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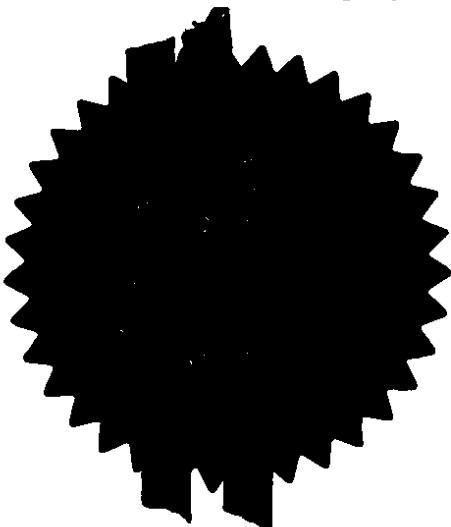
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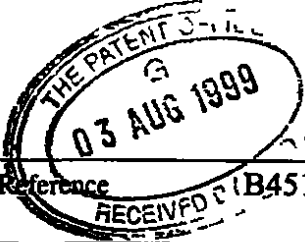
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1 Please give the title of the invention Vaccine Composition

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Claim(s) 2 Description 46

Abstract Drawing(s) 8

8b Which of the following documents also accompanies the application?

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Patents Form 9/77 Preliminary Examination Report

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VACCINE COMPOSITION

FIELD OF THE INVENTION

The present invention relates to the field of Gram-negative bacterial vaccine compositions, their manufacture, and the use of such compositions in medicine. More particularly it relates to the field of novel outer-membrane vesicle (or bleb) vaccines and advantageous methods of rendering these vaccines more effective.

BACKGROUND OF THE INVENTION

Gram-negative bacteria are separated from the external medium by two successive layers of membrane structures. These structures, referred to as the cytoplasmic membrane and the outer membrane (OM), differ both structurally and functionally. The outer membrane plays an important role in the interaction of pathogenic bacteria with their respective hosts. Consequently, the surface exposed bacterial molecules represent important targets for the host immune response, making outer-membrane components attractive candidates in providing vaccine, diagnostic and therapeutics reagents.

Whole cell bacterial vaccines (killed or attenuated) have the advantage of supplying multiple antigens in their natural micro-environment. Drawbacks around this approach are the side effects induced by bacterial components such as endotoxin and peptidoglycan fragments. On the other hand, acellular subunit vaccines containing purified components from the outer membrane may supply only limited protection and may not present the antigens properly to the immune system of the host.

The biochemical nature of the outer-membrane of Gram-negative bacteria has been extensively studied in the model bacterium *Escherichia coli*, where proteins, phospholipids and lipopolysaccharides have been identified as the three major constituents (Danese, P. N. and Silhavy, T. J. 1998 *Annu. Rev. Genet.* 32: 59-94). More generally, these three major constituents are found in the outer-membrane of all Gram-negative bacteria. These molecules are distributed asymmetrically: membrane phospholipids (mostly in the inner leaflet), lipooligosaccharides (exclusively in the outer leaflet) and proteins (inner and outer leaflet lipoproteins, integral or polypeptide membrane proteins). For many bacterial pathogens which impact on human health,

lipopolysaccharide and outer-membrane proteins have been shown to be immunogenic and amenable to confer protection against the corresponding disease by way of immunization

The OM of Gram-negative bacteria is dynamic and, depending on the environmental conditions, can undergo drastic morphological transformations. Among these manifestations, the formation of outer-membrane vesicles or “blebs” has been studied and documented in many Gram-negative bacteria (Zhou, L *et al* 1998 *FEMS Microbiol Lett* 163 223-228). Among these, a non-exhaustive list of bacterial pathogens reported to produce blebs include *Bordetella pertussis*, *Brucella burgdorferi*, *Brucella melitensis*, *Brucella ovis*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Escherichia coli*, *Haemophilus influenzae*, *Legionella pneumophila*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica*. Although the biochemical mechanism responsible for the production of OM blebs is not fully understood, these outer membrane vesicles have been extensively studied as they represent a powerful methodology in order to isolate outer-membrane protein preparations in their native conformation. In that context the use of outer-membrane preparations is of particular interest to develop vaccines against *Neisseria*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Chlamydia*. Moreover, outer membrane blebs combine multiple proteinaceous and non-proteinaceous antigens that are likely to confer extended protection against intra-species variants.

In comparison with the other, more widely used, types of bacterial vaccine (whole cell bacterial and purified subunit vaccines), the inventors will show that outer membrane bleb vaccines may represent the ideal compromise.

The wide-spread use of bacterial subunit vaccines has been due to the intensive study of bacterial surface proteins that have been found to be useful in vaccine applications [for instance *B. pertussis* filamentous hemagglutinin (FHA), fimbriae (FIM) and pertactin (PRN)]. These proteins are loosely associated with the bacterial outer membrane and can be purified from culture supernatant or easily extracted from the bacterial cells. However, it has clearly been shown that structural, integral outer membrane proteins are also protective antigens. Examples are PorA for *N. meningitidis* serogroup B, D15 for *H. influenzae*, OMP CD for *M. catarrhalis*,

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OMP F for *P. Aeruginosa*. Such proteins however have rather specific structural features, particularly the multiple amphipathic β -sheets which complicates their straightforward use as purified (recombinant) subunit vaccines.

In addition, it has become clear that multiple component vaccines are needed (in terms of bacterial surface proteins and integral membrane proteins) to supply a reasonable level of protection. For instance, in the case of *B. pertussis* subunit vaccines multicomponent vaccines (pertussis toxoid, FHA, PRN and other antigens) are superior to mono or bicomponent products.

In order to incorporate integral outer-membrane proteins into such a subunit product native (or near-native) conformational folding of the proteins must be present in the product in order to have a useful immunological effect. The use of excreted outer membrane vesicles or blebs may be an elegant solution to the problem of including protective integral membrane proteins into a subunit vaccine whilst still ensuring that they fold properly.

N. meningitidis serogroup B (menB) excretes outer membrane blebs in sufficient quantities to allow their manufacture on an industrial scale. Such multicomponent outer-membrane protein vaccines from naturally-occurring menB strains have been found to be efficacious in protecting teenagers from menB disease and have become registered in Latin America.

An alternative method of preparing outer-membrane vesicles is via the process of detergent extraction of the bacterial cells. This process not only produces greater yields of blebs, it also reduces the lipopolysaccharide (LPS) (or endotoxin) content of the final product (thus rendering it less reactogenic).

Examples of bacterial species from which bleb vaccines could usefully be made are the following:

Neisseria meningitidis

Neisseria meningitidis (meningococcus) is a Gram-negative bacterium frequently isolated from the human upper respiratory tract. It occasionally causes invasive bacterial diseases such as bacteremia and meningitis. The incidence of meningococcal disease shows geographical, seasonal and annual differences (Schwartz, B., Moore, P. S., Broome, C. V., Clin. Microbiol. Rev. 2 (Supplement)

S18-S24, 1989) Most disease in temperate countries is due to strains of serogroup B and varies in incidence from 1/10/100,000/year total population sometimes reaching higher values (Kaczmarek, E B (1997), Commun Dis Rep Rev 7 R55-9, 1995 Scholten, R J P M , Bijlmer, H A , Poolman, J T et al Clin Infect Dis 16 237-246
 5 1993, Cruz, C , Pavez, G , Aguilar, E , et al Epidemiol Infect 105 119-126, 1990) Age-specific incidences in the two high risk-groups, infants and teenagers reach higher levels

Epidemics dominated by serogroup A meningococci occur, mostly in central Africa, sometimes reaching levels up to 1000/100,000/year (Schwartz, B , Moore
 10 P S , Broome, C V Clin Microbiol Rev 2 (Supplement) S18 S24, 1989) Nearly all cases of meningococcal disease as a whole are caused by serogroup A, B C W-135 and Y meningococci A tetravalent A, C, W-135 Y capsular polysaccharide vaccine is available (Armand, J , Arminjon, F , Mynard, M C , Lafaux, C , J Biol Stand 10 335-339, 1982)

15 The polysaccharide vaccines are currently being improved by way of chemically conjugating them to carrier proteins (Lieberman, J M , Chiu, S S , Wong V K et al JAMA 275 1499-1503, 1996) A serogroup B vaccine is not available since the B capsular polysaccharide is non-immunogenic most likely because it shares structural similarity to host components (Wyle, F A , Artenstein, M S , Brandt,
 20 M L et al J Infect Dis 126 514-522, 1972 Finne, J M , Leinonen, M , Makela P M Lancet ii 355-357, 1983)

For many years efforts have been focused on developing meningococcal outer membrane based vaccines (de Moraes, J C , Perkins, B , Camargo M C et al Lancet 340 1074-1078, 1992, Bjune, G , Hoiby, E A Gronnesby, J K et al 338 1093-1096,
 25 1991) Such vaccines have demonstrated efficacies from 57% - 85% in older children (>4 years) and adolescents Most of these efficacy trials were performed with OMV (outer membrane vesicles, derived by LPS depletion from blebs) vaccines derived from wild-type *N meningitidis* B strains

Many bacterial outer membrane components are present in these vaccines,
 30 such as PorA, PorB, Rmp, Opc, Opa, FrpB and the contribution of these components to the observed protection still needs further definition Other bacterial outer membrane components have been defined (using animal or human antibodies) as

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potentially being relevant to the induction of protective immunity, such as TbpB NspA (Martin D , Cadieux, N , Hamel, J Brodeux, B R , J Exp Med 185 1173-1183, 1997 Lissolo, L , Maitre-Wilmotte, C Dumas, p et al , Inf Immun 63 884-890, 1995) The mechanism of protective immunity will involve antibody mediated bactericidal activity and opsonophagocytosis

The frequency of *Neisseria meningitidis* infections has risen dramatically in the past few decades This has been attributed to the emergence of multiple antibiotic resistant strains, and increased exposure due to an increase in social activities (for instance swimming pools or theatres) It is no longer uncommon to isolate *Neisseria meningitidis* strains that are resistant to some or all of the standard antibiotics This phenomenon has created an unmet medical need and demand for new anti-microbial agents vaccines drug screening methods, and diagnostic tests for this organism

Most adults have developed bactericidal antibodies against most menB strains due to carriage, cross-reactive bacteria, etc The nature of the protective antigens however remains, for the most part, obscure

Moraxella catarrhalis

Moraxella catarrhalis (also named *Branhamella catarrhalis*) is a Gram negative bacterium frequently isolated from the human upper respiratory tract It is responsible for several pathologies, the main ones being otitis media in infants and children, and pneumonia the elderly It is also responsible of sinusitis, nosocomial infections and, less frequently, of invasive diseases

The immune response to *M catarrhalis* is poorly characterized The analysis of strains isolated sequentially from the nasopharynx of babies from 0 to 2 years of age indicates that they frequently get and eliminate new strains with time This indicates that an efficacious immune response against this bacteria is mounted by the colonized children (Faden, HL et al (1994) J Infect Dis 169 1312)

Bactericidal antibodies have been identified in most adults tested (Chapman, AJ et al (1985) J Infect Dis 151 878) Strains of *M catarrhalis* present variations in their capacity to resist serum bactericidal activity in general, isolates from diseased individuals are more resistant than those who are simply colonized (Hol, C et al (1993) Lancet 341 1281, Jordan, KL et al (1990) Am J Med 88 (suppl 5A) 28S) Serum resistance

could therefore be considered as a virulence factor of the bacteria. An opsonizing activity has been observed in the sera of children recovering from otitis media.

The antigens targeted by these different immune responses in humans have not been identified, with the exception of OMP B1, a 84 kDa protein, the expression of which is regulated by iron, and that is recognized by the sera of patients with pneumonia (Sethi S, et al (1995) Infect Immun 63 1516), and of UspA1 and UspA2 (Chen D et al (1999) Infect Immun 67 1310).

A few other membrane proteins present on the surface of *M. catarrhalis* have been characterized using biochemical methods, or for their potential implication in the induction of a protective immunity (for review, see Murphy, TF (1996) Microbiol Rev 60 267). In a mouse pneumonia model, the presence of antibodies raised against some of them (UspA, CopB) favors a faster clearance of the pulmonary infection. Another polypeptide (OMP CD) is highly conserved among *M. catarrhalis* strains, and presents homologies with a porin of *Pseudomonas aeruginosa* which has been demonstrated to be efficacious against this bacterium in animal models.

M. catarrhalis produces outer membrane vesicles (Blebs) these Blebs have been isolated or extracted by using different methods (Murphy T F, Loeb M R 1989 Microb Pathog 6 159-174, Unhanand M, Maciver, I, Ramillo O, Arencibia-Mireles O, Argyle J C, McCracken G H Jr, Hansen E J 1992 J Infect Dis 165 644-650). The protective capacity of such Bleb preparations has been tested in a murine model for pulmonary clearance of *M. catarrhalis*. It has been shown that active immunization with Bleb vaccine or passive transfer of anti-Blebs antibody induces significant protection in this model (Maciver I, Unhanand M, McCracken G H Jr, Hansen, E J 1993 J Infect Dis 168 469-472).

Haemophilus influenzae

Haemophilus influenzae is a non-motile Gram-negative bacterium. Man is its only natural host. *H. influenzae* isolates are usually classified according to their polysaccharide capsule. Six different capsular types designated 'a' through 'f' have been identified. Isolates that fail to agglutinate with antisera raised against one of these six serotypes are classified as nontypeable, and do not express a capsule.

H influenzae type b (Hib) is clearly different from the other types in that it is a major cause of bacterial meningitis and systemic diseases. Nontypeable strains of *H influenzae* (NTHi) are only occasionally isolated from the blood of patients with systemic disease. NTHi is a common cause of pneumonia, exacerbation of chronic bronchitis, sinusitis and otitis media. NTHi strains demonstrate a large variability as identified by clonal analysis, whilst Hib strains as a whole are more homogeneous.

Various proteins of *H influenzae* have been shown to be involved in pathogenesis or have been shown to confer protection upon vaccination in animal models.

Adherence of NTHi to human nasopharyngeal epithelial cells has been reported (Read RC et al 1991 J Infect Dis 163 549). Apart from fimbriae and pili (Brinton CC et al 1989 Pediatr Infect Dis J 8 S54, Kar S et al 1990 Infect Immun 58 903, Gildorf JR et al 1992 Infect Immun 60 374, St Geme JW et al 1991 Infect Immun 59 3366, St Geme JW et al 1993 Infect Immun 61 2233) many adhesins have been identified in NTHi. Among them, two surface exposed high-molecular-weight proteins designated HMW1 and HMW2 have been shown to mediate adhesion of NTHi to epithelial cells (St Geme JW et al 1993 Proc Natl Acad Sci USA 90 2875). Another family of high-molecular-weight proteins has been identified in NTHi strains that lack proteins belonging to the HMW1/HMW2 family. The NTHi 115-kDa Hia protein (Barenkamp SJ, St Geme JW 1996 Mol Microbiol In press) is highly similar to the Hsf adhesin expressed by *H influenzae* type b strains (St Geme JW et al 1996 J Bact 178 6281). Another protein, the Hap protein shows similarity to IgA1 serine proteases and has been shown to be involved in both adhesion and cell entry (St Geme JW et al 1994 Mol Microbiol 14 217).

Five major outer membrane proteins (OMP) have been identified and numerically numbered. Original studies using *H influenzae* type b strains showed that antibodies specific for P1 and P2 OMPs protected infant rats from subsequent challenge (Loeb MR et al 1987 Infect Immun 55 2612, Musson RS Jr et al 1983 J Clin Invest 72 677). P2 was found to be able to induce bactericidal and opsonic antibodies, which are directed against the variable regions present within surface exposed loop structures of this integral OMP (Haase EM et al 1994 Infect Immun 62 3712, Troelstra A et al 1994 Infect Immun 62 779). The lipoprotein

P4 also may induce bactericidal antibodies (Green BA et al 1991 Infect Immun 59 3191)

OMP P6 is a conserved peptidoglycan associated lipoprotein making up 1.5 % of the outer membrane (Nelson MB et al 1991 Infect Immun 59 2658). Later a lipoprotein of about the same molecular weight was recognized called PCP (P6 cross-reactive protein) (Deich RM et al 1990 Infect Immun 58 3388). A mixture of the conserved lipoproteins P4, P6 and PCP did not reveal protection as measured in chinchilla otitis-media model (Green BA et al 1993 Infect Immun 61 1950). P6 alone appears to induce protection in the chinchilla model (Demaria TF et al 1996 Infect Immun 64 5187).

A fimbria protein (Miyamoto N, Bakaletz, LO 1996 Microb Pathog 21 343) has also been described with homology to OMP P5, which itself has sequence homology to the integral *Escherichia coli* OmpA (Miyamoto N, Bakaletz, LO 1996 Microb Pathog 21 343, Munson RS Jr et al 1993 Infect Immun 61 1017). NTHi seem to adhere to mucus by way of fimbriae.

In line with the observations made with gonococci and meningococci, NTHi expresses a dual human transferrin receptor composed of TbpA and TbpB when grown under iron limitation. Anti-TbpB protected infant rats (Loosmore SM et al 1996 Mol Microbiol 19 575). Hemoglobin / haptoglobin receptor also have been described for NTHi (Maciver I et al 1996 Infect Immun 64 3703). A receptor for Haem Hemopexin has also been identified (Cope LD et al 1994 Mol Microbiol 13 868). A lactoferrin receptor is also present amongst NTHi, but is not yet characterized (Schryvers AB et al 1989 J Med Microbiol 29 121). A protein like neisserial FrpB-protein has not been described amongst NTHi.

An 80kDa OMP, the D15 surface antigen, provides protection against NTHi in a mouse challenge model (Flack FS et al 1995 Gene 156 97). A 42kDa outer membrane lipoprotein, LPD is conserved amongst *Haemophilus influenzae* and induces bactericidal antibodies (Akkoyunlu M et al 1996 Infect Immun 64 4586). A minor 98kDa OMP (Kimura A et al 1985 Infect Immun 47 253), was found to be a protective antigen, this OMP may very well be one of the Fe-limitation inducible OMPs or high molecular weight adhesins that have been characterized thereafter. *H. Influenzae* produces IgA1-protease activity (Mulks MH, Shoberg RJ 1994 Meth

Enzymol 235 543) IgA1-proteases of NTHi reveals a high degree of antigenic variability (Lomholt H, van Alphen L Kilian M 1993 Infect Immun 61 4575)

Another OMP of NTHi, OMP26, a 26-kDa protein has been shown to enhance pulmonary clearance in a rat model (Kyd, J M Cripps A W 1998 Infect Immun 66 2272) The NTHi HtrA protein has also been shown to be a protective antigen. Indeed this protein protected Chinchilla against otitis media and protected infant rats against *H influenzae* type b bacteremia (Loosmore S M et al 1998 Infect Immun 66 899)

Outer membrane vesicles (or blebs) have been isolated from *H influenzae* (Loeb M R, Zachary A L, Smith D H 1981 J Bacteriol 145 569-604, Stull T L Mack K Haas J E, Smit J, Smith A L 1985 Anal Biochem 150 471-480) The vesicles have been associated with the induction of blood-brain barrier permeability (Wiwelwey B Hansen E J, Scheld W M 1989 Infect Immun 57 2559-2560), the induction of meningeal inflammation (Mustafa M M Ramilo O Svrogiannopoulos G A Olsen K D, McCracken G H Jr Hansen, E J 1989 J Infect Dis 159 917-922) and to DNA uptake (Concino M F, Goodgal S H 1982 J Bacteriol 152 441-450) These vesicles are able to bind and be absorbed by the nasal mucosal epithelium (Harada T, Shimuzu T, Nishimoto K, Sakakura Y 1989 Acta Otorhinolarygol 246 218-221) showing that adhesins and/or colonization factors could be present in Blebs. Immune response to proteins present in outer membrane vesicles has been observed in patients with various *H influenzae* diseases (Sakakura Y, Harada T Hamaguchi Y Jin C S 1988 Acta Otorhinolarygol Suppl (Stockh) 454 222-226, Harada T, Sakakura Y, Miyoshi Y 1986 Rhinology 24 61-66)

25 *Pseudomonas aeruginosa*

The genus *Pseudomonas* consists of Gram-negative, polarly flagellated straight and slightly curved rods that grow aerobically and do not form spores. Because of their limited metabolic requirements, *Pseudomonas spp* are ubiquitous and are widely distributed in the soil, the air, sewage water and in plants. Numerous species of *Pseudomonas* such as *P aeruginosa*, *P pseudomallei*, *P mallei*, *P maltophilia* and *P cepacia* have also been shown to be pathogenic for human. Among this list, *P aeruginosa* is considered as an important human pathogen since it is

associated with opportunistic infection of immuno-compromised host and is responsible for high morbidity in hospitalized patients. Nosocomial infection with *P. aeruginosa* afflicts primarily patients submitted for prolonged treatment and receiving immuno-suppressive agents, corticosteroids, antimetabolites, antibiotics or radiation.

The *Pseudomonas*, and particularly *P. aeruginosa*, produces a variety of toxins (such as hemolysins, fibrinolysins, esterases, coagulases, phospholipases, endo- and exo-toxins) that contribute to the pathogenicity of these bacteria. Moreover, these organisms have high intrinsic resistance to antibiotics due to the presence of multiple drug efflux pumps. This latter characteristic often complicates the outcome of the disease.

Due to the uncontrolled use of antibacterial chemotherapeutics, the frequency of nosocomial infection caused by *P. aeruginosa* has increased considerably over the last 30 years. In the US, for example, the economic burden of *P. aeruginosa* nosocomial infection is estimated to 4.5 billion US\$ annually. Therefore, the development of a vaccine for active or passive immunization against *P. aeruginosa* is actively needed (for review see Stanislavsky et al. 1997, FEMS Microbiol. Lett. 21: 243-277).

Various cell-associated and secreted antigens of *P. aeruginosa* have been the subject of vaccine development. Among *Pseudomonas* antigens, the mucoid substance, which is an extracellular slime consisting predominantly of alginate, was found to be heterogenous in terms of size and immunogenicity. High molecular mass alginate components (30-300 kDa) appear to contain conserved epitopes while lower molecular mass alginate components (10-30 kDa) possess conserved epitopes in addition to unique epitopes.

Surface-exposed antigens including O-antigens (O-specific polysaccharide of LPS) or H-antigens (flagellar antigens) have been used for serotyping due to their highly immunogenic nature. Chemical structures of repeating units of O-specific polysaccharides have been elucidated and these data allowed the identification of 31 chemotypes of *P. aeruginosa*. Conserved epitopes among all serotypes of *P. aeruginosa* are located in the core oligosaccharide and the lipid A region of LPS and immunogens containing these epitopes induce cross-protective immunity in mice against different *P. aeruginosa* immunotypes. The outer core of LPS was implicated

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DAS

to be a ligand for binding of *P. aeruginosa* to airway and ocular epithelial cells of animals. However, heterogeneity exists in this outer core region among different serotypes. Epitopes in the inner core are highly conserved and have been demonstrated to be surface-accessible, and not masked by O-specific polysaccharide.

5 Vaccines prepared from *P. aeruginosa* ribosomes induce protective immunity in mice, but the efficacy of ribosomal vaccines in humans is not yet known. A number of recent studies indicate the potential of some *P. aeruginosa* antigens that deserve attention as new vaccine candidates.

10 To examine the protective properties of OM proteins, a vaccine containing *P. aeruginosa* OM proteins of molecular masses ranging from 20 to 100 kDa has been used in pre-clinical and clinical trials. This vaccine was efficacious in animal models against *P. aeruginosa* challenge and induced high levels of specific antibodies in human volunteers. Plasma from human volunteers containing anti-*P. aeruginosa* antibodies provided passive protection and helped the recovery of 87% of patients
15 with severe forms of *P. aeruginosa* infection. More recently, a hybrid protein containing parts of the outer membrane proteins OprF (amino acids 190-342) and OprI (amino acids 21-83) from *Pseudomonas aeruginosa* fused to the glutathione-S-transferase was shown to protect mice against a 975-fold 50% lethal dose of *P. aeruginosa* (Knapp et al. 1999. Vaccine 17:1663-1669).

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Although bleb vaccines made from the above bacterial strains (and others) are promising vaccine candidates, the present inventors have realised a number of drawbacks associated with wild-type bleb vaccines (either naturally occurring or chemically made).

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Examples of such problems are the following:

- the presence of immunodominant but variable proteins on the bleb (PorA, TbpB, Opa [*N. meningitidis* B], P2, P5 [non-typeable *H. influenzae*]) – such blebs being effective only against a restricted selection of bacterial species. Type-specificity of the bactericidal antibody response may
30 preclude the use of such vaccines in infancy.
- the presence of unprotective (non relevant) antigens (Rmp, H8,) on the bleb – antigens that are decoys for the immune system.

- the lack of presence of important molecules which are produced conditionally (for instance iron-regulated outer membrane proteins IROMP's, in vivo regulated expression mechanisms) – such conditions are hard to control in bleb production in order to optimise the amount of antigen on the surface
- the low level of expression of protective, conserved antigens (NspA, P6)
- the toxicity of the LPS remaining on the surface of the bleb
- the potential induction of an autoimmune response because of host-identical structures (for example the capsular polysaccharide in *Neisseria meningitidis* serogroup B, the lacto-N-neotetraose in *Neisseria* LPS, saccharide structure within ntH1 LPS, saccharide structures within Pili)

Such problems may prevent the use of bleb vaccines as human vaccine reagents. Accordingly, the present invention provides methods of removing the above problems (for instance by using genetically engineered bacterial strains), and consequently improving the bleb vaccines. Such methods will be especially useful in the generation of new vaccines against bacterial pathogens such as *Neisseria meningitidis*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and others.

The aim of these new bleb vaccines is to focus the immune response on a few conserved antigens or epitopes - formulated in a multiple component vaccine. If such an antigen is an integral OMP the outer membrane vesicles of bleb vaccines will ensure its proper folding. This invention provides methods to optimize the OMP and LPS composition of OMV (bleb) vaccines by deleting immunodominant variable as well as non protective OMPs, by creating conserved OMPs by deletion of variable regions, by upregulating expression of desirable protective OMPs, by eliminating control mechanisms for expression such as iron restriction. In addition, the invention allows the saccharide part of LPS to be optimized genetically to ensure the desired immune response and avoiding host-identical structures. In addition the invention provides for the reduction in toxicity of lipid A by modification of the lipid portion or by changing the phosphoryl composition whilst retaining its adjuvant activity or by immunologically masking it by binding protective antigens to it. Each of these

methods of improvement individually improve the bleb vaccine however a combination of one or more of these methods work in conjunction so as to produce an optimised engineered bleb vaccine

5 SUMMARY OF THE INVENTION

The present invention provides an optimised engineered bleb vaccine preparation from a Gram-negative bacterial strain characterized in that it is manufactured using one or more methods selected from the following group

- 10 a) genetically reducing immunodominant variable antigens within the bleb vaccine comprising the steps of determining the identity of such an antigen engineering a bacterial strain to produce less or none of said antigen and making blebs from said strain,
- 15 b) genetically reducing non protective OMP antigens within the bleb vaccine comprising the steps of determining the identity of such an antigen engineering a bacterial strain to produce less or none of said antigen and making blebs from said strain,
- 20 c) genetically creating conserved OMP antigens within the bleb vaccine comprising the steps of identifying the identity of such an antigen engineering a bacterial strain so as to delete variable regions of the gene encoding said antigen, and making blebs from said strain
- 25 d) genetically upregulating expression of conserved protective OMP antigens within the bleb vaccine comprising the steps of identifying the identity of such an antigen, engineering a bacterial strain so as to remove the repressive control mechanisms of its expression (such as iron restriction) and making blebs from said strain,
- 30 e) genetically upregulating expression of conserved, protective OMP antigens within the bleb vaccine comprising the steps of identifying the identity of such an antigen, engineering a bacterial strain so as to introduce a stronger promoter sequence upstream of the gene encoding said antigen and making blebs from said strain,
- f) genetically modifying the saccharide portion of the bacterial LPS within the bleb vaccine, comprising the steps of identifying a gene involved in the

rendering the saccharide portions of LPS toxic, engineering a bacterial strain so as to reduce the expression of said gene and making blebs from said strain,

g) genetically modifying the lipid A portion of the bacterial LPS within the bleb vaccine comprising the steps of identifying a gene involved in the rendering the lipid A portion of LPS toxic, engineering a bacterial strain so as to reduce the expression of said gene, and making blebs from said strain, or

h) reducing the lipid A toxicity within the bleb vaccine and increasing the levels of protective antigens, comprising the steps of engineering the genome of a bacterial strain to incorporate a gene encoding a Polymyxin A peptide (or a derivative thereof) fused to a protective antigen and making blebs from said strain

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Reactivity of the 735 mAb on different colonies

Figure 2 Reactivities of specific monoclonal antibodies by whole cell Elisa

Figure 3 Schematic representation of the pCMK vectors used to deliver genes, operons and/or expression cassettes in the genome of *Neisseria meningitidis*

Figure 4 Analysis of PorA expression in total protein extracts of recombinant *N meningitidis* serogroupB (H44/76 derivatives) Total proteins were recovered from *cps*- (lanes 3 and 4), *cps-porA* pCMK+ (lanes 2 and 5) and *cps-porA nspA* (lanes 1 and 6) recombinant *N meningitidis* serogroupB strains and were analyzed under SDS-PAGE conditions in a 12% polyacrylamide gel. Gels were stained with Coomassie blue (lanes 1 to 3) or transferred to a nitrocellulose membrane and immuno-stained with an anti-PorA monoclonal antibody

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Figure 5 Analysis of NspA expression in protein extracts of recombinant *N meningitidis* serogroupB strains (H44/76 derivatives) Proteins were extracted from whole bacteria (lanes 1 to 3) or outer-membrane blebs preparations (lanes 4 to 6) separated by SDS-PAGE on a 12% acrylamide gel and analyzed by immuno-blotting using an anti-NspA polyclonal serum Samples corresponding to *cps*- (lanes 1 and 6) *cps*- *porA* *pCMK*+ (lanes 3 and 4) and *cps*- *porA* *nspA* (lanes 2 and 5) were analyzed Two forms of NspA were detected a mature form (18kDa) co-migrating with the recombinant purified NspA, and a shorter form (15kDa)

Figure 6 Analysis of D15/omp85 expression in protein extracts of recombinant *N meningitidis* serogroupB strains (H44/76 derivatives) Proteins were extracted from outer-membrane blebs preparations and were separated by SDS-PAGE on a 12% acrylamide gel and analyzed by immuno-blotting using an anti-omp85 polyclonal serum Samples corresponding to *cps*- (lane 2), and *cps*- *PorA*+ *pCMK*+*Omp85/D15* (lane 1) recombinant *N meningitidis* serogroupB strains were analyzed

Figure 7 General strategy for modulating gene expression by promoter delivery (RS stands for restriction site)

Figure 8 Analysis of outer-membrane blebs produced by recombinant *N meningitidis* serogroupB *cps*- strains (H44/76 derivatives) Proteins were extracted from outer-membrane bleb preparations and were separated by SDS-PAGE under reducing conditions on a 4-20% gradient polyacrylamide gel The gel was stained with Coomassie brilliant blue R250 Lanes 2, 4, 6 corresponded to 5µg of total proteins whereas lanes 3, 5 and 7 were loaded with 10µg proteins

DESCRIPTION OF THE INVENTION

The present invention relates to a general set of tools and methods capable of being used for manufacturing improved, engineered blebs from Gram-negative bacterial strains The invention includes methods used to make recombinant blebs more immunogenic, less toxic and safer for their use in a human and/or animal

vaccine Moreover the present invention also describes specific methods necessary for constructing producing, obtaining and using recombinant, engineered blebs from various Gram-negative bacteria, for vaccine, therapeutic and/or diagnostic purposes By the methods of the invention, the biochemical composition of bacterial blebs can be manipulated by acting upon/altering the expression of bacterial genes encoding products present in or associated with bacterial outer-membrane blebs The production of blebs using a method of genetic modification to increase, decrease or render conditional the expression of one or more genes encoding outer-membrane components are also included in the scope of this invention

For clarity, the term 'expression cassette' will refer herein to all the genetic elements necessary to express a gene or an operon and to produce and target the corresponding protein(s) of interest to outer-membrane blebs, derived from a given bacterial host A non-exhaustive list of these features includes control elements (transcriptional and/or translational), protein coding regions and targeting signals

Moreover, the term "integrative cassette" will refer herein to all the genetic elements required to integrate a DNA segment in given bacterial host A non-exhaustive list of these features includes a delivery vehicle recombinogenic regions, and selectable and counter selectable markers

Aspects of the invention relate to individual methods for making improved engineered blebs, to a combination of such methods, and to the bleb compositions made as a result of these methods Another aspect of the invention relates to the genetic tools used in order to genetically modify a chosen bacterial strain in order to extract improved engineered blebs from said strain

The first two aspects of the invention relate to tools and methods which allow the modulation of expression of one or more endogenous components of bacterial blebs by delivering, for instance, genes, operons or promoters in the genome of a preferred host

1 Vectors for gene and/or operon delivery

An efficient strategy to modulate the composition of a Bleb preparation is to deliver one or more copies of a DNA segment containing an expression cassette into

the genome of a Gram-negative bacterium. A non exhaustive list of preferred bacterial species that could be used as a recipient for such a cassette includes *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*. The gene(s) contained in the expression cassette may be homologous (i.e. exist naturally in the genome of the manipulated bacterium) or heterologous (i.e. do not exist naturally in the genome of the manipulated bacterium). The reintroduced expression cassette may consist of unmodified, 'natural' promoter/gene/operon sequences or engineered expression cassettes in which the promoter region and/or the coding region or both have been altered. A non-exhaustive list of preferred promoters that could be used for expression includes the promoters *porA*, *porB*, *lbpB*, *tbpB*, *pilQ*, *lgt*, *hpuAB* from *N. meningitidis* or *N. gonorrhoeae*, the promoter λ pL, *lac*, *tac*, *araB* of *Escherichia coli* or promoters recognized specifically by bacteriophage RNA polymerase such as the *E. coli* bacteriophage T7. A non-exhaustive list of preferred genes that could be expressed in such a system includes *Neisseria* NspA, Omp85, PilQ, TbpA/B complex, Hsf, PldA, HasR, *Chlamydia* MOMP, HMWP, *Moraxella* OMP106, HasR, PilQ, OMP85, PldA, *Bordetella pertussis* FHA, PRN, PT.

In a preferred embodiment of the invention the expression cassette is delivered and integrated in the bacterial chromosome by means of homologous and/or site specific recombination. Integrative vectors used to deliver such genes and/or operons can be conditionally replicative or suicide plasmids, bacteriophages, transposons or linear DNA fragments obtained by restriction hydrolysis or PCR amplification. Integration is preferably targeted to chromosomal regions dispensable for growth *in vitro*. A non exhaustive list of preferred loci that can be used to target DNA integration includes the *porA*, *porB*, *opa*, *opc*, *rmp*, *omp26*, *lecA*, *cps*, *lgtB* genes of *Neisseria meningitidis* and *Neisseria gonorrhoeae*, the *P1*, *P5*, *hmv1/2*, *IgA-protease*, *fimE* genes of NTHi, the *lecA1*, *lecA2*, *omp106*, *uspA1*, *uspA2* genes of *Moraxella catarrhalis*. Alternatively, the expression cassette used to modulate the expression of bleb component(s) can be delivered into a bacterium of choice by means of episomal vectors such as circular/linear replicative plasmids, cosmids, phasmids, lysogenic bacteriophages or bacterial artificial chromosomes. Selection of the recombination event will be selected by means of selectable genetic marker such as

genes conferring resistance to antibiotics (for instance kanamycin, erythromycin, chloramphenicol, or gentamycin), genes conferring resistance to heavy metals and/or toxic compounds or genes complementing auxotrophic mutations (for instance *pin*, *leu*, *met*, *aro*)

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2 Vector for promoter delivery and modulation

Another particularly preferred aspect of the invention relates to modifying the composition of blebs by altering *in situ* the regulatory region controlling the expression of gene(s) and/or operon(s) of interest. This alteration includes partial or
10 total replacement of the endogenous promoter controlling the expression of a gene of interest, with one conferring a distinct transcriptional activity. This distinct transcriptional activity may be conferred by variants (point mutations, deletions and/or insertions) of the endogenous control regions, by naturally occurring or modified heterologous promoters or by a combination of both. Such alterations will
15 preferably confer a transcriptional activity stronger than the endogenous one, resulting in an enhanced expression of the gene(s)/operon(s) of interest (up-regulation). Such a method is particularly useful for enhancing the production of immunologically relevant Bleb components such as outer-membrane proteins and lipoproteins.

Alternatively, such alterations can confer a transcriptional activity weaker than
20 the endogenous one, resulting in a decreased expression of the gene(s)/operon(s) of interest (down-regulation). This second method is particularly useful for decreasing the production of deleterious molecules such as cross-reactive bacterial polysaccharide (as found in *N. meningitidis* serogroup B), toxic lipopolysaccharide and non-protective immunodominant antigens.

25 In a third method concerning this approach, such alterations confer a conditional transcriptional activity, resulting in a conditional expression of the gene(s)/operon(s) of interest (down-regulation). This third approach is useful in the expression of molecules that are toxic to or not well supported by the bacterial host.

A preferred embodiment of this aspect of the invention is when the promoter
30 region is delivered and integrated in the bacterial chromosome by means of homologous and/or site specific recombination. Integrative vectors used to deliver such promoter regions can be conditionally replicative or suicide plasmids,

bacteriophages, transposons or linear DNA fragments obtained by restriction hydrolysis or PCR amplification. Selection of the recombination event is selected by means of selectable genetic marker such as genes conferring resistance to antibiotics (for instance kanamycin, erythromycin, chloramphenicol, or gentamycin) genes
5 conferring resistance to heavy metals and/or toxic compounds or genes complementing auxotrophic mutations (for instance *pur leu met aro*)

A third aspect of the invention relates to methods for enriching bleb preparations in molecules that are normally absent or under-represented

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3 Bleb components produced conditionally

The expression of some genes coding for certain bleb components is carefully regulated. The production of the components is conditionally modulated and depends upon various metabolic and/or environmental signals. Such signals include for
15 example, iron-limitation, modulation of the redox potential, pH and temperature variations, nutritional changes. Some examples of bleb components known to be produced conditionally include iron-regulated outer-membrane proteins from *Neisseria* and *Moraxella* (for instance TbpB, LbpB), and substrate-inducible outer-membrane porins. The present invention covers the use of the genetic methods
20 described previously (under gene and/or promoter delivery) to render constitutive the expression such molecules. Indeed, an approach to overcome the influence of environmental signal upon the expression of gene(s) of interest consists in modifying/replacing its corresponding control region so that it becomes constitutively active. Alternatively, another approach consists in delivering to the genome an
25 additional copy of the gene/operon of interest which is placed artificially under the control of a constitutive promoter.

4 Expression of foreign/heterologous proteins in outer-membrane blebs

Outer-membrane bacterial blebs represent a very attractive system to produce,
30 isolate and deliver recombinant proteins for vaccine, therapeutic and/or diagnostic uses. A fourth aspect of this invention is in respect of the expression, production and targeting of foreign, heterologous proteins (not synthesized naturally by the host

bacterium) to the outer-membrane and the use of the bacteria to produce recombinant blebs. A non-exhaustive list of bacteria that can be used as a recipient host for production of recombinant blebs includes *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*. The gene expressed in such a system can be of viral, bacterial, fungal, parasitic or higher eukaryotes origin. In that context, a preferred application of the invention includes the expression of *Moraxella*, *Haemophilus* and/or *Pseudomonas* outer-membrane proteins (integral polytopic and/or lipoproteins) in *Neisseria meningitidis* recombinant blebs.

A particularly preferred application of this aspect is in the field of the prophylaxis or treatment of sexually-transmitted diseases (STDs). It is often difficult for practitioners to determine whether the principal cause of a STD is due to gonococcus or *Chlamydia trachomatis* infection. These two organisms are the main causes of salpingitis – a disease which can lead to sterility in the host. It would therefore be useful for a STD to be vaccinated against or treated with a combined vaccine which could disease caused by both organisms. The Major Outer Membrane Protein (MOMP) of *C. trachomatis* has been shown to be the target of protective antibodies. However, the structural integrity of this integral membrane protein is important for inducing such antibodies. In addition, the epitopes recognised by these antibodies are variable and define more than 10 serovars. The previously described aspect of this invention allows the proper folding of one or more membrane proteins within a bleb outer membrane preparation. The engineering of a gonococcal strain expressing multiple *C. trachomatis* MOMP serovars in the outer membrane, and the production of blebs therefrom produces a single solution to the multiple problems of correctly folded membrane proteins, the presentation of sufficient MOMP serovars to protect against a wide spectrum of serovars, and the simultaneous prophylaxis/treatment of gonococcal infection (and consequently the non-requirement of practitioners to initially decide which organism is causing particular clinical symptoms – both organisms can be vaccinated against at an early stage thus allowing the treatment of the STD at a very early stage).

Targeting of heterologous proteins to outer-membrane blebs

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The expression of some heterologous proteins in bacterial blebs may require the addition of outer-membrane targeting signal(s). The preferred method to solve this problem is by creating genetic fusion between a heterologous gene and a gene coding for a resident OMP as a specific approach to target recombinant proteins to blebs. Most preferably, the heterologous gene is fused to the signal peptides sequences of such an OMP.

5 Anchoring heterologous proteins to outer-membrane blebs

A fifth aspect of this invention covers the use of genetic sequences encoding polymyxin B peptides (or analogues thereof) as a means to target fusion proteins to the outer-membrane. Polymyxin B is a cyclic peptide composed of non tRNA-encoded amino acids (produced by Gram-positive actinomycetal organisms) that binds very strongly to the Lipid A part of LPS present in the outer-membrane. This binding decreases the intrinsic toxicity of LPS (Endotoxin activity). Peptides mimicking the structure of Polymyxin B and composed of canonical (tRNA encoded) amino acids have been developed and also bind lipid A with a strong affinity. These peptides have already been used for detoxifying LPS in order to use it as a vaccine. One of these peptide called SEAP-2 (Nterminus-Lys-Thr-Lys-Cys-Lys-Phe-Leu-Lys-Lys-Cys-Cterminus) was shown to be very promising in that respect (Molecular Mapping and detoxifying of the Lipid A binding site by synthetic peptides (1993) Rustici, A, Velucchi, M, Faggioni, R, Sironi M, Ghezzi, P, Quataert, S, Green, B and M Porro *Science* 259, 361-365). The present aspect provides an improvement to this use by describing the use of DNA sequences coding for the SEAP-2 peptide (or derivatives therefrom), fused genetically to a gene of interest (encoding a protective antigen) as a mean to target the corresponding recombinant protein to the outer-membrane of a preferred bacterial host (whilst at the same time reducing the toxicity of the LPS). This targeting strategy might be of particular interest in the case of genes encoding proteins that are not normally targeted to the outer-membrane. This methodology should allow the isolation of recombinant blebs enriched in the foreign/heterologous protein of interest. Preferably such a peptide targeting signal allows the enrichment of outer membrane blebs in one or several proteins of interest, which are naturally not found in that given subcellular localization. A non exhaustive

list of bacteria that can be used as a recipient host for such a production of recombinant blebs includes *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*

5 An alternative preferred embodiment is that SAEP-2-tagged recombinant proteins may be made separately, and may be attached at a late stage to a bleb preparation

A further embodiment of this aspect is that this system can be used in a new expression system for producing recombinant proteins in general. The SAEP-2 peptide tag could be used for affinity purification of the protein to which it is attached using a column containing immobilised lipid A molecules

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In further aspects, this invention relates to methods used for removing undesirable molecules from recombinant bleb preparations

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6 Detoxification of LPS

This invention relates to methods of genetically detoxifying the LPS present in blebs. Lipid A is the primary component of LPS responsible for cell activation. Many mutations in genes involved in this pathway lead to essential phenotypes. However mutations in the genes responsible for the terminal modifications steps lead to temperature-sensitive (*htrB*) or permissive (*msbB*) phenotypes. Mutations in these genes result in altered toxic activity of lipid A. Indeed, the non-lauroylated (*htrB*) or non-myristoylated (*msbB*) lipid A are less toxic than the wild-type lipid A (Clementz T, Zhou Z, Raetz C R H 1997 *J Biol Chem* 272 10353-10360, Clementz T, Bednarski J J, Raetz C R H 1996 *J Biol Chem* 272 12095-12102, Sommerville J E Jr, Cassiano L, Bainbridge B, Cunningham M D, Darveau R 1996 *J Clin Invest* 99 359-365, Karow M, Georgopoulos C 1992 *J Bacteriol* 174 702-710). Mutations in the lipid A 4'-kinase encoding gene (*lpxK*) also alters the toxic activity of lipid A (Garret T, Kadamas J L, Raetz C R H 1997 *J Biol Chem* 272 21855-21864, Garret, T A, Que, N L S, Raetz, C R H 1998 *J Biol Chem* 273 12457-12465)

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Mutations in the PhoP-PhoQ regulatory system, which is a phospho-relay two component regulatory system (for PhoP constitutive phenotype, PhoP^c) or low

Mg²⁺ environmental or culture conditions (that activate the PhoP-PhoQ regulatory system) lead to the addition of aminoarabinose on the 4'-phosphate and 2-hydroxymyristate replacing myristate (hydroxylation of myristate) This modified lipid A displays reduced ability to stimulate E-selectin expression by human endothelial cells and TNF- α secretion from human monocytes (Guo L., Lim K B, Gunn J S, Bainbridge B, Darveau R P, Hackett M, Miller, S I 1997 *Science* 276 250-253)

LPS toxic activity could also be altered by introducing mutations in genes/loci involved in polymyxin B resistance (such resistance has been correlated with addition of aminoarabinose on the 4 phosphate of lipid A) These genes/loci could be *pmrE* that encodes a UDP-glucose dehydrogenase, or a region of antimicrobial peptide-resistance genes common to many enterobacteriaceae which could be involved in aminoarabinose synthesis and transfer The gene *pmiF* that is present in this region encodes a dolicol-phosphate manosyl transferase (Gunn J S, Kheng, B L, Krueger J, Kim K, Guo L, Hackett M, Miller S I 1998 *Mol Microbiol* 27 1171-1182)

The use of polymyxin B resistant strains also leads to the preparation of Blebs with reduced LPS toxicity For instance meningococcal mutant strains resistant to polymyxin B express a LPS with reduced toxicity (van der Ley, P, Hamstra HJ, Kramer, M, Steeghs, L, Petrov, A and Poolman, JT 1994 *In* Proceedings of the ninth international pathogenic Neisseria conference The Guildhall, Winchester, England) The addition of synthetic peptides that mimic the binding activity of polymyxin B (as previously described) to the Bleb preparation may also be done in order to reduce LPS toxic activity (Rustici, A, Velucchi, M, Faggioni R, Sironi, M, Ghezzi, P, Quataert, S, Green, B and Porro M 1993 *Science* 259 361-365, Velucchi, M, Rustici, A, Meazza, C, Villa, P, Ghezzi, P and Porro, M 1997 *J Endotox Res* 4)

7 Variable and non-protective immunodominant antigens

Many surface antigens are variable among bacterial strains and as a consequence are protective only against a limited set of closely related strains A seventh aspect of this invention covers the reduction in expression, or, preferably, the deletion of the gene(s) encoding variable surface protein(s) which would result in a

bacterial strain producing blebs with a stronger cross-reactivity potential due to a higher influence to the immune response by conserved proteins retained on the outer membranes. Examples of such variable antigens include *Neisseria* pilin (PilC) which undergoes antigenic variations as well as PorA, Opa, TbpB, FrpB. *H. influenzae* P2 P5, pilin, IgA1-protease, *Moraxella* CopB, OMP106

Most of these proteins are integral OMPs and their variability may be limited to one or few of their surface exposed loops. Another aspect of this invention covers the deletion of DNA regions coding for these surface exposed loops which would lead to the expression of an integral OMP containing conserved surface exposed loops. Surface exposed loops of *H. influenzae* P2 and P5 are preferred examples of proteins that could be turned into cross-reactive antigens by using such a method.

The deletion of genes which *in vivo*, may easily be expressed or not by the bacterium is another aspect of this invention – as proteins encoded by such genes are not always present in the bacteria, the presence of such proteins in the bleb preparations may also be detrimental to the effectiveness of the vaccine. A preferred example is, *Neisseria* Opc protein expression which can be turned off in response to the immune selection induced by an Opc containing bleb vaccine. Such anti-Opc immunity would only have limited protective capacity as infecting organism could become Opc-. *H. influenzae* HgpA and HgpB are other preferred examples of such proteins.

8 Cross-reactive capsular polysaccharides

The isolation of bacterial outer-membrane blebs from capsulated Gram-negative bacteria often results in the co-purification of capsular polysaccharide. In some cases, this ‘contaminant’ material may prove useful since polysaccharide may enhance the immune response conferred by other bleb components. In other cases however, the presence of contaminating polysaccharide material in bacterial bleb preparations may prove detrimental to the use of the blebs in a vaccine. For instance it has been shown at least in the case of *N. meningitidis* that the serogroup B capsular polysaccharide does not confer protective immunity and is susceptible to induce an adverse auto-immune response in humans. Consequently, another aspect of this invention is to engineer the bacterial strain used to be free of capsular polysaccharide,

and making blebs from such a strain. The blebs will then be suitable for use in humans. A particularly preferred example of such a bleb preparation is one from *N. meningitidis* serogroup B devoid of capsular polysaccharide.

This may be achieved by using modified bleb production strains in which the genes necessary for capsular biosynthesis and/or export have been impaired. Inactivation of the gene coding for capsular polysaccharide biosynthesis or export can be achieved by mutating (point mutation, deletion or insertion) either the control region, the coding region or both. Moreover, inactivation of capsular biosynthesis genes may also be achieved by antisense over-expression or transposon mutagenesis. A preferred method is the deletion of some or all of the *Neisseria meningitidis* *cps* genes required for polysaccharide biosynthesis and export. For this purpose the replacement plasmid pMF121 (described in Frosh et al 1990, *Mol Microbiol* 4 1215-1218) can be used to deliver a mutation deleting the *cpsCAD* gene cluster.

The Production of Blebs

The manufacture of bleb preparations from any of the aforementioned strains may be achieved by any of the methods well known to a skilled person. Preferably the methods disclosed in EP 301992, US 5,597 572, EP 1 1243 or US 4,271,147 are used.

Most preferably the method described in Example 8 is used.

The combination of methods 1-8 to make an optimised bleb vaccine

The aforementioned methods of the invention (1-8) can each be used to improve the effectiveness of the bleb vaccine finally produced. Preferably one such method is used, more preferably 2, 3, 4, 5, 6 or 7 of the methods are used, and most preferably all 8 of the methods are used in order to manufacture the bleb vaccine. As each additional method is used in the manufacture of the bleb vaccine, each improvement works in conjunction with the other methods used in order to make an optimised engineered bleb preparation.

General method for the analysis of the non-coding flanking region of a bacterial gene, and its exploitation for modulated expression of the gene in blebs

The non-coding flanking regions of the a specific gene contains regulatory elements important in the expression of the gene This regulation takes place both at the transcriptional and translational level The sequence of these regions either upstream or downstream of the open reading frame of the gene, can be obtained by DNA sequencing (preferred examples of such regions are the NspA, D15, Hsf-like PilQ and Hap gene upstream regions of *N meningitidis* shown in SEQ ID NO 2-6 respectively) This sequence information allows the determination of potential regulatory motifs such as the different promoter elements, terminator sequences inducible sequence elements, repressors, elements responsible for phase variation the Shine-Dalgarno sequence, regions with potential secondary structure involved in regulation, as well as other types of regulatory motifs or sequences

This sequence information allows the modulation of the natural expression of the gene in question The upregulation of the gene expression may be accomplished by altering the promoter, the Shine Dalgarno sequence, potential repressor or operator elements, or any other elements involved Likewise, downregulation of expression can be achieved by similar types of modifications Alternatively, by changing phase variation sequences, the expression of the gene can be put under phase variation control, or may be uncoupled from this regulation In another approach, the expression of the gene can be put under the control of one or more inducible elements allowing regulated expression Examples of such regulation include, but are not limited to, induction by temperature shift, addition of inductor substrates like selected carbohydrates or their derivatives, trace elements, vitamins, co-factors, metal ions etc

Such modifications as described above can be introduced by several different means The modification of sequences involved in gene expression can be done *in vivo* by random mutagenesis followed by selection for the desired phenotype Another approach consists in isolating the region of interest and modifying it by random mutagenesis, or site-directed replacement, insertion or deletion mutagenesis The modified region can then be reintroduced into the bacterial genome by homologous recombination, and the effect on gene expression can be assessed In another approach, the sequence knowledge of the region of interest can be used to replace or delete all or part of the natural regulatory sequences In this case, the regulatory region targeted is isolated and modified so as to contain the regulatory elements from another

gene, a combination of regulatory elements from different genes, a synthetic regulatory region, or any other regulatory region, or to delete selected parts of the wild-type regulatory sequences. These modified sequences can then be reintroduced into the bacterium via homologous recombination into the genome.

- 5 In a preferred example, the expression of the gene can be modulated by exchanging its promoter with a stronger promoter (through isolating the upstream sequence of the gene [for instance SEQ ID NO 1-6], in vitro modification of this sequence and reintroduction into the genome by homologous recombination). Upregulated expression can be obtained in both the bacterium as well as in the outer
10 membrane vesicles shed (or made) from the bacterium.

- In other preferred examples, the described approaches can be used to generate recombinant bacterial strains with improved characteristics for vaccine applications. These can be, but are not limited to, attenuated strains, strains with increased expression of selected antigens, strains with knock-outs (or decreased expression) of
15 genes interfering with the immune response, strains with modulated expression of immunodominant proteins, strains with modulated shedding of outer-membrane vesicles.

EXAMPLES

The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples are illustrative, but do not limit the invention.

Example 1 Construction of a *Neisseria meningitidis* serogroup B strain lacking capsular polysaccharides

The plasmid pMF121 (Frosch et al., 1990) has been used to construct a *Neisseria meningitidis* B strain lacking the capsular polysaccharide. This plasmid contains the flanking regions of the gene locus coding for the biosynthesis pathway of the group B polysaccharide (B PS), and the erythromycin resistance gene. Deletion of the B PS resulted in loss of expression of the group B capsular polysaccharide as well as a deletion in the active copy of *galE* leading to the synthesis of galactose deficient LPS.

Strain transformation

Neisseria meningitidis B H44/76 strain (B 15 P1 7, 16, Los 3,7,9) was selected for transformation. After an overnight CO₂ incubation on MH plate (without erythromycin), cells were collected in liquid MH containing 10 mM MgCl₂ (2 ml were used per MH plate) and diluted up to an OD of 0.1 (550 nm). To this 2 ml solution, 4 µl of the plasmid pMF121 stock solution (0.5 µg/ml) were added for a 6 hours incubation period at 37°C (with shaking). A control group was done with the same amount of *Neisseria meningitidis* B bacteria, but without addition of plasmid. After the incubation period, 100 µl of culture, as such, at 1/10, 1/100 and 1/1000 dilutions, were put in MH plates containing 5, 10, 20, 40 or 80 µg erythromycin/ml before incubation for 48 hours at 37°C.

Colony blotting

After plate incubation, 20 colonies were grown and selected from the 10 and 20 µg erythromycin/ml MH plates, while there was no colony growth in the control group without plasmid transformation. The H44/76 wild type strain was unable to grow in

the selected erythromycin plates (10 to 80 µg erythromycin/ml) The day after all the visible colonies were placed on new MH plates without erythromycin in order to let them grow Afterwards, they were transferred onto nitrocellulose sheets (colony blotting) for presence of B polysaccharide Briefly, colonies were blotted onto a nitrocellulose sheet and rinsed directly in PBS-0.05 % Tween 20 before cell inactivation for 1 hour at 56°C in PBS-0.05% Tween 20 (diluant buffer) Afterwards the membrane was overlaid for one hour in the diluant buffer at room temperature (RT) Then sheets were washed again for three times 5 minutes in the diluant buffer before incubation with the anti-B PS 735 Mab (Boehringer) diluted at 1/3000 in the diluant buffer for 2 hours at RT After a new washing step (3 times 5 minutes) the monoclonal antibody was detected with a biotinylated anti-mouse Ig from Amersham (RPN 1001) diluted 500 times in the diluant buffer (one hour at RT) before the next washing step (as described above) Afterwards, sheets were incubated for one hour at RT with a solution of streptavidin-peroxidase complex diluted 1/1000 in the diluant buffer After the last three washing steps using the same method, nitrocellulose sheets were incubated for 15 min in the dark using the revelation solution (30 mg of 4-chloro-1-naphthol solution in 10 ml methanol plus 40 ml PBS and 30 mcl of H₂O₂ 37% from Merck) The reaction was stopped with a distilled water-washing step

20 Whole cell Elisas

Whole cell Elisas were also done using the two transformed colonies ('D' and 'R') and the wild type strain (H44/76) as coated bacteria (20 µg protein/ml), and a set of different monoclonal antibodies used to characterize *Neisseria meningitidis* strains The following Mabs were tested anti-B PS (735 from Dr Frosch), and the other Mabs from NIBSC anti-B PS (Ref 95/750) anti-P1 7 (A-PorA, Ref 4025), anti-P1 16 (A-PorA, Ref 95/720), anti-Los 3,7,9 (A-LPS, Ref 4047), anti-Los 8 (A-LPS, Ref 4048), and anti-P1 2 (A-PorA Ref 95/696)

Microtiter plates (Maxisorp, Nunc) were coated with 100 µl of the recombinant meningococcal B cells solution overnight (ON) at 37°C at around 20 µg/ml in PBS Afterwards plates are washed three times with 300 µl of 150 mM NaCl - 0.05 % Tween 20, and were overlaid with 100 µl of PBS-0.3 % Casein and incubated for 30 min at room temperature with shaking Plates were washed again

using the same procedure before incubation with antibodies. Monoclonal antibodies (100 µl) were used at different dilutions (as shown in Figure 2) in PBS-0.3 % Casein - 0.05 % Tween 20 and put onto the microplates before incubation at room temperature for 30 min with shaking, before the next identical washing step. 100 µl of the anti-mouse Ig (from rabbit, Dakopatts E0413) conjugated to biotin and diluted at 1/2000 in PBS-0.3 % Casein - 0.05 % Tween 20 were added to the wells to detect bound monoclonal antibodies. After the washing step (as before), plates were incubated with a streptavidin-peroxidase complex solution (100 µl of the Amersham RPN 1051) diluted at 1/4000 in the same working solution for 30 min at room temperature under shaking conditions. After this incubation and the last washing step, plates are incubated with 100 µl of the chromogen solution (4 mg orthophenylenediamine (OPD) in 10 ml 0.1 M citrate buffer pH4.5 with 5 µl H₂O₂) for 15 min in the dark. Plates are then read at 490/620 nm using a spectrophotometer.

Results

Figure 1 shows that from the 20 isolated colonies, which were able to grow on the selected medium with erythromycin, only two (the "D" and the "R") colonies were shown negative for presence of B polysaccharide. Among the others, 16 were clearly positive for B PS and still resistant to erythromycin. This indicated that they integrated the plasmid into their genome, but in the wrong orientation, and keeping intact the B PS and LPS gene (no double crossing-over). Positive and negative controls were also tested on the plates, and showed that the H44/76 wild type NmB strain was clearly positive for the B polysaccharide, while meningococcus A (A1) and meningococcus C (C11) strains were clearly negative with this anti-B PS 735 Mab. These results indicate that around 10 % of the selected colonies correctly integrated the plasmid in their genome by making a double crossing-over, while the other strains/colonies were obtained after a simple crossing-over keeping the B PS and LPS genes intact and expressed.

Using whole cell Elisa, results (Figure 2 and Table 1) clearly indicate that the two "D" and "R" transformants (derived from D and R colonies) can not be recognized anymore by the anti-B PS Mabs (735 and 95/750), nor by the anti-Los 3,7,9 and anti-Los 8 Mabs. However, when using specific anti-PorA Mabs, there is a

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clear reaction with the anti-P1 7 and anti-P1 16 Mabs on the cells as also observed in the wild-type strain. No reaction was observed with a non-specific anti-PorA Mab (anti-P1 2 mab). These results confirm that the PorA protein and specifically P1 7 and P1 16 epitopes are still present after transformation, while B polysaccharide and
5 Los 3 7,9 and Los 8 epitopes (LPS) were not

Example 2 Construction of versatile gene delivery vectors (the pCMK series) targeting integration in the *porA* locus of *Neisseria meningitidis*

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A plasmid allowing homologous recombination and stable integration of foreign DNA in the *porA* locus of *Neisseria meningitidis* was constructed. This delivery vector (genes, operons and/or expression cassettes) is useful for constructing *Neisseria meningitidis* strains producing recombinant, improved blebs. Typically
15 such a vector contains at least (1) a plasmid backbone replicative in *E. coli* but not in *Neisseria meningitidis* (a suicide plasmid), (2) at least one but preferably two regions of homology for targeting the integration in a chromosomal locus such as *porA*, (3) Efficient transcriptional (promoter, regulatory region and terminator) and translational (optimised ribosome binding site and initiation codon) signals functional in *Neisseria*
20 *meningitidis*, (4) a multiple cloning site and (5) selectable gene(s) allowing the maintenance of the plasmid in *E. coli* and the selection of integrants in *Neisseria meningitidis*. Additional elements include, for example, uptake sequences to facilitate the entry of foreign DNA in *Neisseria meningitidis* and counter selectable markers such as *sacB*, *rpsL*, *gltS* to enhance the frequency of double cross-over events.

25 A schematic drawing of the vector constructed in this example and designated pCMK is represented in Figure 3. Its corresponding complete nucleotide sequence is shown in SEQ ID NO 1. pCMK derives from a pSL1180 backbone (PharmaciaBiotech, Sweden), a high copy-number plasmid replicative in *E. coli* harbouring the *bla* gene (and thereby conferring resistance to ampicillin). In addition
30 to this, pCMK functionally contains two *porA* flanking regions (*porA*5' and *porA*3' containing a transcription terminator) necessary for homologous recombination, a selectable marker conferring resistance to kanamycin, two uptake sequences, a

porA/lacO chimeric promoter repressed in the *E. coli* host expressing *lacI^q* but transcriptionally active in *Neisseria meningitidis* and a multiple cloning site (5 sites present *NdeI*, *KpnI*, *NheI*, *PstI* and *SphI*) necessary for the insertion of foreign DNA in pCMK

pCMK was constructed as follows. The *porA* 5' and *porA* 3' recombinogenic regions, the *porA/lacO* promoter were PCR amplified using the oligonucleotides listed in Table 2, cloned in pTOPO and sequenced. These DNA fragments were successively excised from pTOPO and re-cloned in pSL1180. The kanamycin resistance cassette was excised from pUC4K (PharmaciaBiotech, Sweden) and was introduced between the *porA* 5' flanking region and the *porA/lacO* promoter region.

Example 3 Construction of a *Neisseria meningitidis* serogroup B strain lacking both capsular polysaccharides and the major immunodominant antigen PorA

Modulating the antigenic content of outer membrane blebs may be advantageous in improving their safety and efficacy in their use in vaccines or diagnostic or therapeutic uses. Components such as the *Neisseria meningitidis* serogroup B capsular polysaccharides should be removed to exclude the risk of inducing autoimmunity (see example 1). Similarly, it is beneficial to suppress the immunodominance of major outer-membrane antigens such as PorA which induce strain-specific bactericidal antibodies but fail to confer cross-protection. To illustrate such an approach, we used the pCMK(+) vector to construct a *Neisseria meningitidis* serogroup B strain lacking both capsular polysaccharides and the immunodominant PorA outer membrane protein antigen. For this purpose, a deletion of the *porA* gene was introduced in the H44/76 *cps*- strain, described in example 1 by means of homologous recombination.

The H44/76 *cps*- strain was prepared competent and transformed with two 2 µg of supercoiled pCMK(+) plasmid DNA as described previously. Aliquot fractions of the transformation mixture (100 µl) were plated on Mueller-Hinton plates supplemented with Kanamycin (200 µg/ml) and incubated at 37°C for 24 to 48 hours. Kanamycin-resistant colonies were selected, restreaked on MH-Kn and grown for an

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additional 24 hours at 37°C. At that stage half of the bacterial culture was used to prepare glycerol stocks (15 % vol/vol) and was kept frozen at -70°C. Another fraction (estimated to be 10⁸ bacteria) was resuspended in 15 µl of distilled water, boiled ten minutes and used as a template for PCR screening. Two *porA* internal primers named PPA1 and PPA2, were synthesized and used to perform PCR amplification on boiled bacterial lysates in the conditions described by the supplier (HiFi DNA polymerase, Boehringer Mannheim GmbH). The thermal cycling used was the following: 25 times (94°C 1min, 52°C 1min, 72°C 3min) and 1 time (72°C 10min, 4°C up to recovery). Since a double crossing-over between pCMK DNA and the chromosomal *porA* locus deletes the region required for #1 and #2 annealing clones lacking a 1170bp PCR amplification fragment were selected as *porA* deletion mutants. These PCR results were further confirmed by analyzing in parallel, the presence of PorA in the corresponding bacterial protein extracts. For that purpose another aliquot of bacteria (estimated to be 5 x 10⁸ bacteria) was resuspended in 50 µl of PAGE-SDS buffer (SDS 5%, Glycerol 30%, Beta-mercaptoethanol 15%, Bromophenol blue 0.3mg/ml, Tris-HCl 250 mM pH6.8), boiled (100°C), frozen (-20°C) / boiled (100°C) three times and was separated by PAGE SDS electrophoresis on a 12.5 % gel. Gels were then stained by Coomassie Brilliant blue R250 or transferred to a nitrocellulose membrane and probed with an anti-PorA monoclonal antibody as described in Maniatis *et al*. As represented in Figure 4, both Coomassie and immunoblot staining confirmed that *porA* PCR negative clones do not produce detectable levels of PorA. This result confirms that the pCMK vector is functional and can be used successfully to target DNA insertion in the *porA* gene, abolishing concomitantly the production of the PorA outer membrane protein antigen.

Example 4 Up-regulation of the NspA outer membrane protein production in blebs derived from a recombinant *Neisseria meningitidis* serogroup B strain lacking functional *porA* and *cps* genes

Enriching bleb vesicles with protective antigens is advantageous for improving the efficiency and the coverage of outer membrane protein-based vaccines. In that

context, recombinant *Neisseria meningitidis* strains lacking functional *cps* and *porA* genes were engineered so that the expressions level of the outer-membrane protein NspA was up-regulated. For that purpose, the gene coding for NspA was PCR amplified using the N01-full-*NdeI* and *NdeI*-3 oligonucleotide primers (see table 2)

5 The conditions used for PCR amplification were those described by the supplier (HiFi DNA polymerase, Boehringer Mannheim, GmbH). Thermal cycling done was the following: 25 times (94°C 1min, 52°C 1min, 72°C 3min) and 1 time (72°C 10min, 4°C up to recovery). The corresponding amplicon was digested with *NdeI* and inserted in the *NdeI* restriction site of the pCMK(+) delivery vector. Insert orientation was checked and recombinant plasmids, designed pCMK(+)-NspA, were purified at a large scale using the QIAGEN maxiprep kit and 2 µg of this material was used to transform a *Neisseria meningitidis* serogroup B strain lacking functional *cps* genes (strain described in example 1). Integration resulting from a double crossing-over between the pCMK(+)-NspA vector and the chromosomal *porA* locus were selected using a combination of PCR and Western blot screening procedures presented in example 3.

Bacteria (corresponding to about 5×10^8 bacteria) were re-suspended in 50 µl of PAGE-SDS buffer, frozen(-20°C) / boiled (100°C) three times and then were separated by PAGE-SDS electrophoresis on a 12.5 % gel. Gels were then stained by Coomassie Brilliant blue R250 or transferred to a nitrocellulose membrane and probed with an anti-NspA polyclonal serum. Both Coomassie (data not shown) and immunoblot staining (see figure 4) confirmed that *porA* PCR negative clones do not produce detectable levels of PorA. The expression of NspA was examined in Whole-cell bacterial lysates (WCBL) or outer-membrane bleb preparations derived from NmB [*cps*- *porA*-] or NmB [*cps*- *porA*- *Nspa*+]. Although no difference was observable by Coomassie staining, immunoblotting with the anti-NspA polyclonal serum detected a 3-5 fold increase in the expression of NspA (with respect to the endogenous NspA level), both in WCBL and outer-membrane bleb preparations (see figure 5). This result confirms that the pCMK(+)-NspA vector is functional and can be used successfully to up-regulate the expression of outer membrane proteins such as NspA, abolishing concomitantly the production of the PorA outer membrane protein antigen.

Example 5 Up-regulation of the D15/Omp85 outer membrane protein antigen in blebs derived from a recombinant *Neisseria meningitidis* serogroup B strain lacking functional *cps* genes but expressing PorA

Certain geographically isolated human populations (such as Cuba) are infected by a limited number of *Neisseria meningitidis* isolates belonging largely to one or few outer membrane protein serotypes. Since PorA is a major outer-membrane protein antigen inducing protective and strain-specific bactericidal antibodies, it is then possible to confer vaccine protection using a limited number of porA serotypes in a vaccine. In such a context, the presence of PorA in outer membrane vesicles may be advantageous strengthening the vaccine efficacy of such recombinant improved blebs. Such PorA containing vaccines, however can be improved still further by increasing the level of other cross-reactive OMPs such as omp85/D15.

In the following example, the pCMK(+) vector was used to up-regulate the expression of the Omp85/D15 outer membrane protein antigen in a strain lacking functional *cps* genes but expressing *porA*. For that purpose, the gene coding for Omp85/D15 was PCR amplified using the D15-*NdeI* and D15-*NorI* oligonucleotide primers. The conditions used for PCR amplification were those described by the supplier (HiFi DNA polymerase, Boehringer Mannheim, GmbH). Thermal cycling done was the following: 25 times (94°C 1min, 52°C 1min, 72°C 3min) and 1 time (72°C 10min, 4°C up to recovery). The corresponding amplicon was inserted in the pTOPO cloning vector according to the manufacturer's specifications and confirmatory sequencing was performed. This Omp85/D15 DNA fragment was excised from pTOPO by restriction hydrolysis using *NdeI/NsiI* and subsequently cloned in the corresponding restriction sites of the pCMK(+) delivery vector. Recombinant plasmids, designed pCMK(+)-D15 were purified on a large scale using the QIAGEN maxiprep kit and 2 µg of this material was used to transform a *Neisseria meningitidis* serogroup B strain lacking functional *cps* genes (strain described in example 1). In order to preserve the expression of *porA*, integration resulting from a single crossing-over (either in Omp85/D15 or in *porA*) were selected by a combination of PCR and Western blot screening procedures. Kanamycin resistant

clones testing positive by *porA*-specific PCR and western blot were stored at -70°C as glycerol stocks and used for further studies

Bacteria (corresponding to about 5×10^8 bacteria) were re-suspended in 50 μl of PAGE-SDS buffer frozen (-20°C) / boiled (100°C) three times and then were
 5 separated by PAGE-SDS electrophoresis on a 12.5 % gel. Gels were then stained by Coomassie Brilliant blue R250 or transferred to a nitrocellulose membrane and probed with an anti-*porA* monoclonal antibody. As represented in Figure 6, both Coomassie and immunoblot staining confirmed that *porA* PCR positive clones produce PorA.

The expression of D15 was examined using outer-membrane bleb preparations
 10 derived from NmB [*cps*-, *porA*-] or NmB [*cps*-, *porA*+, D15+] Coomassie detected a significant increase in the expression of D15 (with respect to the endogenous D15 level), preparations (see Figure 6). This result confirmed that the pCMK(+)-D15 vector is functional and can be used successfully to up-regulate the expression of outer membrane proteins such as D15, without abolishing the production of the major PorA
 15 outer membrane protein antigen.

Example 6 Construction of versatile promoter delivery vectors

Rational The rational of this approach is represented in Figure 7 and can be
 20 summarized in 7 essential steps. Some of these steps are illustrated below with the construction of Vector for up-regulating the expression of NspA and D15/Omp85.

Vector for up-regulating the expression of the NspA gene

Step 1 A DNA region (997bp) located upstream from the NspA coding gene was
 25 discovered (SEQ ID NO 2) in the private Incyte PathoSeq data base containing unfinished genomic DNA sequences of the *Neisseria meningitidis* strain ATCC 13090. Two oligonucleotide primers referred to as PNS1 and PNS2 (see table 2) were designed using this sequence and synthesized. These primers were used for PCR amplification using genomic DNA extracted from the H44/76 strain. **Step 2** The
 30 corresponding amplicons were cleaned-up using the Wizard PCR kit (Promega USA) and submitted to digestion with the *EcoRI/XbaI* restriction enzymes for 24 hours using the conditions described by the supplier (Boehringer Mannheim, Germany). The

corresponding DNA fragments were gel purified and inserted in the corresponding sites of the pUC18 cloning vector **Step 3** Recombinant plasmids were prepared on a large scale and an aliquot fraction was used as a template for inverse PCR amplification Inverse PCR was performed using the PNS4 and PNS5 oligonucleotides using the following thermal cycling conditions 25 times (94°C 1min, 50°C 1min, 72°C 3min) and 1 time (72°C 10min, 4°C up to recovery) Linearized pUC 18 vectors harbouring a deletion in the NspA upstream region insert were obtained

10 Vector for up-regulating the expression of the D15/omp85 gene

Step 1 A DNA region (1000 bp) located upstream from the D15/omp85 coding gene was discovered (SEQ ID NO 3) in the private Incyte PathoSeq database containing unfinished genomic DNA sequences of the *Neisseria meningitidis* strain ATCC 13090 Two oligonucleotide primers referred to as PromD15-51X and PromD15-S2

15 (see table 2) were designed using this sequence and synthesized These primers were used for PCR amplification using genomic DNA extracted from the H44/76 strain

Step 2 The corresponding amplicons were cleaned-up using the Wizard PCR kit (Promega, USA) and submitted to digestion with the *EcoRI/XbaI* restriction enzymes for 24 hours in the conditions described by the supplier (Boehringer Mannheim

20 Germany) The corresponding DNA fragments were gel purified and inserted in the corresponding sites of the pUC18 cloning vector **Step 3** Recombinant plasmids were prepared on a large scale and an aliquot fraction was used as a template for inverse PCR amplification Inverse PCR was performed using the D15-S4 and D15-S5 oligonucleotides using the following thermal cycling conditions 25 times (94°C 1min, 50°C 1min, 72°C 3min) and 1 time (72°C 10min, 4°C up to recovery) 25 Linearized pUC 18 vectors harbouring a deletion in the D15/omp85 upstream region insert were obtained

Example 7 Fermentation processes for producing recombinant blebs

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The examples listed below describe methods for producing recombinant blebs lacking either capsular polysaccharides or capsular polysaccharides and PorA Such a

procedure may be used for a wide range of *Neisseria meningitidis* recombinant strains and may be adapted over an extended scale range

Culture media *Neisseria meningitidis* serogroup B strains were propagated in solid

5 (FNE 004 AA, FNE 010 AA) or liquid (FNE 008 AA) culture media

Components	FNE 004 AA	FNE 008 AA	FNE 010 AA
Agar	18 g/L		18 g/L
NaCl	6 g/L	6 g/L	6 g/L
Na-Glutamate		1.52 g/L	
NaH ₂ PO ₄ 2H ₂ O	2.2 g/L	2.2 g/L	2.2 g/L
KCl	0.09 g/L	0.09 g/L	0.09 g/L
NH ₄ Cl	1.25 g/L	1.25 g/L	1.25 g/L
Glucose	5 g/L	20 g/L	5 g/L
Yeast Extract UF		2.5 g/L	
Soy Pepton	5 g/L	30 g/L	5 g/L
CaCl ₂ 2H ₂ O	0.015 g/L		0.015 g/L
MgSO ₄ 7H ₂ O	0.6 g/L	0.6 g/L	0.6 g/L
Erythromycine	0.015 g/L		
Kanamycine			0.2 g/L

Flask cultivation of *Neisseria meningitidis* serogroup B cps- recombinant blebs

10 This was performed in two steps comprising preculture on solid medium followed by liquid cultivation. **Solid pre-culture** A vial of seed was removed from freezer (-80°C), thawed to room temperature and 0.1 mL was streaked into a Petri dish containing 15 mL of FNE004AA (see above). The Petri dish was incubated at 37°C for 18 ± 2 hours. The surface growth was resuspended in 8 mL of FNE008AA (see
15 above) supplemented with 15 mg/L of erythromycin. **Flask culture** 2 mL of resuspended solid pre-culture were added to a 2 litre flask containing 400 mL of FNE008AA supplemented with 15 mg/L of erythromycin. The flask was placed on a shaking table (200 rpm) and incubated at 37°C for 16 ± 2 hours. The cells were separated from the culture broth by centrifugation at 5000g at 4°C for 15 minutes.

20

Batch mode cultivation of *Neisseria meningitidis* serogroup B cps- recombinant

blebs This was performed in three steps comprising preculture on solid medium

liquid cultivation and batch mode cultivation **Solid pre-culture** A vial of seed was

removed from freezer (-80°C), thawed to room temperature and 0.1 mL was streaked

5 into a Petri dish containing 15 mL of FNE004AA (see above) The Petri dish was

incubated at 37°C for 18 ± 2 hours The surface growth was resuspended in 8 mL of

FNE008AA (see above) supplemented with 15 mg/L of erythromycin **Liquid pre-**

culture 2 mL of resuspended solid pre-culture were added to one 2 liters flask

containing 400 mL of FNE008AA supplemented with 15 mg/L of erythromycin The

10 flask was placed on a shaking table (200 rpm) and incubated at 37°C for 16 ± 2 hours

The content of the flask was used to inoculate the 20 liters fermenter **Batch mode**

culture in fermenter The inoculum (400 mL) was added to a pre-sterilized 20 liters

(total volume) fermenter containing 10 L of FNE008AA supplemented with 15 mg/L

of erythromycin The pH was adjusted to and maintained at 7.0 by the automated

15 addition of NaOH (25% w/v) and H₃PO₄ (25% v/v) The temperature was regulated

at 37°C The aeration rate was maintained at 20 L of air / min and the dissolved

oxygen concentration was maintained at 20% of saturation by the agitation speed

control The overpressure in the fermenter was maintained at 300 g/cm² After 9 ± 1

hours, the culture was in stationary phase The cells were separated from the culture

20 broth by centrifugation at 5000g at 4°C for 15 minutes

Flask cultivation of *Neisseria meningitidis* serogroup B cps- PorA- recombinant

blebs This was performed in two steps comprising preculture on solid medium

followed by liquid cultivation **Solid pre-culture** A vial of seed was removed from

25 freezer (-80°C), thawed to room temperature and 0.1 mL was streaked into a Petri dish

containing 15 mL of FNE010AA (see above) The Petri dish was incubated at 37°C

for 18 ± 2 hours The surface growth was resuspended in 8 mL of FNE008AA (see

above) supplemented with 200 mg/L of kanamycin **Flask culture** 2 mL of

resuspended solid pre-culture were added to a 2 litre flask containing 400 mL of

30 FNE008AA supplemented with 200 mg/L of kanamycin The flask was placed on a

shaking table (200 rpm) and incubated at 37°C for 16 ± 2 hours The cells were

separated from the culture broth by centrifugation at 5000g at 4°C for 15 minutes

Example 8 Isolation and purification of blebs from meningococci devoid of capsular polysaccharide

- Recombinant blebs were purified as described below. The cell paste (42gr) was suspended in 211 ml of 0.1M Tris-Cl buffer pH 8.6 containing 10 mM EDTA and 0.5% Sodium Deoxycholate (DOC). The ratio of buffer to biomass was 5/1 (V/W). The biomass was extracted by magnetic stirring for 30 minutes at room temperature. Total extract was then centrifuged at 20,000g for 30 minutes at 4°C (13,000 rpm in a JA-20 rotor, Beckman J2-HS centrifuge). The pellet was discarded. The supernatant was ultracentrifuged at 125,000g for 2 hours at 4°C (40,000 rpm in a 50.2Ti rotor, Beckman L8-70M ultracentrifuge) in order to concentrate vesicles. The supernatant was discarded. The pellet was gently suspended in 25 ml of 50 mM Tris-Cl buffer pH 8.6 containing 2 mM EDTA, 1.2% DOC and 20% sucrose. After a second ultracentrifugation step at 125,000g for 2 hours at 4°C, vesicles were gently suspended in 44 ml of 3% sucrose and stored at 4°C. All solutions used for bleb extraction and purification contained 0.01% thiomersalate. As illustrated in Figure 8, this procedure yields protein preparations highly enriched in outer membrane proteins such as PorA and PorB.

SEQ ID NO 1

Nucleotide sequence of the pCMK(+) vector

TCTTCCGCTTCTCGCTCACTGACTCGC¹CCGCTCGGTCGTTCCGGCTGCGGCGAGCGGTATCAGCTCAC²CAAAGGCGGT
 AATACGGTTATCCACAGAATCAGGGCATA³CGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCC⁴GGAACCGTA
 5 AAAAGGCCGCGTTGCTGGCGT⁵TTTCCAT⁶AGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCTC⁷AGTCAGAGG
 TGGCG⁸AAACCCGACAGG⁹CTATAAAGAT¹⁰CCAGGCGTTTCCCTCGGAAGCTCCCTCG¹¹GCCTCTCCTCT¹²CCGACCT
 GCCGCTTACCGGATACCTGTCCGCCTTTC¹³CCCTTCGGGAAGCGTGGCGCTT¹⁴TCTCATAGCTCAGCGTGT¹⁵GGTATCTCA
 GTTCGGTGTAGGTCGTTCCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCGTT¹⁶CAGCCCGACCGCTGCGCCT¹⁷ATCCGGT
 AACTATCGTCTTG¹⁸GTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACT¹⁹GGTAACAGG²⁰TTAGCAGAGC
 10 GAGGTATGTAGGCGGTGCTACAGAGTCT²¹GAAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGT²²TTTGGTATCT
 GCGCTCTGTGAAGCCAGTTACCTTCGGA²³AAAGAGT²⁴GGTAGCTCTTGATCCGGCAAACAACCACCGC²⁵GGT²⁶GCCTG
 GGT²⁷TTTTTTGTTTGCAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTCTACGGGGTC
 TGACGCTCAGTGGAACG²⁸AAACTCACGTT²⁹AGGGATT³⁰GGTCATGAGATTATCAAAA³¹GGATCTTCACCTAGATCCCTT
 TAAATTA³²AAAAATGAAGTTTAAATCAATCT³³AAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGT
 15 GAGGCACCTATCTCAGCG³⁴ATCTGTCTATT³⁵CGTTATCCATAGTTGCCTGACTCCCCGTCGTGTAGATA³⁶CTACGATACG
 GGAGGGCTTACCATCTGGCCCCAGTGTGC³⁷ATGATACCGCGAGACCCACGCTCACCGGCTCCAGATT³⁸ATCAGCAATAA
 ACC³⁹AGCCAGCCGGA⁴⁰GGGCGCAGCGCAG⁴¹GTGGTCT⁴²GCAACTTTATCCGCTCCA⁴³CCAGTCTATTA⁴⁴TTGT⁴⁵TGCCGG
 GAAGCTAGAGTAAGTAGTTCCGCCAGTTA⁴⁶GT⁴⁷TTGCGCAACGTTGT⁴⁸TGCCATTGCTACAGGCATCGTGG⁴⁹GT⁵⁰CACGCTC
 GTCGTTTGGTATGGCTT⁵¹CATT⁵²CAGCTCCGTTCCCAACGATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAAAAG
 20 CGGTAGCTCCTTCGGTCTCCGATCGTTG⁵³CAGAAGT⁵⁴AGTTGGCCGAGTGT⁵⁵TATCACTCATGGTTATGGC⁵⁶GCACCTG
 CATAATTCTCTTACTGTATGCCATCCGT⁵⁷AGATGCTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATA
 GTGTATGGCGGACCGAGTTGCTCT⁵⁸TGCCCGCGCTCA⁵⁹TACGGGATAATACCGCGCCACATAGCAGAACTTTA⁶⁰AGTGC
 TCATCAT⁶¹GGAAAACGT⁶²CT⁶³CGGGCGC⁶⁴AACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTT⁶⁵CGATGTAACCCACT
 CGTGCACCCAACTGATCTT⁶⁶CAGCATCTTT⁶⁷ACTTTCCACGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGC
 25 AAAAAAGGGAATAAGGGCGACACGGAAATG⁶⁸TGAATACTCATACTCTTCTTTTCAATATTATTGAAGC⁶⁹TTTATCAGG
 GTTATTGTCTCATGAGCGGATACATATT⁷⁰CTATGTATT⁷¹AGAAAAATAACAAATAGGGGTTCCGCGCAC⁷²TTTCCCCGA
 AAAGTGCCACCTG⁷³CGTCTAAGAAACCAT⁷⁴TTATCATG⁷⁵CATTAACT⁷⁶ATAAAAATAGGCGTATCAG⁷⁷AGGCCCTTTCG
 TCTCGCGCTTTCGGTG⁷⁸TGACGGTGAAA⁷⁹CCTCTGAC⁸⁰CATGACAGCTCCCGGAGACGGTCACAGCTTG⁸¹CTGTAAAGCGG
 ATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGTGTTGGCGGGTGT⁸²CGGGGCT⁸³GGCTTA⁸⁴ACTATGCGGCATCA
 30 GAGCAGATTGTACTGAGAGTGACCATAA⁸⁵ATTGTAA⁸⁶CGTTAATATT⁸⁷TGTTAAAT⁸⁸TCGCGTTAAAT⁸⁹TTGT⁹⁰TAAAT
 CAGCTCATTTT⁹¹TAAACCAATAGGCCGAA⁹²TCGGCAA⁹³ATCCCTTATAAATCAAAGAATAGCCCGAGAT⁹⁴AGGGTGTAGTG
 TTGTTCCAGTTTGGAA⁹⁵CAAGAGTCCACT⁹⁶TTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGC
 GATGGCCCACTACG⁹⁷TGAACCATCACCCAA⁹⁸CAAGTTTT⁹⁹TGGGTGAGGTGCCGTAAAGCACTAAATCGGAACCCATAA
 AGGGAGCCCCGATT¹⁰⁰TAGAGCTTGACGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGG
 35 GCGCTAGGGCGCTGGCAAGTGTAGCGGTC¹⁰¹CGCTGCGCGTAACCAACACACCCGCGCGCTTAATGCGCCGCTACAGGGC
 GCGTACTATGGTTGCTTTGACGTATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATC¹⁰²AGGCCCAT
 TCGCCATT¹⁰³CAGGCTGCGCAACTGTTGGG¹⁰⁴AGGGCGATCGGTGCGGGCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGG
 ATGTGCTGCAAGGCGATTAAAGTTGGGTAACGCCAGGGTTTTCCAGTCACGACGTTGTAAACGACGGCCAGTGCCAAGC
 TTGCCG¹⁰⁵CTGAATACATCCCGTCATTCTCTCAAAAACAGAAAACCAAAATCAGAAACCTAAATCCCCTCATCCCCGCGCA
 40 GCGGGGAATCCAGTCCGTT¹⁰⁶CAGTTTCGGTCA¹⁰⁷TTCCGATAAATCTCTGCTGCTTTTCA¹⁰⁸TTTCTAGATTCCCAC¹⁰⁹TTTCGTG
 GGAATGACGGCGGAAGGGTTTTGGTTTTTCCGATAAATCTTGAGGCATTGAAATTTAGATTCCCCTGCGCGGGAA
 TGACGGCTGTAGATGCCCGATGGTCTTT¹¹⁰AGCGGATTAACAAAAATCAGGACAAGGCGACGAAGCCGACAGACAGTACAG
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CTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCC
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 TCCCCGGGAAAAACGATTCCAGGTATTAGAAGAATATCCTGATTAGGTGAAAAATATTGTTGATGCGCTGGCAGTGTTC
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 GCCCCCCCCCCCCGAGGAGTCTGCGCTTGAATTGTGTTGTAGAAACACAACGTTTGTGAAAAATAAGCTATTGTTT
 15 TATATCAAAATATATCATTTTAAAAATAAGGTTGCGGCATTTATCAGATATTTGTTCTGAAAAATGGTTTTTTCGGGG
 GGGGGGGGTATTTGAAGACGTATCGGGTGTGTCGGGAATTGTGAGCGGATAACAATTCGATGTTTTTAGGTTTTTA
 TCAAAATTACAAAGGAAGCCCATATGCATCCTAGGCCATTAAATATCCGGAGTATACGTAGCCGGCTAACGTTAACAA
 CCGGTACTCTAGACTATAGCTAGCATGCGCAAAATTTAAAGCGCTGATATCGATCGCGCGCAGATCTGATTAATAGGC
 GAAAAACCGCTACGATCAAAATCATCGCGGCGTTGATTATGATTTTTCCAAACGCACCTCCGCCATCGTGTCTGGCGC
 20 TTGGCTGAAACGCATACCGGCATCGGCAACTACACTCAAATTAATGCCGCTCCGTGGTTTGGCCACAAATCTAAA
 TATCGGGGCGGTGACGGATAGCTTTGTTTTGACGGCTTCGCTTCATTCTTTGATTGCAATCTGACTGCCAATCTGC
 TTCAGCCCCAACAAAACCCGGATACGGAAGAAAAACGGCAATAAAGACAGCAAAATACCGTCTGAAAGATTTTCAGACG
 GTATTCGCTATTTGGCTTGGTTTGACATATAGTGAGACCTTGGCAAAATAGTCTGTTAACGAAATTTGACGCATAA
 AAATGCCCAAAATTTTCAATTGCCTAAACCTTCTAATATTGAGCAAAAAGTAGGAAAAATCAGAAAAGTTTTGCA
 25 TTTTGAAATGAGTTGAGCATAAATTTTAGTAACCTATGTTATTGCAAAGGTCTCGAATTGTCATCCCACGCAGGCG
 GGAATCAGTCTGTTCGGTTTCAGTTATTTCCGATAAATTCCTGCTGCGCGCTCTGAAGAATTCGTAATCATGGTCTAG
 CTGTTTCTGTGTGAAATTGTTATCCGCTCACAATCCACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGG
 TGCCTAATGAGTGAGTAACACTACATTAATTGCGTTGCGCTCACTGCCCCGCTTTCAGTCGGGAAACCTGTCTGCCAGC
 TGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTTCGCTATTGGGCGC

SEQ ID NO 2

Nucleotide sequence of DNA region (997 bp) up-stream from the NspA gene in
 the *Neisseria meningitidis* serogroup A strain Z2491

GGAACCGAACACGCCGTTCCGTCATACGCCCGGAAAGGTTTGGCGCAAGACGAAGCCGCCCTCGACATCGAAGACGCGG
 35 TACACGGCGCGCTGGAAAGCGCGGTTTGTCCACTACGAAACATCGGCTTTTGCAGAACCCAGCCATGCAGTGCCGCCAC
 AATTTGAACTACTGGCAGTTCCGGCATTATTTAGGCATAGGCGCGGGCGGCACGGCAAAATTTCTATCCCGACCGCAT
 CGAGCGCACCGTCCGCCCGCCACCCCAACGACTACCTCGCTTAATGCAAAACCGACCGAGCGAAGCCGTGCAACGCA
 AAACCGTCCGCCCGAAGATTTGCCGTTCAATTCATGATGAACGCCCTGCGCCTGACCGACGGCGTACCCACCGCGATG
 TTGCAGGAGCGCACGGGCGTACCGAGTGCCAAAATCATGGCGCAAATCGAAACGGCAAGGCAAAAAGGCTGCTGGAAAC
 40 CGACCCCGCGGTATCCGCCCGACCGAAAAAGGACGCTTGTTTTAAACGATTGCTGCACTGTTTATAGTGGAATTA
 ACAAAAACAGTACGGCGTTGCTCGCTTAGCTCAAAGAGAACGATTCTTAAGGTGCTGAAGCACCAAGTGAATCGGT
 TCCGTACTATCTGCTGCTGCGGCTTCGTGCGCTTGTCTGATTTTTGTAAATCCACTATATAAGCGCAACAAATCG
 GCGGCGCGCCGGGAAACCCCCCGAACGCGTCCGGAATATGCTTATCGATGGAAACCGCAGCCGCATCCCCCGCCGG
 GCGTTTCAGACGGCACAGCCCGCGCGGAATGTCCGACGCTTAAGGCACAGACGCACAAAAAACCGTATGCCTGCAC
 45 CTGCAACAATCCGCAGATACCGCTGTTTTTCCAAACCGTTTGAAGTTTCAACCATCCGCCGCGTGATGCCGCCACCA

CCATTTAACGCAACGCGCGGGTTAACGGCTTTGCCG

SEQ ID NO 3

Nucleotide sequence of DNA region (1000 bp) up-stream from the D15/Omp85 gene in the *Neisseria meningitidis* serogroup B strain ATCC13090

ACCATTGCCGCCCGCGCGGGCTTCCAAAGCGGCGACAAAATACAATCCGTCAACGGCACACCCGTTGCAGATTGGGGCAG
CGCGCAAACCGAAATCGTCTCTCAACCTCGAAGCCGGCAAAGTCGCGTCGGGTTTCAGACGGCATCAGGCGCGC
CCGCACCATCGATGCCGAGGCACGCGGAAGCCGGTAAAAATCGCAAAAAACCAAGGCTACATCGGACTGATGCCCTTTA
AAATCACAACCGTTGCCGGTGGCGTGGAAAAAGGCAGCCCCGCGGAAAAAGCAGGCCCTGAAACCGGGCGACAGGCTGCT
10 GCCGCCGACGGCAAACCCATTACCTCATGGCAAGAATGGGCAAACCTGACCCGCCAAAGCCCCGGCAAAAATCACCCCT
GAACTACGAACGCGCGCGGACAAACCCATACCGCCGACATCCGCCCCGATACTGTGAAACAGCCCGACCACCCCTGATCG
GGCGCGTCGGCCTCCGTCCGCGAGCCGACAGGGCGTGGGACGCGCAAATCCGCGCGAGCTACCGTCCGTCTGTTATCCGC
GCATTCCGGCATGGGCTGGGAAAAACCGTTTCCCACTCGTGGACAACCTCAAATTTTCGGCAAACTAATCGCGGCA
CGCCTCCGTGAGCCATATTTCCGGGCGCGTGACCATTGCCGACATTGCCGGACAGTCCGCCGAACCTCGGCTTGCAAGTT
15 ATTTGGAATTTTGGCACTGGTCAGCATCAGCCTCGGCGTGTGAACCTGCTGCCCGTCCCGGTTTGGACGGCGGCC
CTCGTGTTTTATACGCGCAATGGATACGCGGCAAACCTTTGGGCGAACGCGTCCAAACATCGGTTTGGCGTTCGGGCT
TGCCCTCATGATGCTGATGATGGCGGTGCGCTTCTTCAACGACGTTACCCGGCTGCTCGGTTAGATTTTCGTTTCGGAA
TGCCGTCTGAAACCGCATTCGCGACCACAAGGAACTGACA

20 SEQ ID NO 4

Nucleotide sequence of DNA region (1000 bp) up-stream from the Hsf-like gene from *Neisseria meningitidis*

ATTCCCGCCC-GGCGGGTCCAGAAACGCAACGCAACAGGAATTTATCGGAAAAACAGAAACCTCACCGCCGTCATTC
CCGCAAAAGCGGGAATCTAGAAACACAACGCGCGAGGACTTTATCAGAAAAACAGAAACCCACCGCCGTCATTC
25 AAAAGCGGGTCCAGACCCGTCGGCACGGAACTTACCGGATTAACAGTTTCTTAGATTCCACGTCCTTGTTCCCG
CTTTCGCGGGATGACGCGTATTTAGATTATGGGAATTTATCAGGAATGATTGAATCCATAGAAAAACCAAGGATCTA
TCAGAAAAACGCAACCCCCACCGCGTCATTCCCGCGCAGGCGGGAATCCAGAAACACAACGCGGCAGGACTTATCGG
AAAAAACCGTAACCCACCGACCGTCATTCCCGCAAAAGTTGGAATCCAAACACGCAACGCAACAGGAATTTATCGG
AAACAGAAACCCCCACCGCGTCATTCCCGCGCAGGCGGGAATCCAGAAACACAACGCAACAGGAATTTATCGGAA
30 AGAAACCCACCGACCGTCATTCCCGCAAAAGCGGGAATCCAGCAACCGAAACACAGGAATCTATCAGCAAAACAG
AAACCCCCACCGACCGTCATTCCCGCGCAGGCGGGAATCCAGAAACACAACGCGGCAGGACTTTATCGGAAACGCA
ACCCACCGTCCGTCATTCCCGCAAAAGCTGGAATCCAAACACGCAACGCAACAGGAATTTATCGGAAACGCAAC
CCACCGCGTCATTCCCGCAAAAGCGGGAATCCAGACCCGTCGGCACGGAACTTACCGGATAAACAGTTCTTAGAT
TCCACGTCCCGATTCCGGCTTCGCGGGAATGACGAGATTTAAGTTGGGGAATTTATCAGAAACCCCAACCCCA
35 AAAACGGGCGGATGCGCGACCATCCGCCCCAAACCCGATTTAACCATTCAAACAAACCAAAAGAAAAACAA

SEQ ID NO 5

Nucleotide sequence of DNA region (772 bp) up-stream from the PilQ gene from *Neisseria meningitidis*

GCGATGTCGGGAAGCCTTCTCCGAATCATTACCCCTTGAGTCGCTGAAAATCGCCCAATCTCCGGTAACGGCGGCAT
CATGACGGCAAGAGCAGCATCCTGAACCTCAGTGCCATTGCCACCACCTACCAAGCAAATCCGTAGAAGAGCTTGCCGC
AGAAGCGGCACAAATGCCGAGCAAAATAACTTACGTTAGGGAAACCATGAAACACTATGCCTTACTCATCGCTTTCT
GGCTCTCTCCGCGTGTTCCTAAGGTTCTGAGGACCTAAACGAATGGATGGCACAACGCGACGCGAAGCCAA-GCAGAA
TCATACCTTTCAAGCACCTACCTGCGGTTGCGCCGGTATACAGCCCGCCGAGCTTACAGGGCCGAACGCAATCGAC
45 TTCCGCCGCATGGTAACCGCAAAAAAGGGGAAATGCCCCGACACCAAGCGTATTAAAGAAACGCTGGAAATTCAG
TTTGAAAAATGCGTTATGTGCGCATTTTGAAGTCTGGACAGAAAGTCTCCGGCTTCATCGAGGCTGAAGGATATGTCT
ACACTGTCGGTGTGCGCACTATTGGGACAAAACCTACGGTAGAATCGAAAGCATTACCGACGACAGCATCGTCTGAAC
GAGCTGATAGAAGACAGCAGGGCAACTGGGTTTCCCGTAAAGCAGAACTGCTGTTGAATTTCTCCGACAAACACACCGA
ACAAGCGGCAGCACCTGCCGAGAACAAATTAAGAAGAGGATTACTCCATT

50 SEQ ID NO 6

Nucleotide sequence of DNA region (1000 bp) up-stream from the Hap gene from *Neisseria meningitidis*

```

GTGCGGCAAAACAGCAAAAGCCCGCTGTCGATTGCCTGACCGTCCGCGTCCGTAAACAGCAAGGTGCCACGCGC
GGCTTGGGCGTTTCCACACAAAGCCTCTGCCATCGGCAGCAGGTTTTCCTCCGATAGCGTATCAGCCCACGCCGCC
5 GCGCCCGGGTGGGTAGCGACTGCCGCAATCGTTGGAACGTTATCCGACATAAAACCCCGAAAAACAAACAGCCGCG
ATTATAGCAATGCCGTCTGAAGTCCGACGGTTTGGCTTTCAGACGGCATAAACCGCAATGCTTGAATAATCCGTG
CGCTTGACCTAAATAACCATATGGAAAAACGAAACACATACGCCCTTCCTGCTCGGTAAGGCTCGCTGCTGGGTCTGT
CCATCCCGCAATACCGCCAACGCCCAATCCCGCCGACGATCTCAAAAACATCGGCCGCGATTTCACACGCCCAATC
10 AGAAGCGCGCAATGACCGAAACGCACAGGACAAGGCGCGGCAGGCTGTCGAAACCGTCTCAAACCCCGAGCTTG
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GACTTGGCGCAATCAACGACATTATCAGCAACGGGGCAGACCGCATTATGGCAATGGCGGAAACCAACGCCGTCCG
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GGTGCTTTCGTGATTGGAAGAAGCCGAGACCAAGGCAAAAATTAATTGCAAATCCTAGGGCGTGCTCATATCCGCCCGA
15 ACGCCGAACCGCACATATAGGCACATCCCGCGCGCCGCGGAAGCGGAAGCCGCGCCCACCAACACCCGAATCCCG
TCAGATAAGGAAATA

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235 233

Table 1 Specificities of the monoclonal antibodies tested

Mabs Tested	Directed against	Result
Anti B PS 735	B polysaccharide	++ on the wild type strain () on the D and R mutants
Anti B PS 95/750 from NIBSC	B PS	++ on the wild type strain () on the D and R mutants
Anti P1 7 (NIBSC)	Loop 1 of Porin A	++ on all wild type and mutants strains
Anti P1 16 (NIBSC)	Loop 4 of Porin A	++ on all wild type and mutants strains
Anti Los 579	LPS	++ on the wild type strain () on the D and R mutants
Anti Los 8 (NIBSC)	LPS	+/- on the wild type strain () on the D and R mutants
Anti P1 2 (NIBSC)	Anti Porin A Sero subtype 1 2	() on all wild type and mutants strains

Table 2 *Oligonucleotides used in this work*

Oligonucleotides	Sequence	Remark(s)
PorA5 Fwd	5' CCC AAG CTT GCC GTC TGA ATA CAT CCC GTC ATT CCT CA 3'	HindIII cloning site Uptake sequence (_)
PorA5 Rev	5' -CGA TGC TCG CGA CTC CAG AGA CCT CGT GCG GGC C 3'	XbaI cloning site
PorA3 Fwd	5' GGA AGA TCT GAT TAA ATG GGC GAA AAT ACC AGC TAC GA 3'	BglII cloning site Stop codons (_)
PorA3 Rev	5' GCC GAA TTC TTC AGA CGG C GC AGC AGG AAT TTA TCG G 3'	EcoRI cloning site Uptake sequence (_)
PoLa Rev1	5' GAA TTG TTA TCC GCT CAC AAT TCC GGG CAA ACA CCC GAT AC 3'	
PoLa Rev2	5' GAA TTC CAT ATG ATC GGC TTC CTT TTG TAA ATT TGA TAA AAA CCT AAA AAC ATC GAA TTG TTA TCC GCT C 3'	Xdel cloning site
PorAlacO Fwd	5' AAG CTC TGC AGG AGG TCT GCG CTT GAA TTG 3'	PstI cloning site
PorAlacO Rev	5' CTT AAG GCA TAT GGG CTT CCT TTT GTA A 3'	Xdel cloning site
PPA1	5' GCG GCC GTT GCC GAT GTC AGC C 3'	
PPA2	5' GGC ATA GCT GAT GCG TGG AAC TGC 3'	
N full 01	5' -GGG AAT TCC ATA TGA AAA AAG CAC TTG CCA CAC 3'	Xdel cloning site
Nde NspA 3	5' GGA ATT CCA TAT GTC AGA ATT TGA CGC GCA C 3'	Xdel cloning site
PNS1	5' CCG CGA ATT CGG AAC CGA ACA CGC CGT TCG 3'	EcoRI cloning site
PNS1	5' CGT CTA GAC GTA GCG GTA TCC GGC TGC 3'	XbaI cloning site
PromD15 S1X	5' GGG CGA ATT CGC GGC CGC CGT CAA CGG CAC ACC CGT TG 3'	EcoRI and XbaI cloning sites
PromD15 S2	5' GCT CTA GAG CGG AAT GCG GTT TCA GAC G 3'	XbaI cloning site
PNS4	5' AGC TTT ATT TAA ATC CTT AAT TAA CGC GTC CGG AAA ATA TGC TTA TC_34	SwaI and PaeI cloning sites
PNS5	5' AGC TTT GTT TAA ACC CTG TTC CGC TGC TTC GGC 3'	PmeI cloning site
D15 S4	5' GTC CGC ATT TAA ATC CTT AAT TAA GCA GCC GGA CAG GGC GTG G 3'	SwaI and PaeI cloning sites
D15 S5	5' AGC TTT GTT TAA AGG ATC AGG GTG TGG TCG GGC 3'	PmeI cloning site

We Claim

1 An engineered bleb vaccine preparation from a Gram-negative bacterial strain
5 characterized in that it is manufactured using one or more methods selected from the following group

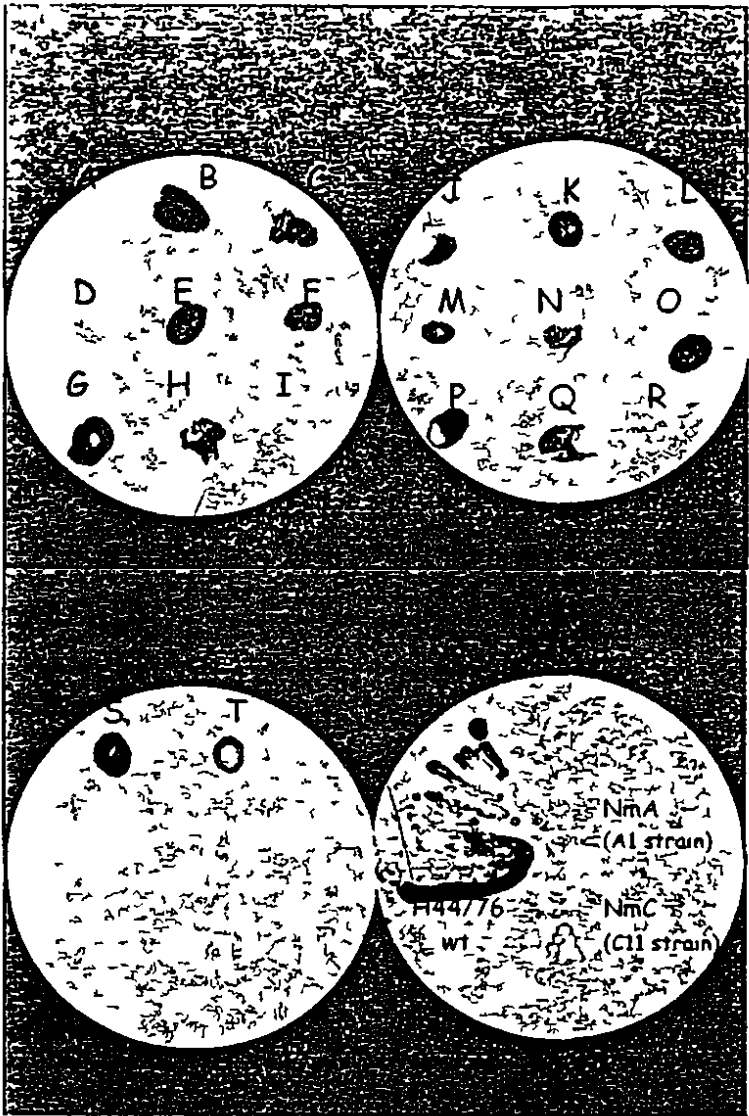
- 10 a) genetically reducing immunodominant variable antigens within the bleb vaccine comprising the steps of determining the identity of such an antigen, engineering a bacterial strain to produce less or none of said antigen, and making blebs from said strain,
- b) genetically reducing non protective OMP antigens within the bleb vaccine comprising the steps of determining the identity of such an antigen engineering a bacterial strain to produce less or none of said antigen and making blebs from said strain,
- 15 c) genetically creating conserved OMP antigens within the bleb vaccine comprising the steps of identifying the identity of such an antigen engineering a bacterial strain so as to delete variable regions of the gene encoding said antigen, and making blebs from said strain,
- 20 d) genetically upregulating expression of conserved protective OMP antigens within the bleb vaccine comprising the steps of identifying the identity of such an antigen, engineering a bacterial strain so as to remove the repressive control mechanisms of its expression, and making blebs from said strain
- 25 e) genetically upregulating expression of conserved, protective OMP antigens within the bleb vaccine comprising the steps of identifying the identity of such an antigen, engineering a bacterial strain so as to introduce a stronger promoter sequence upstream of the gene encoding said antigen, and making blebs from said strain
- 30 f) genetically modifying the saccharide portion of the bacterial LPS within the bleb vaccine, comprising the steps of identifying a gene involved in the rendering the saccharide portions of LPS toxic, engineering a bacterial

strain so as to reduce the expression of said gene, and making blebs from said strain

5 g) genetically modifying the lipid A portion of the bacterial LPS within the bleb vaccine, comprising the steps of identifying a gene involved in the rendering the lipid A portion of LPS toxic engineering a bacterial strain so as to reduce the expression of said gene, and making blebs from said strain, or

10 h) reducing the lipid A toxicity within the bleb vaccine and increasing the levels of protective antigens, comprising the steps of engineering the genome of a bacterial strain to incorporate a gene encoding a Polymyxin A peptide (or a derivative thereof) fused to a protective antigen, and making blebs from said strain

Figure 1



238-17236

Figure 2

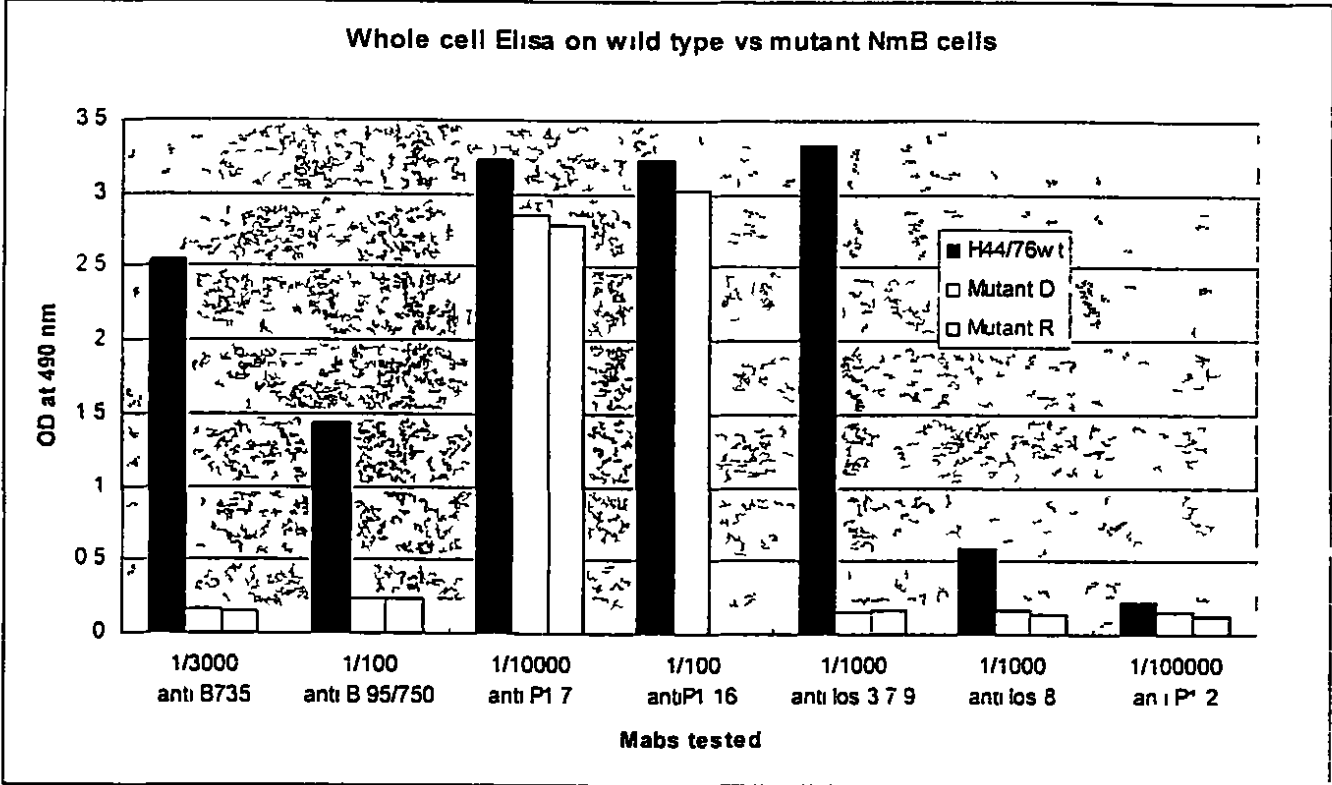


Figure 3

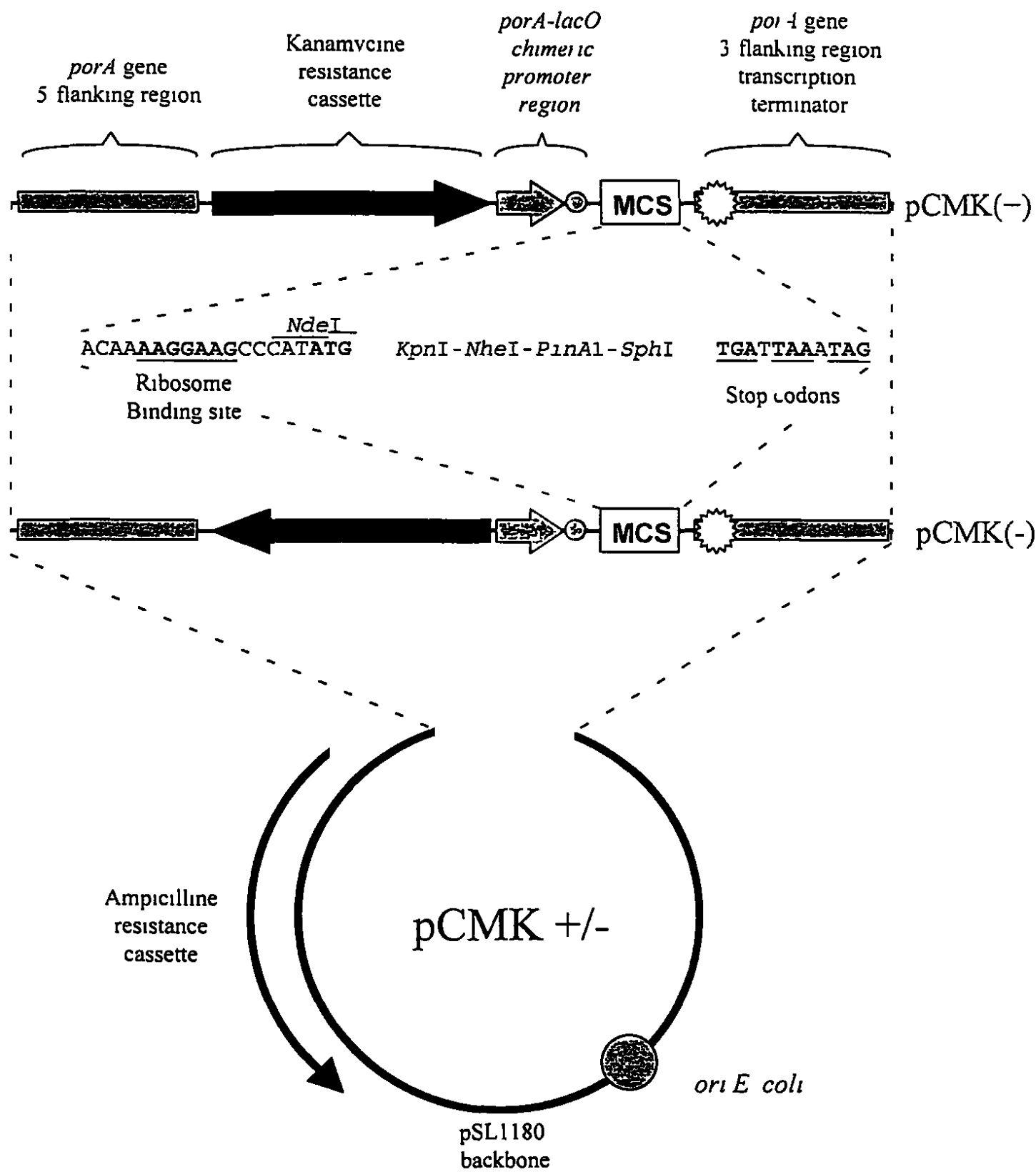
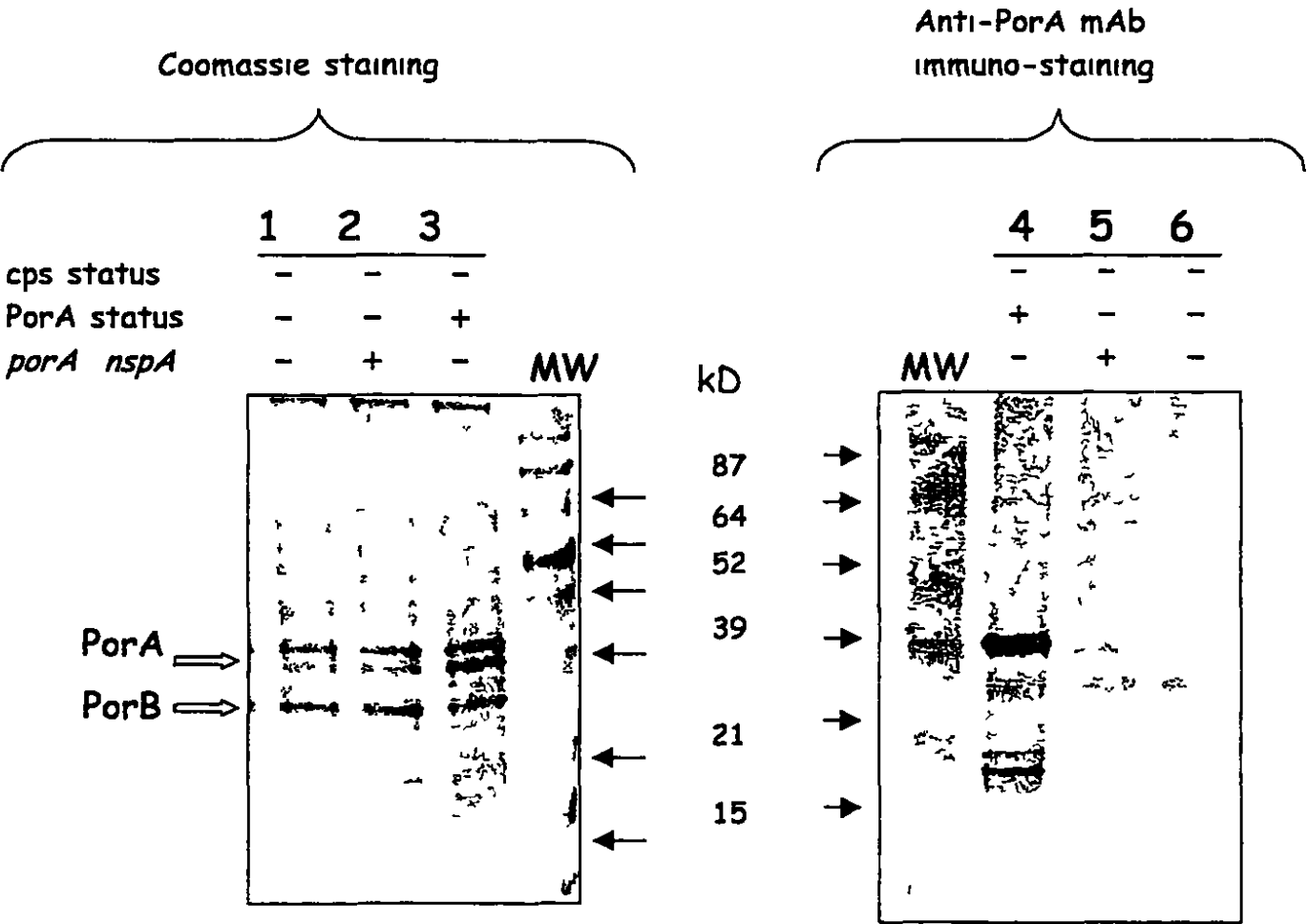


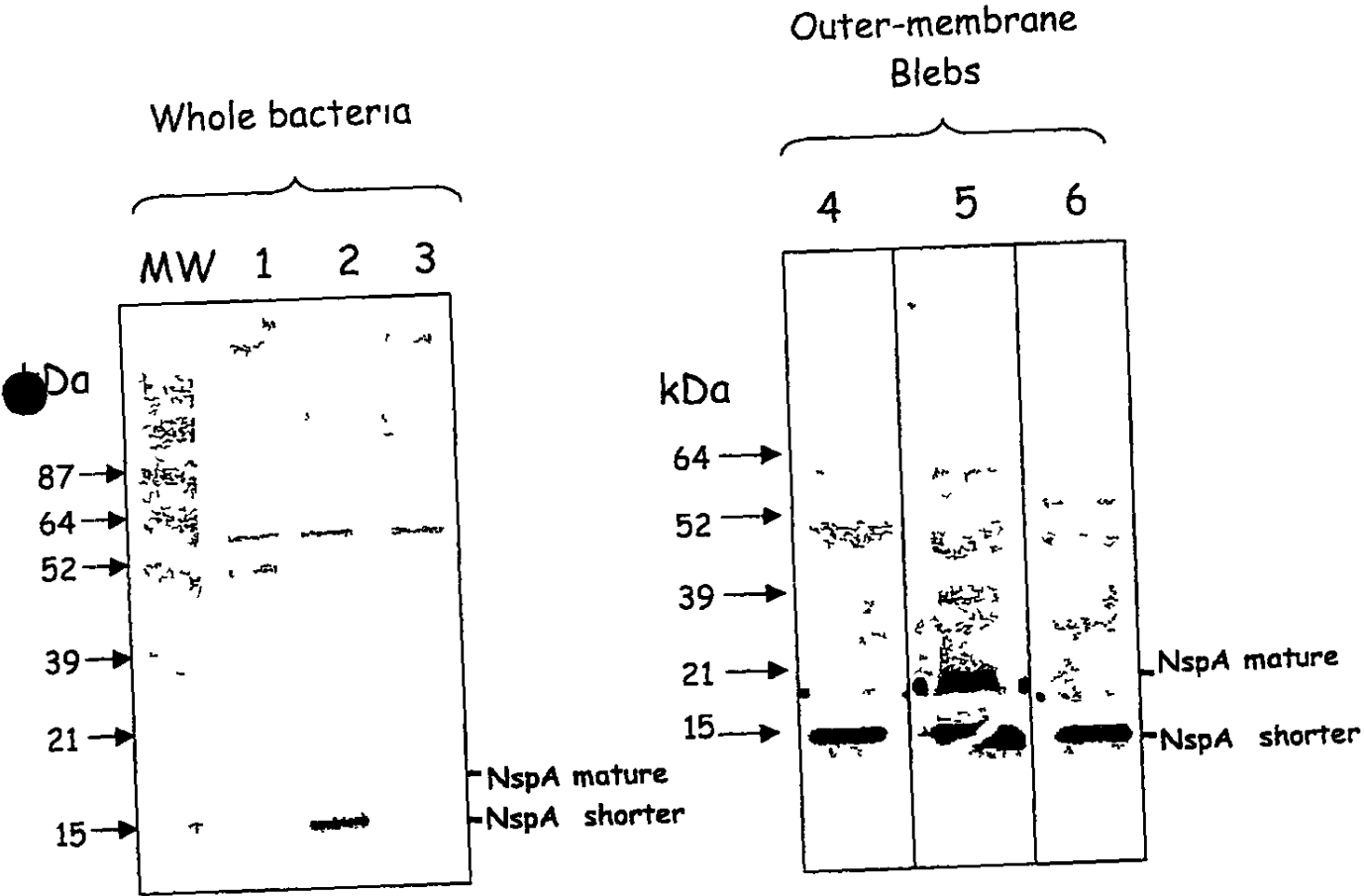
Figure 4



241-AR39

B-5189

Figure 5



247 210

Figure 6

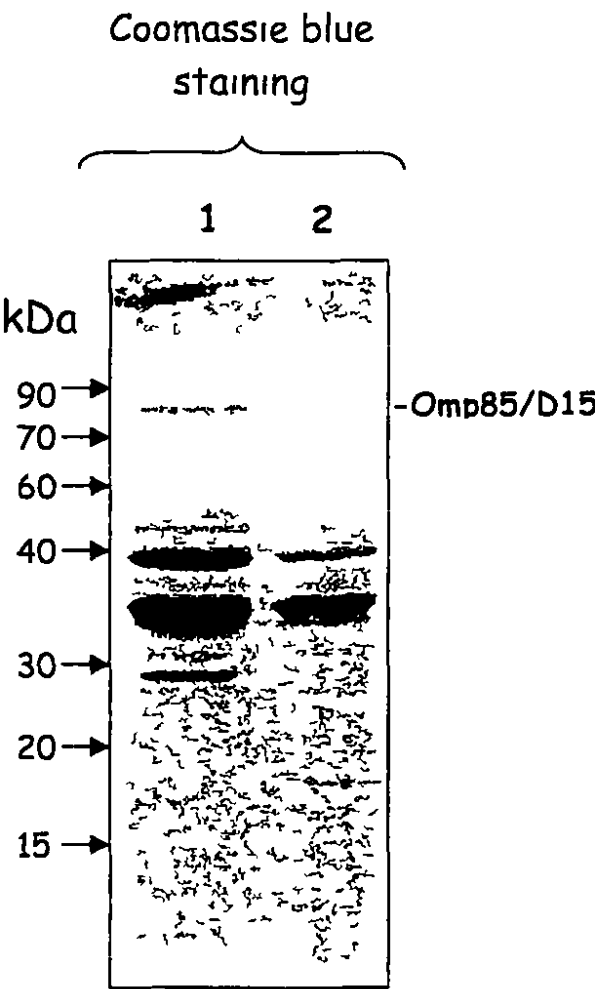
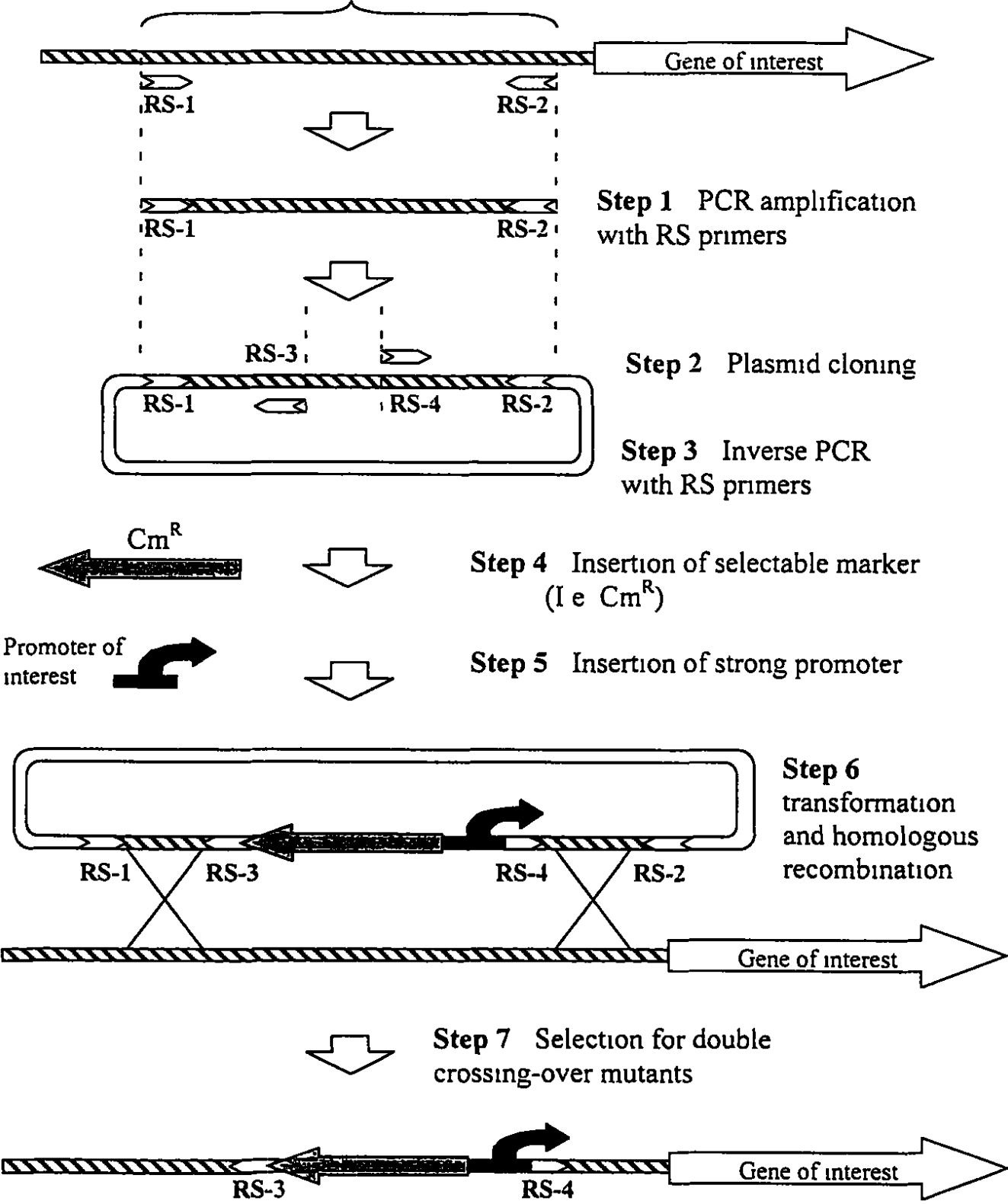


Figure 7



~~244~~ ~~242~~

SDS-PAGE gel showing protein profiles of *E. coli* strains. Molecular weight markers (kD) are indicated on the left: 175, 83, 62, 47.5, 32.5, 25, 16.5, 6.5. Lanes are numbered 1 to 7 at the top. On the right, arrows point to bands labeled PorA and PorB. Lane 1 is a molecular weight marker. Lanes 2-7 show protein profiles of different *E. coli* strains. PorA and PorB bands are prominent in lanes 2-7.

245 243

LEGALIZACIONES

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El suscrito, un funcionario debidamente autorizado conforme a los Articulos 74(1) y (4) de la Ley de Desreglamentacion y Contratacion de 1994 para firmar y expedir certificados en nombre del Contralor General, certifico que la copia adjunta es una copia correcta de los documentos archivados originalmente con respecto a la solicitud de patente que allí se identifica

De acuerdo con las Reglamentaciones de Patentes de 1982 (Re-registro de Compañias), si una compañía nombrada en este certificado y en los documentos adjuntos se ha vuelto a registrar segun la Ley de Compañias de 1980 con el mismo nombre con el que fue registrada inmediatamente antes del re-registro, salvo la sustitucion o inclusion como la ultima parte del nombre de las palabras "sociedad anonima" o su equivalente en gales, las referencias al nombre de la compañía y sus documentos adjuntos serán tratadas como referencias al nombre con el cual fue re-registrada

De acuerdo con las reglas, las palabras "sociedad anonima" (o en ingles *public limited company*) pueden ser reemplazadas por las letras p l c, plc P L C o PLC

El re-registro bajo la Ley de Compañias no constituye una nueva entidad legal sino simplemente somete a la compañía a ciertas normas de ley adicionales

(FIRMA) R MAHONEY

FECHA 21 de julio de 2000

(SELLO DE LACRE)

246 A-244

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Referencia No B45189 No 9918319 6 03 de agosto de 1999

OFICINA DE PATENTES Solicitud de adjudicacion de una Patente

Forma 1/77

Ley de Patentes de 1977

1 TITULO DEL INVENTO "COMPOSICION DE VACUNA"

2 INFORMACIÓN DEL SOLICITANTE

2a Nombre SmithKline Beecham Biologicals S A

 Pais BELGICA

2b (N/D)

2c Direccion Rue de l Institut 89, B-1330 Rixensart

 Codigo Postal

 PAIS BELGICA

 ADP No 5781117001

3 Ha nombrado Ud a alguien que le represente en su solicitud? SI

 Nombre del Agente MARCUS J W DALTON

 SMITHKLINE BEECHAM

 CORPORATE INTELLECTUAL PROPERTY

 SMITHKLINE BEECHAM PLC

 2 HORIZONS COURT, Bldg 2

 BRENTFORD, MIDDLESEX

 Código Postal TW8 9EP

 No ADP 5776299002

4 Numero de Referencia MJWD/MJL/ND/B45189

5 Solicita Ud que esta solicitud sea tratada como si hubiera sido radicada en la
 fecha de presentacion de una solicitud anterior NO

9918319 6 -ago 00

6 No aplica

7 Es usted el unico inventor o inventor conjunto? NO

8 Cuantas hojas describen esta solicitud? 46 - Reivindicaciones - 2

Resumen - - Dibujos - 8

9 Solicitud Solicitamos la adjudicacion de una patente teniendo en cuenta esta solicitud

(FIRMA) MARCUS J W DALTON Fecha 03/08/99

Favor devolver la forma diligenciada y sus anexos y duplicados junto con su valor a

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25 Southampton Buildings
London
WC2A 1AY

7A 246

COMPOSICION DE VACUNA

REIVINDICACIONES

1 Una preparacion de vacuna en globulo fabricada de una cepa bacteriana Gram-negativa, caracterizada en que es fabricada utilizando uno o mas metodos seleccionados del siguiente grupo

- a) reducir geneticamente antigenos variables inmunodominantes dentro de la vacuna de globulo, que comprende los pasos de determinar la identidad de ese antígeno, fabricar una cepa bacteriana para producir menos o ninguno de dicho antígeno y hacer globulos de dicha cepa,
- b) reducir geneticamente antigenos OMP no protectores dentro de la vacuna de globulo, que comprende los pasos de determinar la identidad de dicho antígeno, fabricar una cepa bacteriana para producir menos o ninguno de dicho antígeno, y hacer globulos de dicha cepa,
- c) crear geneticamente antigenos OMP conservados dentro de la vacuna de globulo, que comprende los pasos de identificar la identidad de dicho antígeno, fabricando una cepa bacteriana con el fin de eliminar regiones variables del gen que codifica dicho antígeno, y hacer globulos de dicha cepa,
- d) regular geneticamente la expresion de antigenos OMP protectores conservados dentro de la vacuna de globulo, que comprende los pasos de identificar la identidad de dicho antígeno, fabricar una cepa bacteriana con el fin de eliminar los

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mecanismos de control represivos de su expresion, y hacer globulos de dicha cepa

- e) regular genéticamente la expresion de antígenos OMP protectores conservados dentro de la vacuna de globulo, que comprende los pasos de identificar la identidad de dicho antígeno, fabricar una cepa bacteriana con el fin de introducir una corriente ascendente mas fuerte de secuencias promotoras que codifiquen dicho antígeno, y hacer globulos de dicha cepa
- f) modificar genéticamente la porcion sacarida del LPS bacteriano dentro de la vacuna de globulo, que comprende los pasos de identificar un gen involucrado en la produccion de porciones sacaridas de LPS toxico, fabricar una cepa con el fin de reducir la expresion de dicho gen y hacer globulos de dicha cepa,
- g) modificar genéticamente la porcion del lipido A del LPS bacteriano dentro de la vacuna de globulo, que comprende los pasos de identificar un gen involucrado en la produccion de la porcion de lipido A de LPS toxico, fabricar una cepa bacteriana con el fin de reducir la expresion de dicho gen y hacer globulos de dicha cepa, o
- h) reducir genéticamente la toxicidad del lipido A dentro de la vacuna de globulo y aumentar los niveles de antígenos protectores, que comprende los pasos de fabricar el genoma de una cepa bacteriana para incorporar un gen que codifique un peptido de Polimixin A (o un derivado de lo mismo) fusionado a un antígeno protector, y hacer globulos de dicha cepa