal; diez aves del grupo 2 se inocularon con SM69 por alimentación forzada por vía oral; diez aves del grupo 3 se inocularon con una vacuna con *AroA* de *S. Typhimurium* (Poulvac® ST) por vía intratraqueal; y cinco aves del grupo 4 se inocularon con PBS por vía intratraqueal.

Los pollos en los grupos 1 y 2 se inocularon con el patrón SM69 sembrado por vía intratraqueal o alimentación forzada por vía oral, respectivamente, con el valor real de $2,3 \times 10^8$ cfu/0,2 ml por ave. A los pollos del grupo 3 se les administró $2,2 \times 10^8$ cfu/0,2 ml por ave de Poulvac® ST por vía intratraqueal. Los pollos del grupo 4 se administraron por vía intratraqueal con 0,2 ml de PBS por ave.

Tras la inoculación, las aves de los grupos 1 y 2 del tratamiento se colocaron en un separador y las de los grupos 3 y 4 en otro separador.

Tras las inoculaciones, se observó la mortalidad cada día hasta 21 días después de la inoculación. Se registró también el peso corporal de todas las aves al final del periodo de estudio (21 días). Se utilizaron Poulvac® ST y PBS como referencias del procedimiento intratraqueal.

Durante el periodo de observación de 21 días, un ave del grupo de tratamiento intratraqueal SM69 (grupo 1) murió por un saco vitelino infectado. No se asoció

ninguna mortalidad con la inoculación de SM69, lo que indica que la cepa SM69 es segura en el valor probado, $2,3 \times 10^8$ cfu por ave por las vías intratraqueal y de alimentación forzada por vía oral. Como cabía esperar, no se observó ninguna muerte ya sea en las aves tratadas con Poulvac® ST al valor de $2,2 \times 10^8$ cfu por ave o en las aves tratadas con PBS, lo que indica que el estudio era válido (Tabla 9).

Se comparó el peso corporal entre los grupos en un análisis de modelo de varianza (ANOVA) con el peso corporal como variable dependiente y el tratamiento se incluyó como una variable independiente. Se hicieron comparaciones de los grupos utilizando la prueba de Tukey para comparaciones múltiples. El nivel de significación se fijó a una $p < 0,05$. El estudio se consideró válido porque los pollos de referencia (grupo de referencia con PBS) permanecieron sanos y libres de signos clínicos o de enfermedades o mortalidad en todo el estudio.

No hubo diferencias significativas en el peso corporal final en los pollos a los que se les administró SM69 por inoculación intratraqueal o de alimentación forzada por vía oral, Poulvac® ST o PBS (Tabla 9). Aun cuando no se creó ninguna referencia de las aves en cada grupo a un día de vida, era improbable que hubiera una diferencia significativa en el peso corporal inicial entre los cuatro grupos ya que las aves se colocaron al azar en cada uno de los cuatro grupos de tratamiento.

Puede sacarse en conclusión que SM69 es segura cuando se administra a un valor probado de $2,3 \times 10^8$ cfu por ave a las 2 semanas de vida, ya sea por vía de inoculación intratraqueal o por alimentación forzada por vía oral.

Evaluación de la seguridad de SM73 en pollos de 1 día de vida inoculados por las vías intratraqueal y de alimentación forzada por vía oral

Se evaluó la seguridad de la cepa SM73 mutante por eliminación de *S. Enteritidis* (Δ guaB Δ flicC) en pollos SPF por las vías intratraqueal y de alimentación forzada por vía oral. Se utilizó la mortalidad como criterio principal y el peso corporal como criterio secundario para la determinación de la seguridad.

Todas las aves a las 2 semanas de vida se agruparon por las patas y se asignaron al azar a uno de los cuatro grupos de tratamiento incluidos en este estudio (Grupo 1: SM73-IT, grupo 2: SM73-OG, grupo 3: Poulvac ST-IT y grupo 4: PBS-IT). Diez aves del grupo 1 se inocularon con SM73 por vía intratraqueal; diez aves del

grupo 2 se inocularon con SM73 por alimentación forzada por vía oral; diez aves del grupo 3 se inocularon con una vacuna con AroA⁻ de *S. Typhimurium* (Poulvac[®] ST) por vía intratraqueal; y cinco aves del grupo 4 se inocularon con PBS por vía intratraqueal. Se utilizaron un total de 4 separadores (uno para cada grupo) en el que los pollos se alojaron durante el estudio.

Se inocularon los pollos de un día de vida. Los pollos en los grupos 1 y 2 se inocularon con el patrón SM73 sembrado por vía intratraqueal o alimentación forzada por vía oral, respectivamente, con un valor real de $2,5 \times 10^7$ cfu/0,2 ml por ave. A los pollos del grupo 3 se les administró Poulvac[®] ST por vía intratraqueal con $2,1 \times 10^7$ CFU/0,2 ml por ave. Los pollos del grupo 4 se administraron por vía intratraqueal con 0,2 ml de PBS por ave.

Se observó la mortalidad y se registró también el peso corporal tal como se describió anteriormente para SM69. Se utilizaron otra vez Poulvac[®] ST y PBS como referencias del procedimiento intratraqueal.

Durante el periodo de observación de 21 días, no se registró ninguna mortalidad para ningún ave en absoluto. No hubo ninguna diferencia en el peso corporal final de las aves inoculadas con PBS-IT, Poulvac ST-IT y SM73-OG. El peso corporal medio de los grupos SM73-IT fue significativamente inferior en comparación con el grupo SM73-OG ($p = 0,0009$) pero esto es debido más probablemente a un error experimental.

Del presente experimento puede concluirse que SM73 es seguro cuando se administra al valor probado de $2,5 \times 10^7$ CFU por ave y un día de edad por vía intratraqueal o alimentación forzada por vía oral.

Se ha realizado un depósito según el Tratado de Budapest en la BCCM/LMG Culture Collection, Laboratorium voor Microbiologie, K.L. Ledeganckstraat 35, B-9000 Gante (Bélgica) para los microorganismos siguientes: SM69 de *Salmonella Enteritidis* con el número de depósito LMG P-21641 (fecha de depósito: 9 de agosto de 2002); SM73 de *S. Enteritidis* con el número de depósito LMG P-21642 (fecha de depósito: 9 de agosto de 2002), SM86 de *S. Typhimurium* con el número de depósito LMG P-21646 (fecha de depósito: 28 de agosto de 2002) y *S. Typhimurium* con el número de depósito LMG P-21643 (fecha de depósito: 9 de agosto de 2002) . Los depósitos se han realizado en nombre del Prof. J.-P. Hernalsteens, dirección anterior: Vrije Universiteit Brussel, Laboratorium Genetische Virologie, Paardenstraat 65, B-1640

Y3

- 32 -

Sint-Genesius-Rhode, dirección actual: Vrije Universiteit Brussel, Onderzoeksgroep
Genetische Virologie, Pleinlaan 2, B-1050 Bruselas, Bélgica.

24

Tabla 1: Secuencias de cebador

SEC ID n°	Cebador	Secuencia
1	GuaB2	5' CGTTCAGCG CAACAGCCG TTGT 3'
2	GuaB3	5' GGCTGCGAT GCGAGGTAG TA 3'
3	GuaB4	5' GGTGATCCG GCGGTCAAC GTCAGGGCTT CTTTA 3'
4	GuaB5	5' TTGACGCCG GATCACCA AGAGTCCCG AACTA 3'
5	GuaB6	5' GCAACAACTC CTGCTGGTA 3'
6	GuaB7	5' AGACCGAGA TCACTTATC 3'
7	GuaB10	5' AGGAAGTTG AGAGATAA 3'
8	P1	5' GTGTAGGCTG GAGCTGCTC 3'
9	P2	5' CATATGAATA TCCTCCTTAG 3'
10	FLiCP1	5' ATGGCACAAG TCATTAATAC AAACAGCCTG TCGCTGGTTG ACCAGAATA ATGTGTAGG TGGAGTGCT TC 3'
11	FLiCP2	5' CGCATTAACG CAGTAAAGAG AGGACGTTT GCGAACCTG GTTMCCTGC GCCACATATG AATATCCTCC TTAG 3'
12	FLiC1	5' ATGGCACAAG TCATTAATAC AAACAG 3'
13	FLiC2	5' CGCATTAACG CAGTAAAGAG AGGAC 3'
14	FLiC3	5' TATCGGCAAT CTGGAGGCAA 3'
15	FLjBAP1	5' ATGGCACAAG TAATCAACAC TAACAGTCTG TCGTGTGTA CCCAGAATAA CTGTGTAGG TGGAGTGCT TC 3'
16	FLjHAP2	5' TTATTCAGCG TAGTCCGAG ACGTGATCCT GCTCACCCAG TCAACATAA CCATATGAT ATCCTCCTTA G 3'
17	FLjBA5	5' CAGCGTAGTC CGAGACGTG ATC 3'
18	FLjBA6	5' ACACTAACAG TCTGTCGCTG CT 3'

Tabla 2: Prueba de virulencia en ratones BALB/c del mutante SM20 por eliminación de Δ guaB de *S. Enteritidis*

Infección		Supervivencia	Día de muerte	Estado de los ratones
Cepa	Dosis			
Primer experimento				
referencia negativa: leche	/	11/11	/	Sin síntomas de enfermedad
referencia positiva: 76Sa88 de S. Enteritidis	2,1×10 ⁸	0/5	7, 7, 8, 8, 9	
referencia de la vacuna: ΔaroA SM50 de S. Enteritidis	2,5×10 ⁸	10/10	/	Sin síntomas de enfermedad
AguaB SM20 de S. Enteritidis	5,1×10 ⁸	9/10	13	Síntomas de enfermedad entre el 7º y el 14º día tras la infección
Segundo experimento				
referencia negativa: leche	/	4/4	/	Sin síntomas de enfermedad
referencia positiva: 76Sa88 de S. Enteritidis	1,4×10 ⁸	0/3	8, 9, 9	
referencia de la vacuna: ΔaroA SM50 de S. Enteritidis	2,1×10 ⁸	3/3	/	Sin síntomas de enfermedad
AguaB SM20 de S. Enteritidis	1,9×10 ⁸	3/3	/	Sin síntomas de enfermedad

87

Tabla 3: Prueba de provocación de ratones vacunados con el mutante SM20 por eliminación de guaB de S. Enteritidis

Vacunación		Prueba de provocación		Supervivencia	Día de muerte	Estado de los ratones
Cepa	Dosis	Cepa	Dosis			
Primer experimento						
referencia negativa: leche	/	referencia negativa: leche	/	6/6	/	Sin síntomas de enfermedad
referencia negativa: leche	/	76Sa88 de <i>Enteritidis</i>	$1,5 \times 10^8$	0/5	7, 8, 8, 8, 9	Síntomas de enfermedad a partir del 5º día tras la prueba de provocación
Referencia de la vacuna: ΔaroA SM50 de <i>S. Enteritidis</i>	$2,5 \times 10^8$	76Sa88 de <i>Enteritidis</i>	$1,5 \times 10^8$	3/5	9, 13	Síntomas de enfermedad entre el 7º y el 14º día tras la prueba de provocación
ΔguaB SM20 de <i>S. Enteritidis</i>	$5,1 \times 10^8$	76Sa88 de <i>Enteritidis</i>	$1,5 \times 10^8$	5/5	/	Los ratones son menos activos entre el día 11º y el 14º tras la prueba de provocación
Segundo experimento						
referencia negativa: leche	/	referencia negativa: leche	/	2/2	/	Sin síntomas de enfermedad
referencia negativa: leche	/	76Sa88 de <i>Enteritidis</i>	$1,5 \times 10^8$	0/2	9, 18	
Referencia de la vacuna: ΔaroA SM50 de <i>S. Enteritidis</i>	$2,1 \times 10^8$	76Sa88 de <i>Enteritidis</i>	$1,5 \times 10^8$	1/3	9, 21	Síntomas de enfermedad entre el día 7º y el 21º tras la prueba de provocación
ΔguaB SM20 de <i>S. Enteritidis</i>	$1,9 \times 10^8$	76Sa88 de <i>Enteritidis</i>	$1,5 \times 10^8$	2/3	10	Síntomas de enfermedad a partir del 9º día tras la infección

Tabla 4: Prueba de virulencia en ratones BALB/c de los mutantes SM20 y SM21 de *S. Enteritidis*

Infección		Supervivencia	Día de muerte	Estado de los ratones
Cepa	Dosis			
Primer experimento				
Referencia negativa: no infectada		11/11	/	Asintomático
Referencia positiva: 76a88 de <i>S. Enteritidis</i>		0/5	7, 7, 8, 8, 9	Síntomas graves del 5º día en adelante
<i>ΔguaB</i> SM20 de <i>S. Enteritidis</i>		9/10	/	Síntomas leves a graves, a partir del 7º día hasta el 17º día
<i>ΔguaB ΔflicC</i> SM21 de <i>S. Enteritidis</i>		10/10	/	Asintomático
Segundo experimento				
Referencia negativa: no infectada		4/4	/	Asintomático
Referencia positiva: 76a88 de <i>S. Enteritidis</i>		0/3	8, 9, 9	Síntomas graves del 6º día en adelante
<i>ΔguaB</i> SM20 de <i>S. Enteritidis</i>		3/3	/	Asintomático
<i>ΔguaB ΔflicC</i> SM21 de <i>S. Enteritidis</i>		3/3	/	Asintomático

Tabla 5: Prueba de provocación de los ratones vacunados con los mutantes SM20 y SM21 de S. Enteritidis

Vacunación		Prueba de provocación			Supervivencia	Día de muerte	Estado de los ratones
Cepa	Dosis	Cepa	Dosis				
Primer experimento							
Referencia negativa: leche	/	Cepa 76Sa88 natural de S. Enteritidis	1,6×10 ⁸	0/5	7, 8, 8, 8, 9	Síntomas graves del 6º día en adelante	
Cepa 76aS88 natural de S. Enteritidis	2,1×10 ⁸	Cepa 76Sa88 natural de S. Enteritidis	1,6×10 ⁸	/		/	
ΔguaB SM20 de S. Enteritidis	5,1×10 ⁸	Cepa 76Sa88 natural de S. Enteritidis	1,6×10 ⁸	4/4	8, 8, 19	Asintomático	
ΔguaB ΔflicC SM21 de S. Enteritidis	4,3×10 ⁸	Cepa 76Sa88 natural de S. Enteritidis	1,6×10 ⁸	5/5		Asintomático	
Segundo experimento							
Referencia negativa: leche	/	Cepa 76Sa88 natural de S. Enteritidis	1,5×10 ⁸	0/2	9, 18	Síntomas graves del 6º día en adelante	
Cepa 76aS88 natural de S. Enteritidis	1,4×10 ⁸	Cepa 76Sa88 natural de S. Enteritidis	1,5×10 ⁸	/	/	/	
ΔguaB SM20 de S. Enteritidis	1,9×10 ⁸	Cepa 76Sa88 natural de S. Enteritidis	1,5×10 ⁸	2/3	10	Ningún síntoma, excepto uno con síntomas graves	
ΔguaB ΔflicC SM21 de S. Enteritidis	3,2×10 ⁸	Cepa 76Sa88 natural de S. Enteritidis	1,5×10 ⁸	3/3	/	Asintomático	

Tabla 6: Prueba de virulencia en ratones BALB/c de los mutantes SM71, SM73 y SM69 de *S. Enteritidis*

Infección		Supervivencia	Día de muerte	Estado de los ratones
Cepa	Dosis			
Referencia negativa: no infectada	/	4/4	/	Asintomático
Referencia positiva: 76a88 de <i>S. Enteritidis</i>	$3,7 \times 10^8$	0/3	7, 8, 9	Síntomas graves del 5º día en adelante
Δ flicC SM71 de <i>S. Enteritidis</i>	$1,4 \times 10^8$	0/3	6, 8, 8	Síntomas graves del 4º día en adelante
Δ guaB SM69 de <i>S. Enteritidis</i>	$7,6 \times 10^8$	5/5	/	Síntomas leves, a partir del 11º día hasta el 18º día
Δ guaB Δ flicC SM73 de <i>S. Enteritidis</i>	$1,2 \times 10^8$	5/5	/	Actividad reducida, a partir del 11º día hasta el 13º día

Tabla 7: Prueba de provocación de los ratones vacunados con los mutantes SM71, SM73 y SM69 de S. Enteritidis

Vacunación		Prueba de provocación		Supervivencia	Día de muerte	Estado de los ratones
Cepa	Dosis	Cepa	Dosis			
Referencia negativa: leche	/	Cepa 76Sa88 natural de S. Enteritidis	$3,1 \times 10^8$	0/4	8, 8, 8, 9	Síntomas graves del 5º día en adelante
Cepa 76aS88 natural de S. Enteritidis	$3,7 \times 10^8$	Cepa 76Sa88 natural de S. Enteritidis	$3,1 \times 10^8$	/	/	/
$\Delta fliC$ SM71 de S. Enteritidis	$1,4 \times 10^8$	Cepa 76Sa88 natural de S. Enteritidis	$3,1 \times 10^8$	/	/	/
$\Delta guaB$ SM69 de S. Enteritidis	$7,6 \times 10^8$	Cepa 76Sa88 natural de S. Enteritidis	$3,1 \times 10^8$	2/5	8, 8, 19	Síntomas graves del 5º día en adelante
$\Delta guaB \Delta fliC$ SM73 de S. Enteritidis	$1,2 \times 10^8$			5/5	/	Asintomático

Tabla 8: Evaluación de seguridad del mutante SM69 de *S. Enteritidis* en pollos de un día de vida

Infección	Grupo	N	Valor	Vía	Supervivencia	Día de muerte (DPI)
Cepa						
SM69 de <i>S. Enteritidis</i>	1: SM69-IT	10	1,3×10 ⁸ cfu/0,2 ml	intratraqueal	4/10*	0, 2, 3, 5, 13
SM69 de <i>S. Enteritidis</i>	2: SM69-OG	10	1,3×10 ⁸ cfu/0,2 ml	alimentación forzada por vía oral	8/10**	0, 5
Referencia negativa: PBS	3: PBS-IT	10	PBS – 0,2 ml	intratraqueal	10/10	-
Referencia negativa: PBS	4: PBS-OG	10	PBS – 0,2 ml	alimentación forzada por vía oral	10/10	-

IT Intratraqueal

5

OG Alimentación forzada por vía oral

DPI Días tras la inoculación

*

En el grupo 1, 1 ave murió durante la inoculación; 1 ave murió a los 3, 5 y 13 DPI; y 2 aves a los 2 DPI respectivamente

**

En el grupo 2, 1 ave murió durante la inoculación; y 1 ave murió a los 5 días DPI.

91

92

Tabla 9: Evaluación de seguridad del mutante SM69 de Salmonella en pollos de 2 semanas de vida

Infección		Grupo	N	Valor	Vía	Supervivencia	Día de muerte (DPI)	Peso medio (kg)	Peso normal
Cepa									
SM69 de S. Enteritidis		1: SM69-IT	10	2,3×10 ⁸ cfu/0,2 ml	IT	9/10*	13	0,429	0,064
SM69 de S. Enteritidis		2: SM69-OG	10	2,3×10 ⁸ cfu/0,2 ml	OG	10/10	-	0,420	0,044
Referencia de la vacuna: Poulvac® ST**		3: Poulvac-IT	10	2,2×10 ⁸ cfu/ 0,2 ml	IT	10/10	-	0,423	0,046
Referencia negativa: PBS		4: PBS-IT	5	PBS – 0,2 ml	OG	5/5	-	0,388	0,019

IT Intratraqueal

OG Alimentación forzada por vía oral

DPI Días tras la inoculación

* Muerte debida a infección del saco vitelino

** Vacuna AroA⁻ con microbios vivos de S. Typhimurium contra S. Typhimurium

Tabla 10: Experimentos de virulencia con cepas mutantes de *S. Typhimurium* en ratones BALB/c. Inoculación oral con aproximadamente 10⁸ células de la cepa 1491S96 de *S. Typhimurium*

Infección	Supervivencia (días de muerte)	Estado de los ratones
Cepa (1491S96 de <i>S. Typhimurium</i>)		
SM2 natural	0/3 (9, 9, 10)	Síntomas de la enfermedad a partir del 4º día en adelante
Δ <i>fliC</i> SM91	0/5 (9, 10, 11, 15, 15)	Síntomas de la enfermedad a partir del 5º día en adelante
Δ <i>fliBA</i> SM90	0/5 (8, 10, 10, 11, 34)	Síntomas de enfermedad grave a partir del 7º día en adelante
Δ <i>fliBA</i> Δ <i>fliC</i> SM83	0/5 (8, 9, 12, 14, 15)	Síntomas de enfermedad grave a partir del 7º día en adelante
Δ <i>guaB</i> SM86	2/5* (2, 2, 2)	Síntomas de enfermedad leve a partir del 9º día hasta la prueba de provocación
Δ <i>guaB</i> Δ <i>fliBA</i> SM87	5/5	Ningún síntoma
Δ <i>guaB</i> Δ <i>fliC</i> Δ <i>fliBA</i> SM89	5/5	Ningún síntoma
Referencia (no infectada)	4/4	Ningún síntoma

* muertos tras una lucha

Tabla 11: Experimentos de protección con cepas mutantes de *S. Typhimurium* en ratones BALB/c. Vacunación oral con aproximadamente 10⁸ células de la cepa 1491S96 de *S. Typhimurium*

Vacunación		Prueba de provocación			
Cepa	1491S96	Cepa	Dosis	Supervivencia	Estado de los ratones
de <i>S. Typhimurium</i>		(1491S96 de <i>S. Typhimurium</i>)		(días de muerte)	
Δ guaB SM86		1491S96 de <i>S. Typhimurium</i>	1,3×10 ⁷	2/2	Síntomas leves hasta 14 días, después un ratón presentó síntomas evidentes, el otro sanó de nuevo
Δ guaB Δ fliJBA SM87		1491S96 de <i>S. Typhimurium</i>	1,3×10 ⁷	5/5	Actividad reducida, entre el 8° día y el 10° día
Δ guaB Δ fliC Δ fliJBA SM89		1491S96 de <i>S. Typhimurium</i>	1,3×10 ⁷	5/5	Actividad reducida, entre el 8° día y el 10° día
referencia negativa: leche		1491S96 de <i>S. Typhimurium</i>	1,3×10 ⁷	0/4	Síntomas graves a partir del 6° día en adelante

CPY

Tabla 12: Experimentos de virulencia con las cepas mutantes 1491S96 de *S. Typhimurium* en ratones BALB/c

Infección	Dosis	Supervivencia (días de muerte)	Estado de los ratones
Cepa (1491S96 de <i>S. Typhimurium</i>)			
SM2 natural	$0,8 \times 10^8$	1/4 (11, 13, 14)	Síntomas de enfermedad a partir del 6° día en adelante
Δ guaB SM86	$0,8 \times 10^8$	5/5	Síntomas leves el día 13 y 14
Δ guaB Δ fliC SM91	$2,5 \times 10^8$	5/5	Sin síntomas
Δ guaB Δ fliBA SM87	$1,5 \times 10^8$	5/5	Sin síntomas
Δ guaB Δ fliC Δ fliBA SM89	$1,7 \times 10^8$	5/5	Sin síntomas
Referencia (no infectada)	-	5/5	Sin síntomas

Tabla 13: Experimentos de protección con las cepas mutantes 149S96 de *S. Typhimurium* en ratones BALB/c

Vacunación		Prueba de provocación		Dosis	Supervivencia (días de la muerte)	Estado de los ratones
Cepa (1491S96 de <i>S. Typhimurium</i>)	Dosis	Cepa (1491S96 de <i>S. Typhimurium</i>)		Dosis	Supervivencia (días de la muerte)	Estado de los ratones
Δ guaB SM86	$0,8 \times 10^8$	1491S96 de <i>S. Typhimurium</i>		$2,7 \times 10^8$	5/5	Actividad reducida desde el 6° día hasta el 16° día
Δ guaB Δ fliC SM91	$2,5 \times 10^8$	1491S96 de <i>S. Typhimurium</i>		$2,7 \times 10^8$	5/5	Actividad reducida desde el 7° día en adelante; síntomas leves desde el 8° día hasta el 14° día
Δ guaB Δ fliBA SM87	$1,5 \times 10^8$	1491S96 de <i>S. Typhimurium</i>		$2,7 \times 10^8$	3/5 (10, 28)	Síntomas leves entre el 6° día y el 16° día
Δ guaB Δ fliC Δ fliBA SM89	$1,7 \times 10^8$	1491S96 de <i>S. Typhimurium</i>		$2,7 \times 10^8$	4/5 (19)	Actividad reducida entre el 8° día y el 16° día
Referencia (no infectada)	-	1491S96 de <i>S. Typhimurium</i>		$2,7 \times 10^8$	0/5 (8, 9, 10, 11, 16)	Síntomas graves desde el 6° día en adelante

91

REIVINDICACIONES

- 5 1. Cepa mutante atenuada de una bacteria que infecta especies veterinarias, en la que dicho mutante contiene por lo menos una primera modificación genética y por lo menos una segunda modificación genética, dicha primera modificación en uno o más genes con movilidad, y dicha segunda modificación en uno o más genes implicados en la supervivencia o proliferación de la bacteria en el hospedador.
- 10 2. Cepa mutante según cualquiera de las reivindicaciones anteriores, en la que la especie veterinaria es un ave de corral.
- 15 3. Cepa mutante según cualquiera de las reivindicaciones anteriores, que es una cepa de *Salmonella entérica* o de *Escherichia coli*.
4. Cepa mutante según cualquiera de las reivindicaciones anteriores, en la que el gen con movilidad es un gen que codifica la flagelina.
- 20 5. Cepa mutante según la reivindicación 4, con una mutación en los genes *fliC* y/o *fljB* o *fljBA*.
6. Cepa mutante según la reivindicación 4 ó 5, que es incapaz de aglomerarse en el medio LB que contiene agar-agar al 0,4%.
- 25 7. Cepa mutante según cualquiera de las reivindicaciones anteriores, en la que el gen implicado en la supervivencia es un gen constitutivo o un gen con virulencia.
- 30 8. Cepa mutante según la reivindicación 7, en la que el gen constitutivo que está inactivado es el gen *guaB*.

9. Cepa mutante según la reivindicación 8, en la que la cepa mutante contiene una mutación por eliminación que altera la función del gen *guaB*.

5 10. Mutante según la reivindicación 8 ó 9, que es incapaz de formar de nuevo nucleótidos de guanina.

11. Cepa mutante según cualquiera de las reivindicaciones anteriores, en la que dicha cepa mutante codifica y expresa un xenoantígeno.

10 12. Cepa mutante según cualquiera de las reivindicaciones anteriores, que es una cepa atenuada de *S. Enteritidis* o de *S. Typhimurium*.

15 13. Cepa mutante según cualquiera de las reivindicaciones anteriores, en la que las modificaciones genéticas se introducen en la cepa progenitora 76Sa88 tipo 4 del fago *S. Enteritidis*.

14. Cepa mutante según la reivindicación 13, que es la cepa SM73 atenuada de *S. Enteritidis* con el número de depósito LMG P-21642.

20 15. Cepa mutante según cualquiera de las reivindicaciones 1 a 12, en la que las modificaciones genéticas se introducen en una cepa progenitora 1491S96 de *S. Typhimurium*.

25 16. Cepa mutante según la reivindicación 15, que es la cepa SM89 atenuada de *S. Typhimurium* con el número de depósito LMG P-21643.

17. Mutante según cualquiera de las reivindicaciones anteriores, que contiene una modificación genética en el gen *guaB* y una modificación genética en el gen *fliC*.

30 18. Mutante según cualquiera de las reivindicaciones anteriores, que contiene una modificación genética en el gen *guaB* y una modificación genética en los genes *fliBA*.

94

- 48 -

19. Mutante según cualquiera de las reivindicaciones anteriores, que contiene una modificación genética en el gen *guaB*, una modificación genética en el gen *fliC* y una modificación genética en los genes *fliBA*.

5 20. Cepa mutante según cualquiera de las reivindicaciones anteriores, en la que la modificación se selecciona de entre el grupo constituido por una inserción, una eliminación y/o una sustitución de uno o más nucleótidos en dichos genes.

10 21. Vacuna para inmunizar una especie veterinaria contra una infección bacteriana, comprendiendo dicha vacuna: una cantidad farmacéuticamente eficaz o inmunizadora de una cepa mutante atenuada según cualquiera de las reivindicaciones anteriores; y un vehículo o diluyente farmacéuticamente aceptable.

15 22. Vacuna según la reivindicación 21, en la que dicha cepa mutante codifica y expresa un xenoantígeno.

20 23. Vacuna según la reivindicación 21 ó 22, en la que dicha cepa mutante comprende un plásmido, que codifica y expresa, en una célula eucariótica, un gen extraño.

24. Procedimiento de inmunización de una especie veterinaria contra una infección bacteriana, comprendiendo dicho procedimiento la etapa siguiente:

25 - administrar a una especie veterinaria que lo necesita un agente inmunizador de una cepa mutante según cualquiera de las reivindicaciones 1 a 20 y/o una vacuna según cualquiera de las reivindicaciones 21 a 23.

25. Procedimiento según la reivindicación 24, en el que la especie veterinaria son aves de corral.

30 26. Procedimiento según cualquiera de las reivindicaciones 24 ó 25, en el que la cepa mutante es una cepa atenuada de *S. enterica* o de *E. coli*.

150

27. Procedimiento según cualquiera de las reivindicaciones 24 a 26, en el que la cepa mutante y/o la vacuna se administra por vía oral, nasal o parenteral.

5 28. Utilización de una cepa mutante según cualquiera de las reivindicaciones 1 a 20 destinada a la preparación de una vacuna destinada a la prevención y/o al tratamiento de infecciones en especies veterinarias, preferentemente las aves de corral.

10 29. Procedimiento para una diferenciación serológica entre los animales vacunados y los animales infectados por una cepa natural, en el que los animales vacunados han sido inmunizados con una cepa mutante en la que un gen de flagelina está inactivado, comprendiendo dicho procedimiento las etapas siguientes:

- 15
- ensayar en animales la presencia de anticuerpos contruidos contra la flagelina,
 - diferenciar los animales infectados de los animales vacunados basándose en la presencia o ausencia de dichos anticuerpos.

20 30. Procedimiento según la reivindicación 29, en el que dichos anticuerpos son generados por un animal infectado por una cepa natural, no obstante no por un animal que ha sido vacunado con una cepa mutante en la que el gen de flagelina está inactivado, tal como un mutante según cualquiera de las reivindicaciones 4 a 20.

25 31. Procedimiento según cualquiera de las reivindicaciones 29 a 30, en el que la presencia de dichos anticuerpos indica la presencia de cepas naturales y por lo tanto de infección.

30 32. Procedimiento según cualquiera de las reivindicaciones 29 a 31, en el que los animales infectados por *Salmonellae* se distinguen de los animales que han sido inmunizados con una vacuna con virus vivos atenuada según cualquiera de las reivindicaciones 21 a 23.

101

- 50 -

33. Procedimiento según cualquiera de las reivindicaciones 29 a 32, en el que se analizan en los animales los anticuerpos contruidos contra *FliC*.

5 34. Procedimiento según cualquiera de las reivindicaciones 29 a 32, en el que el animal es una especie veterinaria, preferentemente aves de corral, más preferentemente un pollo.

10

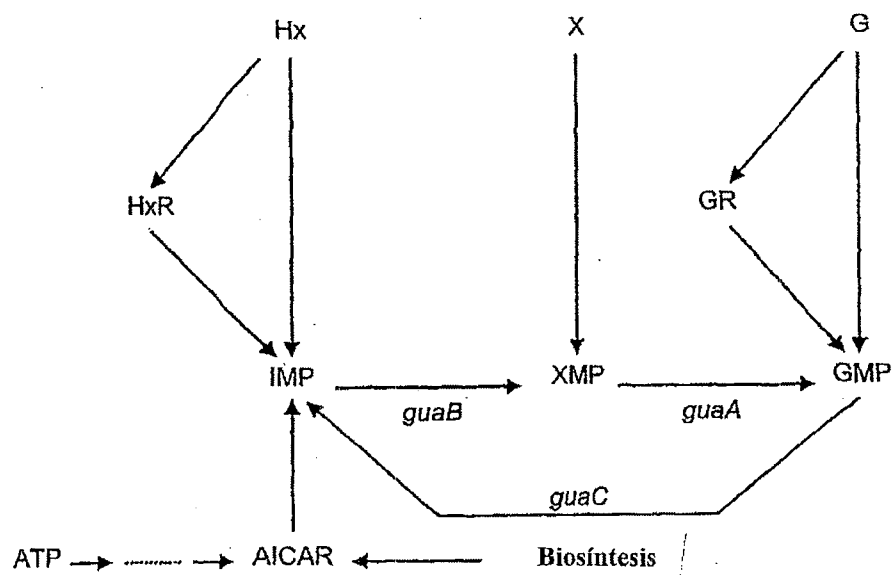


Fig. 1

109

CTGATCGAACAAAGCCTTCGGCCTGCAGTTTGGCTTTTAGCTGCTCATATTTCTGCTGCAA ~ 60
CAAGCCTTCGCCCGCGGGCTGCATACTTTCGGCGATGATTTGATAATCGCCCGCGCGGCTC - 120
GTACAGCGTAATGTTGGCGCGTACCAGCACCTGCTGCCCGTGTGCGGSCGGAACGTAC - 180
CCGGCGATTGCTGTTACGGAACATCGCACAGCGCACCTGAGCGGTATCGTCTTTGAGCGT - 240
AAAGTACCAGTGGCCCCGACGAGGCTGCGTGAAATAGAAATCTCGCCGCTGATCCATAC - 300
CTGTCCCATCTCCGTCTAACAGCAGACGAACCGTCTGGTTAAGGCGGCTTACGGTAAA - 360
AATTGAGGAAGTTTGAGAGGATAACATGTGAGCGGGATCAAATTCTAAATCAGCAGGTTA - 420
TTCAATCGATAGTAACCTGCTCACGGGGGATCGCAAGCACTATTTGCAAAAAAATGTAGA - 480
TGCAACCGATTACGTTCTGTATAATGCCGCGGGAATATTTATTAACTCCAGGCGAGAT - 540
ATTGCCCATGCTACGTATCGCTAAAGAAGCTCTGACGTTTGACGACGTCTCTCTTGTTC - 600
CGCTCACTCCACCGTTTTCGCGAATACTGCCGATCTCAGCACGCGAGTTGACGAAAACTAT - 660
TCGTCTGAATATTCTATGCTTTCTGCGGCGATGGACACCGTGACGGAAGCGCGCTGGC - 720
AATTGCCCTGGCCAGGAAGGCGGCATTGGTTTATCCACAAAAACATGTCTATTGAGCG - 780
CCAGGCGGAAGAAGTTCCGCCGCTGAAGAAACACGAGTCCGGCGTAGTGACCGACCCGCA - 840
GACCGTCTGCAACCAACACCGTTGCATGAAGTGAAAGCCCTGACCGAGCGTAAACGGTTT - 900
TGCGGGCTATCCGGTGGTGACTGAAGATAACGAGCTGGTGGGTATCATCACCGGTCGTGA - 960
CGTGCGTTTTGTGACTGACCTGAACCAGCCGGTGAGTGTCTACATGACACCGAAAGAGCG - 1020
TCTGGTGACCGTTCTGTGAAGGCGAAGCCCGTGAAGTCTGCTGGCAAAAATGCACGAAAA - 1080
ACGCGTAGAAAAAGCGCTGGTCTGTGATGATAACTTCCATCTGCTTGGCATGATTACCGT - 1140
AAAAGATTTCCAGAAAGCGGAACGTAAACCAAACTCCTGTAAAGATGAGCAGGCGCGTTT - 1200
ACGTGTGGCGCGCGCGGTCCGCCGAGGCGCGGGCAACGAAGAGCGCGTTGACCGCGTGGT - 1260
GGCGGCGAGGCGTTGACGTACTGCTGATCGACTCCTCTCACGGTCACTCTGAAGCGGTGT - 1320
GCAACGTATCCGTGAGACCGGTGCTAAATATCCTGACCTGCAATCATCGGCGGGAACGT - 1380
TGCGACGGGCGCAGGCGCTCGCGCACTGGCGGAAGCCGGTTGCAACGCGGTGAAAGTGGG - 1440
TATCGGCCCGGGTTCCATCTGTACGACTCGTATCGGGACTGCTGTGGGCGTTCGCGAGAT - 1500
CACCGCTGTTTCTGACGCGGTGGAAGCGCTGGAAGGCACCGGGATTCCGGTTATCGCTGA - 1560
CGGCGGTATCCGTTTCTCCGNNACATCGCCAAAGCCATCGCCGAGGCGCGAGCGCGGT - 1620
AATGGTGGGTTCTATGCTGGCCGTTACCGAAGAATCNCGGGCGAAATCGAACTCTACCA - 1680
GGGCGGTTCTTACAAATCTTATCGCGGTATGGGTTCTCTGGGCGCGATGTCCAAAGGTTT - 1740
CTCCGACCGTTACTTCCAGAGCGACAACGCCGCCGACAACTGGTGCCGGAAGGTATCGA - 1800
AGGCCGCGTAGCCTATAAAGGTCGCTGAAAGAGATCATTCACCAGCAGATGGGCGGCCCT - 1860
GCGCTCCTGTATGGGGCTGACCGGTTGTGCTACCATCGACGAACTGCGTACTAAAGCGGA - 1920
GTTTGTGCGTATCAGCGGTGCGGGTATCCAGGAAAGCCAGTTCACGACGTGACCATCAC - 1980
CAAAGAGTCCCCGAACCTACCGTCTGGGCTCCTGATTTTCTTCGCCCGACCTTCGCGTCGG - 2040
GCGATTTATTTAATCTGTTTCACTTGCTCGGAATAAGCGTCAATGACGGAACATTCA - 2100
TAAGCATCGCATCCTCATCTGGACTTCGGTTCTCAGTACACTCAACTGGTTGCGCGCCG - 2160
CGTGCGTGAGCTGGGTGTTTACTGTGAACGTGTGGGCGTGGGATGTGACAGAAGCACAAAT - 2220
TCGTGACTTCAACCCAAGCGGCATTATTCTTCCGGCGGCCCGGAAAGCACCACCGAAGA - 2280
AAACAGCCCGCGCGCGCGCAGTATGCTTTGAAGCAGGCGTGCCGGTATTTGGCGTTTG - 2340
CTATGGTATGCAGACCATGGCGATGCAGCTTGGCGGTGATGTAGAAGGTTCTAATGAGCG - 2400
TGAATTTGGTTATGCGCAGGTGGAAGTGTGACCGACAGCGCGCTGGTTCCGCGTATTGA - 2460
AGATTCCTGACCGCAGACGGCAAACCGCTGCTGGACGTGTGGATGAGCCACGGCGATAA - 2520
AGTGACGGCGATTCCGTCGACCTTCTGACCGTCCGCCAGCAGGAGCTGCCCGTTTCGC - 2580
CATTATGGCCAACGAAGAAAAACGCTTCTACGCGGTACAGTTCCACCCGGAAGTGAAGTCA - 2640
CACCCGCCAGGGTATGCGCATGCTGGAGCGTTTTGTACCGGATATCTGCCAGTGTGAAGC - 2700
GTTGTGGACGCGCGGAAGATCATCGATGACGCCGTGGCGCGCATTCGCGAGCAGGTAGG - 2760
CGACGATAAAGTGATCCTCGGTCTCTCCGGCGCGGTGGATTCTTCGTCACCGCCATGCT - 2820
GCTGCACCGCGCGATCGGTAAAAATCTGACCTGTGTTTTCGTCGACACACGGCCGTGGCG - 2880
CCTGAACGAAGCCGAGCAGGTGA -2903 (SEQ. ID NO: 19)

Fig. 2

104

quaB3
1 GGCTGCGATTGGCGAGGTAGTAGTCCATCGCCATGCCAGACGCTGCTGGCGGANCTGGA

quaB6
61 TCTGACGCAACAACCTCTGCTGGTTACGGCTGACAATTTCTGCCGCTGCTGATGGCGTAG
121 CGCGCGCAGGTGGCGACAAAATCAGCTATCGTGACGTCCGTTTCGTGACCGACGGCGCT
181 GACCACCGGAATGCGGCTGGCAAAAATCGCCCGCGCCACGCGTTCGTGCTTAAACTCCA
241 CAAATCTTCCAGCGAACCGCGCGCGCGCCGACGATCAGTACATCACATTGCGCGCGCGC
301 GTTCGCCAGTTCGATAGCACGAACGATCTGCCCCGCGCGCTCGTCCCTGGACTGCGGT
361 TGGATAGATAATAACGGGCAGGGATGGGTACGACGCTTTAGCACGTGGAGAATATCGTG
421 CAGCGCGCGCGCGGTTTTCGAAGTGATCACCCCAACGCAGTGGGCGGGGAGGGCAACGG
481 CTGTTTATGCTGCTGATCGAACAAGCCTTCGGGCTGCAGTTTGGCTTTTAGCTGCTCATA
541 TTTCTGCTGCAACAAGCCTTCGCGCGCGGCTGCATACCTTCGGCGATGATTTGATAATC
601 GCCGCGCGGCTCGTACAGCGTAATGTTGGCGCGTACCAGCACCTGCTGCCCGTGTGCGG
661 GCGGAACGTACCCGGCGATTGCTGTTACGGAACATCGCACAGCGCACCTGAGCGGTATC
721 GTCTTIGAGCGTAAAGTACCAGTGGCGCGACGCGAGGCTGCGTGAAATTAGAAATCTCGCC
781 GCTGATCCATACCTGTCCCATCTCCTGTTCTAACAGCAGACGAACCGTCTGGTTAAGGCG

quaB10
841 GCTTACGGTAAAAATTGAGGAAGTTTGAGAGGATAACATGTGAGCGGGATCAAATTCTAA
901 ATCAGCAGGTTATTCAATCGATAGTAACCTGCTCACGGGGGATCGCAAGCACTATTTGCA
961 AAAAAATGTAGATGCAACCGATTACGTTCTGTATAATGCCGCGGCAATATTTATTAACCT

quaB4
1021 CCCAGGTGAGATATTGCCCATGCTACGTATCGCTAAAGAAGCTCTGACGTTTGACGCCCCG

quaB5
1081 GGATCACCAAAGAGTCCCGAAGTACCGTCTGGGCTCCTGATTTTCTTCGCCCGACCTTC
1141 GCGTCGGGCGATTTATTTAATCTGTTTCACTTGCCTCGGAATAAGCGTCAATGACGGAAA
1201 ACATTCAATAGCATCGCATCCTCATTCCTGGACTTCGGTTCTCAGTACACTCAACTGGTTG
1261 CGCGCCGCGTGCCTGAGCTGGGTGTTTACTGTGAAGTGTGGGCGTGGGATGTGACAGAAG
1321 CACAAATTCGTGACTTCAACCAAGCGGCATTATTCTTTCCGGCGCGCCGGAAGCACCA
1381 CCGAAGAAAACAGCCCGCGCGCGCGCAGTATGCTTTGAAGCAGGCGTGCCGGTATTTG
1441 GCGTTTGCTATGGTATGCAGACCATGGCGATGCAGCTTGGCGGTCATGTAGAAGTTCTA
1501 ATGAGCGTGAATTTGGTTATGCGCAGGTGCAAGTGTGACCGACAGCGCGTGGTTTCGCG
1561 GTATTGAAGATTCCCTGACCGCAGACGGCAAACCGCTGCTGGACGTGTGGATGAGCCACG
1621 GCGATAAAGTGACGGCGATTCCGTCCGACTTCGTGACCGTCGCCAGCACCGAGAGCTGCC
1681 CGTTCGCCATTATGGCCAACGAAGAAAAACGCTTCTACGGCGTACAGTTCACCCGGAAG
1741 TGACTCACACCCGCCAGGGTATGCGCATGCTGGAGCGTTTTGTACGCGATATCTGCCAGT
1801 GTGAAGCGTTGTGGACGCCGCGGAAGATCATCGATGACGCCGTGGCGCGCATTCGCGAGC

quaB7
1861 AGGTAGGCGACGATAAAGTGATCCTCGGTCTCTCCGGCGGCGTGGATTCTTCCGTCACCG

quaB2
1921 CCATGCTGCTGCACCGCGGATCGGTAAAAATCTGACCTGTGTTTTCGTGACAACGGCC
1981 TGTGCGCCTGAACG

(SEQ ID NO: 20)

Fig. 3

105

1 CATGTGTGAGCGGGATCAAATTCTAAATCAGCAGGTTATTCAATCGATAG
51 TAACCTGCTCACGGGGGATCGCAAGCACTATTGCAAAAAAATGTAGAT
101 GCAACCGATTACGTTCTGTATAATGCCCGCAATATTTATTACCTCCCA
151 GGTGAGATAATCGCCATGTACGTATCGCTAAAGAAGCCCTGACGTTTGAC
P1
201 GCCCGTGTAGGCTGAGCTGCTTCTGAAGTTCTTATACTTTCTAGAGAATA
P2
251 GGAAGTCCCGAATAGGAACTAAGGAGGATATTTCATATGGGATCACCAAAG
301 ATCCCCGAAGTACCGTCTGGGCTCTGATTTTCTTCGCCCGACCTTCGCG
(SEQ. ID NO: 21)

Fig. 4

1 ATTTGCAAAA AAATGTAGAT GCAACCGATT ACGTTCTGTA TAATGCCGCG GCAATATTTA
61 TTAACCTCCC AGGTGAGATA TTGCCCATGC TACGTATCGC TAAAGAAGCC CTGACGTTTG
121 ACGACGTCCT CTTGTTTCCC GCTCACTCCA CCGTTTGGCC GAATACTGCC GATCTCAGCA
181 CGCAGTTGAC GAAAACTATT CGTCTGAATA TTCTATGCT TTCTGCGGCG ATGGACACCG
241 TGACGGGAAGC GCGCCTGGCA ATTGCCCTGG CCCAGGAAGG CGGCATTGGT TTTATCCACA
301 AAAACATGTC CATTGAGCGC CAGGCGGAAG AAGTTCCGCG CGTGAAGAAA CACGAGTCCG
361 GCGTAGTGAC CGACCCGCAG ACCGTCCTGC CAACCAACAC GTTGCATGAA GTGAAAGCCC
421 TGACCGAGCG TAACGGTTT GCGGGCTATC CGGTGGTGAC TGAAGATAAC GAGCTGGTGG
481 GTATCATCAC CGGTCGTGAC GTGCGTTTTG TGACTGACCT GAACCAAGCC GTTAGCGTTT
541 ACATGACGCC GAAAGAGCGT CTGGTGACCG TTCGTGAAGG CGAAGCCCGT GAAGTCGTGC
601 TGGCAAAAAT GCACGAAAAA CGCGTAGAAA AAGCGCTGGT CGTTGATGAT AACTTCCATC
661 TGCTTGGCAT GATTACCGTA AAAGATTTC AGAAAGCGGA ACGTAAACCA AACTCCTGCA
721 AAGATGAGCA GGGCCGTTTA CGTGTCCGCG CGGCGGTCGG CGCAGGCGCG GGCAACGAAG
781 AGCGCGTTGA CGCGCTGGTG GCGGCAGGCG TTGACGTA CTGATCGAC TCCTCTCACG
841 GTCATTGAGA AGGCGTGTG CAACGTATCC GTGAAACCCG TGCTAAATAT CTTGACCTGC
901 AAATCATCGG CGGCAACGTC GCGACAGGCG CAGGCGCTCG CGCACTGGCG GAAGCCGGTT
961 GCAGCGCGGT GAAAGTCGGT ATTGGCCCGG GTTCCATCTG TACCACTCGT ATCGTGAAGT
1021 GCGTGGGCGT TCCGCAGATC ACCGCTGTT CTGACGCACT TGAAGCGCTG GAAGGTACCG
1081 GTATTCCGGT TATCGCTGAC GCGCGTATCC GTTTCTCCGG CGACATAGCC AAAGCGATTG
1141 CCGCAGGTGC AAGCGCGTA ATGGTGGGTT CCATGCTGGC GGGTACGGAA GAATCCCCGG
1201 GCGAAATCGA ACTCTACCAG GCGCGTTCTT ACAATCTTA CCGCGGCATG GGCTCGCTGG
1261 GTGCGATGTC CAAAGGTTCC TCCGACCGTT ACTTCCAGAG CGACAACGCC GCTGACAAAC
1321 TGGTGCCGGA AGGTATCGAA GGTGCGGTAG CCTATAAAGG TCGCCTGAAA GAGATCATTC
1381 ACCAGCAGAT GGGCGGCCTG CGCTCCTGTA TGGGCTGAC CGGTTGTGCT ACCATCGACG
1441 AACTGCGTAC TAAAGCGGAG TTTGTGCGTA TCAGCGGTGC GGGCATTCAG GAAAGCCACG
1501 TTCACGACGT GACCATCACC AAAGAGTCCC CGAACTACCG TCTGGGCTCC TGA
(SEQ. ID NO: 22)

Fig. 5

106

CTATGGCACAAGTCATTAATACAAACAGCCTGTCGCTGGTTGACCCAGAATAATGTGTAG
P1 →
GCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATAGGAAC
TTCGGAATAGGAACTAAGGAGGATATTCATATGTGGCGCAGGCGAACCAGGT
← P2
TCCGCAAAACGTCCTCTCTTTACTGGCGTTAATGCG
(SEQ. ID NO: 23)

Fig. 6

1 AGAATAACTGTGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGA
P1 →
51 GAATAGGAACTTCGGAATAGGAACTAAGGAGGATATTCATATGGTTATGT
← P2
101 TTGACTGGGTGAGCAGGATCACGTCTTCGGACTACGCTGT
(SEQ. ID NO: 24)

Fig. 7

108

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS -- BCCM COPIA
RECOGIDA- LGM

Página 1 del formulario BCCM/LMG/BP/4 02-132 Recibo en caso de un depósito original

Tratado de Budapest sobre el Reconocimiento Internacional del Depósito de Microorganismos con fines
del procedimiento en materia de patentes

Recibo en el caso de un depósito original emitido según la Regla 7.1 por la Autoridad internacional de
depósito BCCM/LMG identificada en la parte inferior de la siguiente página

Formulario Internacional BCCM/LMG/BP/4/02-132

Para: Nombre del depositante: Prof. J.P. Hernalsteens

Dirección: Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Identificación del microorganismo:

I.1 Referencia de la identificación proporcionada por el depositante:

SM69

I.2 Número de acceso proporcionado por la Autoridad internacional de depósito:

LMG P-21641

7/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGM

Página 2 del formulario BCCM/LMG/BP/4 02-132 Recibo en caso de un depósito original

II. Descripción científica y/o designación taxonómica propuesta

El microorganismo identificado con I está acompañado por:

(marcar con una cruz el recuadro (s) adecuado):

☒

una descripción científica

☒

una designación taxonómica propuesta

III. Recepción y aceptación

La presente Autoridad Internacional acepta el microorganismo identificado con el símbolo I anterior, que fue recibido por la misma en (fecha del depósito original)

9 de agosto de 2002

IV. Autoridad Internacional de depósito

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS (BCCM)
Laboratorium voor Microbiologie – Bacterienverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-900 Gent, Bélgica

Firma(s) de la persona(s) facultada(s) para representar la Autoridad internacional de depósito o de funcionario (s) autorizado (s):

(firma ilegible)

Dr. D. Janssens, comisario IDA

Fecha: 5 de septiembre de 2002

109

8/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGM

Página 1 del formulario BCCM/LMG/BP/4 02-132 Declaración de viabilidad

Tratado de Budapest sobre el Reconocimiento Internacional del Depósito de Microorganismos
con fines del procedimiento en materia de patentesDeclaración de viabilidad según la Regla 10.2 por la Autoridad internacional de depósito
Autoridad BCCM/LMG identificada en la siguiente páginaFormulario internacional BCCM/LMG/BP/9/ 02-132

Para: La parte a la que se le concede la declaración de viabilidad:

Nombre: Prof. J.P. Hernalsteens

Dirección: Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Depositante:

I.1 Nombre: Prof. J.P. Hernalsteens

I.2 Dirección: Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

II. Identificación del microorganismo

II.1 Número de acceso proporcionado por la Autoridad internacional de depósito

LMG P-21641

II.2 Fecha del depósito original (o en caso de haberse realizado un nuevo depósito o una
transferencia, la fecha relevante más reciente)

9 de agosto de 2002

III. Declaración de viabilidad

La viabilidad del microorganismo identificada con el símbolo II fue comprobada el

9 de agosto de 2002

(Proporcionar la fecha. En los casos referidos en la Regla 10.2(a)(II) y (III), es necesario hacer
referencia a la comprobación de viabilidad más reciente)

En dicha fecha, dicho microorganismo era: (marcar el recuadro aplicable con una cruz)

☒ viable☒ Ya no es viable

W

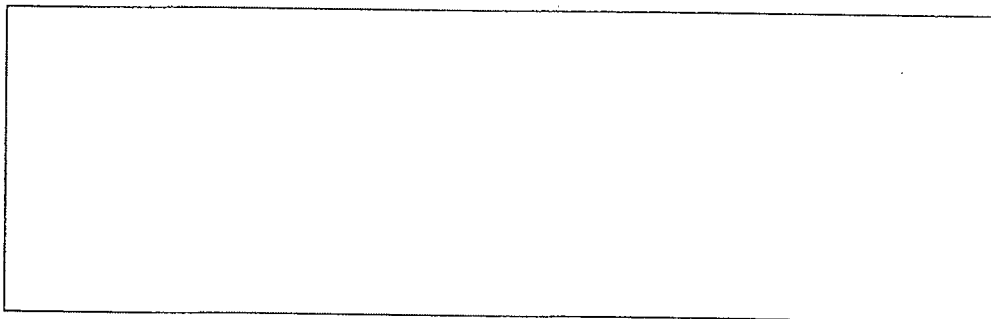
9/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS -- BCCM COPIA
RECOGIDA- LGM

Página 2 del formulario BCCM/LMG/BP/4 02-132 Declaración de viabilidad

IV. Condiciones bajo las cuales se ha llevado a cabo la comprobación de viabilidad:

(Rellenar en caso de que se haya exigido la información y si los resultados de la comprobación fueran negativos)



V. Autoridad internacional de depósito

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS (BCCM)
Laboratorium voor Microbiologie – Bacterienverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Bélgica

Firma(s) de la persona(s) facultada(s) para representar la Autoridad internacional de depósito o de funcionario (s) autorizado (s):

(firma ilegible)

Dr. D. Janssens, comisario IDA

Fecha: 5 de septiembre de 2002

Figura 8

11

10/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGM

Página 1 del formulario BCCM/LMG/BP/4 02-132 Recibo en caso de un depósito original

Tratado de Budapest sobre el Reconocimiento Internacional del Depósito de Microorganismos
con fines del procedimiento en materia de patentes

Declaración de viabilidad según la Regla 10.2 por la Autoridad internacional de depósito
Autoridad BCCM/LMG identificada en la siguiente página

Formulario internacional BCCM/LMG/BP/9/ 02-132

Para: Nombre del depositante: Prof. J.P. Hernalsteens

Dirección: Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Identificación del microorganismo

I.1 Referencia de la identificación proporcionada por el depositante:

SM73

I.2 Número de acceso proporcionado por la Autoridad internacional de depósito:

LMG P-21642

11/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGMPágina 1 del formulario BCCM/LMG/BP/4 02-133 Recibo en caso de un depósito original

II. Descripción científica y/o designación taxonómica propuesta

El microorganismo identificado con I está acompañado por:

(marcar con una cruz el recuadro (s) adecuado):

☒

una descripción científica

☒

una designación taxonómica propuesta

III. Recepción y aceptación

La presente Autoridad Internacional acepta el microorganismo identificado con el símbolo I anterior, que fue recibido por la misma en (fecha del depósito original)

9 de agosto de 2002

IV. Autoridad internacional de depósito

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS (BCCM)

Laboratorium voor Microbiologie – Bacteriënverzameling (LMG)

Universiteit Gent

K.L. Ledeganckstraat 35

B-900 Gent, Bélgica

Firma(s) de la persona(s) facultada(s) para representar la Autoridad internacional de depósito o de funcionario (s) autorizado (s):

(firma ilegible)

Dr. D. Janssens, comisario IDA

Fecha: 5 de septiembre de 2002

117

12/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGM

Página 1 del formulario BCCM/LMG/BP/4 02-133 Declaración de viabilidad

Tratado de Budapest sobre el Reconocimiento Internacional del Depósito de Microorganismos
con fines del procedimiento en materia de patentesDeclaración de viabilidad según la Regla 10.2 por la Autoridad internacional de depósito
Autoridad BCCM/LMG identificada en la siguiente páginaFormulario internacional BCCM/LMG/BP/9/ 02-133

Para: La parte a la que se le concede la declaración de viabilidad:

Nombre: Prof. J.P. Hernalsteens

Dirección: Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Depositante:

I.1 Nombre: Prof. J.P. Hernalsteens

I.2 Dirección: Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

II. Identificación del microorganismo

II.1 Referencia de la identificación proporcionada por la Autoridad internacional de
depósito:
LMG P-21642II.2 Fecha del depósito original (o en caso de haberse realizado un nuevo depósito o una
transferencia, la fecha relevante más reciente)

9 de agosto de 2002

III. Declaración de viabilidad

La viabilidad del microorganismo identificada con el símbolo II fue comprobada el

9 de agosto de 2002

(Proporcionar la fecha. En los casos referidos en la Regla 10.2(a)(II) y (III), es necesario hacer
referencia a la comprobación de viabilidad más reciente)

En dicha fecha, dicho microorganismo era: (marcar el recuadro aplicable con una cruz)

☒

viable

☐

Ya no es viable

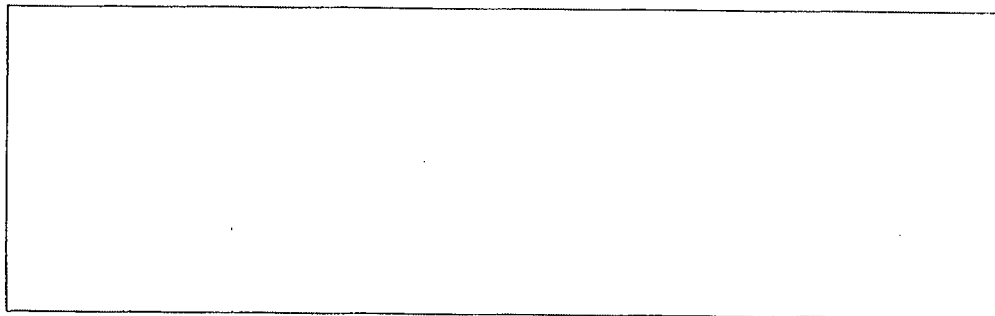
24

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGM

Página 2 del formulario BCCM/LMG/BP/4 02-133 Declaración de viabilidad

IV. Condiciones bajo las cuales se ha llevado a cabo la comprobación de viabilidad:

(Rellenar en caso de que se haya exigido la información y si los resultados de la comprobación fueran negativos)



V. Autoridad internacional de depósito

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS (BCCM)
Laboratorium voor Microbiologie – Bacterienverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-900 Gent, Bélgica

Firma(s) de la persona(s) facultada(s) para representar la Autoridad internacional de depósito o de funcionario (s) autorizado(s):

(firma ilegible)

Dr. D. Janssens, comisario IDA

Fecha: 5 de septiembre de 2002

Figura 9

14

14/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGM

Página 1 del formulario BCCM/LMG/BP/4 02-134 Recibo en el caso de un depósito original

Tratado de Budapest sobre el Reconocimiento Internacional del Depósito de Microorganismos
con fines del procedimiento en materia de patentes

Recibo en el caso de un depósito original emitido según la Regla 7.1 por la Autoridad
internacional de depósito BCCM/LMG identificada en la parte inferior de la siguiente página

Formulario internacional BCCM/LMG/BP/9/ 02-134

Para: Nombre del depositante: Prof. J.P. Hernalsteens

Dirección: Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

V. Identificación del microorganismo:

I.1 Referencia de la identificación proporcionada por el depositante:

SM89

I.2 Número de acceso proporcionado por la Autoridad internacional de depósito:

LMG P-21643

11p

15/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGMPágina 2 del formulario BCCM/LMG/BP/4 02-134 Recibo en caso de un depósito original

II. Descripción científica y/o designación taxonómica propuesta

El microorganismo identificado con I está acompañado por:

(marcar con una cruz el recuadro (s) adecuado):

☒

una descripción científica

☒

una designación taxonómica propuesta

III. Recepción y aceptación

La presente Autoridad Internacional acepta el microorganismo identificado con el símbolo I anterior, que fue recibido por la misma en (fecha del depósito original)

9 de agosto de 2002

IV. Autoridad Internacional de depósito

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS (BCCM)
Laboratorium voor Microbiologie – Bacterienverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Bélgica

Firma(s) de la persona(s) facultada(s) para representar la Autoridad internacional de depósito o de funcionario (s) autorizado (s):

(firma ilegible)

Dr. D. Janssens, comisario IDA

Fecha: 5 de septiembre de 2002

117

16/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGMPágina 1 del formulario BCCM/LMG/BP/4 02-134 Declaración de viabilidadTratado de Budapest sobre el Reconocimiento Internacional del Depósito de Microorganismos
con fines del procedimiento en materia de patentesDeclaración de viabilidad según la Regla 10.2 por la Autoridad internacional de depósito
Autoridad BCCM/LMG identificada en la siguiente páginaFormulario internacional BCCM/LMG/BP/9/ 02-134

Para: La parte a la que se le concede la declaración de viabilidad:

Nombre: Prof. J.P. Hernalsteens

Dirección: Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Depositante:

I.1 Nombre: Prof. J.P. Hernalsteens

I.2 Dirección: Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

II. Identificación del microorganismo

II.1 Número de acceso proporcionado por la Autoridad internacional de depósito

LMG P-21643

II.2 Fecha del depósito original (o en caso de haberse realizado un nuevo depósito o una
transferencia, la fecha relevante más reciente)

9 de agosto de 2002

III. Declaración de viabilidad

La viabilidad del microorganismo identificada con II fue comprobada el

9 de agosto de 2002

(Proporcionar la fecha. En los casos referidos en la Regla 10.2(a)(II) y (III), es necesario hacer referencia
a la comprobación de viabilidad más reciente)

En dicha fecha, dicho microorganismo era: (marcar el recuadro aplicable con una cruz)

☒

viable

☒

Ya no es viable

118

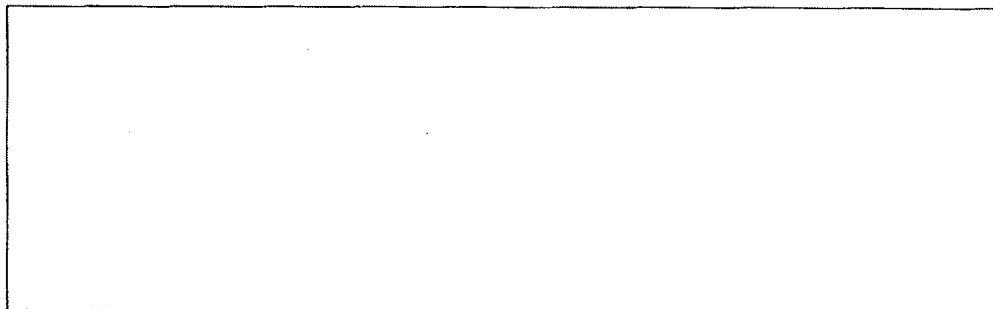
17/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGM

Página 2 del formulario BCCM/LMG/BP/4 02-134 Declaración de viabilidad

IV. Condiciones bajo las cuales se ha llevado a cabo la comprobación de viabilidad:

(Rellenar en caso de que se haya exigido la información y si los resultados de la comprobación fueran negativos)



V. Autoridad internacional de depósito

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS (BCCM)
Laboratorium voor Microbiologie – Bacterienverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-900 Gent, Bélgica

Firma(s) de la persona(s) facultada(s) para representar la Autoridad internacional de depósito o de funcionario (s) autorizado(s):

(firma ilegible)

Dr. D. Janssens, comisario IDA

Fecha: 5 de septiembre de 2002

Figura 10

18/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGMPágina 1 del formulario BCCM/LMG/BP/4 02-135 Recibo en caso de un depósito original

Tratado de Budapest sobre el Reconocimiento Internacional del Depósito de Microorganismos con fines
del procedimiento en materia de patentes

Recibo en el caso de un depósito original emitido según la Regla 7.1 por la Autoridad internacional de
depósito BCCM/LMG identificada en la parte inferior de la siguiente página

Formulario Internacional BCCM/LMG/BP/4/02-135

Para: Nombre del depositante: Prof. J.P. Hernalsteens

Dirección: Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Identificación del microorganismo:

I.1 Referencia de la identificación proporcionada por el depositante:

SM86

I.2 Número de acceso proporcionado por la Autoridad internacional de depósito:

LMG P-21646

19/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGMPágina 2 del formulario BCCM/LMG/BP/4 02-132 Recibo en caso de un depósito original

II. Descripción científica y/o designación taxonómica propuesta

El microorganismo identificado con I está acompañado por:

(marcar con una cruz el recuadro (s) adecuado):

☒

una descripción científica

☒

una designación taxonómica propuesta

III. Recepción y aceptación

La presente Autoridad Internacional acepta el microorganismo identificado con el símbolo I anterior, que fue recibido por la misma en (fecha del depósito original)

8 de agosto de 2002

IV. Autoridad internacional de depósito

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS (BCCM)
Laboratorium voor Microbiologie – Bacterienverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Bélgica

Firma(s) de la persona(s) facultada(s) para representar la Autoridad internacional de depósito o de funcionario (s) autorizado (s):

(firma ilegible)

Dr. D. Janssens, comisario IDA

Fecha: 5 de septiembre de 2002

20/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGM

Página 1 del formulario BCCM/LMG/BP/4 02-135 Declaración de viabilidad

Tratado de Budapest sobre el Reconocimiento Internacional del Depósito de Microorganismos
con fines del procedimiento en materia de patentesDeclaración de viabilidad según la Regla 10.2 por la Autoridad internacional de depósito
Autoridad BCCM/LMG identificada en la siguiente páginaFormulario internacional BCCM/LMG/BP/9/ 02-135

Para: La parte a la que se le concede la declaración de viabilidad:

Nombre: Prof. J.P. Hernalsteens

Dirección: Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Depositante:

I.1 Nombre: Prof. J.P. Hernalsteens

I.2 Dirección: Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

II. Identificación del microorganismo

II.1 Número de acceso proporcionado por la Autoridad internacional de depósito

LMG P-21646

II.2 Fecha del depósito original (o en caso de haberse realizado un nuevo depósito o una
transferencia, la fecha relevante más reciente)

28 de agosto de 2002

III. Declaración de viabilidad

La viabilidad del microorganismo identificada con II fue comprobada el

28 de agosto de 2002

(Proporcionar la fecha. En los casos referidos en la Regla 10.2(a)(II) y (III), es necesario hacer
referencia a la comprobación de viabilidad más reciente)

En dicha fecha, dicho microorganismo era: (marcar el recuadro aplicable con una cruz)

☒

viable

☐

Ya no es viable

12 ✓

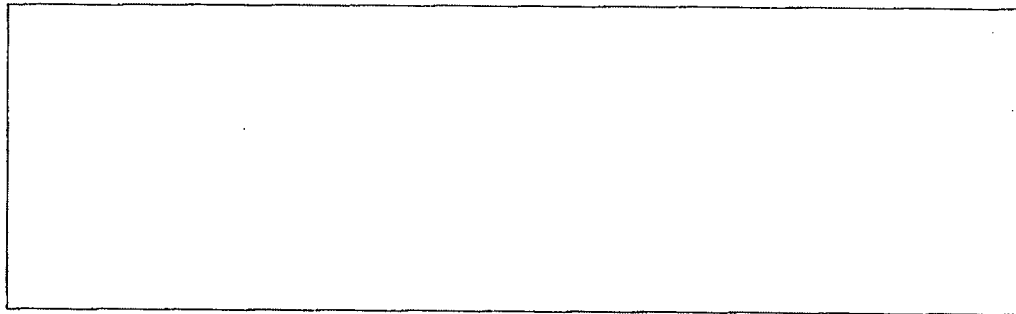
21/21

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGM

Página 2 del formulario BCCM/LMG/BP/4 02-135 Declaración de viabilidad

IV. Condiciones bajo las cuales se ha llevado a cabo la comprobación de viabilidad:

(Rellenar en caso de que se haya exigido la información y si los resultados de la comprobación fueran negativos)



V. Autoridad internacional de depósito

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS (BCCM)
Laboratorium voor Microbiologie – Bacterienverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-900 Gent, Bélgica

Firma(s) de la persona(s) facultada(s) para representar la Autoridad internacional de depósito o de funcionario (s) autorizado (s):

(firma ilegible)

Dr. D. Janssens, comisario IDA

Fecha: 5 de septiembre de 2002

Figura 11

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:

PRONOVEM-OFFICE VAN MALDEREN
Attn. Van Malderen, Joëlle
Avenue Josse Goffin 158
B-1082 Brussels
BELGIQUE

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT AND
THE WRITTEN OPINION OF THE INTERNATIONAL
SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

Date of mailing (day/month/year) 13/12/2006	
Applicant's or agent's file reference BPFDAH002AWO	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/BE2006/000020	International filing date (day/month/year) 20/03/2006
Applicant VRIJE UNIVERSITEIT BRUSSEL	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally two months from the date of transmittal of the International Search Report.

Where? Directly to the International Bureau of WIPO, 34 chemin des Colombettes
1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 338.82.70

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.
3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

- ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.
- ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Reminders

Shortly after the expiration of 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. These comments would also be made available to the public but not before the expiration of 30 months from the priority date.

Within 19 months from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later); otherwise, the applicant must, within 20 months from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

In respect of other designated Offices, the time limit of 30 months (or later) will apply even if no demand is filed within 19 months.

See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the International Searching Authority  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 Michela Digiusto
--	--

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the *PCT Applicant's Guide*, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report and the written opinion of the International Searching Authority, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only (see *PCT Applicant's Guide*, Volume I/A, Annexes B1 and B2).

The attention of the applicant is drawn to the fact that amendments to the claims under Article 19 are not allowed where the International Searching Authority has declared, under Article 17(2), that no international search report would be established (see *PCT Applicant's Guide*, Volume I/A, paragraph 296).

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

124

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference BPFDAH002AWO	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/BE2006/000020	International filing date (day/month/year) 20/03/2006	(Earliest) Priority Date (day/month/year)
Applicant VRIJE UNIVERSITEIT BRUSSEL		

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 5 sheets.

☒ 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
☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

b. ☒ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, see Box No. I.

2. ☐ Certain claims were found unsearchable (See Box No. II)

3. ☐ Unity of invention is lacking (see Box No. III)

4. With regard to the title,

- ☐ the text is approved as submitted by the applicant
☒ the text has been established by this Authority to read as follows:

LIVE ATTENUATED SALMONELLA VACCINE

5. With regard to the abstract,

- ☒ the text is approved as submitted by the applicant
☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the drawings,

- a. the figure of the drawings to be published with the abstract is Figure No. 1
☒ as suggested by the applicant
☐ as selected by this Authority, because the applicant failed to suggest a figure
☐ as selected by this Authority, because this figure better characterizes the invention
b. ☐ none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No.

PCT/BE2006/000020

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. type of material

☒

a sequence listing

☐

table(s) related to the sequence listing

b. format of material

☒

on paper

☒

in electronic form

c. time of filing/furnishing

☒

contained in the international application as filed

☒

filed together with the international application in electronic form

☐

furnished subsequently to this Authority for the purpose of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/BE2006/000020

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/20 G01N33/53 A61K39/108 A61K39/112 A61P31/04

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)

C12N G01N A61K A61P

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, BIOSIS, EMBASE, MEDLINE, SCISEARCH, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KODAMA CHIE ET AL: "Salmonella flagellin is not a dominant protective antigen in oral immunization with attenuated live vaccine strains" INFECTION AND IMMUNITY, vol. 72, no. 4, April 2004 (2004-04), pages 2449-2451, XP002408910 ISSN: 0019-9567 page 2450 ----- -/--	1-7,12, 13,15, 20,21

☒ Further documents are listed in the continuation of Box C.

☒ 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" 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

23 November 2006

Date of mailing of the international search report

13/12/2006

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

Domingues, Helena

INTERNATIONAL SEARCH REPORT

International application No

PCT/BE2006/000020

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MATSUI HIDENORI ET AL: "Oral immunization with ATP-dependent protease-deficient mutants protects mice against subsequent oral challenge with virulent Salmonella enterica serovar Typhimurium." INFECTION AND IMMUNITY, vol. 71, no. 1, January 2003 (2003-01), pages 30-39, XP002408911 ISSN: 0019-9567 page 31	1-7,12, 13,15, 20,21
X	----- MANN B J ET AL: "Protection in a gerbil model of amebiasis by oral immunization with Salmonella expressing the galactose/N-acetyl d-galactosamine inhibitable lectin of Entamoeba histolytica" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 15, no. 6-7, April 1997 (1997-04), pages 659-663, XP004064528 ISSN: 0264-410X page 659	1-7, 11-13, 15,20-23
X	----- US 2004/120962 A1 (CURTISS ROY [US] ET AL) 24 June 2004 (2004-06-24) page 6; table 1 examples 3,4	1-7, 11-13, 15,20-23
Y	----- CURTISS R ET AL: "NONRECOMBINANT AND RECOMBINANT AVIRULENT SALMONELLA VACCINES FOR POULTRY" VETERINARY IMMUNOLOGY AND IMMUNOPATHOLOGY, AMSTERDAM, NL, vol. 54, no. 1/4, November 1996 (1996-11), pages 365-372, XP000876519 ISSN: 0165-2427 the whole document	1-34
Y	----- BARBEZANGE CYRIL ET AL: "Some safety aspects of Salmonella vaccines for poultry: In vivo study of the genetic stability of three Salmonella typhimurium live vaccines" FEMS MICROBIOLOGY LETTERS, vol. 192, no. 1, 1 November 2000 (2000-11-01), pages 101-106, XP002408912 ISSN: 0378-1097 the whole document	1-34
	----- -/--	

INTERNATIONAL SEARCH REPORT

International application No
PCT/BE2006/000020

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MCFARLAND W C ET AL: "EFFECT OF DIFFERENT PURINE AUXOTROPHIC MUTATIONS ON MOUSE-VIRULENCE OF A VI-POSITIVE STRAIN OF SALMONELLA-DUBLIN AND OF TWO STRAINS OF SALMONELLA-TYPHIMURIUM" MICROBIAL PATHOGENESIS, ACADEMIC PRESS LIMITED, NEW YORK, NY, US, vol. 3, no. 2, 1987, pages 129-142, XP002376858 ISSN: 0882-4010 table 1 page 135, paragraph 1; table 2 page 138, paragraph 3 -----	1-34

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/BE2006/000020

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2004120962 A1	24-06-2004	NONE	

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY
(PCT Rule 43bis.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.
PCT/BE2006/000020

International filing date (day/month/year)
20.03.2006

Priority date (day/month/year)

International Patent Classification (IPC) or both national classification and IPC
INV. C12N1/20 G01N33/53 A61K39/108 A61K39/112 A61P31/04

Applicant
VRIJE UNIVERSITEIT BRUSSEL

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA:



European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Date of completion of
this opinion

see form
PCT/ISA/210

Authorized Officer

Domingues, Helena

Telephone No. +49 89 2399-7810



Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:
 - ☒ the international application in the language in which it was filed
 - ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).
2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:
 - a. type of material:
 - ☒ a sequence listing
 - ☐ table(s) related to the sequence listing
 - b. format of material:
 - ☒ on paper
 - ☒ in electronic form
 - c. time of filing/furnishing:
 - ☒ contained in the international application as filed.
 - ☒ filed together with the international application in electronic form.
 - ☐ furnished subsequently to this Authority for the purposes of search.
3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
4. Additional comments:

128

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/BE2006/000020

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of

☐ the entire international application

☒ claims Nos. 24-34

because:

☒ the said international application, or the said claims Nos. 24-34 with regard to IA relate to the following subject matter which does not require an international search (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed (*specify*):

☐ no international search report has been established for the whole application or for said claims Nos.

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.

☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.

☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rules 13~~ter~~.1(a) or (b).

☐ a meaningful opinion could not be formed without the tables related to the sequence listings; the applicant did not, within the prescribed time limit, furnish such tables in electronic form complying with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions, and such tables were not available to the International Searching Authority in a form and manner acceptable to it.

☐ the tables related to the nucleotide and/or amino acid sequence listing, if in electronic form only, do not comply with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions.

☐ See Supplemental Box for further details

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims	8-10,14,16-19
	No: Claims	1-7,11,12,13,15,20-23
Inventive step (IS)	Yes: Claims	8-10,14,16-19
	No: Claims	1-7, 11,12,13,15, 20-34
Industrial applicability (IA)	Yes: Claims	1-23 (for 24-34 see separate sheet)
	No: Claims	

2. Citations and explanations
see separate sheet

129

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING
AUTHORITY (SEPARATE SHEET)

International application No.

PCT/BE2006/000020

1. Concerning section III

Claims 29-34 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(I) PCT).

2. Concerning section V

2.1 Industrial applicability (Art. 33(4) PCT)

For the assessment of the present claims 29-34 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

2.2 Novelty (Art. 33(2) PCT) and inventive step (Art. 33(3)PCT)

2.2.1 D1-D4 disclose an attenuated mutant strain of *S. Typhimurium* comprising a modification in one or more motility genes and a second modification in one gene involved in the survival of proliferation of the bacterium in the host. Concretely, D1 describes strains *S. Typhimurium* which are deficient in ClpXP, FliC and FljB (CS2056) or in ClpX and FliA (CS2026). Oral immunization of mice with these strains resulted in protection against infection with a virulent *S. Typhimurium* strain (pg. 2450). The authors conclude that FliC and FljB are not dominant protective antigens (pg. 2451). D2 also describes the CS2056 strain mentioned above

D3 refers to a strain of *Salmonella dublin* which is aromatic vitamin dependent (aro) and has a modification in fliC (SL5928). This attenuated strain was transformed with a portion of the GalNAc lectin of *E. histolytica* and used to orally immunize gerbils.

D4 discloses a strain of *S. Typhimurium* containing a deletion of fliC and phoP (χ 8613) and its use to immunize mice against the PspA antigen of *S. penumoniae*.

Attention is drawn to the fact that ClpX, aro- and phoP are regarded as genes involved in the survival of proliferation of the bacterium in the host. In view of this, D1 and D2 are novelty destroying for **claims 1-7, 12, 13, 15, 20 and 21**. D3 and D4 also preclude the recognition of novelty for these claims and for **claims 11, 22 and 23**.

2.2.2 D4 (pg. 365-6) **and D5** (pg. 101) refer to the vulnerability of poultry to *S. enteritidis* and *S. typhimurium* infection and to the spread of infection through the food chain into humans. As stated above, D1 describes the protection of mice against *S. typhimurium* infection by oral immunization with a CIXP, FliC and FljB deficient strain. The technical problem, with regard to this document can be defined as the provision of a method of immunizing a veterinary species, namely poultry, against *S. enterica* or *E. coli* infection. Given the positive results described in D1 for mice immunization, the skilled person would have felt motivated to try the vaccines described therein in poultry thus arriving that the subject-matter of claims 24-28.

The skilled person faced with the problem of providing a method for a serological distinction between animals vaccinated with the vaccine described in D1 and animals infected with the wild type strain would need no inventive skill to conclude that the presence of antibodies against flagellin (particularly FliC and FljB) would be indicative of wild type infection, since the strain used in the vaccine is FliC and FljB deficient. Hence, claims 29-34 do not appear to involve inventive merit.

2.2.3 The available prior art does not appear to disclose *S. typhimurium* or *S. enteritidis* strains with a mutation in the fliC and/or fljB or fljBA genes and a mutation in the guaB gene. **D7** discloses *S. typhimurium* strains comprising a block in the guaB gene that had reduce virulence in mice inoculated with 2.5×10^5 cfu but significant virulence in mice inoculated at 2.5×10^7 cfu. **Claims 8-10, 14 and 16-19** thus appear to be novel. It may also be possible to recognise inventive merit for the subject-matter of these claims, since: i) the available prior art seems to provide no indication to combine the particular mutations under discussion ii) examples 4 and 5 show that $\Delta\text{guaB } \Delta\text{fliC}$, $\Delta\text{guaB } \Delta\text{fljBA}$ and $\Delta\text{guaB } \Delta\text{fliC } \Delta\text{fljBA}$ mutants are highly attenuated and have low virulence when orally administered to mice (tables 10-12) at a dosage of approximately 10^8 cells; iii) examples 6 and 7 show that *S. enteritidis* $\Delta\text{guaB } \Delta\text{fliC}$ is safe when administered to chicks at the age of one day at a titer of 2.5×10^7 .

In view of the above, it appears that claims 8 -10, 14 and 16-19 might serve as a basis for acceptable claims.

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF RECEIPT
OF SEARCH COPY

(PCT Rule 25.1)

To: PRONOVEM-OFFICE VAN MALDEREN Attn. Van Malderen, Joëlle Avenue Josse Goffin 158 B-1082 Brussels BELGIUM	REÇU 22.05.2006 OFFICE VAN MALDEREN
--	---

Date of mailing (day/month/year)	19/05/2006
-------------------------------------	------------

Applicant's or agent's file reference BPFDAH002AWO		IMPORTANT NOTIFICATION	
International application No. PCT/BE2006/000020	International filing date(day/month/year) 20/03/2006	Priority date (day/month/year)	
Applicant VRIJE UNIVERSITEIT BRUSSEL			

1. Where the International Searching Authority and the receiving Office are not the same office:

The applicant is hereby notified that the search copy of the international application was received by this International Searching Authority on the date indicated below.

Where the International Searching Authority and the receiving Office are the same office:

The applicant is hereby notified that the search copy of the international application was received on the date indicated below.

04/05/2006 (date of receipt).

- ☒ The search copy was accompanied by a nucleotide and/or amino acid sequence listing or tables related thereto in electronic form.
- ☐ The search copy contained a nucleotide and/or amino acid sequence listing or tables related thereto in electronic form.
- Time limit for establishment of international search report and written opinion of the International Searching Authority**
The applicant is informed that the time limit for establishing the international search report and the written opinion of the International Searching Authority is three months from the date of receipt indicated above or nine months from the priority date, whichever time limit expires later (Rules 42.1 and 43bis.1(a)).
- A copy of this notification has been sent to the International Bureau and, where the first sentence of paragraph 1 applies, to the receiving Office.

Name and mailing address of the International Searching Authority  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 ISA/EP
--	------------------------------

131

PATENT COOPERATION TREATY

780552

From the RECEIVING OFFICE

To:
VAN MALDEREN JOËLLE
OFFICE VAN MALDEREN

AVENUE JOSSE GOFFIN 158

1082 BRUXELLES

PCT

NOTIFICATION OF THE INTERNATIONAL
APPLICATION NUMBER AND OF THE
INTERNATIONAL FILING DATE

(PCT Rule 20.5(c))

Date of mailing
(day/month/year) 14.04.2006

Applicant's or agent's file reference
BPFDAH002AWO

IMPORTANT NOTIFICATION

International application No. PCT/BE2006/000020	International filing date (day/month/year) 20 March 2006 (20.03.2006)	Priority date (day/month/year)
--	--	--------------------------------

Applicant VRIJE UNIVERSITEIT BRUSSEL

Title of the invention Live attenuated vaccine

1. The applicant is hereby notified that the international application has been accorded the international application number and the international filing date indicated above.

2. The applicant is further notified that the record copy of the international application:

- ☒ was transmitted to the International Bureau on 07-04-2006
- ☐ has not yet been transmitted to the International Bureau for the reason indicated below and a copy of this notification has been sent to the International Bureau*:
- ☐ because the necessary national security clearance has not yet been obtained.
- ☐ because (reason to be specified):

* The International Bureau monitors the transmittal of the record copy by the receiving Office and will notify the applicant (with Form PCT/IB/301) of its receipt. Should the record copy not have been received by the expiration of 14 months from the priority date, the International Bureau will notify the applicant (Rule 22.1(c)).

Name and mailing address of the receiving Office OFFICE / PIIE / BRUXELLES	Authorized officer Madame DE WULF M.M.
Facsimile No. 02 / 277 52 62	Telephone No. 02 / 277 67 79

182

REÇU
04.05.2006
OFFICE VAN MALDEREN

PATENT COOPERATION TREATY

PCT/BE2006/000020

PCT

NOTIFICATION OF RECEIPT OF
RECORD COPY

(PCT Rule 24.2(a))

From the INTERNATIONAL BUREAU

To:

VAN MALDEREN, Joëlle
pronovem-OFFICE VAN MALDEREN
Avenue Josse Goffin 158
1082 Brussels
BELGIQUE

Date of mailing (day/month/year) 28 April 2006 (28.04.2006)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference BPFDAH002AWO	International application No. PCT/BE2006/000020

The applicant is hereby **notified** that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

VRIJE UNIVERSITEIT BRUSSEL (for all designated States except US)
DE GREVE, Henri Marcel Jozef et al (for US)

International filing date: 30 March 2006 (30.03.2006)

Priority date(s) claimed: None

Date of receipt of the record copy by the International Bureau: 20 April 2006 (20.04.2006)

List of designated Offices:

AP: BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW

EA: AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

EP: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR

OA: BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG

National: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW

ATTENTION: The applicant should carefully check the data appearing in this notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau. In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

- time limits for entry into the national phase - see updated important information (as of April 2002)
- requirements regarding priority documents (if applicable)

A copy of this notification is being sent to the receiving Office and to the International Searching Authority.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Suarez Javier Facsimile No. +41 22 338 71 30 Telephone No. +41 22 338 80 58
---	---

INFORMATION ON ENTERING THE NATIONAL PHASE

The applicant is reminded that the "national phase" must be entered before each of the designated Offices indicated on the cover sheet of this notification by paying national fees and furnishing translations, as prescribed by Articles 22 and 39 and the applicable national laws. In addition, the applicant may also have to comply with other special requirements applicable in certain Offices. It is the applicant's responsibility to ensure that the necessary steps to enter the national phase are taken in a timely fashion. Most Offices do not issue reminders to applicants in connection with the entry into the national phase.

The applicable time limit for entering the national phase will, subject to what is said in the following paragraph, be 30 MONTHS from the priority date, not only in respect of any elected Office if a demand for international preliminary examination is filed before the expiration of 19 months from the priority date (see Article 39(1)), but also in respect of any designated Office, in the absence of filing of such demand, where Article 22(1) as modified with effect from 1 April 2002 applies in respect of that designated Office. For further details, see *PCT Gazette* No. 44/2001 of 1 November 2001, pages 19926, 19932 and 19934, as well as the *PCT Newsletter*, October and November 2001 and February 2002 issues.

In practice, time limits other than the 30-month time limit will continue to apply, for various periods of time, in respect of certain designated or elected Offices. For regular updates on the applicable time limits (20, 21, 30 or 31 months, or other time limit), Office by Office, refer to the *PCT Gazette* ("Section IV" part published on a weekly basis), to the *PCT Newsletter* (on a monthly basis) and to the relevant National Chapters in Volume II of the *PCT Applicant's Guide* (the paper version of which is updated usually twice a year and the Internet version of which is updated usually on a weekly basis). Finally, a cumulative table of all applicable time limits for entering the national phase is available from WIPO's Internet site, via links from various pages of the site, including those of the *Gazette*, *Newsletter* and *Guide*, at <http://www.wipo.int/pct/en/index.html>.

Information about the requirements for filing a demand for international preliminary examination is set out in the *PCT Applicant's Guide*, Volume I/A, Chapter IX. Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination (at present, all PCT Contracting States are bound by Chapter II).

REQUIREMENTS REGARDING PRIORITY DOCUMENTS

For applicants who have not yet complied with the requirements regarding priority documents, the following is recalled.

Where the priority of an earlier national, regional or international application is claimed, the applicant must submit a copy of the said earlier application, certified by the authority with which it was filed ("the priority document") to the receiving Office (which will transmit it to the International Bureau) or directly to the International Bureau, before the expiration of 16 months from the priority date, provided that any such priority document may still be submitted to the International Bureau before the date of international publication of the international application, in which case that document will be considered to have been received by the International Bureau on the last day of the 16-month time limit (Rule 17.1(a)).

Where the priority document is issued by the receiving Office, the applicant may, instead of submitting the priority document, request the receiving Office to prepare and transmit the priority document to the International Bureau. Such request must be made before the expiration of the 16-month time limit and may be subjected by the receiving Office to the payment of a fee (Rule 17.1(b)).

If the priority document concerned is not submitted to the International Bureau and if the request to the receiving Office to prepare and transmit the priority document has not been made (and the corresponding fee, if any, paid) within the applicable time limit indicated under the preceding paragraphs, any designated State may disregard the priority claim, provided that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances (Rule 17.1(c)).

Where several priorities are claimed, the priority date to be considered for the purposes of computing the 16-month time limit (and all other PCT time limits) is the filing date of the earliest application whose priority is claimed (Article 2(xi)(b)).

131

RECU
04.08.2008
pronovem - Bruxelles

PATENT COOPERATION TREATY

WO 2007/112518
PCT/BE2006/000020

From the INTERNATIONAL BUREAU

SECOND AND SUPPLEMENTARY NOTICE
INFORMING THE APPLICANT OF THE
COMMUNICATION OF THE INTERNATIONAL
APPLICATION (TO DESIGNATED OFFICES
WHICH APPLY THE 30 MONTH TIME
LIMIT UNDER ARTICLE 22(1))
(PCT Rule 47.1(c))

To:
VAN MALDEREN, Joëlle pronovem-OFFICE VAN MALDEREN Avenue Josse Goffin 158 1082 Brussels BELGIQUE

Date of mailing (day/month/year) 24 July 2008 (24.07.2008)		IMPORTANT NOTICE
Applicant's or agent's file reference BPFDAH002AWO		
International application No. PCT/BE2006/000020	International filing date (day/month/year) 20 March 2006 (20.03.2006)	Priority date (day/month/year)
Applicant VRIJE UNIVERSITEIT BRUSSEL et al		

1. **ATTENTION:** For any designated Office(s), for which the time limit under Article 22(1), as in force from 1 April 2002 (30 months from the priority date), **does not apply**, please see Form PCT/IB/308(First Notice) issued previously.
2. Notice is hereby given that the following designated Office(s), for which the time limit under Article 22(1), as in force from 1 April 2002, **does apply**, has/have requested that the communication of the international application, as provided for in Article 20, be effected under Rule 93bis.1. The International Bureau has effected that communication on the date indicated below:
11 October 2007 (11.10.2007)

AU, AZ, BY, CN, CO, DZ, EP, HU, KG, KP, KR, MD, MK, MZ, NA, NG, PG, RU, SY, TM, US

In accordance with Rule 47.1(c-bis)(i), those Offices will accept the present notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

3. The following designated Offices, for which the time limit under Article 22(1), as in force from 1 April 2002, **does apply**, have not requested, as at the time of mailing of the present notice, that the communication of the international application be effected under Rule 93bis.1 :

AE, AG, AL, AM, AP, AT, BA, BB, BG, BR, BW, BZ, CA, CR, CU, CZ, DE, DK, DM, EA, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, ID, IL, IN, IS, JP, KE, KM, KN, KZ, LC, LK, LR, LS, LT, LV, LY, MA, MG, MN, MW, MX, NI, NO, NZ, OA, OM, PH, PL, PT, RO, SC, SD, SE, SG, SK, SL, SM, TJ, TN, TR, TT, UA, UZ, VC, VN, YU, ZA, ZM, ZW

In accordance with Rule 47.1(c-bis)(ii), those Offices accept the present notice as conclusive evidence that the Contracting State for which that Office acts as a designated Office does not require the furnishing, under Article 22, by the applicant of a copy of the international application.

4. **TIME LIMITS** for entry into the national phase

For the designated or elected Office(s) listed above, the applicable time limit for entering the national phase will, **subject to what is said in the following paragraph**, be **30 MONTHS** from the priority date.

In practice, time limits other than the 30-month time limit will continue to apply, for various periods of time, in respect of certain of the designated or elected Office(s) listed above. For regular updates on the applicable time limits (30 or 31 months, or other time limit), Office by Office, refer to the *PCT Gazette*, the *PCT Newsletter* and the *PCT Applicant's Guide*, Volume II, National Chapters, all available from WIPO's Internet site, at <http://www.wipo.int/pct/en/index.html>.

It is the applicant's sole responsibility to monitor all these time limits.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Nora Lindner
Facsimile No. +41 22 338 82 70	e-mail: pt11.pct@wipo.int

171

DUPLICATA

BPFDAH002AWO

1/5

PCT REQUEST

Original (for SUBMISSION)

0	For receiving Office use only	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	
0-4	Form PCT/RO/101 PCT Request	
0-4-1	Prepared Using	PCT-SAFE [EASY mode] Version 3.51.002.177 MT/FOP 20060101/0.20.4rc.2.7
0-5	Petition The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	Receiving Office (specified by the applicant)	Industrial Property Office (Belgium) (RO/BE)
0-7	Applicant's or agent's file reference	BPFDAH002AWO
I	Title of Invention	LIVE ATTENUATED VACCINE
II	Applicant	
II-1	This person is	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	VRIJE UNIVERSITEIT BRUSSEL
II-5	Address	Pleinlaan 2 B-1050 BRUSSELS Belgium
II-6	State of nationality	BE
II-7	State of residence	BE
III-1	Applicant and/or inventor	
III-1-1	This person is	applicant and inventor
III-1-2	Applicant for	US only
III-1-4	Name (LAST, First)	DE GREVE, Henri Marcel Jozef
III-1-5	Address	Jef Lambeauxlaan 11 B-1060 SINT-GILLIS Belgium
III-1-6	State of nationality	BE
III-1-7	State of residence	BE

OFFICE
20.03.2006
ENTREE
INGELIJDEN 24.03.06

PCT REQUEST

Original (for SUBMISSION)

0	For receiving Office use only	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	
0-4	Form PCT/RO/101 PCT Request	
0-4-1	Prepared Using	PCT-SAFE [EASY mode] Version 3.51.002.177 MT/FOP 20060101/0.20.4rc.2.7
0-5	Petition The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	Receiving Office (specified by the applicant)	Industrial Property Office (Belgium) (RO/BE)
0-7	Applicant's or agent's file reference	BPFDAH002AWO
I	Title of Invention	LIVE ATTENUATED VACCINE
II	Applicant	
II-1	This person is	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	VRIJE UNIVERSITEIT BRUSSEL
II-5	Address	Pleinlaan 2 B-1050 BRUSSELS Belgium
II-6	State of nationality	BE
II-7	State of residence	BE
III-1	Applicant and/or inventor	
III-1-1	This person is	applicant and inventor
III-1-2	Applicant for	US only
III-1-4	Name (LAST, First)	DE GREVE, Henri Marcel Jozef
III-1-5	Address	Jef Lambeauxlaan 11 B-1060 SINT-GILLIS Belgium
III-1-6	State of nationality	BE
III-1-7	State of residence	BE

136

BPFDAH002AWO

2/5

PCT REQUEST

Original (for SUBMISSION)

III-2	Applicant and/or inventor	
III-2-1	This person is	applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	ADRIAENSEN, Connie Theresia
III-2-5	Address	Leiekaaai 33 B-9000 GHENT Belgium
III-2-6	State of nationality	BE
III-2-7	State of residence	BE
III-3	Applicant and/or inventor	
III-3-1	This person is	applicant and inventor
III-3-2	Applicant for	US only
III-3-4	Name (LAST, First)	HERNALSTEENS, Jean-Pierre Ernest Clément
III-3-5	Address	François Desmedtstraat 156 B-1150 SINT-PIETERS WOLUWE Belgium
III-3-6	State of nationality	BE
III-3-7	State of residence	BE
IV-1	Agent or common representative; or address for correspondence The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name (LAST, First)	VAN MALDEREN, Joëlle
IV-1-2	Address	pronovem-OFFICE VAN MALDEREN Avenue Josse Goffin 158 1082 Brussels Belgium
IV-1-3	Telephone No.	+32 2 426 38 10
IV-1-4	Facsimile No.	+32 2 426 37 60
IV-1-5	e-mail	vanmalderen@pronovem.com
IV-2	Additional agent(s)	additional agent(s) with same address as first named agent
IV-2-1	Name(s)	VAN MALDEREN, Michel; VAN MALDEREN, Eric; VANDERSTEEN, Pieter; VAN GYSEL, Stefan; HECK, Dieter; VENITE-AUORE, Olessia; SCHOOF, Hilde

PCT REQUEST

Original (for SUBMISSION)

V	DESIGNATIONS		
V-1	The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.		
VI-1	Priority Claim	NONE	
VII-1	International Searching Authority Chosen	European Patent Office (EPO) (ISA/EP)	
VIII	Declarations	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	-	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-	
IX	Check list	number of sheets	electronic file(s) attached
IX-1	Request (including declaration sheets)	5	✓
IX-2	Description	52	-
IX-3	Claims	4	-
IX-4	Abstract	1	✓
IX-5	Drawings	21	-
IX-7	TOTAL	83	

138

BPFDAH002AWO

4/5

PCT REQUEST

Original (for SUBMISSION)

	Accompanying Items	paper document(s) attached	electronic file(s) attached
IX-8	Fee calculation sheet	✓	-
IX-15	Separate indications concerning deposited microorganism or other biological material	✓	-
IX-16(A)	Sequence listing in electronic form		
IX-16(A) - (ii)	additional copies including, where applicable, the copy for the purposes of international search under Rule 13ter	-	3 Diskette
IX-17	PCT-SAFE physical media	-	✓
IX-18	other	Statement according to Rule 27a (2) EPC	
IX-18	other	Statement according to Rule 28 (4) EPC	
IX-18	other	Deposit receipts for LMG P-21642 form BP/4/02-133 & BP/9/02-133	
IX-18	other	Deposit receipts for LMG P-21643 form BP/4/02-134 & BP/9/02-134	
IX-18	other	Deposit receipts for LMG P-21646 form BP/4/02-135 & BP/9/02-135	
IX-18	other	Deposit receipts for LMG P-21641 form BP/4/02-132 & BP/9/02-132	
IX-19	Figure of the drawings which should accompany the abstract	1	
IX-20	Language of filing of the international application	English	
X-1	Signature of applicant, agent or common representative		
X-1-1	Name (LAST, First)	VAN GYSEL, Stefan	
X-1-2	Name of signatory		
X-1-3	Capacity		

PCT REQUEST

Original (for SUBMISSION)

FOR RECEIVING OFFICE USE ONLY

10-1	Date of actual receipt of the purported international application	
10-2	Drawings:	
10-2-1	Received	
10-2-2	Not received	
10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/EP
10-6	Transmittal of search copy delayed until search fee is paid	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	
------	--	--

138

The present invention is related to double and triple attenuated mutant strains of a bacterium infecting veterinary species such as *Salmonella enterica* and/or (a pathogenic) *Escherichia coli*. The mutants of the invention contain at least one first genetic modification and at least one second genetic modification, said first modification in one or more motility genes, and said second modification in one or more genes involved in the survival or the proliferation of the pathogen in the host.

The present invention further relates to live attenuated vaccines based on such mutants for preventing amongst others Salmonellosis and/or an infection by an *E. coli* pathogen in a veterinary species.

Statement according to Rule 28 (4) EPC

The applicant informs the European Patent Office that,

(a) until the publication of the mention of the grant of the European patent or, where applicable,

(b) for twenty years from the date of filing if the application has been refused or withdrawn or deemed to be withdrawn,

the availability referred to in paragraph 3 shall be effected only by the issue of a sample to an expert nominated by the requester.

Date : 20/03/2006

Stefan VAN GYSEL
(Authorized representative)

FDAH02AWO.ST25.txt
SEQUENCE LISTING

<110> Fort Dodge Animal Health
<120> LIVE ATTENUATED SALMONELLA VACCINE
<130> BP.FDAH.002A/EP
<160> 24
<170> PatentIn version 3.3
<210> 1
<211> 24
<212> DNA
<213> Artificial Sequence
<220>
<223> GuaB2 primer
<400> 1
cgttcaggcg caacaggccg ttgt 24

<210> 2
<211> 22
<212> DNA
<213> Artificial Sequence
<220>
<223> GuaB3 primer
<400> 2
ggctgcgatt ggcgaggtag ta 22

<210> 3
<211> 35
<212> DNA
<213> Artificial Sequence
<220>
<223> GuaB4 primer
<400> 3
ggatgatcccg ggcgtcaaac gtcagggctt cttta 35

<210> 4
<211> 35
<212> DNA
<213> Artificial Sequence
<220>
<223> GuaB5 primer
<400> 4
ttgacgcccg ggatcaccaa agagtccccg aacta 35

<210> 5
<211> 20
<212> DNA
<213> Artificial Sequence
<220>
<223> GuaB6 primer

140

FDAH02AWO.ST25.txt

<400> 5
gcaacaactc ctgctggtta 20

<210> 6
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> GuaB7 primer

<400> 6
agaccgagga tcactttatc 20

<210> 7
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> GuaB10 primer

<400> 7
aggaagtttg agaggataa 19

<210> 8
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> P1 primer

<400> 8
gtgtaggctg gagctgcttc 20

<210> 9
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> P2 primer

<400> 9
catatgaata tcctccttag 20

<210> 10
<211> 72
<212> DNA
<213> Artificial Sequence

<220>
<223> FliCP1 primer

<400> 10
atggcacaag tcattaatac aaacagcctg tcgctgggtg acccagaata atgtgtaggc 60
tggagctgct tc 72

<210> 11
<211> 74

<212> DNA
 <213> Artificial Sequence
 <220>
 <223> FliCP2 primer
 <400> 11
 cgcattaacg cagtaaagag aggacgtttt gcggaacctg gttmgcctgc gccacatatg 60
 aatatcctcc ttag 74
 <210> 12
 <211> 26
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> FliC1 primer
 <400> 12
 atggcacaag tcattaatac aaacag 26
 <210> 13
 <211> 25
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> FliC2 primer
 <400> 13
 cgcattaacg cagtaaagag aggac 25
 <210> 14
 <211> 20
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> FliC3 primer
 <400> 14
 tatcggcaat ctggaggcaa 20
 <210> 15
 <211> 72
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> FljBAP1 primer
 <400> 15
 atggcacaag taatcaacac taacagtctg tcgctgctga cccagaataa ctgtgtaggc 60
 tggagctgct tc 72
 <210> 16
 <211> 71
 <212> DNA
 <213> Artificial Sequence
 <220>

641

FDAH02AWO.ST25.txt

<223> FljBAP2 primer

<400> 16
ttattcagcg tagtccgaag acgtgacct gctcaccag tcaaacataa ccatatgaat 60
atcctcctta g 71

<210> 17
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> FljBA5 primer

<400> 17
cagcgtagtc cgaagacgtg atc 23

<210> 18
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> FLjBA6 primer

<400> 18
acactaacag tctgtcgctg ct 22

<210> 19
<211> 2903
<212> DNA
<213> Salmonella Enteritidis

<220>
<221> misc_feature
<223> Contig 1294 of the S. Enteritidis genome

<220>
<221> misc_feature
<222> (1581)..(1583)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (1657)..(1657)
<223> n is a, c, g, or t

<400> 19
ctgatcgaac aagccttcgg cctgcagttt ggcttttagc tgctcatatt tctgctgcaa 60
caagccttcg cccgcgggct gcatactttc ggcgatgatt tgataatcgc cgcgcggctc 120
gtacagcgta atgttggcgc gtaccagcac ctgctgcccg tgctgcgggc ggaacgtcac 180
ccggcgattg ctgttacgga acatcgcaca gcgcacctga gcggtatcgt ctttgagcgt 240
aaagtaccag tggcccgcgc caggctgcgt gaaattagaa atctcgccgc tgatccatac 300
ctgtcccac tctgtttcta acagcagacg aaccgtctgg ttaaggcggc ttacggtaaa 360
aattgaggaa gtttgagagg ataacatgtg agcgggatca aattctaaat cagcaggtta 420
ttcaatcgat agtaacctgc tcacggggga tcgcaagcac tatttgcaaa aaaatgtaga 480

tgcaaccgat	tacgttctgt	ataatgccgc	gggaatattt	attaacctcc	caggcgagat	540
attgcccattg	ctacgtatcg	ctaaagaagc	tctgacgttt	gacgacgtcc	tccttgttcc	600
cgctcactcc	accgttttgc	cgaatactgc	cgatctcagc	acgcagttga	cgaaaactat	660
tcgtctgaat	attcctatgc	tttctgcggc	gatggacacc	gtgacggaag	cgcgctggc	720
aattgccctg	gcccaggaag	gcggcattgg	ttttatccac	aaaaacatgt	ctattgagcg	780
ccaggcgga	gaagtctgcc	gcgtgaagaa	acacgagtcc	ggcgtagtga	ccgacccgca	840
gaccgtcctg	ccaaccacca	cgttgcatga	agtgaagacc	ctgaccgagc	gtaacggttt	900
tgcgggctat	ccggtggtga	ctgaagataa	cgagctggtg	ggtatcatca	ccggtcgtga	960
cgtgcgtttt	gtgactgacc	tgaaccagcc	ggtgagtgtc	tacatgacac	cgaaagagcg	1020
tctggtgacc	gttcgtgaag	gcgaagcccc	tgaagtcgtg	ctggcaaaaa	tgcacgaaaa	1080
acgcgtagaa	aaagcgctgg	tcgttgatga	taacttccat	ctgcttggca	tgattaccgt	1140
aaaagatttc	cagaaagcgg	aacgtaaacc	aaactcctgt	aaagatgagc	agggccgttt	1200
acgtgtcggc	gcggcggtcg	gcgcaggcgc	gggcaacgaa	gagcgcgttg	acgcgctggt	1260
ggcggcaggc	gttgacgtac	tgctgatcga	ctcctctcac	ggtcactctg	aaggcgtggt	1320
gcaacgtatc	cgtgagacgc	gtgctaaata	tcctgacctg	caaatcatcg	gcgggaacgt	1380
tgcgacgggc	gcaggcgctc	gcgcactggc	ggaagccggt	tgcaacgcgg	tgaaagtggg	1440
tatcgccccg	ggttccatct	gtacgactcg	tatcgggact	ggtgtgggcg	ttccgcagat	1500
caccgctggt	tctgacgcgg	tggaagcgct	ggaaggcacc	gggattccgg	ttatcgctga	1560
cggcggtatc	cgtttctccg	nnnacatcgc	caaagccatc	gccgcaggcg	cgagcgcggt	1620
aatggtgggt	tctatgctgg	ccggtaccga	agaatcnccg	ggcgaaatcg	aactctacca	1680
gggccgttct	tacaaatctt	atcgcggtat	gggttctctg	ggcgcgatgt	ccaaagggtc	1740
ctccgaccgt	tacttccaga	gcgacaacgc	cgccgacaaa	ctggtgccgg	aaggatcga	1800
aggccgcgta	gcctataaag	gtcgcctgaa	agagatcatt	caccagcaga	tgggcggcct	1860
gcgctcctgt	atggggctga	ccggttggtc	taccatcgac	gaactgcgta	ctaaagcgga	1920
gtttgtgcgt	atcagcggtg	cgggtatcca	ggaaagccac	gttcacgacg	tgaccatcac	1980
caaagagtcc	ccgaactacc	gtctgggctc	ctgattttct	tcgcccgacc	ttcgcgtcgg	2040
gcgattttatt	taatctgttt	cacttgccctc	ggaataagcg	tcaatgacgg	aaaacattca	2100
taagcatcgc	atcctcattc	tggacttcgg	ttctcagtac	actcaactgg	ttgcgcgccg	2160
cgtgcgtgag	ctgggtgttt	actgtgaact	gtgggcgtgg	gatgtgacag	aagcacaaat	2220
tcgtgacttc	aaccaagcg	gcattattct	ttccggcggc	ccggaaagca	ccaccgaaga	2280
aaacagcccc	cgcgcgccgc	agtatgtctt	tgaagcaggc	gtgccggtat	ttggcgtttg	2340
ctatggtatg	cagaccatgg	cgatgcagct	tggcggtcat	gtagaagggt	ctaattgagcg	2400
tgaatttggt	tatgcgcagg	tcgaagtgtt	gaccgacagc	gcgctgggtc	gcggtattga	2460
agattccctg	accgcagacg	gcaaaccgct	gctggacgtg	tggatgagcc	acggcgataa	2520

h4C

FDAH02AWO.ST25.txt

agtgacggcg attccgtccg acttcgtgac cgtcgccagc accgagagct gcccgttcgc 2580
cattatggcc aacgaagaaa aacgcttcta cggcgtagagc ttccacccgg aagtgactca 2640
caccgcgagc ggtatgcgca tgctggagcg ttttgtacgc gatattctgcc agtgtgaagc 2700
gttgtggacg ccggcggaaga tcatcgatga cgccgtggcg cgcattcgcg agcaggtagg 2760
cgacgataaa gtgatcctcg gtctctccgg cggcgtaggac tcttcctgca ccgccatgct 2820
gctgcaccgc gcgatcggtg aaaatctgac ctgtgttttc gtcgacaacg gcctgttgcg 2880
cctgaacgaa gccgagcagg tga 2903

<210> 20
<211> 1995
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequence of the delta-guaB fragment of S. Enteritidis cloned in pUC18

<220>
<221> misc_feature
<222> (55)..(55)
<223> n is a, c, g, or t

<400> 20
ggctgcgatt ggcgaggtag tagtccatcg ccatgccagc acgctgctgg cgganctgga 60
tctgacgcaa caactcctgc tggttacggc tgacaatttc tgccgctgct gatggcgtag 120
cgcgcgagcgc tcggcgacaa aatcagctat cgtgacgtcc gtttcgtgac cgacggcgct 180
gaccaccgga atgcggctgg caaaaatcgc ccgcgccacg cgttcgtcgt taaaactcca 240
caaatcttcc agcgaaccgc cgccgcgccc gacgatcagt acatcacatt cgccgcgcgc 300
gttcgccagt tcgatagcac gaacgatctg ccccggcgcg tcgtcgccct ggactgcggt 360
tggatagata ataacgggca gggatgggtc acgacgcttt agcacgtgga gaatatcgctg 420
cagcgcgcgc ccggttttcg aagtgatcac cccaacgcag tgggccgggg agggcaacgc 480
ctgtttatgc tgctgatcga acaagccttc ggctgcgagt ttggctttta gctgctcata 540
tttctgctgc aacaagcctt cgccgcgagg ctgcatactt tcggcgatga tttgataatc 600
gccgcgcggc tcgtacagcg taatgttggc gcgtaccagc acctgctgcc cgtgctgcgc 660
gcggaacgtc acccggcgat tgctgttacg gaacatcgca cagcgcacct gagcggatc 720
gtctttgagc gtaaagtacc agtggcccgga cgcaggctgc gtgaaattag aaatctcgcc 780
gctgatccat acctgtccca tctcctgttc taacagcaga cgaaccgtct ggttaaggcg 840
gcttacggta aaaattgagg aagtttgaga ggataacatg tgagcgggat caaattctaa 900
atcagcaggt tattcaatcg atagtaacct gctcacgggg gatcgcaagc actatttgca 960
aaaaaatgta gatgcaaccg attacgttct gtataatgcc gcggcaatat ttattaacct 1020
cccaggtag atattgccca tgctacgtat cgctaaagaa gctctgacgt ttgacgcccg 1080

ggatcaccaa agagtccccg aactaccgtc tgggctcctg attttcttcg cccgaccttc	1140
gcgtcgggcg atttatttaa tctgtttcac ttgcctcgga ataagcgtca atgacggaaa	1200
acattcataa gcatcgcac ctcattctgg acttcgggtc tcagtacact caactgggtg	1260
cgcgccgcgt gcgtgagctg ggtgtttact gtgaactgtg ggcgtgggat gtgacagaag	1320
cacaaattcg tgacttcaac ccaagcggca ttattctttc cggcggccccg gaaagcacca	1380
ccgaagaaaa cagcccgcgc gcgcccgcagt atgtctttga agcaggcgtg ccggtatttg	1440
gcgtttgcta tggatatgag accatggcga tgcagcttg cggtcagtga gaaggttcta	1500
atgagcgtga atttggttat gcgcaggctc aagtgttgac cgacagcgcg ctggttcgcg	1560
gtattgaaga ttccctgacc gcagacggca aaccgctgct ggacgtgtgg atgagccacg	1620
gcgataaagt gacggcgatt ccgtccgact tcgtgaccgt cgccagcacc gagagctgcc	1680
cgttcgccat tatggccaac gaagaaaaac gcttctacgg cgtacagttc cccccggaag	1740
tgactcacac ccgccagggt atgcgcatgc tggagcggtt tgtacgcgat atctgccagt	1800
gtgaagcggt gtggacgccg gcgaagatca tcgatgacgc cgtggcgcgc attcgcgagc	1860
aggtaggcga cgataaagtg atcctcggtc tctccggcgg cgtggattct tccgtcaccg	1920
ccatgctgct gcaccgcgcg atcggtaaaa atctgacctg tgttttcgtc gacaacggcc	1980
tgttgcgcct gaacg	1995

<210> 21
 <211> 349
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Nucleotide sequence of the S. Enteritidis PCR fragment, which includes the guaB deletion, obtained using primer GuaB10

<400> 21	
catgtgtgag cgggatcaaa ttctaaatca gcaggttatt caatcgatag taacctgctc	60
acgggggatc gcaagcacta ttgcaaaaa aaatgtagat gcaaccgatt acgttctgta	120
taatgcccgg caatatttat taacctccca ggtgagatat tcgccatgta cgtatcgcta	180
aagaagccct gacgtttgac gcccggtgag gctggagctg cttcgaagtt cctatacttt	240
ctagagaata ggaactccgg aataggaact aaggaggata ttcatatggg atcaccaaag	300
atccccgaac taccgctctg gctctgattt tcttcgcccc accttcgcg	349

<210> 22
 <211> 1553
 <212> DNA
 <213> Salmonella Typhimurium

<220>
 <221> misc_feature
 <223> guaB gene of S. Typhimurium LT2, section 117 of 220 of the complete genome

<400> 22

167

FDAH02AWO.ST25.txt

atttgcaaaa aaatgtagat gcaaccgatt acgttctgta taatgccgcg gcaatattta 60
ttaacctccc aggtgagata ttgcccattg tacgtatcgc taaagaagcc ctgacgtttg 120
acgacgtcct ccttgttccc gctcactcca ccgttttgcc gaatactgcc gatctcagca 180
cgcagttgac gaaaactatt cgtctgaata ttcctatgct ttctgcggcg atggacaccg 240
tgacggaagc gcgcctggca attgccctgg cccaggaagg cggcattggt tttatccaca 300
aaaacatgtc cattgagcgc caggcggaag aagttcgccg cgtgaagaaa cagagtcgcg 360
gcgtagtgac cgacccgcag accgtcctgc caaccaccac gttgcatgaa gtgaaagccc 420
tgaccgagcg taacggtttt gcgggctatc cggtggtgac tgaagataac gagctggtgg 480
gtatcatcac cggtcgtgac gtgcgttttg tgactgacct gaaccagccg gttagcgttt 540
acatgacgcc gaaagagcgt ctggtgaccg ttcgtgaagg cgaagcccgt gaagtcgtgc 600
tggtcaaaaat gcacgaaaaa cgcgtagaaa aagcgtggt cgttgatgat aacttccatc 660
tgcttggcat gattaccgta aaagatttcc agaaagcgga acgtaaacca aactcctgca 720
aagatgagca gggccgttta cgtgtcggcg cggcggtcgg cgaggcgcg ggcaacgaag 780
agcgcgttga cgcgctggtg gcggcaggcg ttgacgtact gctgatcgac tcctctcacg 840
gtcattcaga aggcgtggtg caacgtatcc gtgaaacccg tgctaaatat cctgacctgc 900
aaatcatcgg cggcaacgtc gcgacaggcg caggcgctcg cgactggcg gaagccggtt 960
gcagcgcggt gaaagtcggt attggcccgg gttccatctg taccactcgt atcgtgactg 1020
gcgtgggcgt tccgcagatc accgctgttt ctgacgcagt tgaagcgtg gaaggtaccg 1080
gtattccggt tatcgctgac ggcggtatcc gtttctccgg cgacatagcc aaagcgattg 1140
ccgcaggtgc aagcgcggta atggtgggtt ccatgctggc gggtacggaa gaatccccgg 1200
gcgaaatcga actctaccag ggccgttctt acaaacttta ccgcggcatg ggctcgctgg 1260
gtgcgatgtc caaaggttcc tccgaccgtt acttccagag cgacaacgcc gctgacaaac 1320
tggtgccgga aggtatcgaa ggtcgcgtag cctataaagg tcgcctgaaa gagatcattc 1380
accagcagat gggcggcctg cgctcctgta tggggctgac cggttgtgct accatcgacg 1440
aactgcgtac taaagcggag tttgtgcgta tcagcgggtc gggcattcag gaaagccacg 1500
ttcacgacgt gaccatcacc aaagagtccc cgaactaccg tctgggctcc tga 1553

<210> 23
<211> 193
<212> DNA
<213> Artificial Sequence

<220>
<223> Nucleotide sequence obtained after sequencing the PCR fragment amplified with primers Flic1-Flic2 on total DNA of the mutants SM73 and SM89, using primers Flic1 and Flic2

<400> 23
ctatggcaca agtcattaat acaaacagcc tgtcgctggt tgacccagaa taatgtgtag 60
gctggagctg cttcgaagtt cctatacttt ctagagaata ggaacttcgg aataggaact 120

aaggaggata ttcatatgtg gcgcaggcga accaggttcc gcaaaacgtc ctctctttac 180
tggcgттаат gcg 193

<210> 24
<211> 140
<212> DNA
<213> Artificial Sequence

<220>
<223> Nucleotide sequence obtained after sequencing the PCR fragment
amplified with primers FljBA6-FljBA5 on total DNA of the S.
Typhimurium mutant SM48, using primer FljBA6

<400> 24
agaataactg tgtaggctgg agctgcttcg aagttcctat actttctaga gaataggaac 60
ttcggaaatag gaactaagga ggatattcat atggttatgt ttgactgggt gagcaggatc 120
acgtcttcgg actacgctgt 140

144

KOPIE

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the
International Depositary Authority BCCM/LMG identified at the bottom of next page

International Form BCCM/LMG/BP/4/02-132.....

To: Name of the depositor : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Identification of the microorganism:

I.1 Identification reference given by the depositor:

SM69

I.2 Accession number given by the International Depositary Authority:

LMG P-21641

II. Scientific description and/or proposed taxonomic designation

The microorganism identified under I above was accompanied by :

(mark with a cross the applicable box(es)):

- ☒ a scientific description
☒ a proposed taxonomic designation

III. Receipt and acceptance

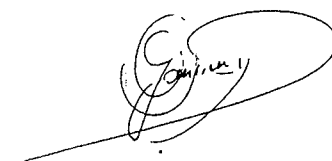
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

August 9, 2002

IV. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):



Dr. D. Janssens, curator IDA

Date :

September 5, 2002

141

KOPIE

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Viability statement Issued pursuant to Rule 10.2 by the International Depositary
Authority BCCM/LMG identified on the following page

International Form BCCM/LMG/BP/9/.....02-132

To: Party to whom the viability statement is issued:

Name : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Depositor:

I.1 Name : Prof. J.-P. Hernalsteens

I.2 Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

II. Identification of the microorganism

II.1 Accession number given by the International Depositary Authority:

LMG P-21641

II.2 Date of the original deposit (or where a new deposit or a transfer has been made, the most recent relevant date) :

August 9, 2002

III. Viability statement.

The viability of the microorganism identified under II above was tested on

: August 9, 2002

(Give date. In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test).

On that date, the said microorganism was: (mark the applicable box with a cross)

☒ viable

☐ no longer viable

KOPIE

IV. Conditions under which the viability test has been performed:

(Fill in if the information has been requested and if the results of the test were negative).

V. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority
of authorized official(s) :



Dr. D. Janssens, curator IDA

Date : September 5, 2002

146

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION

Page 1 of Form BCCM/LMG/BP/4/...02-133. Receipt in the case of an original deposit

KOPIE

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the
International Depositary Authority BCCM/LMG identified at the bottom of next page

International Form BCCM/LMG/BP/4/...02-133...

To: Name of the depositor : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Identification of the microorganism:

I.1 Identification reference given by the depositor:

SM73

I.2 Accession number given by the International Depositary Authority:

LMG P-21642

KOPIE

II. Scientific description and/or proposed taxonomic designation

The microorganism identified under I above was accompanied by :

(mark with a cross the applicable box(es)):

- ☒ a scientific description
☒ a proposed taxonomic designation

III. Receipt and acceptance

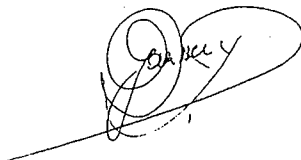
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

August 9, 2002

IV. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):



Dr. D. Janssens, curator IDA

Date : September 5, 2002

147

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION

Page 1 of Form BCCM/LMG/BP/9/.....02-133 Viability statement

KOP/10

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Viability statement issued pursuant to Rule 10.2 by the International Depositary
Authority BCCM/LMG identified on the following page

International Form BCCM/LMG/BP/9/.....02-133

To: Party to whom the viability statement is issued:

Name : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Depositor:

I.1 Name : Prof. J.-P. Hernalsteens

I.2 Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

II. Identification of the microorganism

II.1 Accession number given by the International Depositary Authority:

LMG P-21642

II.2 Date of the original deposit (or where a new deposit or a transfer has been made, the most recent relevant date) :

August 9, 2002

III. Viability statement.

The viability of the microorganism identified under II above was tested on

: August 9, 2002

(Give date. In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test).

On that date, the said microorganism was: (mark the applicable box with a cross)

☒ viable

☐ no longer viable

KOPIE

IV. Conditions under which the viability test has been performed:

(Fill in if the information has been requested and if the results of the test were negative).

V. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) :



Dr. D. Janssens, curator IDA

Date

: September 5, 2002

148

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION

Page 1 of Form BCCM/LMG/BP/4/02-135
Receipt in the case of an original deposit

K O P I E

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the
International Depositary Authority BCCM/LMG Identified at the bottom of next page

International Form BCCM/LMG/BP/4/02-135...

To: Name of the depositor : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Identification of the microorganism:

I.1 Identification reference given by the depositor:

SM86

I.2 Accession number given by the International Depositary Authority:

LMG P-21646

KOPIE

II. Scientific description and/or proposed taxonomic designation

The microorganism identified under I above was accompanied by :

(mark with a cross the applicable box(es)):

- ☒ a scientific description
☒ a proposed taxonomic designation

III. Receipt and acceptance

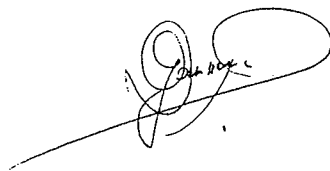
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

August 28, 2002

IV. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):



Dr. D. Janssens, curator IDA

Date : September 5, 2002

KOPIE

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Viability statement Issued pursuant to Rule 10.2 by the International Depositary
Authority BCCM/LMG Identified on the following page

International Form BCCM/LMG/BP/9/.....02-135

To: Party to whom the viability statement is issued:

Name : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Depositor:

I.1 Name : Prof. J.-P. Hernalsteens

I.2 Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

II. Identification of the microorganism

II.1 Accession number given by the International Depositary Authority:

LMG P-21646

II.2 Date of the original deposit (or where a new deposit or a transfer has been made, the most recent relevant date) :

August 28, 2002

III. Viability statement.

The viability of the microorganism identified under II above was tested on

: August 28, 2002

(Give date. In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test).

On that date, the said microorganism was: (mark the applicable box with a cross)

☒ viable

☐ no longer viable

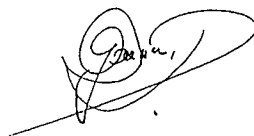
IV. Conditions under which the viability test has been performed:

(Fill in if the information has been requested and if the results of the test were negative).

V. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or
of authorized official(s) :



Dr. D. Janssens, curator IDA

Date

: September 5, 2002

150

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION

Page 1 of Form BCCM/LMG/BP/4/02-134 Receipt in the case of an original deposit

KOPIE

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the
International Depositary Authority BCCM/LMG identified at the bottom of next page

International Form BCCM/LMG/BP/4/02-134

To: Name of the depositor : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Identification of the microorganism:

I.1 Identification reference given by the depositor:

SM89

I.2 Accession number given by the International Depositary Authority:

LMG P-21643

II. Scientific description and/or proposed taxonomic designation

The microorganism identified under I above was accompanied by :

(mark with a cross the applicable box(es)):

- ☒ a scientific description
☒ a proposed taxonomic designation

III. Receipt and acceptance

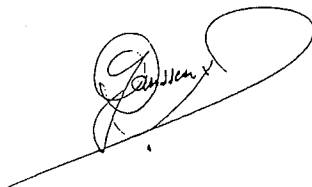
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

August 9, 2002

IV. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):



Dr. D. Janssens, curator IDA

Date : September 5, 2002

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure

Viability statement Issued pursuant to Rule 10.2 by the International Depositary
Authority BCCM/LMG Identified on the following page

International Form BCCM/LMG/BP/9/...02-134

To: Party to whom the viability statement is issued:

Name : Prof. J.-P. Hernalsteens

Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Depositor:

I.1 Name : Prof. J.-P. Hernalsteens

I.2 Address : Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B-1640 Sint-Genesius-Rode

II. Identification of the microorganism

II.1 Accession number given by the International Depositary Authority:

LMG P-21643

II.2 Date of the original deposit (or where a new deposit or a transfer has been made, the most recent relevant date) :

August 9, 2002

III. Viability statement.

The viability of the microorganism identified under II above was tested on

: August 9, 2002

(Give date. In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test).

On that date, the said microorganism was: (mark the applicable box with a cross)

☒ viable

☐ no longer viable

KOPIE

IV. Conditions under which the viability test has been performed:

(Fill in if the information has been requested and if the results of the test were negative).

V. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) :



Dr. D. Janssens, curator IDA

Date : September 5, 2002

152 152

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 08-099559- -00000-0000

Fecha: 2008-09-19 16:24:47
Tra. 11 PATENTEIYII
Act. 411 PRESENTACION

Dep. 2020 NUECREACIONE
Eve: 378 FASENACIONALI
Folios: 151



a y Comercio
TENDENCIA

REQUISITOS MINIMOS PARA ADMISION A TRAMITE PCT

PATENTE DE INVENCION ☒

MODELO DE UTILIDAD ☐

- ◆ Indicación de que se solicita la concesión de una patente. ☒
- ◆ Datos de identificación del solicitante o de la persona que presenta la solicitud. ☒
- ◆ Descripción de la invención. ☒
- ◆ Dibujos de ser estos pertinentes. ☒
- ◆ Comprobante de pago de las tasas establecidas. ☒

COMPLETA ☒

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 trazado. ☐
- ◆ Comprobante de pago de las tasas establecidas. ☐

COMPLETA ☐

INCOMPLETA ☐

Sandra Rodríguez
Firma Responsable

Fecha Sep 19 08

Carrera 13, No. 27-00 Piso 5, 7 y 10
Corrutadori. 3 82 08 40
Fax: 350 52 20
E-mail: info@sic.gov.co
www.sic.gov.co
Bogotá, D.C., Colombia