



DELEGATURA PROPIEDAD INDUSTRIAL

División de Nuevas Creaciones

PCT

SOLICITUD

PATENTE DE INVENCION

ENTRADA EN BASE NACIONAL

08-99562

21. EXPEDIENTE No.

54. TITULO VACUNA DE SALMONELA CON VIRUS VIVOS ATENUADA

51. CLASIFICACION INTERNACIONAL

VRIJE UNIVERSITEIT BRUSSEL

71. SOLICITANTE

BRUSELAS, BELGICA

DOMICILIO

JUAN PABLO CADENA SARMENTO

74. APODERADO

22. SANTAFE DE BOGOTÀ, D.C.

PETITORIO

AS: 108.465



SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 08-099562- -00000-0000

Fecha: 2008-09-19 16:26:25 Dep. 2020 NUECREACION
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 122

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/BE2006/000019

Fecha 20-03-2006

Publicación Internacional No. WO 2007/106956 A1

Fecha 27-09-2007

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, 151

Fecha 19-09-2008

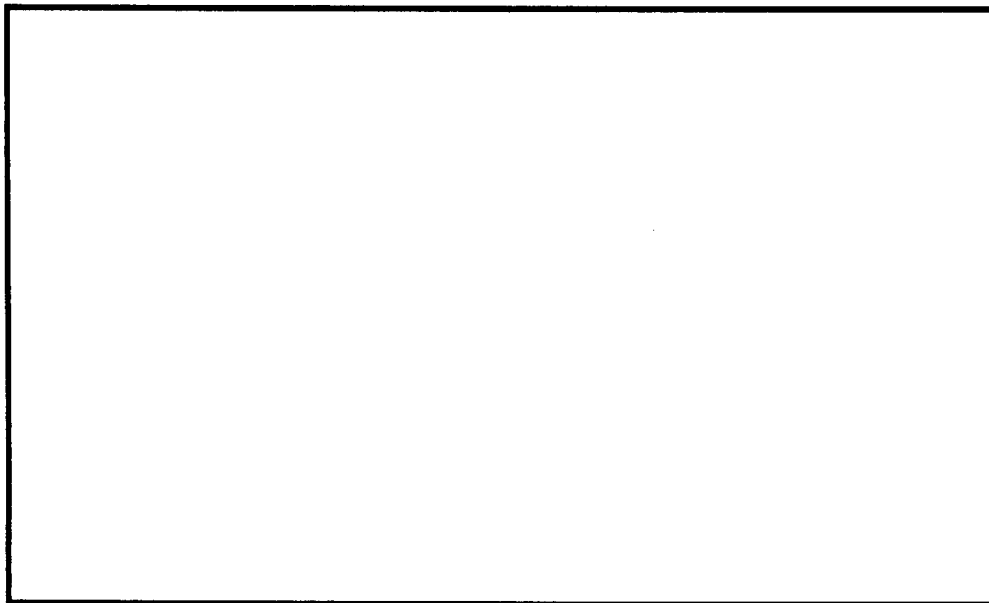
Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.

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.

Instrucciones para la presentación de los anexos, ver reverso de esta página.

108 465

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 900176089-2

RECIBO OFICIAL DE CAJA : 08 - 66,151
FECHA : AGOSTO 20 DE 2008

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 08-099562- -00000-0000

Fecha: 2008-09-19 16:26:25 Dep. 2020 NUECREACION
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 122

***** CONSIGNACION *****

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

***** CONCEPTO *****

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. _____

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

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
27 September 2007 (27.09.2007)

PCT

(10) International Publication Number
WO 2007/106956 A1

(51) International Patent Classification:

CI2N 1/20 (2006.01) A61K 39/112 (2006.01)
A61K 39/108 (2006.01) A61P 31/04 (2006.01)

(21) International Application Number:

PCT/BE2006/000019

(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).

(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). HERNAL-
STEENS, Jean-pierre, Ernest, Clément [BE/BE];
François Desmedtstraat 156, B-1150 Sint-Pieters-Woluwe
(BE).

(74) Agents: VAN MALDEREN, Joëlle et al.; pronovem-OF-
FICE VAN MALDEREN, Avenue Josse Goffin 158,
B-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.

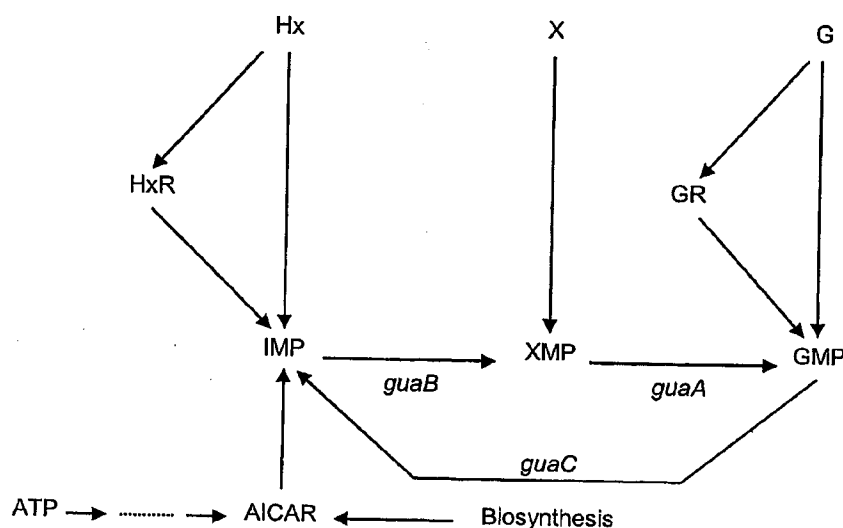
(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IS, IT, LT, 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
- with (an) indication(s) in relation to deposited biological
material furnished under Rule 13bis separately from the
description
- with sequence listing part of description published sepa-
rately in electronic form and available upon request from
the International Bureau

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: LIVE ATTENUATED SALMONELLA VACCINE



(57) Abstract: The present invention relates to attenuated *guaB* deletion mutants of a bacterium infecting veterinary species, more in particular *Salmonella enterica*, to their use and production. The present invention further relates to live attenuated vaccines based on such mutants for preventing bacterial infections, and more in particular Salmonellosis, in a veterinary species, more in particular poultry.

WO 2007/106956 A1

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.

State of the art

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

[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 *Salmonella enterica*, with *S. enterica* serovar Typhimurium (*S. Typhimurium*) and *S. enterica* serovar Enteritidis (*S. Enteritidis*) as the most common serovars.

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

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

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

[0011] Live attenuated *Salmonella* vaccines are potentially superior to inactivated preparations owing to:
(i) their ability to induce cell-mediated immunity in
15 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 cost; and (vi) their possible use as carriers for the
20 delivery of recombinant antigens to the immune system.

[0012] Few live attenuated *Salmonella* vaccines are actually on the market because results with attenuated mutant strains have not always been good.

[0013] For example, poultry has been vaccinated with
25 aro- or cAMP mutants, yet many chicks died shortly after vaccination. As the infection by *Salmonella* often occurs very early, vaccination of very young chicks is crucial. However, at this age these are very sensitive to *Salmonella*, possibly due to the immaturity of their immune
30 system. In addition to poor protection of the vaccinated chicks, a prolonged excretion of the vaccine strains was often observed.

[0014] Vaccination of birds with the Megan[®]Vacl strain, that carries deletions in the *cya* and *crp* genes (US

Patents 5,389,368; US 5,855,879; US 5,855,880) results in a reduction of the number of birds from which a virulent *Salmonella* challenge strain can be isolated 7 days after challenge. However, it does not provide a full protection

5 (<http://www.meganhealth.com/meganvac.html>).

[0015] There is thus still a need for improved live attenuated *Salmonella* vaccine strains, and for improved live attenuated vaccine strains of bacteria infecting veterinary species in general.

10 [0016] Information in literature on the influence of *guaB* mutations on the virulence of *Salmonella* and other bacteria is sparse.

[0017] McFarland and Stocker (1987, *Microbial pathogenesis* 3:129-141) reported on the reduced virulence

15 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 for *S. Typhimurium* and 10^4 cfu for *S. dublin*), however, these authors reported a high lethality, resulting from the multiplication of the auxotrophic strain.

20 [0018] Wang et al. (2001, *Infection and Immunity* 69:4734-4741) reported on pre-clinical trials with a vaccine against typhoid fever in humans. The vaccine of Wang et al. comprised a Δ *guaBA* mutant of *Salmonella typhi*. This attenuated strain, however, showed a significant

25 residual virulence in mice.

[0019] International patent application WO 99/58146 and US Patent 6,190,669 disclose *Salmonella typhi* vaccine strains harboring a Δ *guaBA* mutation, which are used as a live vector to deliver foreign antigens.

30

Aims of the invention

[0020] An object of the present invention is to provide attenuated *Salmonella enterica* strains.

[0021] Another object of the present invention is to provide a live attenuated vaccine against Salmonellosis and methods of prevention based thereon.

[0022] Yet another object of the present invention is to provide live attenuated strains which are useful as live vector and as DNA-mediated vaccines expressing foreign antigens. Such strains are highly suitable for the development of vaccines including polyvalent vaccines.

[0023] Still another object of this invention is to provide a method to achieve a *S. enterica* deletion mutant for use in a live attenuated vaccine.

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

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

Summary of the invention

[0026] A first aspect of the invention relates to an attenuated *Salmonella enterica* mutant strain which is incapable of forming de novo guanine nucleotides, and wherein said mutant contains a deletion mutation in the *guaB* gene.

[0027] The principle as demonstrated here for *S. enterica* can be applied to any organism that can use the guanine nucleotide as an intermediary. An aspect of the invention therefore relates to an attenuated mutant strain of a bacterium infecting veterinary species that contains a deletion mutation in the *guaB* gene. The term "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 a deletion mutation in the *guaB* gene. The

bacterium infecting veterinary species may be a Gram-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 infectious disease in the veterinary species.

[0028] The mutants of the invention fail to express a functional GuaB gene product. In other words the *guaB* gene function is impaired, whereby an auxotrophic attenuated strain is obtained.

[0029] The mutant strain of the invention, carrying a deletion mutation in the *guaB* gene, can not grow on Minimal A medium, unless this medium is supplemented with 0.3mM guanine, xanthine, guanosine or xanthosine.

[0030] The present invention in particular aims to provide attenuated *S. Enteritidis* and *S. Typhimurium* strains.

[0031] Preferably the deletion mutation in the *guaB* gene is introduced into parent strain *S. Enteritidis* phage type 4 strain 76Sa88 or into parent strain *S. Typhimurium* 1491S96.

[0032] One of the attenuated *S. enterica* strains obtained according to the invention is a *S. Enteritidis* strain SM69 having the deposit number LMG P-21641. Another example is the *S. Typhimurium* strain SM86 having the deposit number LMG P-21646.

[0033] The attenuated mutant strains of the invention are highly suitable for use in a live attenuated vaccine including a polyvalent or multivalent vaccine. The attenuated mutant strain of the invention may encode and express a foreign antigen and may as such be used as a live vector and/or as a DNA-mediated vaccine.

[0034] A second aspect of the invention concerns a

pharmaceutical composition or a vaccine for immunizing a veterinary species against a bacterial infection (e.g. Salmonellosis caused by *Salmonellae*) comprising:

- a pharmaceutically effective or an immunizing amount
5 of a mutant strain according to the invention, which is incapable of forming de novo guanine nucleotides due to a deletion mutation in the *guaB* gene; and

- a pharmaceutically acceptable carrier or diluent.
Preferred compositions are those comprising a *Salmonella*
10 *enterica* mutant strain according to the invention.

[0035] The attenuated strain of the invention may transfer DNA encoding a foreign antigen in a eukaryotic cell. This foreign antigen may be encoded by and expressed from a plasmid comprised by the attenuated mutant strain
15 of the invention.

[0036] 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
20 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.

[0037] The attenuated strains of the invention, and
25 pharmaceutical compositions or vaccines comprising same, are highly suitable for immunizing an animal or veterinary species against a bacterial infection (e.g. Salmonellosis) and possibly other diseases (in the case of a multivalent vaccine).

30 [0038] A further aspect of the invention therefore concerns a method of immunizing animals, preferably veterinary species, more preferably poultry such as chicken against an infection by a bacterium (e.g.

Salmonellosis caused by *Salmonellae*), 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.

[0039] Examples of veterinary species to be immunized against Salmonellosis: poultry, small or heavy livestock such as chicken, turkey, ducks, quails, guinea fowl, pigs, sheep, young calves, cattle etc.

[0040] In general an appropriate dose is administered to these animals, preferably via the oral, nasal or parenteral route.

[0041] A last aspect of the invention relates to a 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 disease caused by a pathogen (the infecting bacterium) such as Salmonellosis. Examples of animals or veterinary species to be treated and recommended doses are given above.

25

Detailed description of the invention

[0042] It was surprisingly found that a deletion mutation in the *guaB* gene can lead to attenuated *Salmonella enterica* strains with significantly reduced virulence and capable of inducing an immune response in a livestock animal. Such deletion would attenuate any organism that can use the guanine nucleotide as an intermediary.

[0043] The term "gene" as used herein refers to the

17

coding sequence and its regulatory sequences such as promoter and termination signals.

[0044] The deletion mutant according to the invention is one in which the purine metabolic pathway enzyme IMP
5 dehydrogenase (encoded by *guaB*) is inactivated.

[0045] Such inactivation may be obtained via a deletion by which the *guaB* gene function is impaired, leading to a null-function (no functional gene product formed) of the affected gene(s). A person skilled in the art knows how to
10 obtain such mutants and a simple test can tell whether the *guaB* gene function is impaired. 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,
15 guanosine or xanthosine.

[0046] The invention aims to provide, amongst others, attenuated *S. Enteritidis* and *S. Typhimurium* strains since these are the most common *S. enterica* serovars.

[0047] The present invention provides attenuated
20 strains. The invention provides amongst others attenuated *Salmonella enterica* strains for use, inter alia, as live attenuated vaccines against Salmonellosis, as live vector and/or as DNA-mediated vaccines expressing foreign antigens. As used herein, a "foreign antigen" means an
25 antigen foreign to *Salmonella*.

[0048] 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
30 viral or bacterial vaccine is modified so that it expresses 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*.

[0049] An object of the invention is to provide attenuated strains, like attenuated *S. enterica* strains for use in a live vaccine, possibly a polyvalent or
5 multivalent live vaccine.

[0050] 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
10 of a mutant of the invention (e.g. a *Salmonella enterica* mutant), which is incapable of forming ~~de novo~~ guanine nucleotides, wherein said mutant contains a deletion mutation in the *guaB* gene; and

- a pharmaceutically acceptable carrier or diluent.

15 [0051] Another object of the invention is to provide a live vector vaccine comprising:

- a pharmaceutically effective or an immunizing amount of a mutant of the invention (e.g. a *Salmonella enterica* mutant), which is incapable of forming *de novo* guanine
20 nucleotides, wherein said mutant contains a mutation in the *guaB* gene, and wherein said mutant encodes and expresses a foreign antigen; and

- a pharmaceutically acceptable carrier or diluent.

[0052] The particular foreign antigen employed in the
25 (*S. enterica*) live vector is not critical to the present invention.

[0053] Still another object of the invention is to provide a DNA-mediated vaccine comprising:

- a pharmaceutically effective amount or an immunizing
30 amount of a mutant of the invention (e.g. a *Salmonella enterica* mutant), which is incapable of forming *de novo* guanine nucleotides, wherein said mutant contains a mutation in the *guaB* gene; wherein said mutant contains a

plasmid which encodes and expresses in a eukaryotic cell, a foreign antigen; and

- a pharmaceutically acceptable carrier or diluent.

[0054] Details as to the construction and use of DNA-mediated vaccines can be found in U.S. patent 5,877,159, 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.

[0055] The decision whether to express the foreign antigen in e.g. *S. enterica* (using a prokaryotic promoter in a live vector vaccine) or in the cells invaded by e.g. *S. enterica* (using a 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 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).

[0056] In the vaccines of the present invention, the pharmaceutically effective amount or the immunizing amount of the mutants of the present invention to be administered will vary depending on the age, weight and sex of the subject. By an "immunizing amount" as used herein is in fact meant an amount that is able to induce an immune response in the animal that receives the pharmaceutical composition/vaccine. The immune response invoked may be a humoral, mucosal, local and/or a cellular immune response.

[0057] The particular pharmaceutically acceptable carrier or diluent employed is 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, 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 [0058] The deletion mutants according to the invention have been created via standard homologous recombination techniques, whereby part of the *guaB* gene, for instance part of the *guaB* coding sequence, in a first step is replaced by a resistance gene and flanking FRT sites.
- 10 [0059] 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).

[0060] A particular example of the invention relates for instance to *guaB* deletion mutants of *S. Enteritidis* that comprise a mutated *guaB* gene or coding sequence comprising SEQ ID NO: 12.

20

Short description of the drawings

- [0061] Figure 1 is a schematic representation of the biosynthetic pathway of guanosine monophosphate (adapted from Zalkin and Nygaard, 1996, in "Escherichia coli and
- 25 *Salmonella*, Cellular and Molecular Biology, Second edition", 1996 F.C. Neidhardt ed. ASM Press, Washington D.C., Vol.1, Ch. 34:561-579). AICAR: 5'-phosphoribosyl-4-carboxamide-5-aminoimidazole; ATP: adenosine triphosphate; G: guanine; GMP: guanosine monophosphate; GR: guanosine;
- 30 Hx: hypoxanthine; HxR: hypoxanthine riboside (inosine); IMP: Inosine monophosphate; X: Xanthine, XMP: Xanthosine monophosphate; *guaA*: GMP synthetase, *guaB*: IMP dehydrogenase; *guaC*: GMP reductase.

[0062] Figure 2 represents contig 1294 of the *S.*

Enteritidis genome (SEQ ID NO: 10). The **ATG** initiation codon and **TGA** termination codon of the *guaB* gene are in bold.

[0063] Figure 3 represents the sequence of the *ΔguaB* fragment of *S. Enteritidis* cloned in pUC18 (SEQ ID NO: 11). The primers that were used are indicated by horizontal 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.

[0064] Figure 4 represents the nucleotide sequence of the *S. Enteritidis* PCR fragment, which includes the *guaB* deletion, obtained after sequencing, using primer GuaB10 (SEQ ID NO: 12). 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 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.

[0065] Figure 5 represents the *guaB* gene of *S. Typhimurium* LT2, section 117 of 220 of the complete genome (SEQ ID NO: 13). The **ATG** initiation codon and **TGA** termination codon of the *guaB* gene are in bold.

[0066] Figures 6-7 represent the deposit receipts of SM69 and SM86 respectively.

[0067] The invention will be described in further details in the following examples and embodiments by reference to the enclosed drawings. Particular embodiments and examples are not in any way intended to limit the scope of the invention as claimed.

Examples

Example 1: auxotrophic mutation affects the *guaB* gene

[0068] An auxotrophic insertion mutant of a wild type *S. Enteritidis* was obtained via insertion mutagenesis.
5 Only when supplemented with 0.3 mM guanine, xanthine, guanosine or xanthosine could the mutant strain grow on Minimal A medium.

[0069] These data strongly suggest that the auxotrophic mutation of the strain affects the *guaB* gene, encoding the
10 enzyme IMP dehydrogenase (EC 1.1.1.205). This enzyme converts inosine-5'-monophosphate (IMP) into xanthosine monophosphate (XMP) as indicated in Figure 1.

[0070] An insertion mutant can revert, thereby restoring the pathogenicity of the strain. This limits its
15 applicability in a live attenuated vaccine. In that aspect deletion mutants are preferred. *guaB* deletion 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.

20

Example 2: *guaB* deletion mutants

Construction of *guaB* deletion mutants

[0071] *guaB* deletion mutants were created according to the method for generating deletion mutations in the genome
25 of *Escherichia coli* K12 (Datsenko and Wanner, 2000, PNAS 97:6640-5, incorporated by reference herein).

[0072] This method relies on homologous recombination, mediated by the bacteriophage λ Red recombinase system, of a linear DNA fragment generated by PCR.

30 [0073] The *guaB* sequence is hereby substituted by an antibiotic resistance gene. This resistance gene is flanked by FRT sites (FLP recognition target sites) and can be excised from the genome by site-specific recombination, mediated by the FLP recombinase.

- [0074] Overlap PCR (Ho et al., 1989, Gene 77:51-59) was applied to construct a linear fragment. The principle relies on the use of two primer sets, one upstream pair (GuaB3-GuaB4; GuaB3: 5' GGCTGCGATT GGCGAGGTAG TA 3', SEQ ID NO 2; GuaB4: 5' GGTGATCCCG GGCGTCAAAC GTCAGGGCTT CTTTA 3', SEQ ID NO 3) and one downstream pair (GuaB5-GuaB2; GuaB5: 5' TTGACGCCCCG GGATCACCAA AGAGTCCCCG AACTA 3', SEQ ID NO 4; GuaB2: 5' CGTTCAGGCG CAACAGGCCG TTGT 3', SEQ ID NO 1) of the *guaB* gene.
- 10 [0075] Both sets contain primers (GuaB4, GuaB5) that are partially complementary and to which a *SmaI* restriction site was added.
- [0076] After annealing of the resulting complementary sequences and chain elongation, PCR with the outward
15 primers (GuaB6-GuaB7; GuaB6: 5' GCAACAACCTC CTGCTGGTTA 3', SEQ ID NO 5; GuaB7: 5' AGACCGAGGA TCACTTTATC 3', SEQ ID NO 6) generated a fragment with a 6 basepair *SmaI* site replacing an 861 basepair internal segment of the *guaB* coding sequence. This Δ *guaB* fragment was cloned in the
20 vector pUC18 (see Figure 3).
- [0077] The chloramphenicol resistance gene (*cat*) with its flanking FRT sequences was amplified using the primers P1 (5' GTGTAGGCTG GAGCTGCTTC 3', SEQ ID NO 8) and P2 (5' CATATGAATA TCCTCCTTAG 3', SEQ ID NO 9) (Datsenko and
25 Wanner, 2000) and plasmid pKD3 DNA (Datsenko and Wanner, 2000) as a template.
- [0078] This PCR fragment was ligated in the *SmaI* site of the cloned Δ *guaB* fragment. The desired fragment was generated using nested primers (GuaB6-GuaB7).
- 30 [0079] The resulting PCR fragment was electroporated into *S. Enteritidis* phage type 4 strain 76Sa88 (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.

[0080] The chloramphenicol resistant transformants were tested on Minimal A medium and on Minimal A medium
5 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.

[0081] The *S. Enteritidis* Δ guaB::catFRT mutant (SM12) was transformed with the temperature sensitive replication
10 plasmid pCP20 by electroporation. The plasmid pCP20 encodes the FLP recombinase, which recognizes the FRT-sites, to remove the cat gene. The resulting strain was named SM20.

[0082] The PCR fragment in which the deletion is
15 located was obtained using total genomic DNA of the mutant SM20 and the primer combination GuaB6-GuaB7 (see Figure 4).

[0083] The Δ guaB mutation was confirmed by sequencing of this fragment, using the primer GuaB10 (5' AGGAAGTTTG
20 AGAGGATAA 3', SEQ ID NO 7).

[0084] The sequences of all above-mentioned primers are given in Table 1.

[0085] To avoid the presence of possible additional
25 mutations, caused by the expression of the Red recombinase system, an isogenic strain was constructed.

[0086] The Δ guaB::catFRT mutation of the mutant SM12 was transduced by bacteriophage P22 HT int⁻ (Davis, R.W., Botstein D. and Roth, J.R. (1980) In *Advanced Bacterial*
30 *Genetics, A manual for genetic engineering*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) to wild type *S. Enteritidis* 76Sa88. The cat gene was removed using the plasmid pCP20. The resulting strain was called SM69 (deposit number LMG P-21641).

[0087] A Δ guaB mutant of *S. Typhimurium* strain 1491S96 was constructed using the same procedure and the same primers. The resulting strains *S. Typhimurium* Δ guaB::catFRT and *S. Typhimurium* Δ guaB were named SM9 and SM19 respectively. SM86 (having the deposit number LMG P-21646) is the isogenic strain obtained therefrom, after transduction by bacteriophage P22 HT int⁻ lysate from strain SM9 and excision of the cat gene using the plasmid pCP20.

10 [0088] The Δ guaB mutants SM19, SM20, SM86 and SM69 are sensitive to bacteriophage P22. This proves the presence of intact lipopolysaccharides (LPS).

Virulence and protection tests with the guaB deletion mutant SM20 in mice

[0089] The virulence of the mutant SM20 in mice was tested by oral infection of 6-8 week old female BALB/c mice 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).

[0090] 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 the 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.

22

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

[0091] The virulence of the mutants SM69 and SM86 in
5 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.

[0092] The complete data are given in Tables 4-7. These
10 results demonstrate that the Δ *guaB* mutants SM69 and SM86 are strongly attenuated in mice but still show some residual pathogenicity when administered at this high dose. Oral immunization with the mutants induces protective immunity against infection by a high dose of
15 the corresponding pathogenic wild type strain.

Safety evaluation of the *S. Enteritidis* Δ *guaB* deletion mutant SM69 in one-day-old chickens by the intratracheal and oral gavage routes

20 [0093] 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.

[0094] Chicks at one day of age were leg-banded and
25 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 groups 3 and 4 in another isolator.

30 [0095] 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 1.28×10^8 cfu/0.2 ml per bird. Chickens in groups 3 and 4 were administered with 0.2 ml PBS (phosphate buffered

23

saline) per bird by the intratracheal or oral gavage, respectively.

[0096] Following the inoculation of SM69 or PBS, chick mortality was observed daily until 38 days post 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 birds thus died in group 1. In group 2, two birds died in total. One died during inoculation 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.

[0097] This study indicates that the *S. Enteritidis* Δ guaB mutant strain SM69 is not safe when administered at 1.28×10^8 cfu per bird at one day of age by the intratracheal or oral gavage route.

Safety evaluation of the *S. Enteritidis* Δ guaB deletion mutant SM69 in 2-week old chickens by the intratracheal and oral gavage routes

[0098] Safety of the *S. Enteritidis* Δ guaB mutant strain SM69 was then evaluated in 2 week-old specific pathogen free (SPF) 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.

[0099] 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 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

S. Typhimurium AroA⁻ vaccine (Poulvac® ST) by the intratracheal route; and five birds in group 4 were inoculated with PBS by the intratracheal route.

[0100] 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 2.304×10^8 cfu/0.2 ml per bird. Chickens in group 3 were administered with Poulvac® ST by the intratracheal route with 2.19×10^8 cfu/0.2 ml per bird. Chickens in group 4 were administered by the intratracheal route with 0.2 ml PBS per bird.

[0101] After inoculation, the birds from treatment groups 1 and 2 were placed in one isolator and those from groups 3 and 4 in another isolator.

[0102] 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 procedure controls.

[0103] 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, 2.304×10^8 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 2.19×10^8 cfu per bird or in the PBS treated birds, indicating that the study was valid (Table 9).

[0104] 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 a $p < 0.05$. 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.

[0105] There were no significant differences in the
5 final body weight in chickens administered with SM69 by the intratracheal or oral gavage inoculation, Poulvac® ST, or PBS (Table 5). 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
10 initial body weight amongst the four groups since the birds were randomly placed into each of the 4 treatment groups.

[0106] Since no mortality was attributable to the inoculation of SM69, it can be concluded that the *S.*
15 Enteritidis *AguaB* mutant strain, SM69, is safe when administered at the tested titer of 2.304×10^8 cfu/0.2 ml dose per 2-week-old bird, by either the intratracheal or oral gavage inoculation route. SM69 inoculations had no effect on the final body weight of the birds, bird weight
20 being the second safety parameter evaluated here.

[0107] A deposit has been made according to the Budapest Treaty at the BCCM/LMG Culture Collection, Laboratorium voor Microbiologie, K.L. Ledeganckstraat 35,
25 B-9000 Gent (Belgium) for the following micro-organisms: *Salmonella* Enteritidis SM69 under deposit number LMG P-21641 (deposit date: 9 August, 2002) and *S. Typhimurium* SM86 under deposit number LMG P-21646 (deposit date: 28 August, 2002). The deposits have been made in the name of
30 Prof. J.-P. Hernalsteens, previous address: Vrije Universiteit Brussel, Laboratorium Genetische Virologie, Paardenstraat 65, B-1640 Sint-Genesius-Rhode, current address: Vrije Universiteit Brussel, Onderzoeksgroep Genetische Virologie, Pleinlaan 2, B-1050 Brussels,

27

Table 1: Primer sequences

SEQ ID NO	Primer	Sequence
1	GuaB2	5' CGTTCAGGCGCAACAGGCCGTTGT 3'
2	GuaB3	5' GGCTGCCGATTGGCGAGGTAGTA 3'
3	GuaB4	5' GGTGATCCCCGGCGTCAAAACGTCAGGGCTTCTTTA 3'
4	GuaB5	5' TTGACGCCCCGGGATCACCAAGAGTCCCCGAACTA 3'
5	GuaB6	5' GCAACAACCTCCTGCTGGTTA 3'
6	GuaB7	5' AGACCGAGGATCACTTTATC 3'
7	GuaB10	5' AGGAAGTTTGAGAGGATAA 3'
8	P1	5' GTGTAGGCTGGAGCTGCTTC 3'
9	P2	5' CATATGAATATCCTCCTTAG 3'

Table 2: Virulence test in BALB/c mice of the *S. Enteritidis* *guaB* deletion 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> ΔguaB SM20				Disease symptoms between the 7 th and the 14 th day after infection
	5.1×10 ⁸	9/10	13	
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> ΔguaB SM20	1.9×10 ⁸	3/3	/	No disease symptoms

28

Table 3: Challenge of mice vaccinated with the *S. Enteritidis* *guaB* deletion 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	1.5×10 ⁸	0/5	7, 8, 8, 9	Disease symptoms starting on the 5 th day after challenge
Vaccine control: S. Enteritidis ΔaroA SM50	2.5×10 ⁸	S. Enteritidis 76Sa88	1.5×10 ⁸	3/5	9, 13	Disease symptoms between the 7 th and the 14 th day after challenge
S. Enteritidis ΔguaB SM20	5.1×10 ⁸	S. Enteritidis 76Sa88	1.5×10 ⁸	5/5	/	Mice are less active between the 11 th and the 14 th day after challenge.

29

30

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 ΔguaB 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 with the isogenic *S. Enteritidis* *guaB* deletion mutant SM69

Infection		Survival	Day of death	State of the mice
Strain	Dose			
negative control : milk	/	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 <i>guaB</i> SM69	7.6×10^8	5/5	/	Mild symptoms, from day 11 till day 18

Table 5: Challenge of mice vaccinated with the *S. Enteritidis* *guaB* deletion mutant 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> Δ <i>guaB</i> 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

31

Table 6: Virulence experiments with *S. Typhimurium* 1491S96 isogenic mutant strains in BALB/c mice. Oral inoculation with of the mutants of *S. Typhimurium* strain

Infection		Survival	Day of death	state of the mice
Strain	Dose			
First Experiment				
(S.Typhimurium 1491S96)				
Wild type SM2	*	0/3	(9, 9, 10)	Disease symptoms from day 4 onwards
ΔguaB SM86	*	2/5**	(2, 2, 2)	Mild disease symptoms from day 9 until challenge
Negative control: milk	-	4/4		No symptoms
Second Experiment				
Disease symptoms from day 6 onwards				
Wild type SM2	0.8×10^8	1/4	(11, 13, 14)	Weak symptoms on day 13 and 14
ΔguaB SM86	0.8×10^8	5/5		No symptoms
Negative control: milk		5/5		No symptoms

...ulated with approximately 10^8 cells (exact titer not determined)

* inoculated with approximately 10^8 cells (exact titer not determined)

** died after a fight

Table 7: Protection experiments with *S. Typhimurium* isogenic mutant strains in BALB/c mice.
Oral inoculation with approximately 10^8 cells of the *S. Typhimurium* strain 1491S96

Vaccination		Challenge		Survival	Day of death	State of the mice
Strain	Dose	Strain	Dose			
First Experiment						
Δ guaB SM86		<i>S. Typhimurium</i> 1491S96	1.3×10^7	2/2		Mild symptoms until days 14, afterwards one mouse showed clear symptoms, the other was healthy again
Negative control: milk	-	<i>S. Typhimurium</i> 1491S96	1.3×10^7	0/4		Severe symptoms from day 6 onwards
Second Experiment						
Δ guaB SM86	0.8×10^8	<i>S. Typhimurium</i> 1491S96	2.7×10^8	5/5	/	Reduced activity from day 6 till day 16
Negative control: milk	-	<i>S. Typhimurium</i> 1491S96	2.7×10^8	0/5	(8,9,10,11,16)	Severe symptoms from day 6 onwards

* inoculated with approximately 10^8 cells (exact titer not determined)

33

Table 8: Safety evaluation of the *S. Enteritidis* *guaB* deletion mutant SM69 in one-day-old chickens

Infection	Group	N	Titer	Route	Survival	Day of death (DPI)
Strain						
<i>S. Enteritidis</i> SM69	1: SM69-IT	10	1.28x10 ⁸ cfu/0.2 ml	intratracheal	4/10*	0, 2, 3, 5, 13
<i>S. Enteritidis</i> SM69	2: SM69-OG	10	1.28x10 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

* 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 *S. Enteritidis* *guaB* deletion mutant SM69 in 2-week-old chickens

Infection	Group	N	Titer	Route	Survival	Day of death (DPI)	Mean weight (kg)	Std weight
Strain								
<i>S. Enteritidis</i> SM69	1:SM69-IT	10	2.304x10 ⁸ cfu/0.2 ml	IT	9/10*	13	0.429	0.064
<i>S. Enteritidis</i> SM69	2:SM69-OG	10	2.304x10 ⁸ cfu/0.2 ml	OG	10/10	-	0.420	0.044
vaccine control: Poulvac® ST**	3:Poulvac-IT	10	2.19x10 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* AroA⁻ vaccine against *S. Typhimurium*

35

CLAIMS

1. An attenuated mutant strain of bacterium infecting veterinary species, which is incapable of forming
5 de novo guanine nucleotides, wherein said mutant contains a deletion mutation in the *guaB* gene.

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
10 claims, which is a *Salmonella enterica* or a (pathogenic) *Escherichia coli* strain.

4. The mutant strain of any of the preceding claims, wherein said mutant strain encodes and expresses a foreign antigen.

15 5. The mutant strain of any of the preceding claims, which is a *S. Enteritidis* or a *S. Typhimurium* strain.

6. The mutant strain of any of the preceding claims, wherein said mutation is introduced into parent
20 strain *S. Enteritidis* phage type 4 strain 76Sa88.

7. The mutant strain of claim 6 which is *S. Enteritidis* strain SM69 having the deposit number deposit number LMG P-21641.

8. The mutant strain of any of the preceding
25 claims, wherein said mutation is introduced into parent strain *S. Typhimurium* 1491S96.

9. The mutant strain of claim 8 which is *S. Typhimurium* strain SM86 having the deposit number deposit number LMG P-21646.

30 10. A vaccine for immunizing a veterinary species against a bacterial infection comprising:
a pharmaceutically effective or an immunizing amount of a mutant strain according to any of the preceding claims, which is incapable of forming de novo guanine nucleotides

due to a deletion mutation in the *guaB* gene; and a pharmaceutically acceptable carrier or diluent.

11. The vaccine of claim 10, wherein said mutant strain encodes and expresses a foreign antigen.

5 12. The vaccine of claim 10 or 11, wherein said mutant strain comprises a plasmid which encodes and expresses, in a eukaryotic cell, a foreign gene.

10 13. A method of immunizing a veterinary species against a bacterial infection, said method comprising the 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 9 and/or a vaccine according to any of the claims 10 to 12.

15 14. The method of claim 13 wherein the veterinary species is poultry.

15. The method of any of claims 13 or 14 wherein the mutant strain and/or the vaccine is administered via the oral, nasal or parenteral route.

20 16. The attenuated mutant strain according to any of the claims 1 to 9 for use as a medicament.

17. Use of an attenuated mutant strain according to any of the claims 1 to 9 for the preparation of a medicament or a vaccine for the prevention and/or the
25 treatment of an infection by a bacterium in a veterinary species, preferably poultry.

38

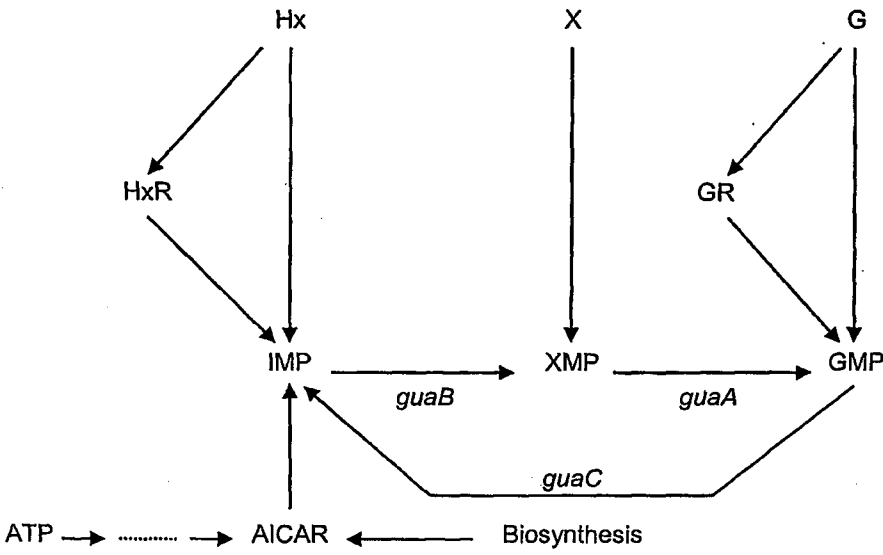


Fig. 1

CTGATCGAACAAAGCCTTCGGCCTGCAGTTTGGCTTTTAGCTGCTCATATTTCTGCTGCAA - 60
 CAAGCCTTCGCCCCGGGGCTGCATACTTTCGGCGATGATTTGATAATCGCCGCGCGGCTC - 120
 GTACAGCGTAATGTTGGCGCGTACCAGCACCTGCTGCCCCTGCTGCGGGCGGAACGTCAC - 180
 CCGGCGATTGCTGTTTACGGAACATCGCACAGCGCACCTGAGCGGTATCGTCTTTGAGCGT - 240
 AAAGTACCAGTGGCCCGACGAGGCTGCGTGAAATTAGAAATCTCGCCGCTGATCCATAC - 300
 CTGTCCCATCTCCTGTTCTAACAGCAGACGAACCGTCTGGTTAAGGCGGCTTACGGTAAA - 360
 AATTGAGGAAGTTTGGAGAGGATAACATGTGAGCGGGATCAAATTTCTAAATCAGCAGGTTA - 420
 TTCAATCGATAGTAACCTGCTCACGGGGGATCGCAAGCACTATTTGCAAAAAAATGTAGA - 480
 TGCAACCGATTACGTTCTGTATAATGCCCGGGGAATATTTATTAACTCCCAGGCGAGAT - 540
 ATTGCCCATGCTACGTATCGCTAAAGAAGCTCTGACGTTTGACGACGTCCTCCTTGTTC - 600
 CGCTCACTCCACCGCTTTTGCCTGAATACTGCCGATCTCAGCACGCGAGTTGACGAAAACTAT - 660
 TCGTCTGAAATATTCCTATGCTTTCTGCGGCGATGGACACCGTGACGGAAGCGCGCCTGGC - 720
 AATTGCCCTGCGCCAGGAAGGCGGCATTGGTTTATCCACAAAAACATGTCTATTGAGCG - 780
 CCAGGCGGAAGAAGTTTCGCGCGGTGAAGAAACAGAGTCCGGCGTAGTGACCGACCCGCA - 840
 GACCGTCTGCAACCAACCACGTTGCATGAAGTGAAAGCCCTGACCGAGCGTAACGGTTT - 900
 TGCGGGCTATCCGGTGGTGACTGAAGATAACGAGCTGGTGGGTATCATCACCGGTCTGTGA - 960
 CGTGCGTTTGTGACTGACCTGAACCAAGCCGTTGAGTGTCTACATGACACCGAAAGAGCG - 1020
 TCTGGTGACCGTTCTGTGAAGGCGAAGCCCGTGAAGTCTGCTGGCAAAAATGCACGAAAA - 1080
 ACGCGTAGAAAAAGCGCTGGTCTGTGATGATAACTTCCATCTGCTTGGCATGATTACCGT - 1140
 AAAAGATTTCCAGAAAGCGGAACGTAAACCAAACTCCTGTAAAGATGAGCAGGGCCGTTT - 1200
 ACGTGTGCGCGCGCGGTGCGGCGCAGGCGCGGCAACGAAGAGCGCGTTGACGCGCTGGT - 1260
 GCGGCGCAGGCGTTGACGTACTGCTGATCGACTCCTCTCACGGTCACTCTGAAGGCGTGT - 1320
 GCAACGTATCCGTGAGACGCGTGCTAAATATCCTGACCTGCAAATCATCGGCGGGAACGT - 1380
 TGCGACGGGCGCAGGCGCTCGCGCACTGGCGGAAGCCGGTTGCAACGCGGTGAAAGTGGG - 1440
 TATCGGCCCGGGTTCCATCTGTACGACTCGTATCGGGACTGGTGTGGGCGTTCCGCGAGAT - 1500
 CACCGCTGTTTCTGACGCGGTGGAAGCGCTGGAAGGCACCGGGATTCCGGTTATCGCTGA - 1560
 CGGCGGTATCCGTTTCTCCGNNNACATCGCCAAAGCCATCGCCGAGGCGCGAGCGCGGT - 1620
 AATGGTGGGTTCCTATGCTGGCCGCTACCGAAGAATCNCCGGGCGAAATCGAACTCTACCA - 1680
 GGGCCGTTCTTACAAATCTTATCGCGGTATGGGTTCTCTGGGCGCGATGTCCAAAGGTTT - 1740
 CTCCGACCGTTACTTCCAGAGCGACAACGCCGCCGACAACTGGTGCCGGAAGGTATCGA - 1800
 AGGCCGCGTAGCCTATAAAGGTCGCTGAAAGAGATCATCACCAGCAGATGGGCGGCCCT - 1860
 GCGCTCCTGTATGGGGCTGACCGGTTGTGCTACCATCGACGAACTGCGTACTAAAGCGGA - 1920
 GTTTGTGCGTATCAGCGGTGCGGGTATCCAGGAAAGCCACGTTACGACGTGACCATCAC - 1980
 CAAAGAGTCCCGAACTACCGTCTGGGCTCCTGATTTTCTTCGCCCCGACCTTCGCGTCGG - 2040
 GCGATTTATTTAATCTGTTTCACTTGCCCTCGGAATAAGCGTCAATGACGGAACATTCA - 2100
 TAAGCATCGCATCCTCATTCTGGACTTCGGTTCTCAGTACACTCAACTGGTTGCGCGCCG - 2160
 CGTGCGTGAGCTGGGTGTTTACTGTGAACTGTGGGCGTGGGATGTGACAGAAGCACAAAT - 2220
 TCGTGACTTCAACCCAAGCGGCATTATTTCTTCCGGCGGCCCGGAAAGCACCACCGAAGA - 2280
 AAACAGCCCGCGCGCGCCGAGTATGTCTTTGAAGCAGGCGTGCCGGTATTTGGCGTTTG - 2340
 CTATGGTATGCAGACCATGGCGATGCAGCTTGGCGGTGATGTAGAAGGTTCTAATGAGCG - 2400
 TGAATTTGGTTATGCGCAGGTGGAAGTGTGACCGACAGCGCGCTGGTTTCGCGGTATTGA - 2460
 AGATTCCTTGACCGCAGACGGCAAACCGCTGCTGGACGTGTGGATGAGCCACGGCGATAA - 2520
 AGTGACGGCGATTCCGTCGACTTCGTGACCGTCGCCAGCACCGAGAGCTGCCCGTTTCGC - 2580
 CATTATGGCCAACGAAGAAAAACGTTCTACGGCGTACAGTTCCACCCGGAAGTGACTCA - 2640
 CACCCGCCAGGGTATGCGCATGCTGGAGCGTTTTGTACGCGATATCTGCCAGTGTGAAGC - 2700
 GTTGTGGACGCCGCGAAGATCATCGATGACGCGGTGGCGCGCATTCGCGAGCAGGTAGG - 2760
 CGACGATAAAGTGATCCTCGGTCTCTCCGGCGCGGTGGATTCTTCCGTACCGCCATGCT - 2820
 GCTGCACCGCGCGATCGGTAAAAATCTGACCTGTGTTTTCTGTCGACAACGGCCTGTTGCC - 2880
 CCTGAACGAAGCCGAGCAGGTGA - 2903 (SEQ ID NO: 10)

Fig. 2

40

quaB3
1 GGCTGCGATTGGCGAGGTAGTAGTCCATCGCCATGCCAGACGCTGCTGGCGGANCTGGA
quaB6
61 TCTGACGCAACAACCTCTGCTGGTTACGGCTGACAATTTCTGCCGCTGCTGATGGCGTAG
121 CGCGCGCAGGTGGCGGACAAAATCAGCTATCGTGACGTCCGTTTCGTGACCGACGGCGCT
181 GACCACCGGAATGCGGCTGGCAAAAATCGCCCGCGCCACGCGTTTCGTGCTTAAACTCCA
241 CAAATCTTCCAGCGAACCGCCCGCGCCGACGATCAGTACATCACATTCGCCCGCGCGC
301 GTTCGCCAGTTCGATAGCACGAACGATCTGCCCCGGCGCGTCGTGCCCCCTGGACTGCGGT
361 TGATAGATAATAACGGGCAGGGATGGGTACGACGCTTTAGCACGTGGAGAAATATCGTG
421 CAGCGCCGCGCGCGTTTTCGAAGTGATACCCCAACGACGTGGGCGGGGAGGGCAACGG
481 CTGTTTATGCTGCTGATCGAACAAGCCTTCGGCCTGCAGTTTGGCTTTTAGCTGCTCATA
541 TTCTGCTGCAACAAGCCTTCGCCCGCGGGCTGCATACTTTGGCGATGATTGATAATC
601 GCCGCGCGGCTCGTACAGCGTAATGTTGGCGCGTACCAGCACCTGCTGCCCGTCTGCGG
661 GCGGAACGTACCCGGCGATTGCTGTTACGGAACATCGCACAGCGCACCTGAGCGGTATC
721 GTCTTTGAGCGTAAAGTACCAGTGGCCCGACGCGAGGCTGCGTGAAATTAGAAATCTCGCC
781 GCTGATCCATACCTGTCCCATCTCTGTTCTAACAGCAGACGAACCGTCTGGTTAAGGCG
quaB10
841 GCTTACGGTAAAAATTGAGGAAGTTTGAGAGGATAACATGTGAGCGGGATCAAATTCTAA
901 ATCAGCAGGTTATTCAATCGATAGTAACCTGCTCACGGGGGATCGCAAGCACTATTTGCA
961 AAAAAATGTAGATGCAACCGATTACGTTCTGTATAATGCCGCGGCAATATTATTAACTT
quaB4
1021 CCCAGGTGAGATATTGCCCATGCTACGTATCGCTAAAGAAGCTCTGACGTTTGACGCCCCG
quaB5
1081 GGATCACCAAAGAGTCCCCGAACCTACCGTCTGGGCTCCTGATTTTCTTCGCCCGACCTTC
1141 GCGTCGGGCGATTATTTAATCTGTTTCACTTGCTCGGAATAAGCGTCAATGACGGAAA
1201 ACATTATAAGCATCGCATCCTCATTCTGGACTTCGGTTCTCAGTACACTCAACTGGTTG
1261 CGCGCCGCGTGCCTGAGCTGGGTGTTTACTGTGAAGTGTGGGCGTGGGATGTGACAGAAG
1321 CACAAATTCGTGACTTCAACCCAAGCGGCATTATTCTTTCCGGCGGCCCGGAAAGCACCA
1381 CCGAAGAAAACAGCCCGCGCGCGCGCGCATATGTCTTTGAAGCAGGCGTGCCGGTATTTG
1441 GCGTTTGCTATGGTATGCAGACCATGGCGATGCAGCTTGGCGGTCTGTAGAAGGTTCTA
1501 ATGAGCGTGAATTTGGTTATGCGCAGGTGGAAGTGTGACCGACAGCGCGCTGGTTTCGCG
1561 GTATTGAAGATTCCCTGACCGCAGACGGCAAACCGCTGCTGGACGTGTGGATGAGCCACG
1621 GCGATAAAGTGACGGCGATTCCGTCCGACTTCGTGACCGTCGCCAGCACCGAGAGCTGCC
1681 CGTTCGCCATTATGGCCAACGAAGAAAAACGCTTCTACGGCGTACAGTTCCACCCGGAAG
1741 TGACTCACACCCGCCAGGGTATGCGCATGCTGGAGCGTTTTGTACGCGATATCTGCCAGT
1801 GTGAAGCGTTGTGGACGCCGCGCAAGATCATCGATGACGCCGTGGCGCGCATTGCGGAGC
quaB7
1861 AGGTAGCGGACGATAAAGTGATCCTCGGTCTCTCCGGCGGCGTGGATTCTTCCGTCACCG
quaB2
1921 CCATGCTGCTGCACCGCGCGATCGGTAAAAATCTGACCTGTGTTTTCGTGCACAACGGCC
1981 TGTTGCGCCTGAACG

(SEQ ID NO: 11)

Fig. 3

41

1 CATGTGTGAGCGGGATCAAATTCTAAATCAGCAGGTTATTCAATCGATAG
51 TAACCTGCTCACGGGGGATCGCAAGCACTATTTGCAAAAAAATGTAGAT
101 GCAACCGATTACGTTCTGTATAATGCCGGCAATATTTATTAACTCCCA
151 GGTGAGATATTCGCCATGTACGTATCGCTAAAGAAGCCCTGACGTTTGAC
P1
201 GCCCCTGTAGGCTGGAGCTGCTTCTGAAGTTCTTACTTTCTAGAGAATA
P2
251 GGAACTCCGGAATAGGAACCTAAGGAGGATATTCATATGGGATCACCAAAG
301 ATCCCCGAACTACCGTCTGGGCTCTGATTTTCTTCGCCCGACCTTCGCG
(SEQ ID NO: 12)

Fig. 4

1 ATTTGCAAAA AAATGTAGAT GCAACCGATT ACGTTCTGTA TAATGCCGCG GCAATATTTA
61 TTAACCTCCC AGGTGAGATA TTGCCCATGC TACGTATCGC TAAAGAAGCC CTGACGTTTG
121 ACGACGTCTT CCTTGTTCCT GCTCACTCCA CCGTTTTGCC GAATACTGCC GATCTCAGCA
181 CGCAGTTGAC GAAACTATT CGTCTGAATA TTCCTATGCT TTCTGCGGCG ATGGACACCG
241 TGACGGAAGC GCGCCTGGCA ATTGCCCTGG CCCAGGAAGG CGGCATTGGT TTTATCCACA
301 AAAACATGTC CATTGAGCGC CAGGCGGAAG AAGTTCGCCG CGTGAAGAAA CACGAGTCCG
361 GCGTAGTGAC CGACCCGCG ACCGTCTGTC CAACCACCAC GTTGCATGAA GTGAAAGCCC
421 TGACCGAGCG TAACGGTTTT GCGGGCTATC CGGTGGTGAC TGAAGATAAC GAGCTGGTGG
481 GTATCATCAC CGGTCTGTGAC GTGCGTTTTG TGAAGTACCT GAACCAGCCG GTTAGCGTTT
541 ACATGACGCC GAAAGAGCGT CTGGTGACCG TTCGTGAAGG CGAAGCCCGT GAAGTCGTGC
601 TGGCAAAAAT GCACGAAAAA CGCGTAGAAA AAGCGCTGGT CGTTGATGAT AACTTCCATC
661 TGCTTGGCAT GATTACCGTA AAAGATTTCC AGAAAGCGGA ACGTAAACCA AACTCCTGCA
721 AAGATGAGCA GGGCCGTTTA CGTGTGCGCG CGGCGGTCCG CGCAGGCGCG GGCACGAAG
781 AGCGCGTTGA CGCGCTGGTG GCGGCAGGCG TTGACGTACT GCTGATCGAC TCCTCTCACG
841 GTCATTCAGA AGGCGTGTG CAACGTATCC GTGAAACCCG TGCTAAATAT CCTGACCTGC
901 AAATCATCGG CGGCAACGTC GCGACAGGCG CAGGCGCTCG CGCACTGGCG GAAGCCGGTT
961 GCAGCGCGGT GAAAGTCGGT ATTGGCCCGG GTTCCATCTG TACCACTCGT ATCGTGACTG
1021 GCGTGGGCGT TCCGAGATC ACCGCTGTTT CTGACGCGAGT TGAAGCGCTG GAAGTACCG
1081 GTATTCCGGT TATCGCTGAC GCGGTATCC GTTCTCTCCG CGACATAGCC AAAGCGATTG
1141 CCGCAGGTGC AAGCGCGGTA ATGGTGGGTT CCATGCTGGC GGGTACGGAA GAATCCCCGG
1201 GCGAAATCGA ACTCTACCA GGCCTTCTT ACAAATCTTA CCGCGGCATG GGCTCGCTGG
1261 GTGCGATGTC CAAAGGTTCC TCCGACCGTT ACTTCCAGAG CGACAACGCC GCTGACAAAC
1321 TGGTGCCGGA AGGTATCGAA GGTGCGGTAG CCTATAAAGG TCGCTGAAA GAGATCATTC
1381 ACCAGCAGAT GGGCGGCCTG CGCTCCTGTA TGGGGCTGAC CGGTTGTGCT ACCATCGACG
1441 AACTGCGTAC TAAAGCGGAG TTTGTGCGTA TCAGCGGTGC GGGCATTGAG GAAAGCCACG
1501 TTCACGACCT GACCATCACC AAAGAGTCCC CGAACTACCG TCTGGGCTCC TGA
(SEQ ID NO: 13)

Fig. 5

5/12

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. Bernalsteens

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

43

WO 2007/106956

PCT/BE2006/000019

6/12

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

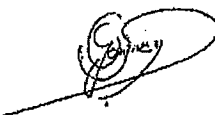
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

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

7/12

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

8/12

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

Fig. 6 (deposit receipt)

9/12

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. Hornalsteens

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:

SX86

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

LMG P-21646

10/12

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

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

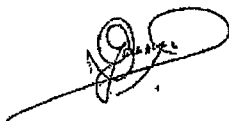
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

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

11/12

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. Bernalsteens

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

I. Depositor:

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

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

49

12/12

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

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. 7 (deposit receipt)

16

PCT/BE2006/000019

0-1	Form PCT/RO/134 (SAFE) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared Using	PCT-SAFE [EASY mode] Version 3.51.002.177 MT/FOP 20060101/0.20.4rc.2.7
0-2	International Application No.	PC T / B E 2 0 0 6 / 0 0 0 0 1 9
0-3	Applicant's or agent's file reference	BPFDAH001AWO

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	20
1-2	line	22
1-3	Identification of deposit	
1-3-1	Name of depositary institution	BCCM Belgian Coordinated Collections of Microorganisms
1-3-2	Address of depositary institution	Federal Public Planning Service Science Policy, 8, rue de la Science, B-1000 Brussels, Belgium
1-3-3	Date of deposit	09 August 2002 (09.08.2002)
1-3-4	Accession Number	BCCM LMG P-21641
1-5	Designated States for Which Indications are Made	all designations
2	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
2-1	page	20
2-2	line	22
2-3	Identification of deposit	
2-3-1	Name of depositary institution	BCCM Belgian Coordinated Collections of Microorganisms
2-3-2	Address of depositary institution	Federal Public Planning Service Science Policy, 8, rue de la Science, B-1000 Brussels, Belgium
2-3-3	Date of deposit	28 August 2002 (28.08.2002)
2-3-4	Accession Number	BCCM LMG P-21646
2-5	Designated States for Which Indications are Made	all designations

FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	yes.
0-4-1	Authorized officer	<i>Harmegnif</i>

51

PCT/BE2006/000019

0-5	This form was received by the International Bureau on:	
0-5-1	Authorized officer	

52

		International application No PCT/BE2006/000019
A. CLASSIFICATION OF SUBJECT MATTER INV. C12N1/20 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 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, 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	WANG JIN YUANG ET AL: "Construction, genotypic and phenotypic characterization, and immunogenicity of attenuated DELTAquaBA Salmonella enterica serovar Typhi strain CVD 915" INFECTION AND IMMUNITY, vol. 69, no. 8, August 2001 (2001-08), pages 4734-4741, XP002409035 ISSN: 0019-9567 cited in the application page 4735; figures 3,4	1-5, 10-12
X	WO 99/58146 A (UNIV MARYLAND [US]) 18 November 1999 (1999-11-18) cited in the application page 34; examples 1,4; table 2 ----- -/-	1-5, 10-12
☒ 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. *G* document member of the same patent family		
Date of the actual completion of the international search 24 November 2006		Date of mailing of the international search report 12/12/2006
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

f3

		International application No PCT/BE2006/000019
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 page 153; tables 1,2	1-17
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, XP002409036 ISSN: 0378-1097 pages 101-2	1-17

76

Information on patent family members			International application No PCT/BE2006/000019		
Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9958146	A	18-11-1999	AT	333285 T	15-08-2006
			AU	754795 B2	28-11-2002
			AU	3740599 A	29-11-1999
			CA	2328056 A1	18-11-1999
			DK	1076566 T3	13-11-2006
			EP	1076566 A1	21-02-2001
			HU	0101747 A2	28-09-2001
			JP	2002514398 T	21-05-2002
			MX	PA00011075 A	24-04-2002
			NO	20005672 A	15-01-2001
			NZ	508110 A	25-10-2002
			PL	344593 A1	05-11-2001
			US	6190669 B1	20-02-2001
			ZA	200006381 A	07-02-2002

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

(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 Patentes:
C12N 1/20 (2006.01) A61K 39/112 (2006.01)
A61K 39/108 (2006.01) A61P 31/04 (2006.01)
- (21) No. Solicitud Internacional: PCT/BE2006/000019
- (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.

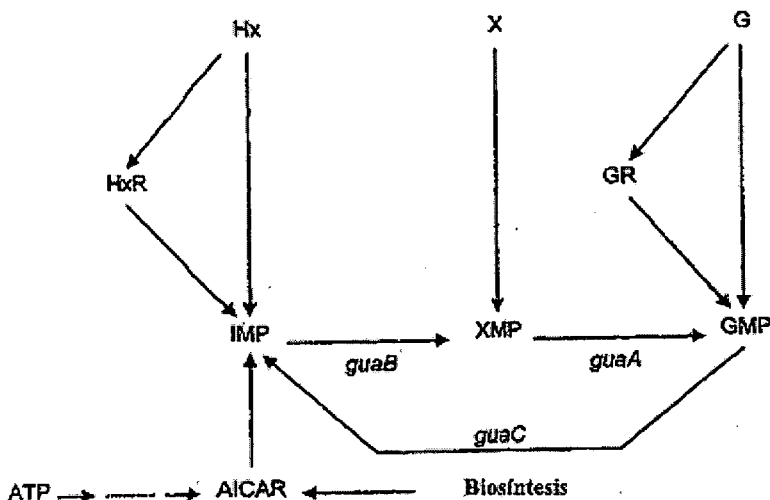
(84) Países designados (a menos que se indique lo contrario para cada clase de protección disponible):
ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasiática (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Europea (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, 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 (una) indicación (es) en relación con el material biológico depositado, proporcionado de conformidad con la Regla 13bis por separado de la descripción
- con el listado de secuencias parte de la descripción publicada separadamente en forma electrónica y disponible bajo petición de la Oficina Internacional.

Para códigos de dos letras y otras abreviaciones, referirse a las "Notas de guía sobre Códigos y Abreviaturas" que aparecen al comienzo de cada edición de la Gaceta del PCT.

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



(57) Extracto: La presente invención se refiere a mutantes atenuadas por eliminación de guaB de una bacteria que infecta especies veterinarias, más en particular, Salmonella enterica, para su uso y producción. La presente invención se refiere a vacunas vivas atenuadas basadas en esos mutantes para la prevención de las infecciones bacterianas, y más en particular Salmonelosis, en especies veterinarias, más en particular, aves de corral.

VACUNA DE SALMONELA CON VIRUS VIVOS ATENUADA

DESCRIPCION

CAMPO DE LA INVENCION

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.

ESTADO DE LA TÉCNICA

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

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.

El 95% de patógenos importantes de *Salmonella* pertenecen a *Salmonella enterica*, con *S. enterica* serovariedad *Typhimurium* (*S. Typhimurium*) y *S. enterica* serovariedad *Enteritidis* (*S. Enteritidis*) como serovariedades más frecuentes.

Las infecciones por *Salmonella* son un problema médico y veterinario grave en todo el mundo y causan preocupación en la industria alimentaria. Los alimentos contaminados no se pueden identificar fácilmente.

El control de la salmonelosis es importante, para evitar las infecciones humanas potencialmente letales y considerables pérdidas económicas para 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.

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

Las vacunas inactivadas 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.

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 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.

Pocas vacunas de *Salmonella* con virus vivos atenuadas están realmente en el mercado porque los resultados con las cepas mutantes atenuadas no han sido siempre buenos.

Por ejemplo, se han vacunado aves de corral con aro- o mutantes de AMPc, incluso muchos pollos muertos poco tiempo después de la vacunación. Como la infección por *Salmonella* con frecuencia se produce muy al principio, es crucial la vacunación de pollos muy jóvenes. Sin embargo, a esta edad éstos son muy sensibles a la *Salmonella*, debido posiblemente a la inmadurez de su sistema inmunitario. Además de la poca protección de los pollos vacunados, se observó a menudo una excreción prolongada de las cepas de la vacuna.

La vacunación de aves con la cepa Megan[®]Vac1, que lleva consigo eliminaciones en los genes *cya* y *crp* (patentes US nº 5.389.368; nº 5.855.879; nº 5.855.880) da como resultado una reducción del número de aves a partir del cual una cepa virulenta de la prueba de provocación con *Salmonella* puede aislarse 7 días después de la prueba de provocación. Sin embargo, esto no proporciona una protección completa (<http://www.meganhealth.com/meganvac.html>).

Existe por lo tanto la necesidad todavía de mejorar las cepas atenuadas de la vacuna de *Salmonella* con virus vivos, y de mejorar las cepas atenuadas de la vacuna con virus vivos de las bacterias que infectan especies veterinarias en general.

La información en la bibliografía sobre la influencia de mutaciones *guaB* sobre la

58

virulencia de *Salmonella* y otras bacterias es escasa.

McFarland y Stocker (1987, *Microbial pathogenesis* 3:129-141) describieron sobre la virulencia reducida de los mutantes por 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*), sin embargo, estos autores describieron una gran letalidad, procedente de la multiplicación de la cepa auxótrofa.

Wang et al. (2001, *Infection and Immunity* 69:4734-4741) describieron una pruebas preclínicas con una vacuna contra la fiebre tifoidea en seres humanos. La vacuna de Wang et al. comprendía un mutante Δ *guaBA* de *Salmonella typhi*. Esta cepa atenuada, sin embargo, presentaba una virulencia residual significativa en ratones.

La solicitud de patente internacional WO 99/58146 y la patente US nº 6.190.669 dan a conocer cepas de la vacuna de *Salmonella typhi* que albergan una mutación Δ *guaBA*, que se utilizan como vector con microbios vivos para administrar xenoantígenos.

OBJETIVOS DE LA INVENCION

Un objetivo de la presente invención consiste en proporcionar cepas de *Salmonella enterica* atenuadas.

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.

Incluso otro objetivo de la presente invención consiste en proporcionar cepas con virus vivos 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.

Aún otro objetivo de la presente invención consiste en proporcionar un método para conseguir un mutante por eliminación de *S. enterica* para su utilización en una vacuna atenuada con virus vivos.

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, aves de corral más en particular.

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

59

SUMARIO DE LA INVENCION

Un primer aspecto de la invención se refiere a una cepa mutante atenuada de *Salmonella enterica* que no puede formar nucleótidos de guanina de nuevo, y en la que dicho mutante contiene una mutación por eliminación en el gen *guaB*.

El principio tal como se demuestra en la presente memoria para *S. enterica* puede aplicarse a cualquier organismo que pueda utilizar el nucleótido de guanina como intermediario. Un aspecto de la invención se refiere por consiguiente a una cepa mutante atenuada de una bacteria que infecta especies veterinarias que contiene una mutación por eliminación en el gen *guaB*. La expresión "especie veterinaria que infecta una bacteria" en el contexto de la invención se refiere en particular a bacterias que son patógenas para especies veterinarias, y que pueden atenuarse por una mutación por eliminación en el gen *guaB*. La especie veterinaria que infecta bacterias puede ser una bacteria Gram-negativa. Para las aves de corral resultan preferidas las bacterias Gram-negativas tales como *Salmonella*, *Pasteurella*, *Escherichia coli*, etc. Son más preferidas *Salmonella enterica* y *E. coli* (patógena). "Patógena para" significa que la bacteria, si no está atenuada, puede producir una enfermedad infecciosa en la especie veterinaria.

Los mutantes de la invención no pueden expresar un producto génico *GuaB* funcional. En otras palabras la función del gen *guaB* está alterada, por lo cual se obtiene una cepa auxótrofa atenuada.

La cepa mutante de la invención, que lleva una mutación por eliminación en el gen *guaB*, no puede crecer en un medio mínimo A, a menos que este medio esté enriquecido con guanina, xantina, guanosina o xantosina 0,3 mM.

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

Preferentemente la mutación por eliminación en el gen *guaB* se introduce en la cepa 76Sa88 del fago tipo 4 *S. Enteritidis* de la cepa progenitora o en la cepa precursora 1491S96 de *S. Typhimurium*.

Una de las cepas atenuadas de *S. enterica* obtenida según la invención es una cepa SM69 de *S. Enteritidis* con el número de registro LMG P-21641. Otro ejemplo es la cepa SM86 de *S. Typhimurium* con el número de registro LMG P-21646.

Las cepas mutantes atenuadas de la invención son muy adecuadas para su

60

utilización en una vacuna atenuada con microbios vivos incluyendo una vacuna polivalente o multivalente. La cepa mutante atenuada de la invención puede codificar y expresar un xenoantígeno y puede como tal utilizarse como vector con microbios vivos y/o como vacuna mediada por el ADN.

En un segundo aspecto de la invención se refiere a una composición farmacéutica o a una vacuna para inmunizar una especie veterinaria contra una infección bacteriana (por ejemplo salmonelosis causada por *Salmonellae*) que comprende:

- una cantidad farmacéuticamente eficaz o inmunizadora de una cepa mutante según la invención, que no puede formar de nuevo nucleótidos de guanina debido a una mutación por eliminación en el gen *guaB*; y
- un vehículo o diluyente farmacéuticamente aceptable. Las composiciones preferidas son las que comprenden una cepa mutante de *Salmonella enterica* según la invención.

La cepa atenuada de la invención puede transferir ADN que codifica a un xenoantígeno en una célula eucariótica. Este xenoantígeno puede ser codificado por un plásmido compuesto por la cepa mutante atenuada de la invención y expresado en el mismo.

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

Las cepas atenuadas de la invención, y las composiciones farmacéuticas o las vacunas que comprenden las mismas, son muy adecuadas para inmunizar un animal o una especie veterinaria contra una infección bacteriana (por ejemplo salmonelosis) posiblemente otras enfermedades (en el caso de una vacuna multivalente).

Otro aspecto de la invención por consiguiente se refiere a un procedimiento de inmunización de animales, preferentemente de especies veterinarias, más preferentemente aves de corral tales como pollos contra una infección por una bacteria (por ejemplo salmonelosis causada por *Salmonellae*), comprendiendo dicho procedimiento

61

la etapa siguiente:

administrar al animal o especie veterinaria necesitado de la misma una cantidad inmunizadora de una cepa mutante atenuada de la invención y/o de una vacuna que comprende la misma, con lo cual se provoca a continuación una respuesta inmunitaria protectora en el animal o especie veterinaria.

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.

En general, se administra una dosis apropiada a estos animales, preferentemente por vía oral, nasal o parenteral.

Un último aspecto 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 infecciosa) tal como la salmonelosis. Los ejemplos de animales o especies veterinarias que van a ser tratados y dosis recomendadas se proporcionan anteriormente.

DESCRIPCIÓN DETALLADA DE LA INVENCION

Se descubrió sorprendentemente que una mutación por eliminación en el gen *guaB* puede conducir a cepas atenuadas de *Salmonella enterica* con virulencia significativamente reducida y que pueden provocar una respuesta inmunitaria en el ganado animal. Dicha eliminación atenuaría cualquier organismo que pueda utilizar el nucleótido guanina como intermediario.

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.

El mutante por eliminación según la invención es en el que está inactivada la enzima IMP deshidrogenasa de la ruta metabólica de la purina (codificada por *guaB*).

Dicha inactivación puede obtenerse mediante una eliminación mediante la cual la función del gen *guaB* se altera, lo que conduce a una función inválida (no se forma ningún producto génico funcional) del gen o genes afectado(s). Un experto en la materia conoce

cómo obtener dichos mutantes y una prueba sencilla puede decir si la función del gen *guaB* está alterada. La cepa mutante que no puede expresar un producto del gen *guaB* funcional no puede desarrollarse en un medio Mínimo A, a menos que este medio esté enriquecido con guanina, xantina, guanosina o xantosina (por ejemplo 0,3 mM).

El objetivo de la invención consiste en proporcionar, entre otras, cepas atenuadas de *S. Enteritidis* y *S. Typhimurium* ya que éstas son las serovariedades más comunes de *S. enterica*.

La presente invención proporciona cepas atenuadas. La invención proporciona entre otras cepas atenuadas de *Salmonella enterica* para su utilización, entre otras, como vacunas activas atenuadas contra la salmonelosis, como vector con microbios vivos y/o como vacunas mediadas por ADN que expresan xenoantígenos. Tal como se utiliza en la presente memoria, un "xenoantígeno" significa un xenoantígeno para la *Salmonella*.

Las vacunas de vector con microbios vivos, denominadas también "vacunas vehículo" y "sistemas de administración de antígenos de virus 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 atenuadas, similares a las cepas atenuadas de *S. enterica* para su utilización en una vacuna con virus vivos posiblemente una vacuna con virus vivos polivalente o multivalente.

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

- una cantidad farmacéuticamente eficaz o un inmunizadora de un mutante de la invención (por ejemplo un mutante de *Salmonella enterica*) que no puede formar de nuevo nucleótidos de guanina, en la que dicho mutante contiene una mutación por eliminación en el gen *guaB*; y
- un vehículo o diluyente farmacéuticamente aceptable.

Otro objetivo de la invención consiste en proporcionar una vacuna con virus vivos

63

de vector que comprende:

- una cantidad farmacéuticamente eficaz o un inmunizadora de un mutante de la invención (por ejemplo un mutante de *Salmonella enterica*), que no puede formar de nuevo nucleótidos de guanina, en la que dicho mutante contiene una mutación en el gen *guaB*, y 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 virus vivos (*S. enterica*) 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 un mutante de la invención (por ejemplo un mutante de *Salmonella enterica*), que no puede formar de nuevo nucleótidos de guanina, en la que dicho mutante contiene una mutación en el gen *guaB*, 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 está incorporada como referencia en la presente memoria en su totalidad. De nuevo, el xenoantígeno específico empleado en la vacuna mediada por ADN no es crítico para la presente invención.

La decisión de si expresar el xenoantígeno por ejemplo, en *S. enterica* (utilizando un activador procariótico en una vacuna de vector con virus vivos) o en las células invadidas por ejemplo por *S. enterica* (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).

En las vacunas de la presente invención, la cantidad farmacéuticamente eficaz o la

cantidad inmunizadora de los mutantes de la presente invención que debe administrarse variará dependiendo de la edad, el peso y el sexo del paciente. "Cantidad inmunizadora" tal como se utiliza en la presente memoria significa de hecho una cantidad que es capaz de provocar una respuesta inmunitaria en el animal que recibe la composición/vacuna farmacéutica. La respuesta inmunitaria provocada puede ser una respuesta humoral, de las mucosas, local y/o celular.

El vehículo o diluyente específico farmacéuticamente aceptable empleado no es crítico para la presente invención y son convencionales en la 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; por ejemplo sacarosa; o polividona.

Los mutantes por eliminación según la invención han sido creados mediante técnicas de recombinación homólogas convencionales, por lo que parte del gen *guaB*, por ejemplo parte de la secuencia de codificación de *guaB*, en la primera etapa está sustituida por un gen con resistencia y secuencias de FRT adyacentes.

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).

Un ejemplo específico de la invención se refiere por ejemplo a mutantes por eliminación de *guaB* de *S. Enteritidis* que comprenden un gen *guaB* mutado o la secuencia de codificación que comprende SEC. ID. nº: 12.

BREVE DESCRIPCIÓN DE LOS DIBUJOS

La Figura 1 es una representación esquemática de la serie de reacciones de la biosíntesis del monofosfato de guanosina (*adaptada de Zalkin y Nygaard, 1996, en "Escherichia coli and Salmonella, Cellular and Molecular Biology, Second edition", 1996 F.C. Neidhardt ed. ASM Press, Washington D.C., vol. 1, ch. 34:561-579*). AICAR: 5'-fosforribosil-4-carboxamida-5-aminoimidazol; ATP: trifosfato de adenosina; G: guanina;

6✓

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: 10). El codón de iniciación **ATG** y el codón de terminación **TGA** del gen *guaB* están en negrita.

La Figura 3 representa la secuencia del fragmento Δ *guaB* de *S. Enteritidis* clonado en pUC18 (SEC. ID. nº: 11). 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: 12). 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. nº: 13). El codón de iniciación **ATG** y el codón de terminación **TGA** del gen *guaB* están en negrita.

Las Figuras 6 y 7 representan las recetas del depósito de SM69 y SM86 respectivamente.

La invención se describirá con mayor detalle en los ejemplos y las formas de realización siguientes haciendo referencia a los dibujos adjuntos. Las formas de realización y ejemplos específicos no pretenden de ninguna manera limitar el alcance de la invención reivindicada.

EJEMPLOS

EJEMPLO 1: la mutación auxótrofa 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,

66

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 microbios 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.

EJEMPLO 2: mutantes por eliminación de *guaB*

Construcción de los mutantes por eliminación de *guaB*

Se crearon mutantes por eliminación de *guaB* según el procedimiento para generar mutaciones por eliminación en el genoma K12 de *Escherichia coli* (Datsenko y Wanner, 2000, PNAS 97:6640-6645, incorporada como referencia en la presente memoria).

Este procedimiento se basa en la recombinación homóloga, mediada por el sistema bacteriófago λ Red recombinasa, de un fragmento lineal de ADN generado por PCR.

La secuencia *guaB* se sustituye de este modo por un gen con resistencia a antibióticos. Este gen con resistencia es adyacente a las secuencias de FRT (secuencias diana de reconocimiento de FLP) y puede escindirse del genoma por recombinación específica para el sitio, mediada por la FLP recombinasa.

Se aplicó PCR por solapamiento (Ho et al., 1989, Gene 77:51-59) para construir un fragmento lineal. El principio se basa en la utilización de dos series de cebador, un par corriente arriba (GuaB3-GuaB4; GuaB3: 5' GGCTGCGATT GGCGAGGTAG TA 3', SEC. ID. nº: 2; GuaB4: 5' GGTGATCCCG GGCGTCAAAC GTCAGGGCTT CTTTA 3', SEC. ID. nº: 3) y un par corriente abajo (GuaB5-GuaB2; GuaB5: 5' TTGACGCCCG GGATCACCAA AGAGTCCCGG AACTA 3', SEC. ID. nº: 4; GuaB2: 5' CGTTCAGGCG CAACAGGCCG TTGT 3', SEC. ID. nº: 1) del gen *guaB*.

Ambas series contienen cebadores (GuaB4 y GuaB5) que son parcialmente

67

complementarios y a los cuales se añade una secuencia de restricción *Sma*I.

Tras la hibridación de las secuencias complementarias resultantes y la elongación de la cadena, la PCR con los cebadores externos (GuaB6-GuaB7; GuaB6: 5' GCAACAACCTC CTGCTGGTTA 3', SEC. ID. nº: 5; GuaB7: 5' AGACCGAGGA TCACTTTATC 3', SEC. ID. nº: 6) generó un fragmento con una secuencia *Sma*I de 6 pares de bases que sustituye a un 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).

El gen con resistencia al cloranfenicol (*cat*) con sus secuencias FRT adyacentes se amplió utilizando los cebadores P1 (5' GTGTAGGCTG GAGCTGCTTC 3', SEC. ID. nº: 8) y P2 (5' CATATGAATA TCCTCCTTAG 3', SEC. ID. nº: 9) (Datsenko y Wanner, 2000) y el ADN plásmido pKD3 (Datsenko y Wanner, 2000) como plantilla.

Este fragmento de PCR se ligó en la secuencia *Sma*I del fragmento Δ *guaB* clonado. El fragmento deseado se generó utilizando cebadores anidados (GuaB6-GuaB7).

El fragmento por PCR resultante se electroporó en la cepa 76Sa88 tipo 4 del fago *S. Enteritidis* (una cepa clínica procedente de un pavo, obtenido 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.

Se probaron transformantes resistentes al cloranfenicol en medio Mínimo A y en medio Mínimo A enriquecido con guanina 0,3 mM. Los mutantes Δ *guaB::catFRT* se confirmaron por PCR utilizando las siguientes combinaciones de cebador: GuaB6-GuaB7, GuaB6-P2, GuaB7-P1 y P1-P2.

El mutante Δ *guaB::catFRT* de *S. Enteritidis* (SM12) se transformó con el plásmido pCP20 de replicación sensible a la temperatura por electroporación. El plásmido pCP20 codifica la FLP recombinasa, que reconoce las secuencias FRT, para eliminar el gen *cat*. La cepa 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 (véase la Figura 4).

La mutación Δ *guaB* se confirmó por secuenciado de este fragmento, utilizando el cebador GuaB10 (5' AGGAAGTTTG AGAGGATAA 3', SEC. ID. nº: 7).

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

68

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.) para 76Sa88 de *S. Enteritidis* natural. Se eliminó el gen *cat* utilizando el plásmido pCP20. La cepa resultante se denominó SM69 (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. Las cepas resultantes $\Delta\text{guaB}::\text{catFRT}$ de *S. Typhimurium* y ΔguaB de *S. Typhimurium* se denominaron SM9 y SM19 respectivamente. SM86 (con el número de depósito LMG P-21646) es la cepa isógena obtenida de la misma, después de la transducción por el lisado del bacteriófago P22 HT *int* de la cepa SM9 y la escisión del gen *cat* utilizando el plásmido pCP20.

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

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

Se probó la virulencia del mutante SM20 en ratones por infección oral de ratones BALB/c hembra de 6 a 8 semanas de vida 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 ΔaroA de *S. Enteritidis* se incluyó en el experimento como referencia de la vacuna. Este mutante lleva una eliminación precisa de la secuencia de codificación *aroA* completa y se construyó por el procedimiento de Datsenko y Wanner (2000).

Los datos completos se dan proporcionan en las Tablas 2 y 3. Estos resultados demuestran que el mutante SM20 de ΔguaB 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*.

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

La virulencia de los mutantes SM69 y SM86 en ratones se probó por infección oral de ratones BALB/c hembra de 6 a 8 semanas de vida. Esto se realizó como se describe a continuación. Las cepas 76Sa88 de *S. Enteritidis* natural y la 1491S96 de *S. Typhimurium* se probaron en paralelo como referencias positivas.

Los datos completos se proporcionan en las Tablas 4 a 7. Estos resultados demuestran que los mutantes SM69 y SM86 de Δ guaB están muy atenuados en ratones pero todavía presentan alguna patogenicidad residual cuando se administran a esta dosis elevada. La inmunización oral con los mutantes produce inmunidad protectora contra infección por una dosis elevada de la correspondiente cepa natural patógena.

Evaluación de seguridad del mutante SM69 por eliminación de *AguaB* de *S. Enteritidis* en pollos de un día de vida por las vías intratraqueal y alimentación forzada por vía oral

El objetivo de este estudio fue evaluar la seguridad de la cepa patrón SM69 mutante de Δ guaB 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.

A los pollos de los grupos 1 y 2 se les inoculó 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 $1,28 \times 10^8$ cfu/0,2 ml por ave. A los pollos en los grupos 3 y 4 se les administró 0,2 ml de PBS (solución salina tamponada con fosfato) por ave por vía intratraqueal o alimentación forzada por vía oral, respectivamente.

70

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 5º 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 ΔguaB de *S. Enteritidis* no es segura cuando se administra a razón de $1,28 \times 10^8$ cfu por ave con un día de vida por vía intratraqueal o por alimentación forzada por vía oral.

Evaluación de la seguridad del mutante SM69 por eliminación de ΔguaB de *S. Enteritidis* en pollos de 2 semanas de vida por las vías intratraqueal y de alimentación forzada por vía oral

Se evaluó a continuación la seguridad de la cepa SM69 mutante de ΔguaB de *S. Enteritidis* en pollos sin patógeno específico (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 $2,304 \times 10^8$ cfu/0,2 ml por ave. A los pollos del grupo 3 se les administró $2,19 \times 10^8$ cfu/0,2 ml por ave de Poulvac® ST por vía intratraqueal. A los pollos del grupo 4 se les administró 0,2 ml de PBS por ave por vía intratraqueal.

71

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, $2,304 \times 10^8$ 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 $2,19 \times 10^8$ 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 5). 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 4 grupos de tratamiento.

Ya que ninguna mortalidad era atribuible a la inoculación de SM69, puede sacarse en conclusión que la cepa mutante SM69 de Δ guaB de *S. Enteritidis* es segura cuando se administra a un valor probado de $2,304 \times 10^8$ cfu/0,2 ml de dosis para aves de 2 semanas de vida, ya sea por vía de inoculación intratraqueal o por alimentación forzada por vía oral. Las inoculaciones de SM69 no tuvieron efecto sobre el peso corporal final de las aves, siendo el peso del ave el segundo parámetro de seguridad evaluado en la presente memoria.

72

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) y SM86 de *S. Typhimurium* con el número de depósito LMG P-21646 (fecha de depósito: 28 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 Sint-Genesius-Rhode, dirección actual: Vrije Universiteit Brussel, Onderzoeksgroep Genetische Virologie, Pleinlaan 2, B-1050 Bruselas, Bélgica.

Tabla 1: Secuencias de los cebadores

SEC. ID. nº:	Cebador	Secuencia
1	GuaB2	5' CGTTCAGGCGCAACAGGCCGTTGT 3'
2	GuaB3	5' GGCTGCGATTGGCGAGGTAGTA 3'
3	GuaB4	5' GGTGATCCCCGGCGGTCAAACGTCAGGGCTTCTTTA 3'
4	GuaB5	5' TTGACGCCCGGGGATCACCAAAGAGTCCCCCGAACTA 3'
5	GuaB6	5' GCAACAACCTCCTGCTGGTTA 3'
6	GuaB7	5' AGACCGAGGATCACCTTTATC 3'
7	GuaB10	5' AGGAAGTTTGAGAGGATAA 3'
8	P1	5' GTGTAGGCTGGAGCTGCTTC 3'
9	P2	5' CATATGAATATCCTCCTTAG 3'

73

Tabla 2: Prueba de virulencia en ratones BALB/c del mutante SM20 por eliminación de *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 <i>S. Enteritidis</i>	2,1×10 ⁸	0/5	7, 7, 8, 8, 9	
referencia de la vacuna: ΔaroA SM50 de <i>S. Enteritidis</i>	2,5×10 ⁸	10/10	/	Sin síntomas de enfermedad
ΔguaB SM20 de <i>S. Enteritidis</i>	5,1×10 ⁸	9/10	13	Síntomas de enfermedad entre el 7° y el 14° día tras la infección
Segundo experimento				
referencia negativa: leche	/	4/4	/	Sin síntomas de enfermedad
referencia positiva: 76Sa88 de <i>S. Enteritidis</i>	1,4×10 ⁸	0/3	8, 9, 9	
referencia de la vacuna: ΔaroA SM50 de <i>S. Enteritidis</i>	2,1×10 ⁸	3/3	/	Sin síntomas de enfermedad
ΔguaB SM20 de <i>S. Enteritidis</i>	1,9×10 ⁸	3/3	/	Sin síntomas de enfermedad

74

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>S. Enteritidis</i>	1,5×10 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>	2,5×10 ⁸	76Sa88 de <i>S. Enteritidis</i>	1,5×10 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>	5,1×10 ⁸	76Sa88 de <i>S. Enteritidis</i>	1,5×10 ⁸	5/5	/	Los ratones son menos activos entre el día 11º y el 14º tras la prueba de provocación

77

Tabla 3 (Continuación)

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

Tabla 4: Prueba de virulencia en ratones BALB/c con el mutante SM69 por eliminación de *guaB* de *S. Enteritidis*

Infección		Supervivencia	Día de muerte	Estado de los ratones
Cepa	Dosis			
referencia negativa: leche	/	4/4	/	Asintomático
referencia positiva: 76Sa88 de <i>S. Enteritidis</i>	$3,7 \times 10^8$	0/3	7, 8, 9	Síntomas graves después del 5º día
Δ <i>guaB</i> SM69 de <i>S. Enteritidis</i>	$7,6 \times 10^8$	5/5	/	Síntomas leves, a partir del 11º día hasta el 18º día

Tabla 5: Prueba de provocación de los ratones vacunados con el mutante SMS69 por eliminación de *guaB* 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 <i>S. Enteritidis</i>	$3,1 \times 10^8$	0/4	8, 8, 8, 9	Síntomas graves del 5º día en adelante
Δ <i>guaB</i> SM69 de <i>S. Enteritidis</i>	$7,6 \times 10^8$	Cepa 76Sa88 natural de <i>S. Enteritidis</i>	$3,1 \times 10^8$	2/5	8, 8, 19	Síntomas graves del 5º día en adelante

27

Tabla 6: Experimentos de virulencia con cepas mutantes isógenas de *S. Typhimurium* en ratones BALB/c.
Inoculación oral con de los mutantes de la cepa de *S. Typhimurium*

Infección		Supervivencia	Día de muerte	Estado de los ratones
Cepa	Dosis			
(S. Typhimurium 1491S96)				
Primer experimento				
SM2 natural	*	0/3	(9, 9, 10)	Sin síntomas de enfermedad del 4° día en adelante
Δ guaB SM86	*	2/5**	(2, 2, 2)	Síntomas leves de enfermedad a partir del 9° día hasta la prueba de provocación
Referencia negativa: leche	-	4/4		Sin síntomas
Segundo experimento				
SM2 natural	$0,8 \times 10^8$	1/4	(11, 13, 14)	Sin síntomas de enfermedad a partir del 6° día en adelante
Δ guaB SM86	$0,8 \times 10^8$	5/5		Síntomas débiles los días 13° y 14°
Referencia negativa: leche		5/5		Sin síntomas

* inoculada con aproximadamente 10^8 células (valor exacto no determinado)
 ** muertos tras una lucha

78

Tabla 7: Experimentos de protección con cepas mutantes isógenas de *S. Typhimurium* en ratones BALB/c. Inoculación oral con aproximadamente 10⁸ células de la cepa 1491S96 de *S. Typhimurium*

Vacunación		Prueba de provocación		Supervivencia	Día de muerte	Estado de los ratones
Cepa 1491S96 de S. Typhimurium	Dosis	Cepa	Dosis			
Primer experimento						
ΔguaB SM86	*	1491S96 de S. Typhimurium	1,3×10 ⁷	2/2		Síntomas leves hasta 14 días, después un ratón presentó síntomas evidentes, el otro sanó de nuevo
referencia negativa: leche	-	1491S96 de S. Typhimurium	1,3×10 ⁷	0/4		Síntomas graves a partir del 6° día en adelante
Segundo experimento						
ΔguaB SM86	0,8 x 10 ⁸	1491S96 de S. Typhimurium	2,7×10 ⁸	5/5	/	Actividad reducida a partir del 6° día hasta el 16° día
referencia negativa: leche	-	1491S96 de S. Typhimurium	2,7×10 ⁸	0/5	(9, 9, 10, 11, 16)	Síntomas graves a partir del 6° día en adelante

* inculada con aproximadamente 10⁸ células (valor exacto no determinado)

Tabla 8: Evaluación de seguridad del mutante SM69 por eliminación de *guaB* 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	1,28×10 ⁸ cfu/0,2 ml	intratraqueal	4/10*	0, 2, 3, 5, 13
SM69 de <i>S. Enteritidis</i>		2: SM69-OG	10	1,28×10 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

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.

80

Tabla 9: Evaluación de seguridad del mutante SM69 por eliminación de *guaB* de *S. Enteritidis* en pollos de 2 semanas de vida

Infección	Grupo	N	Valor	Vía	Supervivencia	Día de muerte (DPI)	Peso medio (kg)	Peso estándar
Cepa								
SM69 de <i>S. Enteritidis</i>	1: SM69-IT	10	2,304×10 ⁸ cfu/0,2 ml	IT	9/10*	13	0,429	0,064
SM69 de <i>S. Enteritidis</i>	2: SM69-OG	10	2,304×10 ⁸ cfu/0,2 ml	OG	10/10	-	0,420	0,044
referencia de la vacuna: Poulvac® ST**	3: Poulvac-IT	10	2,19×10 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*

P/

REIVINDICACIONES

- 5 1. Cepa mutante atenuada de una bacteria que infecta una especie veterinaria, que no puede formar nucleótidos de guanina de nuevo, en la que dicho mutante contiene una mutación por eliminación en el gen *guaB*.
2. Cepa mutante según cualquiera de las reivindicaciones anteriores, en la que la especie veterinaria son las aves de corral.
- 10 3. Cepa mutante según cualquiera de las reivindicaciones anteriores, que es una cepa de *Salmonella enterica* o de *Escherichia coli* (patógena).
4. Cepa mutante según cualquiera de las reivindicaciones anteriores, en la que dicha cepa mutante codifica y expresa un xenoantígeno.
- 15 5. Cepa mutante según cualquiera de las reivindicaciones anteriores, que es una cepa de *S. Enteritidis* o de *S. Typhimurium*.
- 20 6. Cepa mutante según cualquiera de las reivindicaciones anteriores, en la que dicha mutación se introduce en la cepa 76Sa88 del fago tipo 4 de *S. Enteritidis* de la cepa progenitora.
- 25 7. Cepa mutante según la reivindicación 6, que es la cepa SM69 de *S. Enteritidis* con el número de depósito LMG P-21641.
8. Cepa mutante según cualquiera de las reivindicaciones anteriores, en la que dicha mutación se introduce en la cepa progenitora 1491S96 de *S. Typhimurium*.
- 30 9. Cepa mutante según la reivindicación 8, que es la cepa SM86 de *S. Typhimurium* con el número de depósito LMG P-21646.
- 35 10. Vacuna para inmunizar una especie veterinaria contra una infección bacteriana que comprende: una cantidad farmacéuticamente eficaz o inmunizadora de una cepa mutante según cualquiera de las reivindicaciones anteriores, que no puede formar de nuevo nucleótidos de guanina debido a una mutación por eliminación en el

83

gen *guaB*; y un vehículo o diluyente farmacéuticamente aceptable.

5 11. Vacuna según la reivindicación 10, en la que dicha cepa mutante codifica y expresa un xenoantígeno.

12. Vacuna según la reivindicación 10 u 11, en la que dicha cepa mutante comprende un plásmido que codifica y expresa, en una célula eucariótica, un gen extraño.

10 13. Procedimiento de inmunización de una especie veterinaria contra una infección bacteriana, comprendiendo dicho procedimiento la etapa que consiste en: administrar a una especie veterinaria necesitada de la misma una cantidad inmunizadora de una cepa mutante según cualquiera de las reivindicaciones 1 a 9 y/o una vacuna según cualquiera de las reivindicaciones 10 a 12.

15 14. Procedimiento según la reivindicación 13, en el que la especie veterinaria son las aves de corral.

20 15. Procedimiento según cualquiera de las reivindicaciones 13 ó 14, en el que la cepa mutante y/o la vacuna se administra por vía oral, nasal o parenteral.

16. Cepa mutante atenuada según cualquiera de las reivindicaciones 1 a 9, para su utilización como medicamento.

25 17. Utilización de una cepa mutante atenuada según cualquiera de las reivindicaciones 1 a 9, para la preparación de un medicamento o una vacuna para la prevención y/o el tratamiento de una infección por una bacteria en una especie veterinaria, preferentemente las aves de corral.

30

84

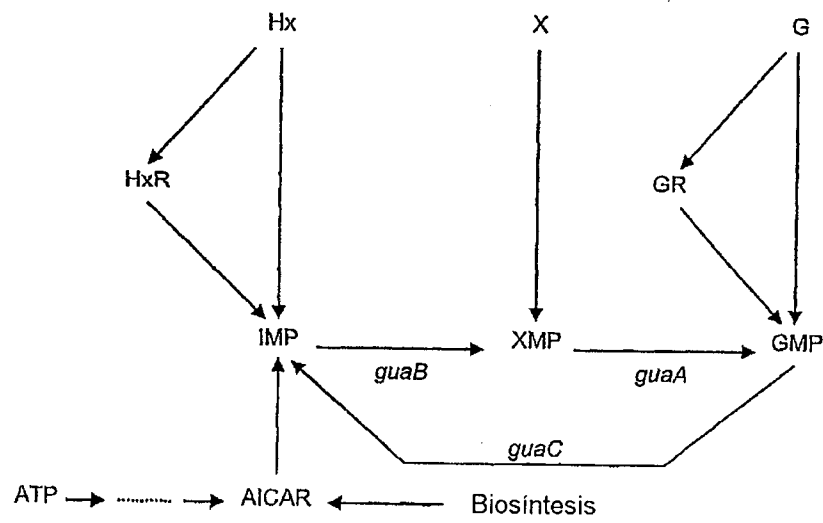


Fig. 1

CTGATCGAACAAGCCTTCGGCCTGCAGTTTGGCTTTTAGCTGCTCATATTTCTGCTGCAA - 60
CAAGCCTTCGCCCGCGGGCTGCATACTTTCGGCGATGATTTGATAATCGCCGCGCGGCTC - 120
GTACAGCGTAATGTTGGCGCGTACCAGCACCTGCTGCCCGTCTGCGGGCGGAACGTCAC - 180
CCGGCGATTGCTGTTACGGAACATCGCACAGCGCACCTGAGCGGTATCGTCTTTGAGCGT - 240
AAAGTACCAGTGGCCCCGACGCGAGGCTGCGTGAATTAGAAATCTCGCCGCTGATCCATAC - 300
CTGTCCCATCTCCTGTTCTAACAGCAGACGAACCGTCTGGTTAAGGCGGCTTACGGTAAA - 360
AATTGAGGAAGTTTGAGAGGATAACATGTGAGCGGGATCAAATTTCTAAATCAGCAGGTTA - 420
TTCAATCGATAGTAACCTGCTCACGGGGATCGCAAGCACTATTTGCAAAAAAATGTAGA - 480
TGCAACCGATTACGTTCTGTATAATGCCGCGGGAATATTTATTAACCTCCAGGCGAGAT - 540
ATTGCCCATGCTACGTATCGCTAAAGAAGCTCTGACGTTTGACGACGTCCCTTGTTC - 600
CGTCACTCCACCGTTTTCGCCGAATACTGCCGATCTCAGCACGCGAGTTGACGAAAATAT - 660
TCGCTGGAATATTCCTATGCTTTCTGCGGCGATGGACACCGTGACGGAAGCGCGCTGGC - 720
AATTGCCCTGGCCCGAGGAAGCGCGCATTTGGTTTATCCACAAAAACATGCTTATTGAGCG - 780
CCAGGCGGAAGAAGTTTCGCCGCGTGAAGAAACAGAGTCCGGCGTAGTGACCGACCCGCA - 840
GACCGTCTGCCAACCACCACGTTGCATGAAGTGAAGCCCTGACCGAGCGTAACGGTTT - 900
TGCGGGCTATCCGGTGGTGACTGAAGATAACGAGCTGGTGGGTATCATCACCGGTCTGTA - 960
CGTGCGTTTGTGACTGACCTGAACAGCCGCTGAGTGTCTACATGACACCGAAAGAGCG - 1020
TCTGGTGACCGTTCTGTAAGGCGAAGCCCGTGAAGTCTGCTGGCAAAAAATGCACGAAAA - 1080
ACGCGTAGAAAAAGCGCTGGTTCGTTGATGATAACTTCCATCTGCTTGGCATGATTACCGT - 1140
AAAAGATTTCCAGAAAGCGGAACGTAAACCAAATCTCTGTAAGATGAGCAGGGCCGTTT - 1200
ACGTGTCGGCGCGCGGTCGGCGCAGGCGCGGGCAACGAAGAGCGCTTGACGCGCTGGT - 1260
GGCGGCAGGCGGTTGACGTAAGTCTGCTGACTCTCTCACGCTCACTCTGAAGGCGTGT - 1320
GCAACGTATCCGTGAGACGCGTGCTAAATATCTGACCTGCAATCATCGCGGGGAACGT - 1380
TGCGACGGGCGCAGGCGCTCGCGCACTGGCGGAAGCCGGTTGCAACGCGGTGAAAGTGGG - 1440
TATCGGCCCGGGTTCCATCTGTACGACTCGTATCGGGACTGGTGTGGGCGTTCCGCGAGAT - 1500
CACCGCTGTTTCTGACGCGGTGGAAGCGCTGGAAGGCACCGGGATTCCGGTTATCGCTGA - 1560
CGGCGGTATCCGTTTCTCCGNNACATCGCCAAAGCCATCGCCGAGGCGGAGCGCGGT - 1620
AATGGTGGGTTCTATGCTGGCCGGTACCGAAGAATCNC CGGCGGAAATCGAACTCTACCA - 1680
GGGCGGTTCTTACAAATCTTATCGCGGTATGGGTTCTCTGGGCGCGATGTCCAAAGGTTT - 1740
CTCCGACCGTTACTTCCAGAGCGACAACGCCCGGACAACTGGTGCCGGAAGGTATCGA - 1800
AGGCCGCGTAGCTATATAAGGTGCGCTGAAAGAGATCATTACAGCAGATGGGCGGCCT - 1860
GCGCTCCTGTATGGGGCTGACCGGTTGTGCTACCATCGACGAACTGCGTACTAAAGCGGA - 1920
GTTTGTGCTATCAGCGGTGCGGTATCCAGGAAGCCACGTTACAGACGTGACCATCAC - 1980
CAAAGAGTCCCCGAATACCGTCTGGGCTCCTGATTTTCTTCGCCCCGACCTTCGCGTCGG - 2040
GCGATTTATTTAATCTGTTTCACTTGCCCTCGGAATAAGCGTCAATGACGGAACATTCA - 2100
TAAGCATCGCATCTCTATTCTGGACTTCGGTTCTCAGTACACTCAACTGGTTGCGCGCCG - 2160
CGTGCGTGAGCTGGGTGTTTACTGTGAAGTGTGGGCGTGGGATGTGACAGAAGCACAAAT - 2220
TCGTGACTTCAACCAAGCGGCATTATTCTTTCCGGCGGCCCGGAAAGCACCACCGAAGA - 2280
AAACAGCCCGCGCGCGCGCAGTATGTCTTTGAAGCAGGCGTGCCCGGTATTTGGCGTTT - 2340
CTATGGTATGCAGACCATGGCGATGCAAGCTTGGCGGTATGTAGAAAGGTTCTAATGAGCG - 2400
TGAATTTGGTTATGCGCAGGTCGAAGTGTGACCGACAGCGCGCTGGTTGCGGTTATTGA - 2460
AGATTCCTTGACCGCAGACGGCAAACCGCTGCTGGACGTGTGGATGAGCCACGGCGATAA - 2520
AGTGACGGCGATTCCGTCCGACTTCGTGACCGTCCGCGAGCAGAGCTGCCCGTTCCG - 2580
CATTATGGCCAACGAAGAAAAACGCTTCTACGGCGTACAGTTCCACCCGGAAGTGAATCA - 2640
CACCCGCCAGGGTATGCGCATGCTGGAGCGTTTGTACGCGATATCTGCCAGTGTGAAGC - 2700
GTTGTGGACGCCGCGGAAGATCATCGATGACCGCGTGGCGCGCATTCGCGAGCAGGTAGG - 2760
CGACGATAAAGTGATCCTCGGCTCTCCGGCGGCGTGGATTCTTCCGTACCGCCATGCT - 2820
GCTGCACCGCGCGATCGGTAAAAATCTGACCTGTGTTTTCTCGCAACGGCCTGTTGCG - 2880
CCTGAACGAAGCCGAGCAGGTGA -2903 (SEC ID n° 10)

Fig. 2

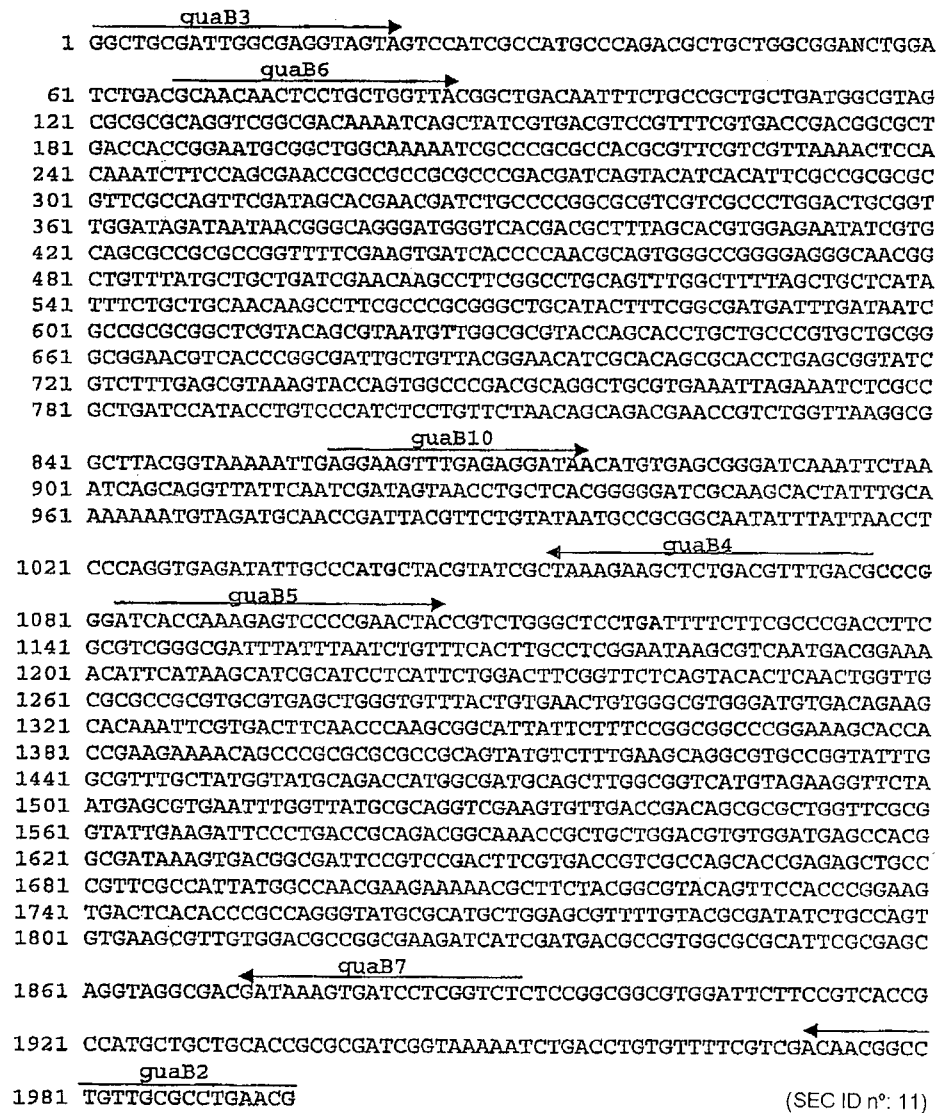


Fig. 3

87

1 CATGTGTGAGCGGGATCAAATTCTAAATCAGCAGGTTATTCAATCGATAG
51 TAACCTGCTCACGGGGGATCGCAAGCACTATTTGCAAAAAAATGTAGAT
101 GCAACCGATTACGTTCTGTATAATGCCCGCAATATTTATTAACCTCCCA
151 GGTGAGATATTGCGCATGTACGTATCGCTAAAGAAGCCCTGACGTTTGAC
P1
201 GCCCCTGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATA
P2
251 GGAACCTCCGGAATAGGAACTAAGGAGGATATTCATATGGGATCACCAAAG
301 ATCCCCGAACTACCGTCTGGGCTCTGATTTTCTTCGCCCACCTTCGCG
(SEC ID n°: 12)

Fig. 4

1 ATTTGCAAAA AAATGTAGAT GCAACCGATT ACGTTCTGTA TAATGCCGCG GCAATATTTA
61 TTAACCTCCC AGGTGAGATA TTGCCCATGC TACGTATCGC TAAAGAAGCC CTGACGTTTG
121 ACGACGTCCT CCTTGTTCCT GCTCACTCCA CCGTTTTCGC GAATACTGCC GATCTCAGCA
181 CGCAGTTGAC GAAAACTATT CGTCTGAATA TTCCTATGCT TTCTGCGGCG ATGGACACCG
241 TGACGGAAGC GCGCCTGGCA ATTGCCCTGG CCCAGGAAGG CGGCATTGGT TTTATCCACA
301 AAAACATGTC CATTGAGCGC CAGGCGGAAG AAGTTCGCCG CGTGAAGAAA CACGAGTCCG
361 GCGTAGTGAC CGACCCGCGAG ACCGTCTTGC CAACCAACAC GTTGCAAGAA GTGAAAGCCC
421 TGACCGAGCG TAACGGTTTT GCGGGCTATC CGGTGGTGAC TGAAGATAAC GAGCTGGTGG
481 GTATCATCAC CGGTCTGTGAC GTGCGTTTTG TGAAGTACCT GAACCAAGCC GTTAGCGTTT
541 ACATGACGCC GAAAGAGCGT CTGGTGACCG TTCGTGAAGG CGAAGCCCGT GAAGTCGTGC
601 TGGCAAAAAT GCACGAAAAA CCGGTAGAAA AAGCGCTGGT CGTTGATGAT AACTTCCATC
661 TGCTTGCCAT GATTACCGTA AAAGATTTC AGAAAGCCGA ACGTAAACCA AACTCCTGCA
721 AAGATGAGCA GGGCCGTTTA CGTGTGCGCG CGGCGGTCGG CGCAGGCGCG GGCAACGAAG
781 AGCGCGTTGA CGCGCTGGTG GCGGCAGGCG TTGACGTACT GCTGATCGAC TCCTCTCAG
841 GTCATTGAGA AGGCGTGTG CAACGTATCC GTGAACCCG TGCTAAATAT CCTGACCTGC
901 AAATCATCGG CGGCAACGTC GCGACAGGCG CAGGCGCTCG CGCACTGGCG GAAGCCGGTT
961 GCAGCGCGGT GAAAGTCGGT ATTGGCCCGG GTTCCATCTG TACCACTCGT ATCGTGAAG
1021 GCGTGGGCGT TCCGAGATC ACCGCTGTTT CTGACGAGT TGAAGCGCTG GAAGGTACCG
1081 GTATTCGGT TATCGCTGAC GCGGCTATCC GTTTCCTCCG CGACATAGCC AAAGCGATTG
1141 CCGCAGGTGC AAGCGCGTA ATGGTGGGTT CCATGCTGGC GGGTACGGAA GAATCCCCGG
1201 GCGAAATCGA ACTCTACCAG GGCCGTTCTT ACAAATCTTA CCGCGGCATG GGCTCGCTGG
1261 GTGCGATGTC CAAAGGTTCC TCCGACCGT ACTTCCAGAG CGACAACGCC GCTGACAAAC
1321 TGGTGCCGGA AGGTATCGAA GGTGCGGTAG CCTATAAAGG TCGCCTGAAA GAGATCATT
1381 ACCAGCAGAT GGGCGGCTG CGTCCTGTA TGGGGCTGAC CGGTTGTGCT ACCATCGACG
1441 AACTGCGTAC TAAAGCGGAG TTTGTGCGTA TCAGCGGTGC GGGCATTGAG GAAAGCCACG
1501 TTCAGACGT GACCATCACC AAAGAGTCCC CGAACTACCG TCTGGGCTCC TGA
(SEC ID n°: 13)

Fig. 5



BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
LGM-COLLECTION

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

89

6/12

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 (los) recuadro(s) adecuado(s)):

- ☒ una descripción científica
- ☒ una designación taxonómica propuesta

III. Recepción y aceptación

La presente Autoridad Internacional de Depósito 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(s) 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

90

7/12

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 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: 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, 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)

X viable
Ya no es viable

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-900 Gent, Bélgica

Firma(s) de la(s) 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 6 (recibo de depósito)

92

9/12

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGM

Página 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

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/ 02-135

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

10/12

93

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS – BCCM COPIA
RECOGIDA- LGM

Página 1 del formulario BCCM/LMG/BP/4 02-135 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 (los) recuadro(s) adecuado(s)):

- ☒ 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)

28 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-9000 Gent, Bélgica

Firma(s) de la(s) 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

74

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 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/ 02-135

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 Referencia de la identificación proporcionada 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)

X viable
Ya no es viable

12/12

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 – Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Bélgica

Firma(s) de la(s) 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 7 (recibo de depósito)

96

0-1	Formulario PCT/RO/134 (SAFE) Indicaciones relacionadas con Los microorganismo(s) depositados o material biológico Material (Norma PCT 13 bis)	
0-1-1	Utilización preparada	PCT-SAFE (modo Easy) Versión 3.51. 002.177 MT/FOP
0-2	Solicitud internacional nº.	PCT/BE 2006/000019
0-3	Referencia del expediente del o del agente	BPFDAH001AWO
1	Las indicaciones realizadas posteriormente relacionadas con otros materiales biológicos relacionados en la descripción en:	
1-1	página	20
1-2	línea	22
1-3	Identificación del depósito	
1-3-1	Nombre de la institución de depósito of	BCCM Belgian Coordinated Collections Microorganisms Federal Public Planning Service Policy, 8, rue de la Science, B-100 Bruselas, Bélgica 9 de agosto de 2002 (09.08.2002) BCM LMG P-21641
1-3-2	Dirección de la institución de depósito Science	
1-3-3	Fecha de depósito	
1-3-4	Número de acceso	
1-5	Estados designados para los cuales se han dado indicaciones	todas las designaciones
2	Las indicaciones realizadas posteriormente relacionadas con otros materiales biológicos relacionados en la descripción en:	
2-1	página	20
2-2	línea	22
2-3	Identificación del depósito	
2-3-1	Nombre de la institución del depósito of	BCCM Belgian Coordinated Collections Microorganisms Federal Public Planning Service Policy, 8, rue de la Science, B-1000 Bruselas, Bélgica
2-3-2	Dirección de la institución del depósito Science	
2-3-3	Fecha de depósito	28 de agosto de 2002 (28.08.2002)
2-3-4	Número de acceso	BCM LMG P-21646
2-5	Estados designados para los cuales se han dado indicaciones	todas las designaciones

97

PARA EL USO EXCLUSIVO DE LA OFICINA DE RECEPCIÓN

- 0-4 Este formulario fue recibido con la
Solicitud internacional: sí
(sí o no)
- 0-4-1 Funcionario autorizado (firma ilegible)
- 0-5 Este formulario fue recibido por la Oficina
Internacional en:
- 0-5-1 Funcionario autorizado:

PATENT COOPERATION TREATY

PCT/BE2006/000019

From the INTERNATIONAL BUREAU

PCTNOTIFICATION OF RECEIPT OF
RECORD COPY

(PCT Rule 24.2(a))

To:

VAN MALDEREN, Joëlle
pronovem-OFFICE VAN MALDEREN
Avenue Josse Goffin 158
B-1082 Brussels
BELGIQUE

Date of mailing (day/month/year)

01 May 2006 (01.05.2006)

IMPORTANT NOTIFICATION

Applicant's or agent's file reference

BPFDAH001AWO

International application No.

PCT/BE2006/000019

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:

20 March 2006 (20.03.2006)

Priority date(s) claimed:

None

Date of receipt of the record copy by the International Bureau:

13 April 2006 (13.04.2006)

List of designated Offices:

P: BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW

A: AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

IP: 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

Additional: 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)

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

Authorized officer

Gaillard Viviane

Facsimile No. +41 22 338 71 30

Telephone No. +41 22 338 98 10

Facsimile No. +41 22 338 82 70

PCT/IB/301 (October 2005)

CT2PMXLF

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)).

99

BP.FDAH.001A/WO

Statement according to Rule 27a (2) EPC

It is hereby stated that the sequence listing enclosed does not include matter which goes beyond the content of the application as filed and that the information recorded on the floppy disk is identical to the information on the written sequence listing as originally filed.

Date: 20/03/2006

Stefan VAN GYSEL
(Authorised representative)

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

100

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

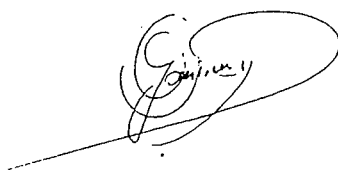
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

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

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

101

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

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

102

~~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

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

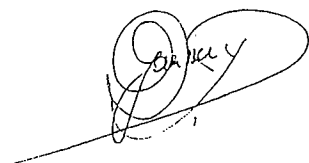
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

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

103

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

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/SP/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

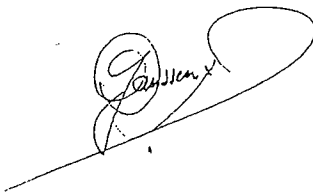
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

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

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

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

106
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

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

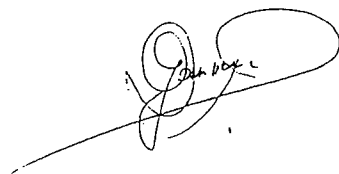
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

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

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

107

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION

Page 2 of Form BCCM/LMG/BP/9/.....02-135 Viability statement

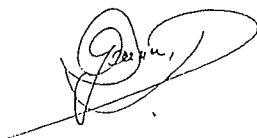
IV. Conditions under which the viability test has been performed:

(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

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

108

<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> 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> 10
ctgatcgaac aagccttcgg cctgcagttt ggcttttagc tgctcatatt tctgctgcaa 60
caagccttcg cccgcgggct gcatactttc ggcgatgatt tgataatcgc cgcgcggctc 120
gtacagcgta atgttggcgc gtaccagcac ctgctgcccc tgctgcgggc ggaacgtcac 180
ccggcgattg ctgttacgga acatcgcaca gcgcacctga gcggtatcgt ctttgagcgt 240
aaagtaccag tggccccgacg caggctgcgt gaaattagaa atctcgccgc tgatccatac 300
ctgtcccatc tcctgttcta acagcagacg aaccgtctgg ttaaggcggc ttacggtaaa 360
aattgaggaa gtttgagagg ataacatgtg agcgggatca aattctaaat cagcaggtta 420
ttcaatcgat agtaacctgc tcacggggga tcgcaagcac tatttgcaaa aaaatgtaga 480
tgcaaccgat tacgttctgt ataatgccg ggaatatattt attaacctcc caggcgagat 540
attgcccattg ctacgtatcg ctaaagaagc tctgacgttt gacgacgtcc tcctgtttcc 600
cgctcactcc accgttttgc cgaatactgc cgatctcagc acgcagttga cgaaaactat 660
tcgtctgaat attcctatgc tttctgcggc gatggacacc gtgacggaag cgcgcctggc 720
aattgccctg gccaggaag gcggcattgg ttttatccac aaaaacatgt ctattgagcg 780
ccaggcgga gaagttcgcc gcgtgaagaa acacgagtcg gccgtagtga ccgacccgca 840
gaccgtcctg ccaaccacca cgttgcatga agtgaaaagcc 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 agggccggtt 1200
acgtgtcggc gcggcggtcg gcgcaggcgc gggcaacgaa gagcgcggtg acgcgctggt 1260
ggcggcaggc gttgacgtac tgctgatcga ctctctcac ggctactctg aaggcgtggt 1320
gcaacgtatc cgtgagacgc gtgctaaata tcctgacctg caaatcatcg gcgggaacgt 1380
tgcgacgggc gcaggcgctc gcgcactggc ggaagccggt tgcaacgcgg tgaaagtggg 1440
tatcgggccc gggtccatct 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
gggcccgttct taaaaatctt atcgcggtat gggttctctg ggcgcgatgt ccaaaggttc 1740
ctccgaccgt tacttccaga gcgacaacgc cgcgcacaaa ctggtgccgg aaggatcga 1800
aggccgcgta gcctataaag gtcgcctgaa agagatcatt caccagcaga tgggcggcct 1860
gcgctcctgt atggggctga ccggttgtgc taccatcgac gaactgcgta ctaaagcgga 1920

109

FDAH001AWO.ST25.txt

gtttgtgcgt atcagcgggtg cgggtatcca ggaaagccac gttcacgacg tgaccatcac 1980
caaagagtcc ccgaactacc gtctgggctc ctgattttct tcgcccgacc ttcgcgtcgg 2040
gcgatttatt taatctgttt cacttgcctc 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 ctaatgagcg 2400
tgaatttggt tatgcgcagg tcgaagtgtt gaccgacagc gcgctgggtc gcggtattga 2460
agattccctg accgcagacg gcaaaccgct gctggacgtg tggatgagcc acggcgataa 2520
agtgcggcg attccgtccg acttcgtgac cgtcgccagc accgagagct gcccgttcgc 2580
cattatggcc aacgaagaaa aacgcttcta cggcgtagac ttccaccgg aagtgactca 2640
caccgccag ggtatgcgca tgctggagcg ttttgtacgc gatatctgcc agtgtgaagc 2700
gttgtggacg ccggcgaaga tcatcgatga cgccgtggcg cgcatcgcg agcaggtagg 2760
cgacgataaa gtgatcctcg gtctctccgg cggcggtgat tcttccgtca ccgccatgct 2820
gctgcaccgc gcgatcggta aaaatctgac ctgtgttttc gtcgacaacg gcctgttgcg 2880
cctgaacgaa gccgagcagg tga 2903

<210> 11
<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> 11
ggctgcgatt ggcgaggtag tagtccatcg ccatgccag acgctgctgg cgganctgga 60
tctgacgcaa caactcctgc tggttacggc tgacaatttc tgccgctgct gatggcgtag 120
cgcgcgaggc 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 tgggccgggg agggcaacgg 480

ctgttttatgc tgctgatcga acaagccttc ggcctgcagt ttggctttta gctgctcata	540
tttctgctgc aacaagcctt cgcccgcggg ctgcatactt tcggcgatga tttgataatc	600
gccgcgcggc tcgtacagcg taatgttggc gcgtaccagc acctgctgcc cgtgctgcgg	660
gcggaacgtc acccggcgat tgctgttacg gaacatcgca cagcgcacct gagcggtatc	720
gtctttgagc gtaaagtacc agtggcccga 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
cccaggtgag atattgcca tgctacgtat cgctaaagaa gctctgacgt ttgacgcccg	1080
ggatcaccaa agagtccccg aactaccgtc tgggctcctg attttcttcg cccgaccttc	1140
gcgtcgggcg atttatttaa tctgtttcac ttgcctcgga ataagcgtca atgacggaaa	1200
acattcataa gcatcgcatc ctcatcttg gacttcggttc tcagtacact caactggttg	1260
cgcgccgcgt gcgtgagctg ggtgtttact gtgaactgtg ggcgtgggat gtgacagaag	1320
cacaaattcg tgacttcaac ccaagcggca ttattctttc cggcggccccg gaaagcacca	1380
ccgaagaaaa cagcccgcgc gcgccgcagt atgtctttga agcaggcgtg ccggtatttg	1440
gcgtttgcta tggatatgag accatggcga tgcagcttgg cggatcatgta 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 caccggaag	1740
tgactcacac ccgccagggt atgcgcatgc tggagcgttt tgtacgcgat atctgccagt	1800
gtgaagcgtt gtggacgccg gcgaagatca tcgatgacgc cgtggcgcgc attcgcgagc	1860
aggtaggcga cgataaagt atcctcggtc tctccggcgg cgtggattct tccgtcaccg	1920
ccatgctgct gcaccgcgcg atcggtaaaa atctgacctg tgttttcgtc gacaacggcc	1980
tgttgcgcct gaacg	1995

<210> 12
 <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> 12
 catgtgtgag cgggatcaaa ttctaaatca gcaggttatt caatcgatag taacctgctc 60
 acgggggatc gcaagcacta ttgcaaaaa aaatgtagat gcaaccgatt acgttctgta 120
 taatgcccgg caatatatat taacctcca ggtgagatat tcgcatgta cgatcgcta 180

110

FDAH001AWO.ST25.txt

aagaagccct gacgtttgac gcccgtgtag gctggagctg cttcgaagtt cctatacttt 240
ctagagaata ggaactccgg aataggaact aaggaggata ttcatatggg atcaccaaag 300
atccccgaac taccgtctgg gctctgattt tcttcgcccc accttcgcg 349

<210> 13
<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> 13
atttgcaaaa aaatgtagat gcaaccgatt acgttctgta taatgccgcg gcaatattta 60
ttaacctccc aggtgagata ttgcccatgc tacgtatcgc taaagaagcc ctgacgtttg 120
acgacgtcct ccttgttccc gtcactcca ccgttttgcc gaatactgcc gatctcagca 180
cgcagttgac gaaaactatt cgtctgaata ttcctatgct ttctgcggcg atggacaccg 240
tgacggaagc gcgcctggca attgccctgg cccaggaagg cggcattggt 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 aagcgctggt cgttgatgat aacttccatc 660
tgcttgcat gattaccgta aaagatttcc agaaagcgga acgtaaacca aactcctgca 720
aagatgagca gggccgttta cgtgtcggcg cggcggtcgg cgcaggcgcg ggcaacgaag 780
agcgcgttga cgcgctggcg gcggcaggcg ttgacgtact gctgatcgac tcctctcacg 840
gtcattcaga aggcgtgttg caacgtatcc gtgaaacccg tgctaaatat cctgacctgc 900
aaatcatcgg cggcaacgtc gcgacaggcg caggcgctcg cgcactggcg gaagccgggt 960
gcagcgcggt gaaagtcggt attggcccgg gttccatctg taccactcgt atcgtgactg 1020
gcgtgggcgt tccgcagatc accgctgttt ctgacgcagt tgaagcgctg gaaggtagcg 1080
gtattccggt tatcgctgac ggcggtatcc gtttctccgg cgacatagcc aaagcgattg 1140
ccgcaggtgc aagcgcggtg atggtgggtt ccatgctggc gggtagcgaa gaatccccgg 1200
gcgaaatcga actctaccag ggccgttctt acaaacttta ccgcggcatg ggctcgctgg 1260
gtgcgatgtc caaaggttcc tccgaccgtt acttcagag cgacaacgcc gctgacaaac 1320
tggtgccgga aggtatcgaa ggtcgcgtag cctataaagg tcgcctgaaa gagatcattc 1380
accagcagat gggcggcctg cgctcctgta tggggctgac cggttgtgct accatcgacg 1440

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) 12/12/2006	
Applicant's or agent's file reference BPFDAH001AWO	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/BE2006/000019	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.

112

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference BPFDAH001AWO	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/BE2006/000019	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/BE2006/000019

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:

113

INTERNATIONAL SEARCH REPORT

International application No
PCT/BE2006/000019

A. CLASSIFICATION OF SUBJECT MATTER			
INV. C12N1/20	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 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, 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	WANG JIN YUANG ET AL: "Construction, genotypic and phenotypic characterization, and immunogenicity of attenuated DELTAguaba Salmonella enterica serovar Typhi strain CVD 915" INFECTION AND IMMUNITY, vol. 69, no. 8, August 2001 (2001-08), pages 4734-4741, XP002409035 ISSN: 0019-9567 cited in the application page 4735; figures 3,4	1-5, 10-12
X	WO 99/58146 A (UNIV MARYLAND [US]) 18 November 1999 (1999-11-18) cited in the application page 34; examples 1,4; table 2 ----- -/--	1-5, 10-12

☒ 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. "8" document member of the same patent family	
Date of the actual completion of the international search 24 November 2006	Date of mailing of the international search report 12/12/2006
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

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 page 153; tables 1,2 -----	1-17
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, XP002409036 ISSN: 0378-1097 pages 101-2 -----	1-17

114

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/BE2006/000019

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
W0 9958146	A	18-11-1999	
		AT 333285 T	15-08-2006
		AU 754795 B2	28-11-2002
		AU 3740599 A	29-11-1999
		CA 2328056 A1	18-11-1999
		DK 1076566 T3	13-11-2006
		EP 1076566 A1	21-02-2001
		HU 0101747 A2	28-09-2001
		JP 2002514398 T	21-05-2002
		MX PA00011075 A	24-04-2002
		NO 20005672 A	15-01-2001
		NZ 508110 A	25-10-2002
		PL 344593 A1	05-11-2001
		US 6190669 B1	20-02-2001
		ZA 200006381 A	07-02-2002

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/BE2006/000019

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 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



115

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/BE2006/000019

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:

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	6-9,13-17
	No: Claims	1-5, 10-12
Inventive step (IS)	Yes: Claims	
	No: Claims	1-17
Industrial applicability (IA)	Yes: Claims	1-17
	No: Claims	

2. Citations and explanations

see separate sheet

1. Concerning section V

Novelty (Art. 33(2) PCT) and inventive step (Art. 33(3) PCT)

D1 (pg. 4735) and D2 (pg. 34, example 1 and table 2) describe an attenuated mutant strain of *S. typhimurium* (CVD 915), which is incapable of forming *de novo* guanine nucleotides, containing a deletion in the *guaBA* operon, i.e., containing a deletion of the *guaB* gene and a deletion in the *guaA* gene. This strain was used to immunize mice intranasally and induced a significant antibody response (D1, Fig. 3). CVD 915 was also used as a vector vaccine expressing tetanus fragment C (D1, Fig. 4; D2 example 4). Intranasal administration of this vaccine to mice induced a strong serum IgG response. The authors of D1 conclude that based on this promising data it will be of interest to proceed to phase 1 clinical trials. In view of this, D1 and D2 are novelty destroying for **claims 1-5 and 10-12**.

The importance of immunizing poultry against *S. enteritidis* and *S. typhimurium* is evident from D4. Therefore, the skilled person when faced with the problem of providing a method of immunizing poultry against these pathogens would turn to D1/D2 and conclude that the use of the strain described therein would be a suitable solution. Hence, **claims 13-17** do not involve inventive merit.

The subject-matter covered by **claims 6-9** does not appear to have been disclosed in the available prior art. It thus appears possible to recognise formal novelty for these claims. However, inventive step would have to be substantiated in view of D1-D3, since the advantages in deleting or disrupting the Δ *guaB* gene in order to obtain attenuated strains of *S. enterica* that can be used as vaccines are evident from these documents.

Should the claims be limited to attenuated strains comprising a deletion affecting *only* the *guaB* gene, the following comments would still be pertinent:

D3 describes *guaB* Tn10 insertion mutants of *S. typhimurium* that had low virulence when administered to mice at a dose of 2.5×10^5 cfu but retained significant virulence when administered at 2.7×10^7 cfu. Similar observations are reported in the application (pg. 16, §90; and pg. 17, §91). The application also states that the Δ *guaB* mutant strain SM69 is not safe when administered at 1.28×10^8 cfu to bird at one day of age but it is safe when administered at two weeks of age (pg. 19, §97; pg. 20, §106). In view of this, it is clear that a Δ *guaB* mutant vaccine is only safe under particular

117

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
BELGIUM

REÇU
19.05.2006
OFFICE VAN MALDEREN

NOTIFICATION OF RECEIPT
OF SEARCH COPY

(PCT Rule 25.1)

Date of mailing
(day/month/year)

15/05/2006

Applicant's or agent's file reference

BPFDAH001AWO

IMPORTANT NOTIFICATION

International application No.

PCT/BE2006/000019

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.

28/04/2006 (date of receipt).

2. ☒ The search copy was accompanied by a nucleotide and/or amino acid sequence listing or tables related thereto in electronic form.
3. ☐ The search copy contained a nucleotide and/or amino acid sequence listing or tables related thereto in electronic form.
4. 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))
5. 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

780070

PATENT COOPERATION TREATY

From the RECEIVING OFFICE

PCT

To:

VAN MALDEREN Joëlle
 Pronovem - Office VAN MALDEREN
 Avenue Josse Goffin 158
 B - 1082 Bruxelles

REÇU

27.03.2006

OFFICE VAN MALDEREN

NOTIFICATION OF THE INTERNATIONAL
 APPLICATION NUMBER AND OF THE
 INTERNATIONAL FILING DATE

(PCT Rule 20.5(c))

Date of mailing
 (day/month/year)

24.03.2006

Applicant's or agent's file reference
 BPFDAH001AWO

IMPORTANT NOTIFICATION

International application No.
 PCT/BE2006/000019

International filing date (day/month/year)
 20 March 2006 (20.03.2006)

Priority date (day/month/year)

Applicant VRIJE UNIVERSITEIT BRUSSEL and al

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 _____
- ☒ 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

OPRI / Bruxelles

Authorized officer

Harmegnies N.

Facsimile No.

Telephone No. +32-2-277 81 00

118

DUPLICATA

BPFDAH001AWO

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	BPFDAH001AWO
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 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
III-1-5	Address	Jef Lambeauxlaan 11 1060 SINT-GILLIS Belgium
III-1-6	State of nationality	BE
III-1-7	State of residence	BE

DEPOT D'UNE REQUETE PCT DEPOSEE CE LUNDI 20/03/2006
DANS LA BOITE AUX LETTRES AVANT MINUIT. VEUILLEZ
NOUS ACCORDER LA DATE DU 20/03/2006.

ORIGINAL
20.03.2006
24

OFFICE VAN MALDEREN

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	BPFDAH001AWO
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 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
III-1-5	Address	Jef Lambeauxlaan 11 1060 SINT-GILLIS Belgium
III-1-6	State of nationality	BE
III-1-7	State of residence	BE

119

BPFDAH001AWO

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	Leiekaai 33 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 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	30	-
IX-3	Claims	2	-
IX-4	Abstract	1	✓
IX-5	Drawings	12	-
IX-7	TOTAL	50	

120

BPFDAH001AWO

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-21641 Forms BP/4/02-132 and BP/9/02-132	
IX-18	other	DEPOSIT RECEIPTS FOR LMG P-21646 Forms BP/4/02-135 & BP/9/02-135	
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		

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	
------	--	--

124

The present invention relates to attenuated *guaB* deletion mutants of a bacterium infecting veterinary species, more in particular *Salmonella enterica*, to their use and production.

The present invention further relates to live attenuated vaccines based on such mutants for preventing bacterial infections, and more in particular Salmonellosis, in a veterinary species, more in particular poultry.

PCT

Original (for SUBMISSION)

0-1	Form PCT/RO/134 (SAFE) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared Using	PCT-SAFE [EASY mode] Version 3.51.002.177 MT/FOP 20060101/0.20.4rc.2.7
0-2	International Application No.	
0-3	Applicant's or agent's file reference	BPFDAH001AWO

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	20
1-2	line	22
1-3	Identification of deposit	
1-3-1	Name of depositary institution	BCCM Belgian Coordinated Collections of Microorganisms
1-3-2	Address of depositary institution	Federal Public Planning Service Science Policy, 8, rue de la Science, B-1000 Brussels, Belgium
1-3-3	Date of deposit	09 August 2002 (09.08.2002)
1-3-4	Accession Number	BCCM LMG P-21641
1-5	Designated States for Which Indications are Made	all designations
2	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
2-1	page	20
2-2	line	22
2-3	Identification of deposit	
2-3-1	Name of depositary institution	BCCM Belgian Coordinated Collections of Microorganisms
2-3-2	Address of depositary institution	Federal Public Planning Service Science Policy, 8, rue de la Science, B-1000 Brussels, Belgium
2-3-3	Date of deposit	28 August 2002 (28.08.2002)
2-3-4	Accession Number	BCCM LMG P-21646
2-5	Designated States for Which Indications are Made	all designations

FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	
0-4-1	Authorized officer	

122

BPFDAH001AWO

2/2

PCT

Original (for SUBMISSION)

FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the international Bureau on:	
0-5-1	Authorized officer	

123

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 08-099562- -00000-0000

Fecha: 2008-09-19 16:26:25 Dep. 2020 NUECREACION
Tra. 11 PATENTEIYII Eve: 378 FASENACIONAL
Act. 411 PRESENTACION Folios: 122



Industria y Comercio
SUPERINTENDENCIA

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 ☐

Sando Rodríguez
Firma Responsable

Fecha SEP 19 08

Carrera 13 No. 27-00 Piso 5, 7 y 10
Conmutador: 3 82 08 40
Fax: 350 52 20
E-mail: info@slc.gov.co
www.slc.gov.co
Bogotá, D.C., Colombia