

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

2020
Bogotá, D.C.,

Abogada
LUZ CLEMENCIA DE PÁEZ

ASUNTO	Radicación	08 106 316
	Trámite	011
	Evento	378
	Actuación	519
	Oficio	1 06 9 4
	Folios	013

Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (Fase Nacional)	☒	Capítulo I	☒ Capítulo II ☐
No. Solicitud Internacional	PCT/IB2007/00100 3		
Fecha de Presentación Internacional	06/Abril/2007		

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 21 a 31	06/Octubre/2008
Reivindicaciones:	Folios 32 a 33	06/Octubre/2008
Prioridad:	IT MI2006A000678	06/Abril/2006

2. Análisis de la prioridad

Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Compuesto peptídico de actividad biológica, su preparación y sus aplicaciones".

Expediente 08 106 316 1er Art 45

El problema planteado en esta solicitud consiste en la necesidad de suministrar nuevas moléculas que tengan propiedades antibióticas.

Para resolver este problema técnico la solicitud en estudio proporciona un compuesto aislado de un cultivo de *Bacillus clausii* caracterizado porque comprende la secuencia de aminoácidos:

El objeto de la presente solicitud se relaciona con la clasificación internacional de patentes (CIP): C07K14/45.

4. No es una invención (Art 15 D 486 literal (b))

El objeto de las reivindicaciones 1 – 6 no se considera una invención, de acuerdo con el Art citado.

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 10 – 12 no es patentable, de acuerdo con el Art 14.

6. De la Solicitud de Patente

Título

El título no corresponde con el objeto de la solicitud porque no permite establecer claramente que la solicitud se refiere a un compuesto peptídico con actividad antimicrobiana aislado de un cultivo de *Bacillus clausii* y su proceso de obtención.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicación 1 reclama un compuesto peptídico por su método de obtención y no por su estructura o secuencia de aminoácidos, lo cual no es aceptable ya que el producto se puede definir por sus características estructurales. Sin embargo se recuerda al solicitante que el producto de las reivindicaciones 1-6 no es patentable puesto que toda materia viva o parte de cualquier ser vivo y las sustancias existentes en la naturaleza no son invenciones. Así, el material biológico por el hecho de haber sido aislado no se considera invención.

El procedimiento de la reivindicación 7 no es conciso porque no establece claramente la forma mediante la cual se llevan a cabo cada una de las etapas reclamadas, por ejemplo no se establece cómo se lleva a cabo la extracción en fase sólida, ni cual es la fracción obtenida que se purifica posteriormente por HPLC. Se sugiere modificar la reivindicación de tal manera que se limite a lo soportado en la descripción y sea posible establecer claramente el alcance de la misma.

7. Determinación del estado de la técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Abril 6 de 2006 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	Journal of Clinical Gastroenterology: July 2004 - Volume 38 - Issue - pp S86-S90 Probiotics, Prebiotics, and New Foods	Bacillus clausii Probiotic Strains: Antimicrobial and Immunomodulatory Activities	Julio/2004
D2	Antimicrob. Agents Chemother. 2001, 45(11): 3156.	In vitro anti-Helicobacter pylori activity of the probiotic strain Bacillus subtilis 3 is due to secretion of antibiotics.	Noviembre/2001

D1 divulga (Folios 90-94) divulga que todas las cepas probadas de B. clausii exhibieron actividad anti-estafilocócica pero no anti-salmonella, y que el compuesto antimicrobiano presente el sobrenadante libre de células de B. clausii O/C demostraron una actividad relativamente restringida, ya que dentro de las bacterias probadas solo se inhibieron las bacterias Gram-positivas incluyendo C. difficile. Enseña que el máximo nivel de producción del antimicrobiano por parte de B. clausii se alcanzó cuando la proporción de esporulación fue 60% (Pág. S88 columna de la izquierda tercer párrafo). El documento D1 adicionalmente revela que se determinó que B. clausii O/C produce al menos una sustancia antimicrobiana la cual secreta al medio y que muestra actividad contra bacterias Gram-positivas. Finalmente, el documento enseña que el B. clausii se cultiva en un medio Mueller Hinton o en un agar MH por 24 a 72 horas a 30°C y que luego se obtiene un sobrenadante que presenta actividad antimicrobiana (Pág. S87 dos columnas y Pág. S88 columna de la izquierda tercera columna y figura 1).

D2 <http://aac.asm.org/content/45/11/3156.full.pdf+html> divulga un método para aislar y purificar un compuesto antimicrobiano secretado por Bacillus subtilis (Pág. 3157).

8. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 9 consiste en: "Composición farmacéutica, caracterizada porque comprende un compuesto según una cualquiera de las reivindicaciones 1 a 6, y al menos un excipiente farmacéuticamente aceptable".

Comparación de las características técnicas **esenciales**, con las del estado de la técnica:

Características esenciales	D1
Composición farmacéutica que comprende:	Composición farmacéutica que comprende:
Un compuesto según una	Un compuesto antimicrobiano

cualquiera de las reivindicaciones 1 a 6	producido por B. clausii.
Al menos un excipiente farmacéuticamente aceptable	Al menos un excipiente farmacéuticamente aceptable

Las características **esenciales** de la composición de la reivindicación 8 habían sido divulgadas previamente en D1, el cual enseña un sobrenadante que contiene medio y el compuesto antimicrobiano secretado por B. clausii (Pág S87 columna de la izquierda última columna, columna de la derecha primera columna; pág. S88 columna de la izquierda tercer y cuarto párrafo).

En consecuencia, la reivindicación 9 no es nueva, según lo divulgado en el documento D1.

Nivel Inventivo (Art18 D486)

La reivindicación 8 consiste en: "Medicamento caracterizado porque comprende un compuesto según una cualquiera de las reivindicaciones 1 a 6".

El documento D1 divulga un sobrenadante que contiene el compuesto antimicrobiano que es secretado por B. clausii y adicionalmente enseña que la sustancia producida por B. clausii O/C es activa contra las cepas de C. difficile probadas. Por tal razón una persona de nivel medio versada en la materia en vista de lo revelado en el documento D1 se vería motivada a incorporar el compuesto antimicrobiano en un medicamento para el tratamiento de diarrea asociada a C. difficile tal y como es revelado en la anterioridad.

En consecuencia, el objeto de la reivindicación 8 se deriva de lo revelado previamente en el estado de la técnica y no contiene nivel inventivo.

Conclusión:

La reivindicación 8 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

La reivindicación 7 consiste en: "Procedimiento para la preparación de un compuesto según una cualquiera de las reivindicaciones 1 a 6, que comprende las fases de

- cultivo de las cepas de Bacillus clausii en un medio apropiado hasta la esporulación de las cepas;
- recogida del sobrenadante del cultivo tras la centrifugación y filtración;
- extracción en fase sólida del cultivo tras centrifugación y filtración;
- si es necesario, purificación de la fracción obtenida por cromatografía líquida de alta resolución (HPLC)".

El proceso reclamado no está conformado por pasos sorprendentes para la persona del oficio medianamente versada en la materia según lo establecido en los documentos D1 y D2 (Documento D1 pág. S87 columna de la izquierda último párrafo y columna de la derecha primer párrafo y pág. S88 tercer párrafo; Documento D2 pág. 3157 columna de la izquierda "isolation and purification") porque

este hubiera inferido sin mucho esfuerzo de su parte, la obtención del sobrenadante y posterior extracción en fase sólida y purificación del compuesto a partir de los conocimientos que en conjunto revelan los documentos D1 y D2.

De manera que el procedimiento divulgado en la actual solicitud es una alternativa a los procedimientos ya conocidos porque este sigue siendo útil para extraer compuestos secretados por microorganismos.

Conclusión:

La reivindicación 7 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

9. Conclusión

Los requisitos de patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Journal of Clinical Gastroenterology: July 2004 - Volume 38 - Issue - pp S86-S90 Probiotics, Prebiotics, and New Foods	9	Novedad
		7 y 8	Nivel inventivo
D2	Antimicrob. Agents Chemother. 2001, 45(11): 3156.	7	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante para dar respuesta y presentarla por escrito.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JOSÉ LUIS SALAZAR LÓPEZ
 Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 20 JUN 2012

Realizado por: Juanita Catalina Rico. 
 Revisado por: Consuelo Leguizamón L. 

Expediente 08 106 316 1er Art 45

Bacillus clausii Probiotic Strains

Antimicrobial and Immunomodulatory Activities

Maria C. Urdaci, PhD, Philippe Bressollier, PhD, and Irina Pinchuk, PhD

Abstract: The clinical benefits observed with probiotic use are mainly attributed to the antimicrobial substances produced by probiotic strains and to their immunomodulatory effects. Currently, the best-documented probiotic bacteria used in human therapy are lactic acid bacteria. In contrast, studies aiming to characterize the mechanisms responsible for the probiotic beneficial effects of *Bacillus* are rare. The current work seeks to contribute to such characterization by evaluating the antimicrobial and immunomodulatory activities of probiotic *B. clausii* strains. *B. clausii* strains release antimicrobial substances in the medium. Moreover, the release of these antimicrobial substances was observed during stationary growth phase and coincided with sporulation. These substances were active against Gram-positive bacteria, in particular against *Staphylococcus aureus*, *Enterococcus faecium*, and *Clostridium difficile*. The antimicrobial activity was resistant to subtilisin, proteinase K, and chymotrypsin treatment, whereas it was sensitive to pronase treatment. The evaluation of the immunomodulatory properties of probiotic *B. clausii* strains was performed in vitro on Swiss and C57 BL/6j murine cells. The authors demonstrate that these strains, in their vegetative forms, are able to induce NOS II synthetase activity, IFN- γ production, and CD4⁺ T-cell proliferation.

Key Words: *Bacillus clausii*, antimicrobial substance, immunostimulation

(*J Clin Gastroenterol* 2004;38:S86–S90)

The use of probiotics to enhance human health has been proposed for many years.¹ Recently, the term *probiotics* was redefined as “live microorganisms administered in an adequate amount which confer a health benefit on the host.”² A significant number of studies have demonstrated the therapeutic efficacy of probiotics when applied to the treatment of several gastrointestinal microbial disorders, including acute diarrhea, antibiotic-associated diarrhea, and traveler’s diarrhea.^{3–5} Be-

side their therapeutic use as pharmaceuticals, probiotics have been extensively included during the past 2 decades in various types of food products (in particular, those based on fermented milk).

So far, the best-documented probiotics are lactic acid bacteria. In contrast, the mechanisms responsible for the beneficial effects of other probiotics, especially the *Bacillus* species, have remained relatively unexplored until fairly recently. *Bacillus* strains for animal nutritional use and for human use (pharmaceutical preparations) include *B. subtilis*, *B. licheniformis*, *B. coagulans*, *B. toyoi* (cereus), *B. natto* (subtilis), *B. clausii*, *B. polyfermentans*, and *B. cereus*.^{6–9}

Many aspects, including safety and functional characteristics, need to be considered during the selection of microorganisms for probiotic design. Functional characteristics include viability and persistence in the gastrointestinal tract, antagonistic properties against bacterial infection, and immunomodulation. The antimicrobial activity of probiotic bacteria was suggested to be one of the probiotic pathogen-inhibiting mechanisms. Moreover, several authors have previously reported that antimicrobial activity of probiotic bacteria in vitro and in vivo is mainly attributed to their capacity to produce antimicrobial substances. Recent reports suggest that the antimicrobial activity of *Lactobacillus* probiotics involves the production of secreted compounds, such as organic acid, hydrogen peroxide, as well as various antibiotics and/or bacteriocins.^{10–13} Our recent study demonstrated that the antibacterial activity of one *B. subtilis* probiotic strain (Biosporin) is the result of the production of 2 distinct antibiotics.⁸

In addition, several studies have demonstrated that orally administered probiotic bacteria can stimulate the cellular and humoral immune systems, resulting in health improvement during intestinal infections.^{14,15} *Bacillus* probiotics have been reported to induce cellular and humoral immune responses in vivo.^{16–18} Recent studies have shown that orally ingested *B. subtilis* spores are immunogenic, can disseminate to the Payer’s patches and mesenteric lymph nodes, and probably affect cytokine production.¹⁹ Moreover, given their immunomodulatory effects, these bacteria were proposed for use as an oral vaccine delivery vehicle.^{19,20} Despite the considerable recent advances in the characterization of *Bacillus* probiotics, the molecular and cellular mechanisms by which

Received for publication January 13, 2004; accepted February 12, 2004.

From Laboratoire de Microbiologie, ENITA-University of Bordeaux, Gradignan, France

Reprints: Maria C. Urdaci, PhD, Laboratoire de Microbiologie, ENITA-University of Bordeaux, 1, Cours du Général de Gaulle, 33175 Gradignan, France (e-mail: m-urdaci@enitab.fr)

Copyright © 2004 by Lippincott Williams & Wilkins

these bacteria exert their immune modulatory action are still unknown.

The *Bacillus* probiotic Enterogermina, which includes 4 strains of *Bacillus*, recently reclassified from *subtilis* to *clausii* and shown to display a low level of intraspecific diversity,²¹ has been reported to exert beneficial clinical effects, notably in the treatment of diarrhea and in the prevention of infectious diseases.^{3,16} However, the precise mechanisms responsible for these effects remain unclear. The current study was designed to analyze the antimicrobial activity of the *B. clausii* strains and to explore their immunomodulatory action through their ability to activate murine antigen presenting cells and T-cell subsets in vitro.

MATERIALS AND METHODS

Bacterial Strains and Culture Media

The 4 *B. clausii* probiotic strains O/C, N/R, SIN, and T (Enterogermina); *B. subtilis* 3 (Biosporine), *B. licheniformis* 31 (Biosporine), *B. cereus* IP 5832 (Bactisubtil), *B. cereus* NT (Biosubtyl), and *B. cereus* DM-423 (Cereobiogen); and the reference strain *B. subtilis* 168 were used in the current study. The strains used as test cultures (in the assays of antimicrobial activity) are listed in Table 1. *Bacillus* strains were cultivated in Mueller Hinton (MH) broth (Difco Laboratory, Detroit, MI)

TABLE 1. Inhibitory Activity Produced by *B. clausii* OC Supernatant for Different Pathogenic and Nonpathogenic Intestinal Bacteria and Other Microorganisms

Strain Tested	Activity*
<i>S. aureus</i> CIP+ 350 53 156	--
<i>Enterococcus faecium</i> LMBA‡ 27323	-
<i>E. faecium</i> LMBA 27323	-
<i>Micrococcus</i> sp LMBA 26	--
<i>Lactococcus lactis</i> ATCC§ 11454	-
<i>L. lactis</i> LMBA 374	-
<i>Clostridium difficile</i> 514¶	—
<i>Escherichia coli</i> LMBA 20684	-
<i>Salmonella enterica</i> serovar: <i>S. typhimurium</i> ATCC 29629	-
<i>S. flexneri</i> LMBA 12225	-
<i>Vibrio cholerae</i> NC1C** 8021	-
<i>C. parvum</i> ATCC 17802	-
<i>Pseudomonas fluorescens</i> LMBA BE1	-
<i>Fusarium oxysporum</i> ox524†	-

*Antimicrobial activity determined by agar diffusion test.

†Collection de l'Institut Pasteur

‡LMBA, laboratoire de Microbiologie et Biochimie Appliquée, ENTIA de Bordeaux, France

§ATCC, American Type Culture Collection.

¶Hopital Pellegrin, Bordeaux, France.

**National Collection of Type Cultures, England

††INRA de Bordeaux

or on MH agar for 24 to 72 hours at 30°C. Lactic acid bacteria were grown in MRS (de Man, Rogosa, and Sharpe) broth (Difco Laboratories) for 24 hours at 37°C. The other bacteria listed in Table 1 were grown in brain heart infusion (BHI) media (Difco). BHI medium supplemented with 2% NaCl was used for the *vibrio* species growth. *Clostridium difficile* was grown in *Brucella* agar (Anaerobe Systems, Morgan Hill, CA) supplemented with 5% defibrinated sheep blood. Finally, the Malta agar was used for fungi cultures. The sporulation percentage determination of *B. clausii* strains grown in different conditions was determined after Gram staining and microscopic observation.

Determination of Antimicrobial Activity

A colony overlay assay was used for the detection of probiotic antimicrobial activity.²² The presence of antagonistic activity of the *Bacillus* probiotic strains was determined as an inhibition of test culture growth around the *Bacillus* spot. An agar well diffusion method was used for analysis of *Bacillus* culture supernatant antimicrobial activity, as described previously.²³ The titer of antimicrobial activity (activity units [AU] per milliliter) was defined as the supernatant with highest dilution showing inhibition of *S. aureus* CIP 350 53 156.

To characterize the antimicrobials produced by probiotic bacteria, we analyzed the thermostability and enzymatic treatment resistance of the antimicrobials present in culture supernatant of the probiotic strains. Analyses were performed as described previously.²⁴ Enzymes (subtilisin, proteinase K, chymotrypsin, pronase, lipase, α -amylase, and lysozyme) were used at final concentrations of 1 mg/ml. The anti-*S. aureus* activity was determined before and after each treatment using an agar well diffusion method.

Evaluation of Immunomodulatory Activity

The murine peritoneal and spleen cells were isolated from female Swiss albino and C57BL/6j mice (6–12 weeks old; Iffa Credo, St. Germain sur l'Abresle, France) as described previously.²⁵ Briefly, cells were washed twice and resuspended in RPMI 1640 (Sigma) medium with supplements containing 10% fetal calf serum (Sigma), 2 mM L-glutamate (Sigma), 2 mM sodium pyruvate (Sigma), 20 mM HEPES (Sigma), and 25 μ g/mL gentamicin (RPMI-FCS). Murine CD4⁺ T cells were purified from the spleen cells by an anti-CD4⁺ magnetic bead selection method (Meltanyi Biotec, CA).

A total of 5×10^5 CFU of probiotic strains were added to 5×10^5 peritoneal or spleen cell cultures. Cocultures were realized in 96 well plates. The plates were incubated 72 hours.

The concentration of nitrite (NO_2^-), a stable oxidized derivative of NO, was determined spectrophotometrically at 540 nm with Griess reagents as previously described.²⁵

IFN- γ production was measured in supernatants of murine spleen cells exposed to *Bacillus* probiotic bacteria using

ELISA (PharMingen, CA) according to the manufacturer's instructions.

T-cell proliferation assay was used to determine whether *Bacillus* probiotic bacteria could activate T-cell proliferation in the presence of antigen presenting cells (APC). Briefly, the assay was performed in 96 well plates, in which 5×10^5 irradiated (3300 rad) naive spleen cells were used (as APC) in coculture with 2×10^5 purified CD4⁺ T cells in the presence or absence of *Bacillus* probiotics (5×10^5 CFU/well). The plates were incubated for 4 days at 37°C, 5% CO₂. Eighteen hours before the end of coculture, the cells were pulsed with [³H]-thymidine (1 µCi; ICN Pharmaceuticals Inc., CA). Finally, [³H]-thymidine incorporation was assessed using a liquid scintillation counter (Beckman Instrument Inc.).

RESULTS

Antimicrobial Activity

The antagonistic activity of *B. clausii* probiotic strains (O C, N-R, S1N, and T) was analyzed using colony overlay assay. In these experiments, *S. aureus* and *Salmonella* strains were used as a test culture. All tested *B. clausii* strains exhibited anti-staphylococcal activity, but not anti-*Salmonella* activity in vitro. The cell-free supernatant of the *B. clausii* O/C strain was used for further characterization of the antimicrobials produced.

The antimicrobial compounds present in the cell-free supernatant of *B. clausii* O/C displayed a relatively narrow activity. Among the tested bacteria, only Gram-positive species, including *C. difficile*, were inhibited. No inhibitory effect was observed against Gram-negative bacteria and fungi (Table 1).

The time course study of antimicrobial production was realized when O C strain was grown in MH broth. The anti-staphylococcal activity of the cell-free supernatant was followed during different growth phases (Fig. 1). Significant production of compounds carrying anti-staphylococcal activity started in the middle of the stationary growth phase (35–43 hours of culture growth) and coincided with sporulation. The maximum level of antimicrobial production (74 U A mL) was achieved when the sporulation rate was 60%.

The antimicrobial compounds present in the cell-free supernatant were relatively thermostable, because activity remained after 30 minutes of incubation at 85°C. Moreover, the cell-free supernatant kept 60% of anti-staphylococcal activity after 30 minutes of heating at 95°C. Treatment of the cell-free supernatant with subtilisin, proteinase K, chymotrypsin, lipase, α-amylase, or lysozyme did not affect its antimicrobial activity. However, the supernatant was inactivated by pronase treatment.

Immunomodulatory Activity

To characterize the immunomodulatory activity of *B. clausii* probiotic strains, these bacteria were screened for

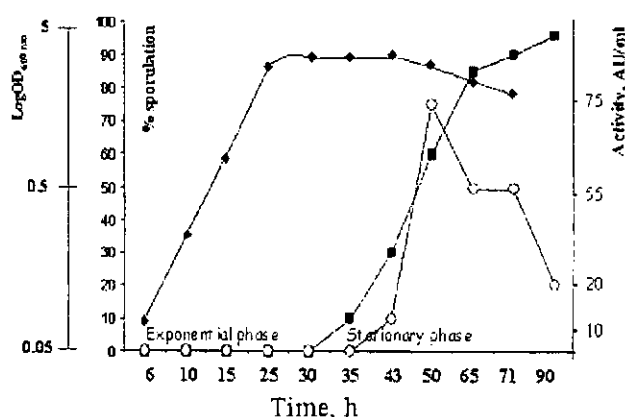


FIGURE 1. Growth and production of an antimicrobial substance by probiotic strain *B. clausii* O/C. During growth, optical density (OD) at 600 nm of culture (◆), percentage of sporulation (■), and anti-*S. aureus* activity in activity units per milliliter (○) was measured.

their ability to stimulate nitrite production in Swiss murine peritoneal cells. Nitrite concentrations were measured in 72-hour coculture supernatants. The vegetative cells of the *B. clausii* strains induced significant levels of nitrite production (approximately 100 µM). Moreover, these levels of stimulation were comparable with those obtained when peritoneal cells were cocultured with *B. subtilis* 3 probiotic strain. Other tested vegetative cells of *Bacillus* probiotics (*B. licheniformis* 31, *B. cereus* IP 5832, *B. cereus* NT, and *B. cereus* DM-423) and the reference strain *B. subtilis* 168 had a lower stimulatory effect (nitrite concentration range, 35–75 µM). This increase in nitrite production was completely abolished when L-N^G-(imino-ethyl)-lysine-dihydrochloride (L-NIL) was added, suggesting that the stimulatory effect occurred through NOS II induction.

NOS II activity in murine APC can be induced by certain cytokines, including INF-γ. We thus analyzed the effect of the four *B. clausii* probiotic strains on INF-γ production by murine C57 BL/6j spleen cells using ELISA. All 4 strains showed a strong stimulatory effect on INF-γ production, which was significantly higher than the effect observed with the *B. subtilis* 168 reference strain (Fig. 2).

Finally, we analyzed the ability of *B. clausii* strains to activate CD4⁺ T-cell proliferation in the presence of APC. The CD4⁺ T cells were purified from C57BL/6j murine spleen cells. The purity of these cells was 96 to 98% (flow cytometry analysis, data not shown). All *B. clausii* strains induced a significant T-cell proliferative response (Table 2). The probiotic strain lymphoproliferation stimulatory effect was comparable with that obtained with concanavalin A.

DISCUSSION

The concept of orally taken nonpathogenic microorganisms for the improvement of one's health is not new. However,

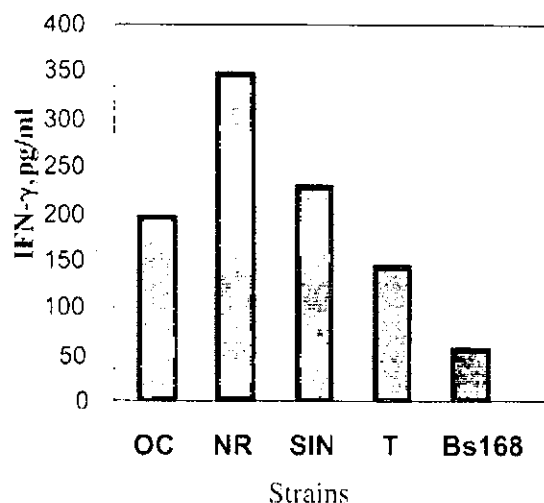


FIGURE 2. Stimulation of IFN-γ production by murine spleen cells induced with *B. clausii* probiotic strains. Spleen cells were isolated from C57BL/6j mice and cocultured with probiotic strain *B. clausii* OC (O/C), *B. clausii* NR (N/R), *B. clausii* SIN (SIN), *B. clausii* T (T), or reference strain *B. subtilis* 168 (Bs168). Analysis of IFN-γ production by murine spleen cells was realized 72 hours later using ELISA.

this idea has not received serious attention until recently. The lack of medical acceptance has been the result of a previous lack of scientifically reported evidence of the beneficial effects of probiotics. During the last decade, clinical studies have lent support to the use of selected probiotic agents for the prevention and treatment of gastrointestinal disorders.²⁶⁻²⁷ (Hart, 2003). However, little is known about the mechanisms responsible for the clinically observed effects. Recent studies indicate that probiotics probably work by multiple mechanisms.^{8,14,15} Furthermore, each agent may have a unique action.

TABLE 2. *B. clausii* Probiotic Strains Stimulate Proliferation of Murine CD4⁺ T Cells

Strain/Immunostimulator	Stimulation of Murine CD4 ⁺ T Cell* Proliferation, Index of Stimulation
<i>B. clausii</i> OC	2.5
<i>B. clausii</i> NR	3.8
<i>B. clausii</i> SIN	2.7
<i>B. clausii</i> T	2.9
<i>B. subtilis</i> 168	1.9
Con A, 2 μg/mL*	3.4

*A total of 5 × 10⁷ CFU bacteria was used for stimulation of 2 × 10⁶ CD4⁺ T cells in the presence of irradiated (3300 rad) naive murine spleen cells (5 × 10⁵ cells). Spleen and CD4⁺ T cells were isolated from C57BL/6j mice.

*Concanavalin A was used as a positive control for CD4⁺ T-cell stimulation.

Bacteria of *Bacillus* genus are known as producers of a large number of bacteriocins and antibiotics. However, little information is available regarding the antimicrobials produced by *Bacillus* probiotic strains. The commercial probiotic *B. polyfermentans* SCD, which has been successfully used for the treatment of long-term intestinal disorders, was described to produce polyfermentacin (proteinase K-sensitive and heat labile bacteriocin).⁷ We also reported that probiotic strain *B. subtilis* 3 (Biosporin) produced at least two antimicrobial substances, one of which was identified as Amicoumacin A.⁸ Moreover, our recent data demonstrate that this strain also produces four other antibiotics of a lipopeptide nature (personal communication). In the current work we demonstrated that all four *B. clausii* strains, which are included in the probiotic Enterogermina, exhibited an antimicrobial activity in vitro. Moreover, we determined that *B. clausii* O/C strain produced at least one antimicrobial substance secreted in the media, which displayed an activity against the Gram-positive bacteria. This substance was characterized as a bacteriocin-like substance, because its antimicrobial activity was sensitive to pronase treatment. However, the time course analysis of the substance production demonstrated that this antimicrobial had secondary-metabolite production kinetics, in contrast to bacteriocins that are considered to be primary metabolites. Therefore we do not exclude that the produced substance could be an antibiotic of polypeptide or lipopeptide nature, because such antibiotics were described to be frequently produced by many *Bacillus* species.^{7,8,23,26}

The use of probiotics for the treatment of primary and recurrent *C. difficile* diarrhea was recently proposed.^{26,28} Interestingly, the antimicrobial substance produced by *B. clausii* O/C strain was active against the *C. difficile* strains tested. This in vitro-observed anti-*C. difficile* effect opens perspectives for the therapeutic benefits of Enterogermina use in the treatment of *C. difficile*-associated diarrhea.

The precise mechanisms by which probiotics improve host defenses and mediate protection are not fully known. There is evidence to suggest that probiotics might also contribute to host health by stimulating both specific and nonspecific host immune responses. Numerous studies concerning the immunomodulatory properties of lactic acid bacteria strains have been recently published.^{14,15} As for *Bacillus* probiotics, little information is available. It has been suggested that the nitrite formation resulting from NOS II activity might be involved in the T_H1/T_H2 balance.²⁹ In our study, all *Bacillus* probiotics tested induced NOS II expression and consequently nitrite production in macrophages, after coculturing the bacteria with murine peritoneal cells. This NOS II induction effect was indirect, because no nitrite production was observed in the coculture of the bacteria with purified macrophages or J774.2 cell line (data not shown).

The immunomodulating activity of lactic acid bacteria may be attributed, in part, to the involvement of a cytokine

network, which plays a pivotal role in coordinating immune function.^{30,31} Muscettola et al.¹⁷ demonstrated that administration of the probiotic Enterogermina, containing 4 *B. clausii* strains, to mice increased ex vivo IFN production. In our study we demonstrated that in vitro each of these 4 *B. clausii* probiotic strains stimulated the production of IFN- γ by murine spleen cells. Moreover, all these strains induced the proliferation of murine CD4⁺ T cells in the presence of irradiated APC. The immunomodulatory capacity of these strains could be the result of the expression of some extracellular and/or cell wall-associated compounds involved in immunostimulation.³² The identification of the compounds responsible for the immunostimulatory capacity of *B. clausii* probiotic strains is currently under investigation.

In conclusion, our results suggest that the beneficial clinical effects observed for Enterogermina can at least be partially the result of the antimicrobial and immunomodulatory activities of the *B. clausii* probiotic strains. These observations provide insights into the mechanisms responsible for *Bacillus* probiotic effects on the host and should encourage further study to understand the physiology of the interaction of these bacteria with the host and the role of different compounds produced by probiotic microorganisms.

REFERENCES

- Sanders ME. Considerations for use of probiotic bacteria to modulate human health. *J Nutr*. 2000;130:384S–390S.
- Food and Agriculture Organization/World Health Organization. Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. 2001. Available at: http://www.fao.org/es/escn/food/foodandfood_probio_en.stm.
- Mazza P. The use of *Bacillus subtilis* as an antidiarrhoeal microorganism. *Boll Chim Farmaceutico*. 1994;133:3–18.
- Shornikova AV, Casas JA, Isolauri E, et al. *Lactobacillus reuteri* as a therapeutic agent in acute diarrhea in young children. *J Pediatr Gastroenterol Nutr*. 1997;24:399–404.
- Rolfe RD. 2002. The role of probiotic cultures in the control of gastrointestinal health. *J Nutr*. 2000;130:398S–402S.
- Hoa N-L, Baccigalupi L, Huxham A, et al. Characterization of *Bacillus* species used for oral bacteriotherapy and bacteriophylaxis of gastrointestinal disorders. *Appl Environ Microbiol*. 2000;66:5241–5247.
- Lee KH, Jun KD, Kim WS, et al. Partial characterization of polyfermentin SC-D, a newly identified bacteriocin of *Bacillus polymyxa*. *Lett Appl Microbiol*. 2001;32:146–151.
- Pinchuk IV, Bressollier P, Verneuil B, et al. In vitro anti-*Helicobacter pylori* activity of the probiotic strain *Bacillus subtilis* 3 is due to secretion of antibiotics. *Antimicrob Agents Chemother*. 2001;45:3156–3161.
- Sanders ME, Morelli L, Tompkins L. Spore formers as human probiotics: *Bacillus*, *Sporolobacterales* and *Brevibacterium*. *Comp Rev Food Sci Food Safety*. 2003;2:101–110.
- Vescevo M, Scolari GL, Caravaggi I, et al. Antimicrobial compounds from *Lactobacillus casei* and *Lactobacillus helveticus*. *New Microbiol*. 1993;16:171–175.
- Mudoko PD, Lambert JR, Hull R, et al. In vitro inhibition of *Helicobacter pylori* NCCTC 11637 by organic acids and lactic acid bacteria. *J Appl Bacteriol*. 1995;79:475–479.
- Bemet-Camard MF, Loevin V, Brassart D, et al. The human *Lactobacillus acidophilus* strain LA1 secretes a nonbacteriocin antibacterial substance(s) active in vitro and in vivo. *Appl Environ Microbiol*. 1997;63:2747–2753.
- Hudault S, Loevin V, Bemet-Camard MF, et al. Antagonistic activity exerted in vitro and in vivo by *Lactobacillus casei* (strain GG) against *Salmonella typhimurium* C5 infection. *Appl Environ Microbiol*. 1997;63:513–518.
- Donnet-Hughes A, Rochat F, Serrant P, et al. Modulation of nonspecific mechanisms of defense by lactic acid bacteria: effective dose. *J Dairy Sci*. 1999;82:863–869.
- Shu Q, Gill HS. Immune protection mediated by the probiotic *Lactobacillus rhamnosus* HN001 (DR20) against *Escherichia coli* O157:H7 infection in mice. *FEMS Immunol Med Microbiol*. 2002;34:59–64.
- Fiorini G, Cimminiello C, Chianese R, et al. *Bacillus subtilis* selectively stimulates the synthesis of membrane bound and secreted IgA. *Chemiotherapy*. 1985;4:310–312.
- Muscettola M, Grasso G, Blach Olszewska Z, et al. Effects of *Bacillus subtilis* spores on interferon production. *Pharmazie Res*. 1992;26:176–177.
- Fan X, Stelter F, Menzel R, et al. Structures in *Bacillus subtilis* are recognized by CD14 in a lipopolysaccharide binding protein-dependent reaction. *Infect Immun*. 1999;67:2964–2968.
- Duc H, Hong HA, Cutting SM. Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen delivery. *Vaccine*. 2003;21:4215–4224.
- Pinchuk IV, Sorokulova IB, Megraud F, et al. Use of *Bacillus subtilis* strain CU1 as a vaccine delivery system for mucosal immunization against *Helicobacter pylori* infection in mice. *Gut*. 2002;51:A49.
- Senesi S, Celandroni F, Tavanti A, et al. Molecular characterization and identification of *Bacillus clausii* strains marketed for use in oral bacteriotherapy. *Appl Environ Microbiol*. 2001;67:834–839.
- Pugsley AP. *Escherichia coli* K12 strains for use in the identification and characterization of colicins. *J Gen Microbiol*. 1985;131:369–376.
- Le Marrec C, Hyronimus B, Bressollier P, et al. Isolation, biochemical and genetic characterisation of coagulins from *Bacillus coagulans* 14, a new pediocin-like bacteriocin. *Appl Environ Microbiol*. 2000;66:5213–5220.
- Hyronimus B, Le Marrec C, Urdaci MC. Coagulin, a bacteriocin-like inhibitory substance produced by *Bacillus coagulans* 14. *J Appl Microbiol*. 1998;85:42–50.
- Gobert AP, Semballa S, Daulouede S, et al. Murine macrophages use oxygen- and nitric oxide-dependent mechanisms to synthesize S-nitrosoalbumin and to kill extracellular trypanosomes. *Infect Immun*. 1998;66:4068–4072.
- Elmer GW. Probiotics: "living drugs." *Am J Health Syst Pharm*. 2001;58:1101–1109.
- Hart AL, Stagg AJ, Kaimm MA. Use of probiotics in the treatment of inflammatory bowel disease. *J Clin Gastroenterol*. 2003;36:111–119.
- Pochapin M. The effect of probiotics on *Clostridium difficile* diarrhea. *Am J Gastroenterol*. 2000;95:S11–S13.
- Mills CD, Kincaid K, Alt JM, et al. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol*. 2000;164:6166–6173.
- Kato I, Tanaka K, Yokokura T. Lactic acid bacterium potently induces the production of interleukin-12 and interferon-gamma by mouse splenocytes. *Int J Immunopharmacol*. 1999;21:121–131.
- Nicnise P, Gleizes A, Forestier F, et al. Influence of intestinal bacterial flora on cytokine (IL-1, IL-6 and TNF-alpha) production by mouse peritoneal macrophages. *Eur Cytokine New*. 1993;4:133–138.
- Lawrence C, Nauciel C. Production of interleukin-12 by murine macrophages in response to bacterial peptidoglycan. *Infect Immun*. 1998;66:4947–4949.

**In Vitro Anti-*Helicobacter pylori* Activity of
the Probiotic Strain *Bacillus subtilis* 3 Is
Due to Secretion of Antibiotics**

Irina V. Pinchuk, Philippe Bressollier, Bernard Verneuil,
Bernard Fenet, Irina B. Sorokulova, Francis Mégraud and
Maria C. Urdaci
Antimicrob. Agents Chemother. 2001, 45(11):3156. DOI:
10.1128/AAC.45.11.3156-3161.2001.

Updated information and services can be found at:
<http://aac.asm.org/content/45/11/3156>

These include:

REFERENCES

This article cites 22 articles, 6 of which can be accessed free at:
<http://aac.asm.org/content/45/11/3156#ref-list-1>

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new
articles cite this article), [more»](#)

Downloaded from <http://aac.asm.org/> on June 7, 2012 by guest

In Vitro Anti-*Helicobacter pylori* Activity of the Probiotic Strain *Bacillus subtilis* 3 Is Due to Secretion of Antibiotics

IRINA V. PINCHUK,^{1,2} PHILIPPE BRESSOLIER,³ BERNARD VERNEUIL,³ BERNARD FENET,⁴
IRINA B. SOROKULOVA,¹ FRANCIS MÉGRAUD,² AND MARIA C. URDADI^{1*}

Laboratoire de Microbiologie et Biochimie Appliquée, ENITA de Bordeaux, 33175 Gradignan Cedex,¹ Laboratoire de Bactériologie, Université Victor Segalen Bordeaux II, 33076 Bordeaux Cedex,² Laboratoire de Génie Enzymatique et Biovalorisation, IUT, 87000 Limoges,³ and Centre de RMN, Université Claude Bernard, Lyon 1, ESPCPE Lyon, 69622 Villerbanne Cedex,⁴ France

Received 1 November 2000/Returned for modification 1 March 2001/Accepted 26 July 2001

A limited number of antibiotics can be used against *Helicobacter pylori* infection, and resistance jeopardizes the success of treatment. Therefore, a search for new agents is warranted. The use of probiotics to enhance gastrointestinal health has been proposed for many years, but the scientific basis of the prophylactic and therapeutic actions of probiotics has not yet been clearly delineated. Probiotic strain *Bacillus subtilis* 3, whose safety has previously been demonstrated, is known to have antagonistic properties against species of the family *Enterobacteriaceae*. In the present study, it was also found to inhibit *H. pylori*. The anti-*H. pylori* activity present in the cell-free supernatant was not related to pH or organic acid concentration. It was heat stable and protease insensitive. At least two antibiotics, detected by thin-layer chromatography (R_f values, 0.47 and 0.85, respectively) and confirmed by high-performance liquid chromatographic analysis, were found to be responsible for this anti-*H. pylori* activity. All *H. pylori* strains tested were sensitive to both compounds. One of these compounds was identified as amicoumacin A, an antibiotic with anti-inflammatory properties. MICs for *H. pylori* determined in solid and liquid media ranged between 1.7 and 6.8 $\mu\text{g/ml}$ and 0.75 and 2.5 $\mu\text{g/ml}$, respectively. The underestimation of MICs determined in solid medium may be due to physicochemical instability of the antibiotic under these test conditions. An additive effect between amicoumacin A and the nonamicoumacin antibiotic against *H. pylori* was demonstrated.

Helicobacter pylori infection is the major cause of chronic gastritis and peptic ulcer disease (16) and is a risk factor for gastric cancer in humans (8). This infection is extremely common throughout the world, and its prevalence increases with age and lower socioeconomic status.

Combinations of several drugs are now widely used for the eradication of *H. pylori*. Numerous clinical trials have indicated that eradication of *H. pylori* by treatment that includes bismuth or antisecretory drugs combined with antibiotics leads to healing of gastritis and drastically decreases the rate of peptic ulcer relapse. However, a limited number of antibiotics can be used, and resistance jeopardizes the success of treatment (15, 19). Therefore, a search for new antimicrobial agents is warranted.

Several authors have previously reported that certain probiotic bacteria, such as *Lactobacillus* spp., exhibit inhibitory activity against *H. pylori* in vitro and in vivo (5, 17). The term "probiotic" was first used by Parker (21) to describe "organisms and substances which contribute to the intestinal microbial balance." This definition has since been narrowed to focus on normal microflora that have a beneficial effect in the prevention and treatment of a diverse spectrum of gastrointestinal disorders such as antibiotic-associated diarrhea and bacterial and viral diarrhea (including diarrhea caused by *Shigella* spp., *Salmonella* spp., rotavirus, and others) (6, 23). Currently, the most well-studied probiotics are the lactic acid bacteria, par-

ticularly lactobacilli, and to a lesser extent, bifidobacteria. Other organisms used or considered for use as probiotics in humans or animals include various *Bacillus* spp., *Bacteroides* spp., *Propionibacterium* spp., and fungi (2, 23, 26). Characterization and study of the mechanisms of action of these microorganisms as probiotics lag significantly behind characterization and study of those of the lactic acid bacteria.

Probiotic strain *Bacillus subtilis* 3 is of interest because of its safety for use in humans (20) and its antagonistic effect in vitro and in vivo against different human bacterial pathogens such as *Campylobacter* spp. (26). Recent reports have suggested that the antimicrobial activity of *Lactobacillus* sp. probiotics involves production of secreted compounds, such as organic acid, hydrogen peroxide, as well as various antibiotics or bacteriocins (1, 5, 12, 25, 27). Therefore, the aim of the present study was to determine the antagonistic activity of probiotic strain *B. subtilis* 3 against *H. pylori* in vitro and the cause of any inhibitory activity.

MATERIALS AND METHODS

Bacteria. All except three of the bacterial strains used as test cultures (see Tables 1 and 2) were grown in Mueller-Hinton (MH) medium at 37°C under aerobic conditions. *H. pylori*, *Campylobacter jejuni*, and *Bacteroides fragilis* strains, however, were cultured on MH agar (Difco Laboratories, Detroit, Mich.) supplemented with 10% defibrinated horse blood (MH&B) and were grown for 48 to 72 h under microaerobic (for *H. pylori* and *C. jejuni*) or anaerobic (for *B. fragilis*) conditions.

Probiotic strain *B. subtilis* 3, originally isolated from animal fodder (IMV National Academy of Sciences, Kiev, Ukraine), is one of the basic components of the commercial probiotic Biosporin (Biofarm, Dnepropetrovsk, Ukraine). It was grown in a liquid starch medium composed of starch (10 g/liter), peptone (5

* Corresponding author. Mailing address: Laboratoire de Microbiologie et Biochimie Appliquée, ENITA de Bordeaux, 1, cours du Général de Gaulle, BP 201, 33175 Gradignan cedex, France. Phone: 33 5 57 35 59 91. Fax: 33 5 57 35 07 39. E-mail: m-urdadi@enitab.fr.

g-liter), and NaCl (0.5 g-liter) for 72 h at 28°C in an aerobic atmosphere on a rotary shaker at 175 rpm.

All strains were stored at -70°C in brucella broth (Difco Laboratories) containing 10% (vol/vol) glycerol.

Antimicrobial testing. (i) **Spotting method.** *B. subtilis* 3 was inoculated as a spot (approximately 5 to 10 mm) on the surface of a starch agar (1.2% [wt/vol]) plate. Controls consisted of uninoculated starch agar plates. After 72 h at 28°C, the bacteria were killed by exposure to chloroform vapor for 15 min, and the plates were overlaid with MH&B containing 24- to 48-h-old *H. pylori* strains at 10^7 CFU/ml (assayed by turbidimetry). All plates were incubated for 24 to 48 h at 37°C in an appropriate atmosphere. The antagonistic activity was detected as the presence of a growth inhibition zone around the spot.

(ii) **Assay for antibacterial substance production.** Assessment of the anti-*H. pylori* activities of culture supernatants and step purification fractions was performed by an agar well diffusion method (5) with MH&B. The inoculum of *H. pylori* strains was approximately 10^7 CFU/ml. Aliquots of 100 µl of these suspensions were inoculated onto agar medium. The wells (diameter, 6 mm) were then cut into each plate. Serial dilutions of each test solution were made, and the wells were filled with 100 µl of native or diluted solution. The plates were incubated at 37°C in a microaerobic atmosphere for 24 to 48 h. The inhibition zones of *H. pylori* were then measured.

The titer of anti-*H. pylori* was defined as the reciprocal of the highest dilution showing inhibition of *H. pylori* and was expressed in activity units (AU) per milliliter.

(iii) **Viability of *H. pylori* SS1.** The viability of *H. pylori* Sydney strain 1 (SS1) in the presence of cell-free supernatant, solid-phase extract, or purified fractions of the antimicrobial substances was determined by the method described by Benet-Camard et al. (13). Briefly, an 18-h-old *H. pylori* culture was resuspended in phosphate-buffered saline (PBS) and centrifuged at $5,500 \times g$ for 5 min. The supernatant was discarded, and the bacteria were washed once with PBS and resuspended (approximately 10^7 CFU/ml) in medium containing 50% (vol/vol) brain heart infusion broth (Difco Laboratories), 40% (vol/vol) Dulbecco modified Eagle's medium (Life Technologies, Cergy-Pontoise, France), and 10% (vol/vol) fetal bovine serum. Then, 4.5 ml of this suspension was incubated with 0.5 ml of a cell-free supernatant, solid-phase extract, or purified fraction of antimicrobial substances at 37°C under microaerobic conditions. In this experiment, the dried solid-phase extract or purified fractions were dissolved in 0.5 ml of starch medium and were used at a final concentration of 200 AU/ml. Initially and then at predetermined intervals, aliquots of the incubated suspension were removed, serially diluted, and plated onto MH&B to determine bacterial colony counts.

Physicochemical and enzymatic treatments. For thermostability assay, lyophilized cell-free supernatant (20 mg/ml in 5 mM Tris HCl buffer [pH 7.0]) was submitted to heat treatment for 15, 30, 45, and 60 min at 100°C. For pH stability, the preparation (20 mg/ml) was incubated for 1 h at 37°C in appropriate buffer solutions (5 mM Tris or 5 mM CH₃COONa adjusted to different pH values with HCl or CH₃COOH, respectively). Sensitivity to enzymes was tested by incubation of the preparation (20 mg/ml in 5 mM Tris HCl buffer [pH 8.3]) for 1 h at 50°C for proteases or at 37°C for lipase, α -amylase, and lysozyme. The enzymes were used at final concentrations of 1 mg/ml. The anti-*H. pylori* activity was determined before and after each treatment by the agar well diffusion method.

Isolation and purification. Cultures of *B. subtilis* 3 grown for 72 h in a starch liquid medium at 28°C in a volume of 8 liters were clarified by filtration through diatomaceous earth (Spendaite N; Spindal, Paris, France). The antimicrobial substances in cell-free filtrates were batch adsorbed on Amberlite XAD7 polystyrene ester resin (Sigma-Médical Chimie, St. Quentin Fallavier, France). After washing of the resin with water and H₂O-isopropanol (80:20 [vol/vol]), antimicrobial compounds were eluted with H₂O-isopropanol (30:70 [vol/vol]) and were then concentrated under vacuum. The dry residue (solid phase extract) was dissolved in H₂O-acetonitrile (90:10 [vol/vol]) containing 0.1% trifluoroacetic acid (TFA). This solvent mixture was used as solvent A. The dissolved residue was loaded onto Nova Pak HR C₁₈ (Waters Div., Millipore Corp., St. Quentin-Yvelines, France) semipreparative reverse-phase high-pressure liquid chromatography (HPLC) column (25 by 100 mm, particle size, 6 µm; pore size, 60 Å) equilibrated at a 18 ml/min flow rate with solvent A. Activity was eluted with a two-step linear gradient of acetonitrile containing 0.07% TFA (solvent B): from 1 to 40 min with 0 to 19% solvent B and then from 40 to 50 min with 19 to 100% solvent B.

Active fractions were pooled, freeze-dried, resuspended in solvent A, and then processed on the same system by using an 80-min linear gradient with from 0 to 20% solvent B. Active fractions (purified antimicrobial substance) were collected, freeze-dried, and stored at -80°C.

All purification steps were performed at room temperature with a 600 E

HPLC system (Waters Division, Millipore Corp.); the absorbance of the eluate was detected at 210 nm.

TLC. Thin-layer chromatography (TLC) analysis of cell-free supernatants and purified antimicrobial fractions was performed on precoated Kieselghur 60 plates (20 by 20 cm; Macherey Nagel, Duren, Germany) with chloroform-methanol-H₂O (65:25:4 [vol/vol/vol]) as a mobile phase. The bands on the TLC chromatogram were marked under UV light at 310 nm. The different bands were scraped separately, dissolved in 1 ml of H₂O-isopropanol (30:70 [vol/vol]), vortexed, and centrifuged for 15 min at $5,500 \times g$. The anti-*H. pylori* activities of the extracts that were obtained were tested by the agar well diffusion method.

Reverse-phase HPLC. Reverse-phase HPLC on a Lichrospher C₁₈ column (4 by 125 mm; particle size, 5 µm; pore size, 100 Å; Merck, Darmstadt, Germany) was used to analyze solid-phase extracts and the purified substance. After sample injection, the column was eluted for 5 min at a flow rate of 1 ml/min with H₂O-acetonitrile (90:10 [vol/vol]) containing 0.1% TFA (solvent A) and was then eluted with a linear gradient with from 0 to 20% solvent B for 30 min. Antibiotic substances in the eluate were detected with a fluorescence detector (Waters Division, Millipore Corp.); the excitation wavelength selected was 312 nm, and emission was measured at 465 nm.

Physicochemical properties. UV-visible antibiotic spectral data were recorded on a 930 Uvikon spectrophotometer (Bio-Tek Instruments, St. Quentin en Yvelines, France). ¹H and ¹³C nuclear magnetic resonance (NMR) spectra of antimicrobial compounds were acquired on a Bruker Spectrospin DRX 500 spectrometer (Bruker Canada Ltd., Milton, Ontario, Canada).

Molecular mass was determined by mass spectrometry in the electrospray mode with a SIEX API 100 spectrometer (Perspective Biosystem, Framingham, United Kingdom). Exact molecular mass determination was performed on a high-resolution chemical ionization mass spectrometer (JMS-AX505; JEOL, USA Inc., Peabody, Mass.).

Determination of MICs. The MIC of the purified antimicrobial substance for different bacterial strains were determined by a dilution method (18) in solid and/or liquid media containing different concentrations of purified antimicrobial substance. The bacterial inoculum was approximately 10^7 CFU/ml. The MIC was considered to be the lowest concentration of antimicrobial substance that caused total inhibition of bacterial growth.

RESULTS

Inhibition of growth of *H. pylori* in vitro by *B. subtilis* 3 and its supernatant. Of the 21 strains of *H. pylori* tested, all were inhibited by *B. subtilis* 3. When the cell-free supernatant of *B. subtilis* 3 was tested, the inhibitory zone for each *H. pylori* isolate ranged from 10 to 16 mm. The diameters of the inhibition zones increased significantly (15 to 25 mm) when the supernatant was concentrated approximately 10-fold.

Effects of heat, pH, and various enzymes on antimicrobial substance(s). The antimicrobial compounds present in the cell-free supernatant were heat stable, and the level of activity remained unchanged after 60 min of incubation at 100°C. This antimicrobial supernatant was also active over a wide range of pH values, from 3 to 10. The activity decreased slightly (20%) at pH 11. Treatment with different enzymes (pepsin, trypsin, proteinase K, lipase, α -amylase, and lysozyme) did not affect the antimicrobial activity of the supernatant.

Study of antibiotic production by TLC analysis. TLC of concentrated cell-free supernatant on silica gel plates yielded five major bands under UV light at 310 nm, with *R_f* values of 0.2, 0.43, 0.47, 0.8, and 0.85. These bands were submitted to an extraction procedure, and the activity of each band against *H. pylori* strains was measured. Only two of them, those with *R_f* values of 0.47 and 0.85, respectively, contained anti-*H. pylori* compounds.

Isolation and characterization. The antimicrobial compounds present in the cell-free filtrate of the *B. subtilis* 3 culture were separated by solid-phase extraction and preparative reverse-phase HPLC assays. Two active fractions (Fractions a