VACCINE COMPOSITION COMPRISING TRANSFERRIN BINDING PROTEIN AND HSF FROM GRAM NEGATIVE BACTERIA

Technical Field

The present invention relates to the field of Gram-negative bacterial immunogenic compositions and vaccines, their manufacture and the use of such compositions in medicine. More particularly, it relates to vaccine compositions comprising both transferrin binding protein and Hsf. The presence of both these antigens leads to the production of higher levels of bactericidal antibodies.

Background

Gram negative bacteria are the causative agents for a number of human pathologies and there is a need for effective vaccines to be developed against many of these bacteria. In particular *Bordetella pertussis*, *Borrelia burgdorferi*, *Brucella melitensis*, *Brucella ovis*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Esherichia coli*, *Haemophilus influenzae*, *Legionella pneumophila*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica* are Gram negative bacteria which cause pathologies which could be treated by vaccination.

Neisseria gonorrhoeae is the etiologic agent of gonorrhea, one of the most frequently reported sexually transmitted diseases in the world with an estimated annual incidence of 62 million cases (Gerbase et al 1998 Lancet 351; (Suppl 3) 2-4). The clinical manifestations of gonorrhea include inflammation of the mucus membranes of the urogenital tract, throat or rectum and neonatal eye infections. Ascending gonococcal infections in women can lead to infertility, ectopic pregnancy, chronic pelvic inflammatory disease and tubo-ovarian abscess formation. Septicemia, arthritis, endocarditis and menigitis are associated with complicated gonorrhea.

The high number of gonococcal strains with resistance to antibiotics contributes to increased morbidity and complications associated with gonorrhea. An attractive alternative to treatment of gonorrhea with antibiotics would be its prevention using vaccination. No vaccine currently exists for *N. gonorrhoeae* infections.

Neisseria meningitidis is an important pathogen, particularly in children and young adults. Septicemia and meningitis are the most life-threatening forms of invasive meningococcal disease (IMD). This disease has become a worldwide health problem because of its high morbidity and mortality.

Thirteen *N. meningitidis* serogroups have been identified based on antigenic differences in the capsular polysaccharides, the most common being A, B and C which are responsible for 90% of disease worldwide. Serogroup B is the most common cause of meningococcal disease in Europe, USA and several countries in Latin America.

Vaccines based on the capsular polysaccharide of serogroups A, C, W and Y have been developed and have been shown to control outbreaks of meningococcal disease (Peltola et al 1985 Pediatrics 76; 91-96). However serogroup B is poorly immunogenic and induces only a transient antibody response of a predominantly IgM isotype (Ala'Aldeen D and Cartwright K 1996, J. Infect. 33; 153-157). There is therefore no broadly effective vaccine currently available against the serogroup B meningococcus which is responsible for the majority of disease in most temperate countries. This is particularly problematic since the incidence of serotype B disease is increasing in Europe, Australia and America, mostly in children under 5. The development of a vaccine against serogroup B meningococcus presents particular difficulties because the polysaccharide capsule is poorly immunogenic owing to its immunologic similarity to human neural cell adhesion molecule. Strategies for vaccine production have therefore concentrated on the surface exposed structures of the meningococcal outer membrane but have been hampered by the marked variation in these antigens among strains.

Further developments have led to the introduction of vaccines made up of outer membrane vesicles which will contain a number of proteins that make up the normal content of the bacterial membrane. One of these is the VA-MENGOC-BC ® Cuban vaccine against *N. meningitidis* serogroups B and C (Rodriguez et al 1999 Mem Inst. Oswaldo Cruz, Rio de Janeiro 94; 433-440). This vaccine was designed to combat an invasive meningococcal disease outbreak in Cuba which had not been eliminated by a

vaccination programme using a capsular polysaccharide AC vaccine. The prevailing serogroups were B and C and the VA-MENGOC-BC ® vaccine was successful at controlling the outbreak with an estimated vaccine efficiency of 83% against serogroup B strains of *N. meningitidis* (Sierra et al 1990 In Neisseria, Walter Gruyter, Berlin, m. Atchman et al (eds) p 129-134, Sierra et al 1991, NIPH Ann 14; 195-210). This vaccine was effective against a specific outbreak, however the immune response elicited would not protect against other strains of *N. meningitidis*.

Subsequent efficacy studies conducted in Latin America during epidemics caused by homologous and heterologous serogroup B meningococcal strains have shown some efficacy in older children and adults but its effectiveness was significantly lower in younger children who are at greatest risk of infection (Milagres et al 1994, Infect. Immun. 62; 4419-4424). It is questionable how effective such a vaccine would be in countries with multistrain endemic disease such as the UK. Studies of immunogenicity against heterologous strains have demonstrated only limited cross-reactive serum bactericidal activity, especially in infants (Tappero et al 1999, JAMA 281; 1520-1527).

A second outer membrane vesicle vaccine was developed in Norway using a serotype B isolate typical of those prevalent in Scandinavia (Fredriksen et al 1991, NIPH Ann, 14; 67-80). This vaccine was tested in clinical trials and found to have a protective efficacy after 29 months of 57% (Bjune et al 1991, Lancet, 338; 1093-1096).

However, the use of outer membrane vesicles in vaccines is associated with some problems. For instance, the OMV contain toxic lipopolysaccharides and they may contain immunodominant antigens which are either strain specific or are expressed variably. Several processes have been described which could be used to overcome some of the problems of outer membrane vesicle preparation vaccines. WO01/09350 describes processes that address some of these problems for instance by reducing toxicity and modifying the antigens present on the outer membrane vesicles.

There are diverse problems with the anti-meningococcal vaccines currently available. The protein based outer membrane vaccines tend to be specific and effective against

only a few strains. The polysaccharide vaccines are also suboptimal since they tend to elicit poor and short immune responses, particularly against serogroup B (Lepow et al 1986; Peltola 1998, Pediatrics 76; 91-96).

Neisseria infections represent a considerable health care problem for which no vaccines are available in the case of *N. gonorrhoeae* or vaccines with limitations on their efficacy and ability to protect against heterologous strains are available in the case of *N. meningitidis*. Clearly there is a need to develop superior vaccines against Neisserial infections that will improve on the efficacy of currently available vaccines and allow for protection against a wider range of strains.

Description of Figures

Figure 1. – A Coomassie stained gel showing expression levels of Hsf, TbpA and NspA in outer membrane vesicle preparations derived from different *N. meningitidis* stains. Lane 1 - molecular weight markers; lane 2 – outer membrane vesicles prepared from strain H44/76 in which capsular polysaccharides were downregulated; lane 3 - outer membrane vesicles prepared from strain H44/76 in which capsular polysaccharides and PorA were downregulated; lane 4 - outer membrane vesicles prepared from strain H44/76 in which capsular polysaccharides and PorA were downregulated and NspA was upregulated; lane 5 - outer membrane vesicles prepared from strain H44/76 in which capsular polysaccharides and PorA were downregulated and Hsf was upregulated; lane 6 - outer membrane vesicles prepared from strain H44/76 in which capsular polysaccharides and PorA were downregulated and TbpA was upregulated; lane 7 - outer membrane vesicles prepared from strain H44/76 in which capsular polysaccharides and PorA were downregulated and TbpA and Hsf were upregulated; lane 8 - outer membrane vesicles prepared from strain H44/76 in which capsular polysaccharides and PorA were downregulated and TbpA and NspA were upregulated.

Detailed description

The present invention discloses a combination of antigens which when combined in an immunogenic composition or vaccine, can induce higher titres of bactericidal

antibodies than that induced by the antigens when administered separately. Preferably the combination of antigens leads to synergistically higher titres of bactericidal antibodies. As bactericidal antibodies closely reflect the efficacy of vaccine candidates, the combination of Tbp and Hsf in vaccines will produce highly effective vaccines. An additional advantage of the invention will be that the combination of the two antigens, Tbp and Hsf, will also enable protection against a wider range of strains.

The invention relates to an immunogenic composition comprising transferrin binding protein and Hsf like protein or antigenic fragments thereof. These proteins are either isolated or preferably purified to at least 30%, 40%, more preferably 50%, 60%, 70%, 80%, 90%, 95% or 99% pure or enriched in a mixture with other antigens. Transferrin binding protein and Hsf like protein may be isolated or derived from the same or different Gram negative bacterial strains.

Isolated means isolated from the protein's natural environment by the hand of man. Purified means purified to at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% pure before the antigen is combined with other components of the immunogenic composition of the invention.

Derived from means that the gene encoding the protein is derived from or the protein is purified from a particular bacterial strain. Therefore derived from includes recombinant proteins produced in a separate expression system if the gene encoding the protein was derived from the named bacteria.

When combined, Tbp and Hsf have been shown to interact advantageously and preferably synergistically to elicit an immune response that is higher in terms of bactericidal activity (for example as measured by serum bactericidal assay or SBA) and preferably higher than the additive response elicited by the antigens individually, more preferably by a factor of at least 1.1, 1.2, 1.5, two, three, four, five, six, seven, eight, nine, most preferably by a factor of at least ten. The addition of both Tbp and Hsf to a vaccine will have considerable advantages over currently available vaccines in eliciting a strong bactericidal immune response and allowing protection against multiple strains.

One embodiment of the invention is an immunogenic composition comprising both transferrin binding protein and Hsf like protein. An immunogenic composition is a composition comprising at least one antigen which is capable of generating an immune response when administered to a host. Tbp and Hsf like protein can be derived from any strain of Gram negative bacteria including *Moraxella catarrhalis*, *Haemophilus influenzae*, Bordetella, Neisseria (including *Neisseria meningitidis* which could be serogroup A, B, C, W135 or Y and *Neisseria gonorrhoeae)* or any of those Gram negative bacteria hereinbefore described. The invention covers immunogenic compositions in which Tbp and Hsf like protein are derived from either the same or different strains of Gram negative bacteria.

Transferrin binding proteins

Transferrin binding protein (Tbp) is a protein or protein complex on the outer membrane of Gram negative bacteria, which binds transferrin. Some proteins in this family will form a beta-barrel anchored in the outer membrane. Structurally, the transferrin binding protein may contain an intracellular N-terminal domain with a TonB box and plug domain, multiple transmembrane beta strands linked by short intracellular and longer extracellular loops. Other examples are lipoproteins which interact to form a complex with the integral membrane protein. Examples of this family of proteins are TbpA and TbpB. The term Tbp encompasses either of these proteins individually or in combination, and a complex formed from TbpA and TbpB. Preferably at least TbpA is present in the immunogenic compositions of the invention.

Two families of TbpB have been distinguished, having a high molecular weight and a low molecular weight respectively. High and low molecular weight forms of TbpB (WO93/06861; EP586266) associate with different families of TbpA (WO93/06861; EP586266; WO92/03467; US5912336) which are distinguishable on the basis of homology. Despite being of the same molecular weight, TbpA are known as the high molecular weight and low molecular weight families because of their association with the high or low molecular weight form of TbpB (Rokbi et al FEMS Microbiol. Lett. 100; 51, 1993). TbpA and TbpB are known to be expressed in a variety of bacteria including *N. meningitidis* (WO93/06861; EP586266; WO92/03467; US5912336), *N.*

gonorrhoeae (WO92/03467; US5912336), *H. influenzae* (Gray-Owen et al Infect. Immun. 1995; 63:1201-1210, Schryvers J. Med. Microbiol. 1989; 29: 121-130; WO95/13370; WO96/40929), *A. pleuropneumoniae, M. cararrhalis* (Mathers et al FEMS Immunol. Med. Microbiol. 1997; 19: 231; Chen et al Vaccine 1999; 18: 109; WO97/13785; WO99/52947) and *P. haemolytica* (Cornelissen et al Infection and Immunity 68; 4725, 2000). TbpA and TbpB have also been referred to as Tbp1 (NMB 0461) and Tbp2 (NMB 0460) respectively (Cornelissen et al Infection and Immunity 65; 822, 1997).

As used herein, Tbp denotes the transferrin binding protein from Gram negative bacteria, including *Moraxella catarrhalis* and *Haemophilus influenzae,* preferably Neisseria, more preferably *N. meningitidis* or *N. gonorrheoea* and most preferably *N. meningitidis* of serotype B. Tbp encompasses both TbpA and TbpB and the high molecular weight and low molecular weight forms of TbpA and TbpB. Tbp encompasses individual proteins described above and complexes of the proteins and any other proteins or complexes thereof capable of binding transferrin.

Although Tbp can refer to either the high or low molecular forms of TbpA or TbpB, it is preferred that both high molecular weight and low molecular weight forms of TbpA and/or TbpB are present in the immunogenic compositions of the invention. Most preferably, high molecular weight and low molecular weight TbpA is present.

It is also thought that instead of, or in addition to, Tbps, other iron acquisition proteins may be included in the immunogenic compositions of the invention. Iron acquisition proteins of *Moraxella catarrhalis* include TbpA, TbpB, Ton-B dependent receptor, CopB (Sethi et al Infect. Immun. 1997; 65: 3666-3671), HasR, OmpB1 and LbpB (Du et al Infect. Immun. 1998; 66:3656-3665; Mathers et al FEMS Immunol. Med. Microbiol. 1997; 19: 231-236; Chen et al Vaccine 1999; 18: 109-118). Iron acquisition proteins of *Haemophilus influenzae* include TbpB, HasR, TonB-dependent receptor, hemoglobin-binding protein, HhuA, HgpA, HgbA, HgbB and HgbC (Cope et al Infect. Immun. 2000; 68: 4092-4101; Maciver et al Infect. Immun. 1996; 64:3703-3712; Jin et al Infect. Immun. 1996; 64:3134-3141; Morton et al J. Gen. Microbiol. 1990; 136:927-933; Schryvers J. Med. Microbiol. 1989; 29: 121-130). Iron aquisition proteins from *Neisseria meningitidis* include Tbp1 (NMB 0461), Tbp2

(NMB 0460), FbpA (NMB 0634), FbpB, BfrA (NMB 1207), BfrB (NMB 1206), LbpA (NMB 1540), LbpB (NMB 1541), Lipo28 also known as GNA2132 (NMB 2132), Sibp (NMB 1882), Ton B dependent receptors (NMB 0964 and NMB 0293) and HmbR (Tettelin et al Science 287; 1809-1815 2000).

Tbp proteins included in the immunogenic compositions of the invention are proteins sharing homology with TbpA and TbpB from *N. meningitidis* as described in WO93/06861 and EP586266; preferably sharing over 40%, 45%, 50%, 60%, 70%, more preferably over 80% or 90%, most preferably over 95%, 96%, 97%, 98%,99% identity with the amino acid sequence of TbpA and TbpB as described in WO93/06861 and EP586266.

Tbp contains several distinct regions. For example, in the case of TbpA from *N. meningitidis* strain H44/76, the amino terminal 186 amino acids form an internal globular domain, 22 beta strands span the membrane, forming a beta barrel structure. These are linked by short intracellular loops and larger extracellular loops. Extracellular loops 2, 3 and 5 have the highest degree of sequence variability and loop 5 is surface exposed. Loops 5 and 4 are involved in ligand binding, and are preferred TbpA fragments for inclusion in the immunogenic compositions of the present invention.

In addition to genetic upregulation techniques described herein, transferrin binding proteins may also be upregulated in Gram negative bacteria when grown under iron limitation conditions as described below. In immunogenic compositions of the invention in which transferrin binding protein is upregulated in an outer membrane vesicle, upregulation is preferably achieved by growth of the host strain under iron limitation conditions. This process will also result in the upregulation of variable iron-regulated proteins, particularly FrpB in Neisserial stains and heme/hemopexin utilisation protein C, HgpA and HgpB in *Haemophilus influenzae*, which may become immunodominant. It is therefore advantageous to downregulate the expression of (and preferably delete the genes encoding) such proteins as described below, to ensure that the immunogenic composition of the invention elicits an immune response against antigens present in a wide range of strains.

Hsf like proteins

Hsf like proteins are autotransporter proteins sharing homology with Hsf of *N. meningitidis* with the sequences found in WO99/31132; preferably sharing over 40%, 50%, 60%, 70%, more preferably over 80%, most preferably over 90%, most preferably over 95%, 96%, 97%, 98%, 99% identity with an Hsf amino acid sequence found in WO99/31132 (preferably SEQ ID NO 2,4,6 or 8). Hsf like proteins are surface exposed proteins and are thought to function as adhesins. These proteins form a multimeric complex and are expressed during infection and colonisation.

Hsf-like proteins are found in many Gram negative bacteria including *Neisseria meningitidis, Neisseria gonorrheoea , Haemophilus influenzae, Moraxella catarrhalis* and *Escherichia coli.* Examples of Hsf-like proteins found in *Neisseria meningitidis* include Hsf (also kown as NhhA – NMB 0992) (WO99/31132), Aida-1 like protein (Peak et al 2000, FEMS Imm. Med. Microbiol. 28; 329), IgA protease, Ssh-2, Hap (WO99/55873), NadA(J. Exp Med. 2002 195; 1445), UspA2 and Tsh. Examples of Hsf-like proteins in *Moraxella catarrhalis* include Hsf, UspA1 (WO93/03761), UspA2(WO93/03761), outer membrane esterase and YtfN. Examples of Hsf-like proteins in *Haemophilus influenzae* include Hia/Hsf (St Geme et al J. Bacteriol. 2000 182: 6005-6013), Hap, IgA1 protease, HMW1, HMW2 (Barenkamp et al Infect. Immun. 1992 60; 1302-1313), YadA, YadAc and YtfN (Hendrixson et al Mol Cell 1998; 2:941-850; St Geme et al Mol Microbiol. 1994; 14:217-233; Grass and St Geme Infect. Immunol. 2001;69; 307-314; St Geme and Cutter J. Bacteriology 2000; 182; 6005-6013). Examples of Hsf-like proteins in *Escherichia coli* include Hsf, Hia, and Hap.

Hsf has a structure that is common to autotransporter proteins. For example, Hsf from *N. meningitidis* strain H44/76 consists of a head region at the amino terminus of the protein (amino acids 52-479) that is surface exposed and contains variable regions (amino acids 52-106, 121-124, 191-210 and 230-234), a neck region (amino acids 480-509), a hydrophobic alpha-helix region (amino acids 518-529) and an anchoring domain in which four transmembrane strands span the outer membrane (amino acids 539-591).

Hsf may refer to the full length polypeptide including the signal sequence that consists of amino acids 1-51. The invention also encompasses Hsf with the signal sequence removed so that the polypeptide would consist of the mature form of Hsf. Other preferred forms of Hsf may be truncated so as to delete variable regions of the protein disclosed in WO01/55182. Preferred variants would include the deletion of one, two, three, four, or five variable regions as defined in WO01/55182. Preferred variants would delete residues from between amino acid sequence 52 through to 237 or would delete amino acids 54 to 237, more preferably deleting residues between amino acid 52 through to 133 or amino acids 55 to 133. It is understood that truncated variants may include or exclude the signal sequence from amino acids 1 to 51 of Hsf. The above sequence and those described below can be truncated or extended by 1, 2, 3, 4, 5, 7, 10, or 15 amino acids at either or both N and C termini.

Where Hsf is used in a subunit vaccine, it is preferred that a portion of the soluble passenger domain is used; for instance the complete domain of amino acids 52 to 479, most preferably a conserved portion thereof for instance amino acids 134 to 479.

Although full length Tbp and/or Hsf like protein (in particular TbpA and Hsf) is preferably used, or natural variants thereof, or such full length sequences lacking no more than 60 amino acids from the N and/or C termini, antigenic fragments of Tbp and/or Hsf like proteins are also included in the immunogenic composition of the invention. These are fragments containing at least 10 amino acids, preferably 20 amino acids, more preferably 30 amino acids, more preferably 40 amino acids or most preferably 50 amino acids, taken contiguously from the amino acid sequence of Tbp and Hsf like protein, preferably TbpA and Hsf. In addition, antigenic fragments denotes fragments that are immunologically reactive with antibodies generated against the *N. meningitidis* Tbp or Hsf like protein, preerably TbpA or Hsf or with antibodies generated by infection of a mammalian host with *N. meningitidis*. Antigenic fragments also includes fragments that elicit an immune response that is specific against Tbp or Hsf like protein, preferably TbpA or Hsf of Gram negative bacteria from which they are derived. Preferably it is protective against infection from the Bacterium from which it is derived, preferably Neisserial infection, more preferably it is protective against *N. meningitidis* infection, most preferably it is protective against *N. meningitidis* serogroup B infection.

Preferred fragments of TbpA include the extracellular loops of TbpA. Using the sequence of TbpA from *N. meningitidis* strain H44/76, these loops correspond to amino acids 200-202 for loop1, amino acids 226-303 for loop 2, amino acids 348-395 for loop 3, amino acids 438-471 for loop 4, amino acids 512-576 for loop 5, amino acids 609-625 for loop 6, amino acids 661-671 for loop 7, amino acids 707-723 for loop 8, amino acids 769-790 for loop 9, amino acids 814-844 for loop 10 and amino acids 872-903 for loop 11. The corresponding sequences, after sequence alignment, in other Tbp proteins would also constitute preferred fragments. Most preferred fragments would include amino acid sequences comprising loop 2, loop 3, loop 4 or loop 5 of Tbp.

Although the preferred fragments of Tbp or TbpA proteins described above relate to *N. meningitidis*, one skilled in the art would readily be able to find the equivalent peptides in Tbp or TbpA proteins from all the above Gram negative strains on the basis of sequence homology, which are also fragments of the invention.

Preferred fragments of Hsf include the entire head region of Hsf, preferably containing amino acids 52-473 of Hsf. Additional preferred fragments of Hsf include surface exposed regions of the head including amino acids 52-62, 76-93, 116-134, 147-157, 157-175, 199-211, 230-252, 252-270, 284-306, 328-338, 362-391, 408-418, 430-440 and 469-479. Most preferred fragments are 134-591for use in a OMV preparation of the invention and 134-479 for use in a subunit composition of the invention.

Although the preferred fragments of Hsf like or Hsf proteins described above relate to *N. meningitidis*, one skilled in the art would readily be able to find the equivalent peptides in Hsf like or Hsf proteins from all the above Gram negative strains on the basis of sequence homology, which are also fragments of the invention.

Also included in the invention are fusion proteins of Tbp and Hsf-like protein, preferably TbpA and Hsf. These may combine both Tbp and Hsf like protein, preferably TbpA and Hsf, or fragments thereof combined in the same polypeptide. Alternatively, the invention also includes individual fusion proteins of Tbp and Hsf

like protein, preferably TbpA and/or Hsf ,or fragments thereof, provided that both Tbp and Hsf like protein, preferably TbpA and Hsf, or fragments thereof are present in the composition of the invention. TbpA or Hsf could for example form a fusion protein with β-galactosidase, glutathione-S-transferase, green fluorescent proteins (GFP), epitope tags such as FLAG, myc tag, poly histidine, or viral/bacterial surface proteins such as influenza virus haemagglutinin, tetaunus toxoid, diphtheria toxoid or CRM197.

Isolated transferrin binding proteins which could be introduced into an immunogenic composition are well known in the art (WO0025811). They may be expressed in a bacterial host, extracted using detergent (for instance 2% Elugent) and purified by affinity chromatography or using standard column chromatography techniques well known to the art (Oakhill et al Biochem J. 2002 364; 613-6). Similarly, the isolation of Hsf could be achieved using techniques well known in the art. Recombinant Hsf could be expressed in *E. coli* or other bacterial strains. The protein could be purified using affinity chromatography. This would be a routine procedure if a tag were introduced into the Hsf sequence.

The terms 'comprising', 'comprise' and 'comprises' herein are intended by the inventors to be optionally substitutable with the terms 'consisting of', 'consist of' and 'consists of', respectively, in every instance.

Vaccine Combinations

The invention relates to combinations of antigens including Tbp and Hsf-like protein, which are effective at eliciting a high bactericidal activity against Gram negative bacteria. Antigenic compositions of the invention may comprise antigens in addition to Tbp and Hsf. They may comprise other protein antigens from Gram negative bacteria, preferably Neisseria and more preferably from *N. meningitidis.*

N. meningitidis

For *N. meningitidis,* the immunogenic compositions of the invention preferably comprise Hsf and TbpA. In a OMV preparation, it is preferred that Hsf and TbpA are

upregulated in the *N. meningitidis* strain from which the OMV is derived. TbpA may be present as either the high or low molecular weight form and preferably both high and low molecular weight forms are represented. Hsf is preferably present in OMVs as a membrane integrated truncate preferably amino acids 134-591. Hsf may also be present as a subunit vaccine preferably as a passenger domain (amino acid 52-479) most preferably as a passenger domain truncate of amino acids 134-479.

Further antigens may be added to the above compositions (or upregulated if presented in a OMV), for example, NspA (WO96/29412), Hap (PCT/EP99/02766), PorA, PorB, OMP85 (also known as D15) (WO00/23595), PilQ (PCT/EP99/03603), PldA (PCT/EP99/06718), FrpB (WO96/31618 see SEQ ID NO:38), FrpA (NMB 0585) or FrpC or a conserved portion in commen to both of at least 30, 50, 100, 500, 750 amino acids (WO92/01460), LbpA and/or LbpB (PCT/EP98/05117; Schryvers et al Med. Microbiol. 1999 32: 1117), FhaB (WO98/02547 SEQ ID NO:38 [nucleotides 3083-9025]), HasR (PCT/EP99/05989), lipo02 (PCT/EP99/08315), MltA (WO99/57280) (NMB0033) and ctrA (PCT/EP00/00135).

Preferred combinations of antigens in an immunogenic composition of the invention include combinations comprising Tbp and Hsf-like protein and FhaB; Tbp and Hsf-like protein and PilQ; Tbp and Hsf-like protein and NspA; Tbp and Hsf-like protein and FrpC; more preferably comprising Tbp and Hsf-like protein and Hap; Tbp and Hsf-like protein and FrpA/C; Tbp and Hsf-like protein and LbpB; Tbp and Hsf-like protein and D15. Most preferably, D15 would be incorporated as part of an outer membrane vesicle preparation.

Moraxella catarrhalis antigens

One or more of the following proteins from *Moraxella catarrhalis* are preferred for incorporation into the immunogenic composition of the invention (preferably where the TbpA and Hsf like proteins are derived from *Moraxella catarrhalis*): OMP106 (WO 97/41731 & WO 96/34960), HasR (PCT/EP99/03824), PilQ (PCT/EP99/03823), OMP85 (PCT/EP00/01468), lipo06 (GB 9917977.2), lipo10 (GB 9918208.1), lipo11 (GB 9918302.2), lipo18 (GB 9918038.2), P6 (PCT/EP99/03038), ompCD, CopB (Helminen ME, et al (1993) Infect. Immun. 61:2003-2010), D15 (PCT/EP99/03822), OmplA1 (PCT/EP99/06781), Hly3

(PCT/EP99/03257), LbpA and LbpB (WO 98/55606), TbpA and TbpB (WO 97/13785 & WO 97/32980), OmpE, UspA1 and UspA2 (WO 93/03761), and Omp21.

Haemophilus influenzae antigens

One or more of the following proteins from *Haemophilus influenzae* are preferred for inclusion in a immunogenic composition of the invention (preferably where the TbpA and Hsf like proteins are derived from *Haemophilus influenzae*): D15 (WO 94/12641), P6 (EP 281673), TbpA, TbpB, P2, P5 (WO 94/26304), OMP26 (WO 97/01638), HMW1, HMW2, HMW3, HMW4, Hia, Hsf, Hap, Hin47, and Hif.

A further aspect of the invention are vaccine combinations comprising the antigenic composition of the invention with other antigens which are advantageously used against certain disease states including those associated with viral or Gram positive bacteria.

In one preferred combination, the antigenic compositions comprising Tbp and Hsf-like protein of the invention are formulated with 1, 2, 3 or preferably all 4 of the following meningococcal capsular polysaccharides or oligosaccharides which may be plain or conjugated to a protein carrier: A, C, Y or W-135. Such a vaccine containing TbpA and Hsf from *N. meningitidis* may be advantageously used as a global meningococcus vaccine. Preferably conjugated meningococcal capsular polysaccharide C, C and Y or A and C are included.

In a further preferred embodiment, the antigenic compositions comprising TbpA and Hsf of the invention, preferably formulated with 1, 2, 3 or all 4 of the plain or conjugated meningococcal capsular polysaccharides (or oligosaccharides) A, C, Y or W-135 as described above, are formulated with a conjugated *H. influenzae* b capsular polysaccharide or oligosaccharide, and/or one or more plain or conjugated pneumococcal capsular polysaccharides or oligosaccharides. Optionally, the vaccine may also comprise one or more protein antigens that can protect a host against *Streptococcus pneumoniae* infection. Such a vaccine may be advantageously used as a meningitis/streptococcus pneumonia vaccine.

In a still further preferred embodiment, the immunogenic composition comprising Tbp and Hsf-like protein of the invention is formulated with capsular polysaccharides derived from one or more of *Neisseria meningitidis*, *Haemophilus influenzae* b, *Streptococcus pneumoniae*, Group A Streptococci, Group B

Streptococci, *Staphylococcus aureus* or *Staphylococcus epidermidis*. In a preferred embodiment, the immunogenic composition would comprise capsular polysaccharides derived from one or more of serogroups A, C, W-135 and Y of *Neisseria meningitidis*. A further preferred embodiment would comprise capsular polysaccharides derived from *Streptococcus pneumoniae*. The pneumococcal capsular polysaccharide antigens are preferably selected from serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F (most preferably from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F). A further preferred embodiment would contain the PRP capsular polysaccharides of *Haemophilus influenzae*. A further preferred embodiment would contain the Type 5, Type 8 or 336 capsular polysaccharides of *Staphylococcus aureus*. A further preferred embodiment would contain the Type I, Type II or Type III capsular polysaccharides of *Staphylococcus epidermidis*. A further preferred embodiment would contain the Type Ia, Type Ic, Type II or Type III capsular polysaccharides of Group B streptocoocus. A further preferred embodiment would contain the capsular polysaccharides of Group A streptococcus, preferably further comprising at least one M protein and more preferably multiple types of M protein.

Preferred pneumococcal proteins antigens are those pneumococcal proteins which are exposed on the outer surface of the pneumococcus (capable of being recognised by a host's immune system during at least part of the life cycle of the pneumococcus), or are proteins which are secreted or released by the pneumococcus. Most preferably, the protein is a toxin, adhesin, 2-component signal tranducer, or lipoprotein of *Streptococcus pneumoniae*, or fragments thereof. Particularly preferred proteins include, but are not limited to: pneumolysin (preferably detoxified by chemical treatment or mutation) [Mitchell *et al.* Nucleic Acids Res. 1990 Jul 11; 18(13): 4010 "Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2.", Mitchell *et al.* Biochim Biophys Acta 1989 Jan 23; 1007(1): 67-72 "Expression of the pneumolysin gene in *Escherichia coli*: rapid purification and biological properties.", WO 96/05859 (A. Cyanamid), WO 90/06951 (Paton et al), WO 99/03884 (NAVA)]; PspA and transmembrane deletion variants thereof (US 5804193 - Briles *et al.*); PspC and transmembrane deletion variants thereof (WO 97/09994 - Briles et al); PsaA and transmembrane deletion variants thereof (Berry & Paton, Infect Immun 1996 Dec;64(12):5255-62 "Sequence

heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*"); pneumococcal choline binding proteins and transmembrane deletion variants thereof; CbpA and transmembrane deletion variants thereof (WO 97/41151; WO 99/51266); Glyceraldehyde–3-phosphate – dehydrogenase (Infect. Immun. 1996 64:3544); HSP70 (WO 96/40928); PcpA (Sanchez-Beato et al. *FEMS Microbiol Lett* 1998, 164:207-14); M like protein, (EP 0837130) and adhesin 18627, (EP 0834568). Further preferred pneumococcal protein antigens are those disclosed in WO 98/18931, particularly those selected in WO 98/18930 and PCT/US99/30390.

The vaccine may also optionally comprise antigens providing protection against one or more of Diphtheria, tetanus and *Bordetella pertussis* infections. The pertussis component may be killed whole cell *B. pertussis* (Pw) or acellular pertussis (Pa) which comprises at least one antigen (and preferably all three) from PT, FHA and 69kDa pertactin. Typically, the antigens providing protection against Diphtheria and tetanus would be Diphtheria toxoid and tetanus toxoid. The toxoids may chemically inactivated toxins or toxins inactivated by the introduction of point mutations.

The vaccine may also optionally comprise one or more antigens that can protect a host against non-typeable *Haemophilus influenzae*, RSV and/or one or more antigens that can protect a host against influenza virus. Such a vaccine may be advantageously used as a global otitis media vaccine.

Preferred non-typeable *H. influenzae* protein antigens include Fimbrin protein (US 5766608) and fusions comprising peptides therefrom (eg LB1 Fusion) (US 5843464 - Ohio State Research Foundation), OMP26, P6, protein D, TbpA, TbpB, Hia, Hmw1, Hmw2, Hap, and D15.

Preferred influenza virus antigens include whole, live or inactivated virus, split influenza virus, grown in eggs or MDCK cells, or Vero cells or whole flu virosomes (as described by R. Gluck, Vaccine, 1992, 10, 915-920) or purified or recombinant proteins thereof, such as HA, NP, NA, or M proteins, or combinations thereof.

Preferred RSV (Respiratory Syncytial Virus) antigens include the F glycoprotein, the G glycoprotein, the HN protein, the M protein or derivatives thereof.

It should be appreciated that antigenic compositions of the invention may comprise one or more capsular polysaccharide from a single species of bacteria.

Antigenic compositions may also comprise capsular polysaccharides derived from one or more species of bacteria.

Such capsular polysaccharides may be unconjugated or conjugated to a carrier protein such as tetatus toxoid, tetanus toxoid fragment C, diphtheria toxoid, CRM197, pneumolysin, Protein D (US6342224), TbpA or Hsf. One embodiment of the invention would contain separate capsular polysaccharides conjugated to TbpA and Hsf.

The polysaccharide conjugate may be prepared by any known coupling technique. For example the polysaccharide can be coupled via a thioether linkage. This conjugation method relies on activation of the polysaccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide may thus be coupled directly or via a spacer group to an amino group on the carrier protein. Preferably, the cyanate ester is coupled with hexane diamine and the amino-derivatised polysaccharide is conjugated to the carrier protein using heteroligation chemistry involving the formation of the thioether linkage. Such conjugates are described in PCT published application WO93/15760 Uniformed Services University.

The conjugates can also be prepared by direct reductive amination methods as described in US 4365170 (Jennings) and US 4673574 (Anderson). Other methods are described in EP-0-161-188, EP-208375 and EP-0-477508.

A further method involves the coupling of a cyanogen bromide activated polysaccharide derivatised with adipic acid hydrazide (ADH) to the protein carrier by Carbodiimide condensation (Chu C. et al Infect. Immunity, 1983 245 256).

Antigenic compositions comprising outer membrane vesicles

A preferred aspect of the present invention is the upregulation, or overexpression, of Tbp and Hsf in an OMV. Gram negative bacteria are separated from the external medium by two successive layers of membrane structures, the cytoplasmic membrane and the outer membrane. The outer membrane of Gram-negative bacteria is dynamic and depending on 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 et al 1998). Among these, a non-exhaustive list of bacterial pathogens reported to produce blebs include: *Bordetella pertussis*, *Borrelia burgdorferi*, *Brucella melitensis*, *Brucella ovis*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Esherichia coli*, *Haemophilus influenzae*, *Legionella pneumophila*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica*. Although the biochemical mechanism responsible for the production of OMV / 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 proteinaceaous and non-proteinaceous antigens that are likely to confer extended protection against intra-species variants.

The outer membrane vesicles of the invention will have Tbp and Hsf-like protein (preferably TbpA and Hsf) upregulated. This is optionally achieved by having Hsf-like protein and Tbp upregulated in outer membrane vesicles derived from a single Gram negative bacterial, preferably Neisserial strain. Hsf-like protein and Tbp may also be upregulated separately in outer membrane vesicles derived from different Gram negative bacterial strains, preferably Neisserial strains. In a preferred embodiment, the different strains of Neisseria in which Tbp and Hsf-like protein, more preferably TbpA and Hsf are upregulated will be a L2 and L3 or L3 and L2 , repectively immunotype of *N. meningitidis*.

The manufacture of bleb preparations from Neisserial 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 11243 or US 4,271,147, Frederikson et al. (NIPH Annals [1991], 14:67-80), Zollinger et al. (J. Clin. Invest. [1979], 63:836-848), Saunders et al. (Infect. Immun. [1999], 67:113-119), Drabick et al. (Vaccine [2000], 18:160-172) or WO 01/09350 (Example 8) are used. In general, OMVs are extracted with a detergent, preferably deoxycholate, and nucleic acids are optionally removed enzymatically. Purification is achieved by ultracentrifugation optionally followed by

size exclusion chromatography. If 2 or more different blebs of the invention are included, they may be combined in a single container to form a multivalent preparation of the invention (although a preparation is also considered multivalent if the different blebs of the invention are separate compositions in separate containers which are administered at the same time [the same visit to a practitioner] to a host). OMV preparations are usually sterilised by filtration through a 0.2 μm filter, and are preferably stored in a sucrose solution (e.g. 3%) which is known to stabilise the bleb preparations.

Upregulation of Tbp and Hsf-like protein within outer membrane vesicle preparations may be achieved by insertion of an extra copy of a gene into the Gram negative bacteria from which the OMV preparation is derived. Alternatively, the promoter of a gene can be exchanged for a stronger promoter in the bacterial strain from which the OMV preparation is derived. Such techniques are described in WO01/09350. Upregulation of a protein will lead to a higher level of protein being present in OMV compared to the level of protein present in OMV derived from unmodified *N. meningitidis* (for instance strain H44/76). Preferably the level will be at least 1.2, 1.5, 2, 3, 4, 5, 7, 10 or 20 times higher.

Where LPS is intended to be an additional antigen in the OMV, a protocol using a low concentration of extracting detergent (for example deoxycholate or DOC) may preferably be used in the OMV preparation method so as to preserve high levels of bound LPS whilst removing particularly toxic, poorly bound LPS. The concentration of DOC used is preferably 0-0.5% DOC, more preferably 0.02%-0.4%, 0.03%-0.3%, 0.04%-0.2%, 0.05%-.15%, 0.05%-0.2% DOC, most preferably around or exactly 0.1% DOC.

"Stronger promoter sequence" refers to a regulatory control element that increases transcription for a gene encoding antigen of interest.

"Upregulating expression" refers to any means to enhance the expression of an antigen of interest, relative to that of the non-modified (i.e., naturally occurring) bleb. It is understood that the amount of 'upregulation' will vary depending on the particular antigen of interest but will not exceed an amount that will disrupt the

membrane integrity of the bleb. Upregulation of an antigen refers to expression that is at least 10% higher than that of the non-modified bleb. Preferably it is at least 50% higher. More preferably it is at least 100% (2 fold) higher. Most preferably, it is at least 3, 4, 5, 7, 10, 20 fold higher. Preferably the level of expression is assessed when blebs have been derived from bacteria grown in iron limited conditions (for instance in the presence of an iron chelator).

Alternatively or additionally, upregulating expression may refer to rendering expression non-conditional on metabolic or nutritional changes, particularly in the case of TbpA, TbpB, LbpA and LbpB.

Again for the purpose of clarity, the terms 'engineering a bacterial strain to produce less of said antigen' or down regulation refers to any means to reduce the expression of an antigen (or the expression of a functional gene product) of interest, relative to that of the non-modified (i.e., naturally occurring bleb), preferably by deletion, such that expression is at least 10% lower than that of the non-modified bleb. Preferably it is at least 50% lower and most preferably completely absent. If the down regulated protein is an enzyme or a functional protein, the downregulation may be achieved by introducing one or more mutations resulting in a 10%, 20%, 50%, 80% or preferably a 100% reduction in enzymatic or functional activity.

The engineering steps required to modulate the expression of Neisserial proteins can be carried out in a variety of ways known to the skilled person. For instance, sequences (e.g. promoters or open reading frames) can be inserted, and promoters/genes can be disrupted by the technique of transposon insertion. For instance, for upregulating a gene's expression, a strong promoter could be inserted via a transposon up to 2 kb upstream of the gene's initiation codon (more preferably 200-600 bp upstream, most preferably approximately 400 bp upstream). Point mutation or deletion may also be used (particularly for down-regulating expression of a gene).

Such methods, however, may be quite unstable or uncertain, and therefore it is preferred that the engineering step is performed via a homologous recombination event. Preferably, the event takes place between a sequence (a recombinogenic region) of at least 30 nucleotides on the bacterial chromosome, and a sequence (a second recombinogenic region) of at least 30 nucleotides on a vector transformed within the strain. Preferably the regions are 40-1000 nucleotides, more preferably 100-800 nucleotides, most preferably 500 nucleotides). These recombinogenic regions

should be sufficiently similar that they are capable of hybridising to one another under highly stringent conditions.

Methods used to carry out the genetic modification events herein described (such as the upregulation or downregulation of genes by recombination events and the introduction of further gene sequences into a Neisserial genome) are described in WO01/09350. Typical strong promoters that may be integrated in *Neisseria* are *porA*, *porB*, *lgtF*, Opa, *p110*, *lst*, and *hpuAB*. PorA and PorB are preferred as constitutive, strong promoters. It has been established that the PorB promoter activity is contained in a fragment corresponding to nucleotides –1 to –250 upstream of the initation codon of *porB*.

Upregulation of expression of iron acquisition proteins by growth under iron limitation conditions

The upregulation of transferrin binding protein in an outer membrane vesicle preparation of the invention is preferably achieved by isolating outer membrane vesicles from a parental strain of Gram negative bacteria grown under iron limitation conditions. A low concentration of iron in the medium will result in increased expression of proteins involved in iron acquisition including TbpA and TbpB. The expression of these proteins is thereby upregulated without the need for recombinantly modifying the gene involved, for instance by inserting a stronger promoter or inserting an additional copy of the gene. The invention would also encompass upregulation of transferrin binding protein by growth in iron limitation medium where the gene has also been recombinantly modified.

Iron limitation is achieved by the addition of an iron chelator to the culture medium. Suitable iron chelators include 2,2-Dipyridil, EDDHA (ethylenediamine-di(o-hydroxyphenylacetic acid) and Desferal (deferoxamine mesylate, Sigma). Desferal is the preferred iron chelator and is added to the culture medium at a concentration of between 10 and 100μM, preferably 25-75μM, more preferably 50-70μM, most preferably at 60μM. The iron content of medium comes primarily from the yeast extract and soy peptone constituents and the amount present may vary between

batches. Therefore different concentrations of Desferal may be optimal to achieve upregulation of iron acquisition proteins in different batches of medium. The skilled artisan should easily be able to determine the optimal concentration. In basic terms, enough iron chelator should be added to the medium to upregulate the expression of the desired iron-regulated protein, but not so much so as to adversely affect the growth of the bacteria.

Preferably, upregulation of transferrin binding protein by growth under iron limited conditions is combined with recombinant upregulation of Hsf like protein so that the outer membrane vesicle of the invention is achieved.

Down regulation/Removal of 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. An aspect of this invention covers outer membrane vesicles comprising Tbp and Hsf like protein, preferably TbpA and Hsf in which the expression of other proteins is reduced, or, preferably, gene(s) encoding variable surface protein(s) are deleted. Such deletion results in a bacterial strain producing blebs which, when administered in a vaccine, have a stronger potential for cross-reactivity against various strains due to a higher influence exerted by conserved proteins (retained on the outer membranes) on the vaccinee's immune system. Examples of such variable antigens include: for *Neisseria* - pili (PilC) which undergoes antigenic variations, PorA, Opa, OpC, PilC, PorB, TbpB, FrpB; for *H. influenzae* - P2, P5, pilin, IgA1-protease; and for *Moraxella* - OMP106.

Other types of gene that could be down-regulated or switched off are genes which, *in vivo*, can easily be switched on (expressed) or off by the bacterium. As outer membrane proteins encoded by such genes are not always present on the bacteria, the presence of such proteins in the bleb preparations can also be detrimental to the effectiveness of the vaccine for the reasons stated above. A preferred example to down-regulate or delete is *Neisseria* Opc protein. Anti-Opc immunity induced by an Opc containing bleb vaccine would only have limited protective capacity as the infecting organism could easily become Opc$^-$. *H. influenzae* HgpA and HgpB are other examples of such proteins.

For example, these variable or non-protective genes may be down-regulated in expression, or terminally switched off. This has the advantage of concentrating the immune system on better antigens that are present in low amounts on the outer surface of blebs.

Methods for downregulation of expression are disclosed in WO01/09350.

By down regulation of an immunodominant outer membrane protein is it meant that levels of expression are decreased and preferably switched off or that mutations and/or deletions of surface exposed immunodominant loops render the outer membrane protein less immunodominant. By down regulation of a protein with enzymatic function it is meant that the level of expression of the protein is decreased or preferably switched off or can mean that the expression of functional enzyme is reduced or preferably eliminated.

Preferred meningococcal strains of bacteria to use in making immunogenic compositions of the invention have downregulation, preferably deletion of 1, 2 or 3 of PorA, OpA and Opc. Preferably PorA and Opa; PorA and OpC; OpA and OpC; PorA and Opa and OpC are downregulated.

Four different Opa genes are known to exist in the meningococcal genome (Aho et al. 1991 Mol. Microbiol. 5:1429-37), therefore where Opa is said to be downregulated in expression it is meant that preferably 1, 2, 3 or (preferably) all 4 genes present in meningococcus are so downregulated. Such downregulation may be performed genetically as described in WO 01/09350 or by seeking readily-found, natural, stable meningococcal strains that have no or low expression from the Opa loci. Such strains can be found using the technique described in Poolman et al (1985 J. Med. Micro. 19:203-209) where cells that are Opa$^-$ have a different phenotype to cells expressing Opa which can be seen looking at the appearance of the cells on plates or under a microscope. Once found, the strain can be shown to be stably Opa$^-$ by performing a Western blot on cell contents after a fermentation run to establish the lack of Opa.

Where upregulation of transferrin binding protein in the outer membrane vesicles of the invention is achieved by growth under iron limitation conditions, variable iron-regulated proteins may also be upregulated. These include FrpB in

Neisseria meningitidis and *Neisseria gonorrhoeae* (Microbiology 142; 3269-3274, (1996); J. Bacteriol. 181; 2895-2901 (1999)), and heme/hemopexin utilisation protein C (J. Bacteriol. 177; 2644-2653 (1995)) and HgpA, HgpB and HgpC (Infect. Immun. 66; 4733-4741 (1998), Infect. Immun. 67; 2729-2739 (1999), Microbiology 145; 905-914 (1999)) in *Haemophilus influenzae*. The inventors have found that it is advantageous to downregulate expression of at least the variable portions of such proteins when iron limitation is used to upregulate transferrin binding protein expression. This is achieved either by using the processes described in WO01/09350 or by deleting the variable part(s) of the protein. This will ensure that the immune response elicited by the immunogenic composition is directed towards antigens that are present in a wide range of strains. Down regulation of FrpB is preferably combined with down regulation of PorA and OpA; PorA and OpC; OpA and OpC; PorA and OpA and OpC in the bleb immunogenic compositions of the invention derived from Gram negative bacterial strains, preferably Moraxella catarrhalis, Haemophilus influenzae or Neisserial (more preferably N. meningitidis) strains.

In an alternative embodiment of the invention, FrpB is downregulated in outer membrane vesicles which have been prepared from Gram negative bacterial strains, preferably Moraxella catarrhalis, Haemophilus influenzae or Neisserial (more preferably N. meningitidis) strains, not necessarily grown under iron limitation conditions.

Detoxification of LPS

The OMVs in the immunogenic composition of the invention may be detoxifed via methods for detoxification of LPS which are disclosed in WO01/09350. In particular, methods for detoxification of LPS involve the downregulation, preferably deletion of htrB and/or msbB enzymes which are disclosed in WO 01/09350. Deletion mutants of these genes are characterised phenotypically by the msbB- mutant LPS losing one secondary acyl chain compared to wild type and the htrB- mutants LPS losing 2 (or both) secondary acyl chains. Such methods are preferably combined with methods of OMV extraction involving low levels of DOC, preferably 0-0.3% DOC, more preferably 0.05-0.2% DOC, most preferably around 0.1% DOC.

Further methods of LPS detoxification include adding to the bleb preparations a non-toxic peptide functional equivalent of polymyxin B [a molecule with high

affinity to Lipid A] (preferably SAEP 2) (see WO 93/14115, WO 95/03327, Velucchi et al (1997) J Endotoxin Res 4: 1-12, and EP 976402 for further details of non-toxic peptide functional equivalents of polymyxin B – particularly the use of the peptide SAEP 2 (of sequence KTKCKFLKKC where the 2 cysteines form a disulphide bridge)).

Cross-reactive polysaccharides

The isolation of bacterial outer-membrane blebs from encapsulated 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, outer membrane vesicles of the invention may be isolated from a bacterial strain for bleb production, which has been engineered such that it is free of capsular polysaccharide. 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. In general, isolation of outer membrane vesicles should be from Gram negative bacterial strains that cannot synthesise capsular pooysaccharides, particularly where the strain is a msbB- mutant described above.

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 (preferably using the homologous recombination techniques described above), or by any other way of decreasing the enzymatic function of such genes. 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* capsular polysaccharide (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* (+ *galE*) gene cluster.

Where the above immunogenic compositions of the invention are derived from a meningococcus B strain, it is further preferred that the capsular polysaccharide (which also contains human-like saccharide structures) is also removed. Although many genes could be switched off to achieve this, the inventors have advantageously shown that it is preferred that the bleb production strain has been genetically engineered to permanently downregulate the expression of functional gene product from the siaD gene (i.e. downregulating α-2-8 polysialyltransferase activity), preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene. Such an inactivation is described in WO 01/09350. The siaD (also known as synD) mutation is the most advantageous of many mutations that can result in removing the human-similar epitope from the capsular polysaccharide, because it one of the only mutations that has no effect on the biosynthesis of the protective epitopes of LOS, thus being advantageous in a process which aims at ultimately using LOS as a protective antigen, and has a minimal effect on the growth of the bacterium. A preferred aspect of the invention is therefore a bleb immunogenic preparation as described above which is derived from an $lgtE^-$ $siaD^-$, an $lgtA^-$ $siaD^-$ or, preferably, an $lgtB^-$ $siaD^-$ meningococcus B mutant strain. The strain itself is a further aspect of the invention.

Although $siaD^-$ mutation is preferable for the above reasons, other mutations which switch off neisserial (preferably meningococcus B) capsular polysaccharide synthesis may be used. Thus bleb production strain can be genetically engineered to permanently downregulate the expression of functional gene product from one or more of the following genes: ctrA, ctrB, ctrC, ctrD, synA (equivalent to synX and siaA), synB (equivalent to siaB) or synC (equivalent to siaC) genes, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene. The $lgtE^-$ mutation may be combined with one or more of these mutations. Preferably the $lgtB^-$ mutation is combined with one or more of these mutations. A further aspect of the invention is therefore a bleb immunogenic preparation as described above which is derived from such a combined mutant strain of meningococcus B. The strain itself is a further aspect of the invention.

Heterogeneity within the oligosaccharide moiety of the LPS generates structural and antigenic diversity among different neisserial strains (Griffiss et al. Inf. Immun. 1987; 55: 1792-1800). This has been used to subdivide meningococcal strains into 12 immunotypes (Scholtan et al. J Med Microbiol 1994, 41:236-243). Immunotypes L3, L7, & L9 are immunologically identical and are structurally similar (or even the same) and have therefore been designated L3,7,9 (or, for the purposes of this specification, generically as "L3"). Meningococcal LPS L3,7,9 (L3), L2 and L5 can be modified by sialylation, or by the addition of cytidine 5'-monophosphate-N-acetylneuraminic acid. Although L2, L4 and L6 LPS are distinguishable immunologically, they are structurally similar and where L2 is mentioned herein, either L4 or L6 may be optionally substituted within the scope of the invention. See M. P. Jennings *et al*, Microbiology 1999, **145**, 3013-3021 and Mol Microbiol 2002, 43:931-43 for further illustration of LPS structure and heterogeneity.

The safety of antibodies raised to L3 or L2 LPS has been questioned, due to the presence of a structure similar to the lacto-N-neotetraose oligosaccharide group (Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-) present in human glycosphingolipids. Even if a large number of people has been safely vaccinated with deoxycholate extracted vesicle vaccines containing residual amount of L3 LPS (G. Bjune *et al*, Lancet (1991), **338**, 1093-1096; GVG. Sierra *et al*, NIPH ann (1991), **14**, 195-210), the deletion of the terminal part of the LOS saccharidic is advantageous in preventing any cross-reaction with structures present at the surface of human tissues. In a preferred embodiment, inactivation of the *lgtB* gene results in an intermediate LPS structure in which the terminal galactose residue and the sialic acid are absent (the mutation leaves a 4GlcNAcβ1-3Galβ1-4Glcβ1- structure in L2 and L3 LOS). Such intermediates could be obtained in an L3 and an L2 LPS strain. An alternative and less preferred (short) version of the LPS can be obtained by turning off the *lgtE* gene. A further alternative and less preferred version of the LPS can be obtained by turning off the lgtA gene. If such an $lgtA^-$ mutation is selected it is preferred to also turn off lgtC expression to prevent the non-immunogenic L1 immunotype being formed.

$LgtB^-$ mutants are most preferred as the inventors have found that this is the optimal truncation for resolving the safety issue whilst still retaining an LPS protective oligosaccharide epitope that can still induce a bactericidal antibody response.

Therefore, immunogenic compositions of the invention further containing L2 or L3 preparations (whether purified or in an isolated bleb) or meningococcal bleb preparations in general are advantageously derived from a Neisserial strain (preferably meningococcal) that has been genetic engineered to permanently downregulate the expression of functional gene product from the lgtB, lgtA or lgtE gene, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene.

A Neisserial locus containing various *lgt* genes, including lgtB and lgtE, and its sequence is known in the art (see M. P. Jennings *et al*, Microbiology 1999, **145**, 3013-3021 and references cited therein, and J. Exp. Med. 180:2181-2190 [1994]).

In bleb preparations, particularly in preparations extracted with low DOC concentrations LPS may be used as an antigen in the immunogenic composition of the invention. It is however advantageous to downregulate/delete/inactivate enzymatic function of either the lgtE, lgtA (particularly in combination with lgtC), or, preferably, lgtB genes/gene products in order to remove human like lacto-N-neotetraose structures. The Neisserial locus (and sequence thereof) comprising the lgt genes for the biosynthesis of LPS oligosaccharide structure is known in the art (Jennings et al Microbiology 1999 145; 3013-3021 and references cited therein, and J. Exp. Med. 180:2181-2190 [1994]). Downregulation/deletion of lgtB (or functional gene product) is preferred since it leaves the LPS protective epitope intact.

In *N. meningitidis* serogroup B bleb preparations of the invention, the downregulation/deletion of both siaD and lgtB is preferred, (although a combination of $lgtB^-$ with any of $ctrA^-$, $ctrB^-$, $ctrC^-$, $ctrD^-$, $synA^-$ (equivalent to $synX^-$ and $siaA^-$), $synB^-$ (equivalent to $siaB^-$) or $synC^-$ (equivalent to $siaC^-$) in a meningococcus B bleb production strain may also be used) leading to a bleb preparation with optimal safety and LPS protective epitope retention.

Immunogenic composition of the invention may comprise at least, one, two, three, four or five different outer membrane vesicle preparations. Where two or more OMV preparations are included, at least one antigen of the invention is upregulated in each OMV. Such OMV preparations may be derived from Neisserial strains of the same species and serogroup or preferably from Neisserial strains of different class, serogroup, serotype, subserotype or immunotype. For example, an immunogenic composition may comprise one or more outer membrane vesicle preparation(s) which

contains LPS of immunotype L2 and one or more outer membrane vesicle preparation which contains LPS of immunotype L3. L2 or L3 OMV preparations are preferably derived from a stable strain which has minimal phase variability in the LPS oligosaccharide synthesis gene locus.

Preferred Neisserial bleb preparations

In addition to Hsf and Tbp, one or more of the following genes (encoding protective antigens) are preferred for upregulation when carried out on a Neisserial strain, including gonococcus, and meningococcus (particularly *N. meningitidis* B): NspA (WO 96/29412), Hap (PCT/EP99/02766), PorA, PorB (NMB 2039), OMP85 (WO 00/23595), PilQ (PCT/EP99/03603), PldA (PCT/EP99/06718), FrpB (WO 96/31618), FrpA/FrpC (WO 92/01460), LbpA/LbpB (PCT/EP98/05117), FhaB (WO 98/02547), HasR (PCT/EP99/05989), lipo02 (PCT/EP99/08315), MltA (WO 99/57280), MafA (NMB 0652), MafB (NMB 0643), Omp26 (NMB 0181), adhesin NMB 0315, adhesin NMB 0995, adhesin NMB 1119, P2086 (NMB 0399), Lipo28 (NMB 2132), NM-ADPRT (NMB 1343), VapD (NMB 1753) and ctrA (PCT/EP00/00135). They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

One or more of the following genes are preferred for downregulation: PorA, PorB, PilC, LbpA, LbpB, Opa, Opc, htrB, msbB and lpxK.

One or more of the following genes are preferred for upregulation: pmrA, pmrB, pmrE, and pmrF.

Preferred repressive control sequences to be modified are: the *fur* operator region (particularly for either or both of the TbpB or LbpB genes); and the DtxR operator region.

One or more of the following genes are preferred for downregulation: galE, siaA, siaB, siaC, siaD, ctrA, ctrB, ctrC, and ctrD.

Immunogenic compositions of the invention may also comprise OMV/bleb preparations derived from Gram negative bacteria including *Pseudomonas aeruginosa*, *Moraxella catarrhalis* and *Haemophilys influenzae* b.

Preferred *Pseudomonas aeruginosa* bleb preparations

In addition to Hsf and Tbp, one or more of the following genes (encoding protective antigens) are preferred for upregulation: PcrV, OprF, OprI. They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

Preferred *Moraxella catarrhalis* bleb preparations

In addition to Hsf and Tbp, one or more of the following genes (encoding protective antigens) are preferred for upregulation: OMP106 (WO 97/41731 & WO 96/34960), HasR (PCT/EP99/03824), PilQ (PCT/EP99/03823), OMP85 (PCT/EP00/01468), lipo06 (GB 9917977.2), lipo10 (GB 9918208.1), lipo11 (GB 9918302.2), lipo18 (GB 9918038.2), P6 (PCT/EP99/03038), ompCD, CopB (Helminen ME, et al (1993) Infect. Immun. 61:2003-2010), D15 (PCT/EP99/03822), OmplA1 (PCT/EP99/06781), Hly3 (PCT/EP99/03257), LbpA and LbpB (WO 98/55606), TbpA and TbpB (WO 97/13785, WO95/13370 & WO 97/32980), OmpE, UspA1 and UspA2 (WO 93/03761), and Omp21. They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

One or more of the following genes are preferred for downregulation: CopB, OMP106, OmpB1, LbpA, and LbpB.

One or more of the following genes are preferred for downregulation: htrB, msbB and lpxK.

One or more of the following genes are preferred for upregulation: pmrA, pmrB, pmrE, and pmrF.

Preferred *Haemophilus influenzae* bleb preparations

In addition to Hsf and Tbp, one or more of the following genes (encoding protective antigens) are preferred for upregulation: D15 (WO 94/12641, WO95/12641), P6 (EP 281673), P2, P5 (WO 94/26304), OMP26 (WO 97/01638), HMW1, HMW2, HMW3, HMW4, Hia, Hap, Hin47, and Hif (all genes in this operon should be upregulated in order to upregulate pilin). They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

One or more of the following genes are preferred for downregulation: P2, P5, Hif, IgA1-protease, HgpA, HgpB, HMW1, HMW2, Hxu, htrB, msbB and lpxK.

One or more of the following genes are preferred for upregulation: pmrA, pmrB, pmrE, and pmrF.

Preferably the immunogenic compositions or vaccines of the invention do not consist of and/or comprise the particular combinations of SEQ IDs listed in the table spanning from page 3, line 18 to page 52, line 2 of WO 00/71725 and/or any individual combination described in the examples 1-11 of WO 00/71725.

Preferably in addition or alternatively any individualised combinations disclosed in WO 01/52885 are not claimed in this invention.

Vaccine Formulations

A preferred embodiment of the invention is the formulation of the immunogenic composition of the invention in a vaccine which may also comprise a pharmaceutically acceptable excipient or carrier.

The manufacture of outer membrane vesicle preparations from any of the aforementioned modified 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 11243 or US 4,271,147 are used. Most preferably, the method described in WO 01/09350 is used.

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York).

The antigenic compositions of the present invention may be adjuvanted in the vaccine formulation of the invention. Suitable adjuvants include an aluminium salt such as aluminum hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium (particularly calcium carbonate), iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

Suitable Th1 adjuvant systems that may be used include, Monophosphoryl lipid A, particularly 3-de-O-acylated monophosphoryl lipid A, and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salt. An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of

QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO96/33739. A particularly potent adjuvant formulation involving QS21 3D-MPL and tocopherol in an oil in water emulsion is described in WO95/17210 and is a preferred formulation.

The vaccine may comprise a saponin, more preferably QS21. It may also comprise an oil in water emulsion and tocopherol. Unmethylated CpG containing oligo nucleotides (WO 96/02555) are also preferential inducers of a TH1 response and are suitable for use in the present invention.

The vaccine preparation of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Thus one aspect of the present invention is a method of immunizing a human host against a disease caused by infection of a gram-negative bacteria, which method comprises administering to the host an immunoprotective dose of the bleb preparation of the present invention.

The amount of antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-100μg of protein antigen, preferably 5-50μg, and most typically in the range 5 - 25μg.

An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

Polynucleotides of the invention

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and

double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications has been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

Another aspect of the invention relates to an immunological/vaccine formulation which comprises one or more polynucleotide(s) encoding Tbp and Hsf-like protein, particularly those which correspond to protein combinations of the invention. Such techniques are known in the art, see for example Wolff *et al.*, *Science*, (1990) *247*: 1465-8.

The expression of Tbp and Hsf-like protein, preferably TbpA and Hsf in such a polynucleotide would be under the control of a eukaryotic promoter, capable of driving expression within a mammalian cell. The polynucleotide may additionally comprise sequence encoding other antigens. Examples of such eukaryotic promoters include promoters from viruses using mammalian cells as host including adenovirus promoters, retroviral promoters. Alternatively, mammalian promoters could be used to drive expression of TbpA an Hsf-like protein.

Antibodies and passive immunisation

Another aspect of the invention is the use of an immunogenic composition comprising TbpA and Hsf-like protein to generate immune globulin which can be used to treat or prevent infection by Gram negative bacteria or preferably Neisseria, more preferably *Neisseria meningitidis* and most preferably *Neisseria meningitidis* serogroup B.

Inocula for polyclonal antibody production are typically prepared by dispersing the antigenic composition in a physiologically tolerable diluent such as saline or other adjuvants suitable for human use to form an aqueous composition. An immunostimulatory amount of inoculum is administered to a mammal and the inoculated mammal is then maintained for a time sufficient for the antigenic composition to induce protective antibodies.

The antibodies can be isolated to the extent desired by well known techniques such as affinity chromatography.

Antibodies can include antiserum preparations from a variety of commonly used animals e.g. goats, primates, donkeys, swine, horses, guinea pigs, rats or man. The animals are bled and serum recovered.

An immune globulin produced in accordance with the present invention can include whole antibodies, antibody fragments or subfragments. Antibodies can be whole immunoglobulins of any class e.g. IgG, IgM, IgA, IgD or IgE, chimeric antibodies or hybrid antibodies with dual specificity to Tbp and Hsf. They may also be fragments e.g. F(ab')2, Fab', Fab, Fv and the like including hybrid fragments. An immune globulin also includes natural, synthetic or genetically engineered proteins that acts like an antibody be binding to specific antigens to form a complex.

A vaccine of the present invention can be administered to a recipient who then acts as a source of immune globulin, produced in response to challenge from the specific vaccine. A subject thus treated would donate plasma from which hyperimmune globulin would be obtained via conventional plasma fractionation methodology. The hyperimmune globulin would be administered to another subject in order to impart resistance against or treat Neisserial infection. Hyperimmune globulins of the invention are particularly useful for treatment or prevention of Neisserial disease in infants, immune compromised individuals or where treatment is required and there is no time for the individual to produce antibodies in response to vaccination.
An additional aspect of the invention is a pharmaceutical composition comprising monoclonal antibodies reactive against TbpA and Hsf which could be used to treat or

presvent infection by Gram negative bacteria or preferably Neisseria, more preferably Neisseria meningitidis and most preferably Neisseria meningitidis serogroup B.

Such pharmaceutical compositions comprise monoclonal antibodies that can be whole immunoglobulins of any class e.g. IgG, IgM, IgA, IgD or IgE, chimeric antibodies or hybrid antibodies with dual specificity to Tbp and Hsf-like protein. They may also be fragments e.g. F(ab')2, Fab', Fab, Fv and the like including hybrid fragments.

Methods of making monoclonal antibodies are well known in the art and can include the fusion of splenocytes with myeloma cells (Kohler and Milstein 1975 Nature 256; 495; Antibodies – a laboratory manual Harlow and Lane 1988). Alternatively, monoclonal Fv fragments can be obtained by screening a suitable phage display library (Vaughan TJ et al 1998 Nature Biotechnology 16; 535). Monoclonal antibodies may also be humanised or part-humanised using techniques that are well-known in the art.

Serum bactericidal assay

The serum bactericidal assay is the preferred method to assess the synergistic relationship between antigens when combined in an immunogenic composition

Such a synergistic response may be characterised by the SBA elicited by the combination of antigens being at least 50%, two times, three times, preferably four times, five times, six times, seven times, eight times, nine times and most preferably ten times higher than the SBA elicited by each antigen separately. Preferably SBA is measured against a homologous strain from which the antigens are derived and preferably also against a panel of heterologous strains. (See below for a representative panel for instance BZ10 (B:2b:P1.2) belonging to the A-4 cluster; B16B6 (B:2a:P1.2) belonging to the ET-37 complex; and H44/76 (B:15:P1.7,16)). SBA is the most commonly agreed immunological marker to estimate the efficacy of a meningococcal vaccine (Perkins et al. J Infect Dis. 1998, 177:683-691). Satisfactory SBA can be acertained by any known method. SBA can be carried out using sera obtained from animal models (see examples 6-9), or from human subjects.

A further preferred method of conducting SBA with human sera is the following. A blood sample is taken prior to the first vaccination, two months after the second vaccination and one month after the third vaccination (three vaccinations in one year being a typical human primary vaccination schedule administered at, for instance, 0, 2 and 4 months, or 0, 1 and 6 months). Such human primary vaccination schedules can be carried out on infants under 1 year old (for instance at the same time as Hib vaccinations are carried out) or 2-4 year olds or adolescents may also be vaccinated to test SBA with such a primary vaccination schedule. A further blood sample may be taken 6 to 12 months after primary vaccination and one month after a booster dose, if applicable.

SBA will be satisfactory for an antigen or bleb preparation with homologous bactericidal activity if one month after the third vaccine dose (of the primary vaccination schedule) (in 2-4 year olds or adolescents, but preferably in infants in the first year of life) the percentage of subjects with a four-fold increase in terms of SBA (antibody dilution) titre (compared with pre-vaccination titre) against the strain of meningococcus from which the antigens of the invention were derived is greater than 30%, preferably greater than 40%, more preferably greater than 50%, and most preferably greater than 60% of the subjects.

Of course an antigen or bleb preparation with heterologous bactericidal activity can also constitute bleb preparation with homologous bactericidal activity if it can also elicit satisfactory SBA against the meningococcal strain from which it is derived.

SBA will be satisfactory for an antigen or bleb preparation with heterologous bactericidal activity if one month after the third vaccine dose (of the primary vaccination schedule) (in 2-4 year olds or adolescents, but preferably in infants in the first year of life) the percentage of subjects with a four-fold increase in terms of SBA (antibody dilution) titre (compared with pre-vaccination titre) against three heterologous strains of meningococcus is greater than 20%, preferably greater than 30%, more preferably greater than 35%, and most preferably greater than 40% of the subjects. Such a test is a good indication of whether the antigen or bleb preparation with heterologous bactericidal activity can induce cross-bactericidal antibodies against various meningococcal strains. The three heterologous strains should preferably have different electrophoretic type (ET)-complex or multilocus sequence typing (MLST) pattern (see Maiden et al. PNAS USA 1998, 95:3140-5) to each other

and preferably to the strain from which the antigen or bleb preparation with heterologous bactericidal activity is made or derived. A skilled person will readily be able to determine three strains with different ET-complex which reflect the genetic diversity observed amongst meningococci, particularly amongst meningococcus type B strains that are recognised as being the cause of significant disease burden and/or that represent recognised MenB hyper-virulent lineages (see Maiden et al. *supra*). For instance three strains that could be used are the following: BZ10 (B:2b:P1.2) belonging to the A-4 cluster; B16B6 (B:2a:P1.2) belonging to the ET-37 complex; and H44/76 (B:15:P1.7,16) belonging to the ET-5 complex, or any other strains belonging to the same ET/Cluster. Such strains may be used for testing an antigen or bleb preparation with heterologous bactericidal activity made or derived from, for instance, meningococcal strain CU385 (B:4:P1.15) which belongs to the ET-5 complex. Another sample strain that could be used is from the Lineage 3 epidemic clone (e.g. NZ124 [B:4:P1.7,4]). Another ET-37 strain is NGP165 (B:2a:P1.2).

Processes for measuring SBA activity are known in the art. For instance a method that might be used is described in WO 99/09176 in Example 10C. In general terms, a culture of the strain to be tested is grown (preferably in conditions of iron depletion – by addition of an iron chelator such as EDDA to the growth medium) in the log phase of growth. This can be suspended in a medium with BSA (such as Hanks medium with 0.3% BSA) in order to obtain a working cell suspension adjusted to approximately 20000 CFU/ml. A series of reaction mixes can be made mixing a series of two-fold dilutions of sera to be tested (preferably heat-inactivated at 56°C for 30 min) [for example in a 50µl/well volume] and the 20000 CFU/ml meningococcal strain suspension to be tested [for example in a 25µl/well volume]. The reaction vials should be incubated (e.g. 37°C for 15 minutes) and shaken (e.g. at 210 rpm). The final reaction mixture [for example in a 100µl volume] additionally contains a complement source [such as 25 % final volume of pretested baby rabbit serum], and is incubated as above [e.g. 37°C for 60 min]. A sterile polystyrene U-bottom 96-well microtiter plate can be used for this assay. A aliquot [e.g. 10 µl] can be taken from each well using a multichannel pipette, and dropped onto Mueller-Hinton agar plates (preferably containing 1 % Isovitalex and 1 % heat-inactivated Horse Serum) and incubated (for example for 18 hours at 37°C in 5 % CO_2). Preferably, individual colonies can be counted up to 80 CFU per aliquot. The following three test samples can be used as controls: buffer + bacteria + complement; buffer + bacteria + inactivated complement;

serum + bacteria + inactivated complement. SBA titers can be straightforwardly calculated using a program which processes the data to give a measurement of the dilution which corresponds to 50 % of cell killing by a regression calculation.

All references or patent applications cited within this patent specification are incorporated by reference herein.

Method of Industrial Application of the Invention

The examples below are carried our 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: Methods for constructing strains of Neisseria meningitidis serogroup B used in outer membrane vesicle preparations

WO01/09350 provides detailed methods for preparing outer membrane vesicles and manipulating the bacterial strains from which the outer membrane vesicles are derived. Where the outer membrane vesicles are to retain lipoproteins such as TbpB and or lipopolysaccharides, methods of isolation with low levels or no deoxycholate are preferred.

Example 2: Up-regulation of the Hsf protein antigen in a recombinant *Neisseiria meningitidis* serogroup B strain lacking functional *cps* genes but expressing PorA.

As described in WO01/09350 examples, in certain countries, the presence of PorA in outer membrane vesicles may be advantageous, and can strengthen the vaccine efficacy of recombinant improved blebs. In the following example, we have used a modified pCMK(+) vector to up-regulate the expression of the Hsf protein antigen in a strain lacking functional *cps* genes but expressing PorA. The original pCMK(+) vector contains a chimeric *porA/lacO* promoter repressed in *E. coli* host expressing $lacI^q$ but transcriptionally active in *Neisseria meningitidis*. In the modified pCMK(+), the native *porA* promoter was used to drive the transcription of the *hsf* gene. The gene coding for Hsf was PCR amplified using the HSF 01-*Nde*I and HSF 02-*Nhe*I oligonucleotide primers, presented in the table below. Because of the sequence of the HSF 01-*Nde*I primer the Hsf protein expressed will contain two methionine residues at the 5' end. The conditions used for PCR amplification were those described by the supplier (HiFi DNA polymerase, Boehringer Mannheim, GmbH). Thermal cycling was the following: 25 times (94°C 1min., 48°C 1min., 72°C 3min.) and 1 time (72°C 10min., 4°C up to recovery). The corresponding amplicon was subsequently cloned in the corresponding restriction sites of pCMK(+) delivery vector. In this recombinant plasmid, designed pCMK(+)-Hsf, we deleted the *lacO*

present in the chimeric *porA/lacO* promoter by a recombinant PCR strategy. The pCMK(+)-Hsf plasmid was used as a template to PCR amplify 2 separate DNA fragments:

-fragment 1 contains the *porA 5'* recombinogenic region, the Kanamycin resistance gene and the *porA* promoter. Oligonucleotide primers used, RP1(*Sac*II) and RP2, are presented in the table below. RP1 primer is homologous to the sequence just upstream of the *lac* operator.

-fragment 2 contains the Shine-Dalgarno sequence from the *porA* gene, the *hsf* gene and the *porA 3'* recombinogenic region. Oligonucleotide primers used, RP3 and RP4(*Apa*I), are presented in the table below. RP3 primer is homologous to the sequence just downstream of the *lac* operator. The 3' end of fragment 1 and the 5'end of fragment 2 have 48 bases overlapping. 500ng of each PCR (1 and 2) were used for a final PCR reaction using primers RP1 and RP4. The final amplicon obtained was subcloned in pSL1180 vector restricted with *Sac*II and *Apa*I. The modified plasmid pCMK(+)-Hsf was purified at a large scale using the QIAGEN maxiprep kit and 2 µg of this material was used to transform a *Neisseiria meningitidis* serogroup B strain lacking functional *cps* genes. In order to preserve the expression of *porA*, integration resulting from a single crossing-over was 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 separated by PAGE-SDS electrophoresis on a 12.5 % gel. The expression of Hsf was examined in Whole-cell bacterial lysates (WCBL) derived from *NmB* [Cps-, PorA+] or *NmB* [Cps-, PorA+, Hsf+]. Coomassie staining detected a significant increase in the expression of Hsf (with respect to the endogenous Hsf level). This result confirms that the modified pCMK(+)-Hsf vector is functional and can be used successfully to up-regulate the expression of outer membrane proteins, without abolishing the production of the major PorA outer membrane protein antigen.

Oligonucleotides used in this work

Oligonucleotides	Sequence	**Remark(s)**
Hsf 01-Nde	5'- GGA ATT **CCA TAT GAT** GAA CAA AAT ATA CCG C-3'	*Nde*I **cloning site**
Hsf 02-Nhe	5'-GTA GCT **AGC TAG CTT** ACC ACT GAT AAC CGA C -3'	*Nhe* I **cloning site**
GFP-mut-Asn	5'-AAC TGC AGA **ATT AAT** ATG AAA GGA GAA GAA CTT TTC-3'	*Asn*I **cloning site** Compatible with *Nde*I
GFP-Spe	5'-GAC ATA **CTA GTT** TAT TTG TAG AGC TCA TCC ATG -3'	*Spe*I **cloning site** Compatible with *Nhe*I
RP1 (SacII)	5'- TCC **CCG CGG** GCC GTC TGA ATA CAT CCC GTC-3'	*Sac*II **cloning site**
RP2	5'-CAT ATG GGC TTC CTT TTG TAA ATT TGA GGG CAA ACA CCC GAT ACG TCT TCA-3'	
RP3	5'-AGA CGT ATC GGG TGT TTG CCC TCA AAT TTA CAA AAG GAA GCC CAT ATG -3'	
RP4(ApaI)	5'-GGG TAT TCC **GGG CCC** TTC AGA CGG CGC AGC AGG -3'	*Apa*I **cloning site**

Example 3: Up-regulation of the *N. meningitidis* serogroup B *tbpA* gene by promoter replacement.

The aim of the experiment was to replace the endogenous promoter region of the *tbpA* gene by the strong *porA* promoter, in order to up-regulate the production of the TbpA antigen. For that purpose, a promoter replacement plasmid was constructed using *E. coli* cloning methodologies. A DNA region (731bp) located upstream from the *tbpA* coding sequence was discovered in the private Incyte PathoSeq data base of the *Neisseria meningitidis* strain ATCC 13090. This DNA contains the sequence coding for TbpB antigen. The genes are organized in an operon. The *tbpB* gene will be deleted and replaced by the *CmR*/porA promoter cassette. For that purpose, a DNA fragment of 3218bp corresponding to the 509bp 5' flanking region of *tbpB* gene, the 2139bp *tbpB* coding sequence, the 87bp intergenic sequence and the 483 first nucleotides of *tbpA* coding sequence was PCR amplified from *Neisseria meningitidis* serogroup B genomic DNA using oligonucleotides **BAD16** (5'- GGC CTA GCT

AGC CGT CTG AAG CGA TTA GAG TTT CAA AAT TTA TTC-3') and **BAD17** (5'-GGC CAA GCT TCA GAC GGC GTT CGA CCG AGT TTG AGC CTT TGC-3') containing uptake sequences and *Nhe*I and *Hind*III restriction sites (underlined). This PCR fragment was cleaned with a High Pure Kit (Boerhinger Mannheim, Germany) and directly cloned in a pGemT vector (Promega, USA). This plasmid was submitted to circle PCR mutagenesis (Jones & Winistofer (1992)) in order to (i) insert suitable restriction sites allowing cloning of a *CmR*/PorA promoter cassette and (ii) to delete 209bp of the 5' flanking sequence of *tbpB* and the *tbpB* coding sequence. The circle PCR was performed using the **BAD 18** (5'-TCC CCC GGG AAG ATC TGG ACG AAA AAT CTC AAG AAA CCG-3') & the **BAD 19** (5'-GGA AGA TCT CCG CTC GAG CAA ATT TAC AAA AGG AAG CCG ATA TGC AAC AGC AAC ATT TGT TCC G -3') oligonucleotides containing suitable restriction sites *Xma*I, *Bgl*II and *Xho*I (underlined). The *CmR*/PorA promoter cassette was amplified from the pUC D15/Omp85 plasmid previously described, using primers **BAD21** (5'- GGA AGA TCT CCG CTC GAG ACA TCG GGC AAA CAC CCG-3') & **BAD20** (5'- TCC CCC GGG AGA TCT CAC TAG TAT TAC CCT GTT ATC CC-3') containing suitable restriction sites *Xma*I, *Spe*I, *Bgl*II and *Xho*I (underlined). This PCR fragment was cloned in the circle PCR plasmid. This plasmid will be used to transform *Neisseria meningitidis* serogroup B (*cps*-) and (*cps- porA*-) strains. Integration by double crossing-over in the upstream region of *tbpA* will direct the insertion of the *porA* promoter directly upstream of the *tbpA* ATG.

Example 4: Construction of a *N. meningitidis* serogroup B strain up-regulated for the expression of two antigens: TbpA and Hsf.

The aim of the experiment was to up-regulate the expression of TbpA and Hsf simultaneously in the same *N. meningitidis* serogroup B strain. The production of TbpA was up-regulated by replacing its endogenous promoter region by the strong *porA* promoter (promoter replacement). In this context, the *tbpB* gene, located upstream of *tbpA* is deleted, and the TbpB protein no longer present in the outer-membrane. The expression of Hsf was up-regulated by insertion (homologous recombination) of a second copy of the corresponding gene at the *porA* locus (gene delivery). Both strains have been described in a separate patent referred to as WO01/09350. The selection markers used in both strategies (Cm^R or Kan^R) allowed the combination of both integrations into the same chromosome.

Total genomic DNA was extracted from the recombinant *Nm*.B *cps*-/TbpA+/PorA+ strain by the Qiagen Genomic tip 500-G protocol. Ten µg of DNA was restricted o/n with *Dra*III restriction enzyme and used to transform *Neisseria meningitidis* serogroup B by the classical transformation protocol. Cells used for transformation were either recombinant *Nm*B *cps*-/Hsf+/PorA+ (homologous recombination by 1 crossing over into the *porA* locus) or recombinant *Nm*B *cps*-/Hsf+/PorA- (Allelic exchange/homologous recombination by 2 crossing over into the *porA* locus). They were plated over-night on GC agar containing 200µg/ml kanamycine, diluted to DO_{650}= 0.1 in GC liquid medium 10mM $MgCl_2$, and incubated 6 hours at 37°C under vigorous agitation with 10µg of *Dra*III restricted genomic DNA. Recombinant *Neisseria meningitidis* resulting from a double crossing over event (PCR screening) were selected on GC medium containing 200µg/ml kanamycin and 5µg/ml chloramphenicol and analyzed for TbpA and Hsf expression in OMV preparations. As represented in Figure 1, the production of both TbpA and Hsf was significantly increased in the OMV prepared from the TbpA/Hsf recombinant *Nm*B strain when compared to the OMV prepared from the control *Nm*B *cps*- strains. The level of over expression of each protein in the dual recombinant is comparable with the level of expression obtained in the corresponding single recombinants. The level of over expression of TbpA and Hsf was comparable in PorA+ and PorA- strains (data not shown). All together, these data demonstrate that: (i) expression of TbpA and Hsf can be jointly and concomitantly up-regulated into *N. meningitidis* and (ii) recombinant blebs enriched for TbpA and Hsf can be obtained and used for immunization.

Analysis of Hsf and TbpA content of Outer Membrane Vesicles

Coommassie blue stained SDS-PAGE

15µg of protein in outer membrane vesicle preparations with up-regulation of Hsf or TbpA or both Hsf and TbpA, were diluted in a sample buffer containing β-mercaptoethanol and heated at 95°C for 10 minutes. The samples were then run on SDS-PAGE polyacrylamide gel (Novex 4-20% Tris-glycine 1.5 mm 2Dwell SDS Page), stained in Coomassie blue for one hour and destained in several washes of destain. Results are shown in Figure 1, which shows that the level of Hsf and TbpA

are considerably higher in outer membrane vesicle preparations, derived from *N. meningitidis* where their level of expression had been enhanced.

Example 5: Immunogenicity of OMVs with upregulation of Hsf and/or TbpA

Groups of 20 mice were immunised three times with OMV by the intra-muscular route on days 0, 21 and 28. Each innoculation was made up of 5µg (protein content) of OMVs formulated on AlPO4 with MPL. The OMVs were derived from *N. meningitidis* strain H44/76, engineered so that capsular polysaccharides and PorA were down regulated. A comparison was made of OMVs in which Hsf, TbpA, both Hsf and TbpA or neither were upregulated. On day 41, blood samples were taken for analysis by ELISA or by serum bactericidal assay.

ELISA to detect antibodies against Hsf

96 well microplates (Nunc, Maxisorb) were coated overnight at 4°C with 100 µl of 1 µg/ml of specific antigen in PBS. After washing with NaCl 150 mM Tween 20 0.05%, plates were saturated with 100 µl of PBS-BSA 1% under shaking at room temperature for 30 minutes. Between each step (performed under shaking at room temperature during 30 min and with PBS-BSA 0.2% as diluant buffer), reagents in excess were removed by washing with NaCl-Tween 20. One hundred micro-liters of diluted serum samples were added per micro-well. Bound antibodies were recognized by a biotinylated anti-mouse Ig (Prosan) (1/2000). The antigen-antibody complex was revealed by incubation with streptavidin-biotinylated peroxidase conjugate (Amersham) (1/4000). OrthoPhenileneDiamine/H_2O_2 (4 mg/10 ml citrate buffer 0.1M pH 4.5 + 5 µl H_2O_2) is used to reveal the assay. Plates were incubated for 15 min at room temperature in the dark before stoping the reaction by addition of 50 µl of 1N HCl . The absorbance was read at 490nm.

	Titre Mid-Point (on pooled sera)
g1, blebs TbpA-HSF, IM	15471
g2, blebs TbpA, IM	15.41
g3, blebs HSF, IM	14508
g4, blebs CPS(-)PorA(-), IM	-
g5, MPL/AlPO4, IM	-

The results shown in the table above, show that high and equivalent antibody titres against Hsf were raised by immunisation with OMVs with upregulation of Hsf or both Hsf and TbpA. Virtually no antibody against Hsf could be detected in sera raised after inoculation with adjuvant alone or OMV in which neither Hsf nor TbpA had been upregulated or OMV in which only TbpA had been upregulated.

Example 6: Serum Bactericidal Activity of antisera raised against OMVs with up-regulation of Hsf and/or TbpA

The serum bactericidal activity of antisera from the mice inoculated with OMVs with upregulation of Hsf, TbpA, both Hsf and TbpA or without upregulation were compared in assays using either the homologous strain H44/76 or the heterologous strain Cu385. The serum bactericidal assay has been shown to show good correlation with the protection and is therefore a good indication of how effective a candidate composition will be in eliciting a protective immune response.

Neisseria meningitidis serogroup B wild type strains (H44/76 strain =B:15 P1.7,16 L3,7,9 and CU385 strain =B: 4 P1.19,15 L3,7,9) were cultured overnight on MH + 1% Polyvitex + 1% horse serum Petri dishes at 37°C + 5% CO2. They were sub-cultured for 3 hours in a liquid TSB medium supplemented with 50 µM of Desferal (Iron chelator) at 37°C under shaking to reach an optical density of approximately 0.5 at 470 nm.

Pooled or individual serum were inactivated for 40 min at 56°C. Serum samples were diluted 1/100 in HBSS-BSA 0.3% and then serially diluted two fold (8 dilutions) in a volume of 50 µl in round bottom microplates.

Bacteria, at the appropriate OD, were diluted in HBSS-BSA 0.3% to yield 1.3 10e4 CFU per ml. 37.5 µl of this dilution was added to the serum dilutions and microplates were incubated for 15 minutes at 37°C under shaking. Then, 12.5 µl of rabbit complement were added to each well. After 1 hour of incubation at 37°C and under shaking, the microplates were placed on ice to stop the killing.

Using the tilt method, 20µl of each well were platted on MH + 1% Polyvitex + 1% horse serum Petri dishes and incubated overnight at 37°C +CO2. The CFU's were counted and the percent of killing calculated. The serum bactericidal titer is the last dilution yielding ≥ 50% killing.

	H44/76		CU385	
OMV	GMT	% responders	GMT	% responders
CPS(-) PorA (-)	93	30%	58	5%
CPS(-) PorA (-) Hsf	158	40%	108	20%
CPS(-) PorA (-) TbpA	327	60%	147	30%
CPS(-) PorA (-) Hsf - TbpA	**3355**	**100%**	**1174**	**80%**

Similar results to those shown in the above table were obtained in two other similar experiments.

A dramatic increase in the bactericidal titres (GMT) against the homologous strain and a heterologous strain were seen after vaccination with OMV in which both Hsf and TbpA were upregulated. By comparison, bactericidal GMTs measured on mice vaccinated with Hsf or TbpA upregulated OMVs were similar to those obtained with mice vaccinated with control OMVs.

The benefit of double up-regulation was also clearly observed in the percentage of mice producing a significant level of bactericidal antibodies (titres greater than 1/100), particularly in experiments using the heterologous strain.

Example 7: Effect of mixing anti-Hsf and anti-TbpA sera on bactericidal activity

Groups of 20 mice were immunised three times with OMV by the intra-muscular route on days 0, 21 and 28. Each inoculation was made up of 5µg (protein content) of OMVs formulated on AlPO4 with MPL. The OMVs were derived *from N. meningitidis* strain H44/76, engineered so that capsular polysaccharides and PorA were down regulated. One group of mice was immunised with control OMVs in which there was no up-regulation of proteins. In a second group, Hsf expression was up-regulated, in a third group TbpA expression was up-regulated and in a fourth group, the expression of both Hsf and TbpA was up-regulated.

The sera were pooled, either using sera from mice in the same group or by mixing sera isolated from the group in with Hsf alone or TbpA alone had been up-regulated. Serum bactericidal activity was measured for each of the pooled sera and the results are shown in the table below.

SBA done on pooled sera from mice immunized with	SBA titer
TbpA-Hsf blebs	774
TbpA blebs	200
Hsf blebs	50
CPS(-) PorA(-) blebs	50
Mix anti-TbpA + anti-Hsf sera	1162

The results in the above table show that mixing of anti-Hsf and anti-TbpA antisera resulted in a much higher serum bactericidal activity than was achieved by either antisera individually. The synergistic effect seems to be achieved by the presence of antibodies against both Hsf and TbpA.

Example 8: Truncated Hsf proteins may combine synergistically with TbpA

A series of truncated Hsf constructs were made using standard molecular biology procedures. These include a construct that encodes amino acids 1 to 54 which contains the signal sequence of Hsf and amino acids 134 to 592 of Hsf (Tr1Hsf). A second truncated Hsf contained amino acids 1-53 of the signal sequence of Hsf followed by amino acids 238-592 of Hsf (Tr2Hsf). These two truncated Hsf constructs and full length Hsf were introduced into *N. Meningitidis* B strain MC58 siaD-, Opc-, PorA- so that their expression would be up-regulated and outer membrane vesicles were produced using the methods described above.

The outer membrane vesicle preparations were adsorbed onto Al(OH)3 and injected into mice on days 0, 21 and 28. On day 42, the mice were bled and sera prepared. The sera were mixed with sera from mice vaccinated with up-regulated TbpA OMVs and serum bactericidal assays were performed as described above.

Results

Group	Serum Bactericidal titres H44/76	CU385
MC58 PorA+ siaD+	25600	25600
MC58 PorA- siaD- Hsf	1530	800
MC58 PorA- siaD- Tr1Hsf	1015	1360
MC58 PorA- siaD- Tr2Hsf	50	50
Negative control	50	50
TbpA + MC58 PorA+ siaD+	25600	24182
TbpA + MC58 PorA- siaD- Hsf	2595	1438
TbpA + MC58 PorA- siaD- Tr1Hsf	**4383**	**2891**
TbpA + MC58 PorA- siaD- Tr2Hsf	1568	742
TbpA + Negative control	778	532

The results shown in the above table reveal that the first truncation (Tr1Hsf) elicits an immune response which is capable of combining with antisera against TbpA to produce a larger serum bactericidal activity than when full length Hsf is used. However, the extent of the truncation is important and the truncation produced in Tr2 has a deleterious effect compared to the full length Hsf. The enhanced bactericidal activity of Tr1Hsf was seen against both the strains used.

Example 9: Serum bactericidal activity of antibodies against TbpA, Hsf and a third meningococcal protein

N. meningitidis strain H66/76 in which PorA and capsular polysaccharides were down regulated as described above, was used as the background strain for up-regulating TbpA and Hsf, LbpB, D15, PilQ or NspA using the procedure described above. Outer membrane vesicles were prepared from each strain as described above. Recombinant FhaB, FrpC, FrpA/C and Hap were made using techniques well known in the art as described in PCT/EP99/02766, WO92/01460 and WO98/02547.

The outer membrane vesicle preparations and recombinant proteins were adsorbed onto Al(OH)3 and injected into mice on days 0, 21 and 28. On day 42, the mice were

bled and sera prepared. The sera against TbpA and Hsf up-regulated OMVs were mixed with sera from mice vaccinated with up-regulated LbpB, D15, PilQ or NspA OMVs or recombinant FhaB, FrpC, FrpA/C or Hap and serum bactericidal assays were performed as described above.

Results

Results are shown in the table below. In assays using the homologous H44/76 stain, the addition of antibodies against a third meningococcal antigen, with the exception of FrpC, did not produce a serum bactericidal titre higher than that produced using antibodies against TbpA and Hsf alone.

However, the addition of antibodies against a third antigen was advantageous in serum bactericidal assays using a heterologous strain. Antibodies against D15 (OMP85), Hap, FrpA/C and LbpB were particularly effective at increasing the serum bactericidal titre against the CU385 strain.

	Serum Bactericidal Titre	
Antisera Mix	H44/76	CU385
anti–TbpA-Hsf and nonimmune sera	5378	2141
anti-TbpA-Hsf and anti-FhaB	5260	2563
anti-TbpA-Hsf and anti-Hap	4577	5150
anti-TbpA-Hsf and anti-FrpA/C	5034	4358
anti-TbpA-Hsf and anti-LbpB	5400	4834
anti-TbpA-Hsf and anti-D15	4823	4657
anti-TbpA-Hsf and anti-PilQ	4708	2242
anti-TbpA-Hsf and anti-NspA	4738	2518
anti-TbpA-Hsf and anti-FrpC	6082	2300

Example 10: Effect of FrpB KO in outer membrane vesicles on their ability to elicit a bactericidal immune response in homologous and heterologous strains

Two strains of H44/76 N. meningitidis were used to prepare outer membrane vesicle preparations as described in WO01/09350, using a 0.1% DOC extraction so that the LOS content was around 20%. Strain B1733 is siaD(-), PorA(-), has upregulation of Tr1 Hsf (example 8) and lgtB is knocked out. Strain B1820 B1733 is siaD(-), PorA(-), has upregulation of Tr1 Hsf , lgtB is knocked out and FrpB is also knocked out. Both strains were cultured in media supplemented with 60μM Desferal so that iron regulated proteins such as LbpA/B and TbpA/B are upregulated.

The bleb preparations were adsorbed onto Al(OH)3 and 5μg were injected intramuscularly into groups of 30 mice on day 0 and day 21. Blood samples were taken on day 28.

Serum bactericidal assays were carried out on three L3 strains (the homologous wild type strain H44/76 and two heterologous L3 strains; NZ124 and M97250687), as described in example 6.

Results

Blebs used for inoculation	H44/76		M97250687		NZ124	
	GMT	SC	GMT	SC	GMT	SC
B1733	1518	30/30	151	11/30	70	4/29
B1820	781	19/30	1316	24/30	276	19/30

GMT indicates the geometric mean titre of the sera in the SBA.
SC indicates the number of mice seroconverting (SBA titre >1/100).

The results clearly show that FrpB KO (B1820) blebs induce a better heterologous cross-bactericidal response than FrpB(+) blebs (B1733). The SBA titres were higher and a higher proportion of mice seroconverted in strains M97250687 and NZ124. The results in the homologous strain was not quite as good when FrpB was deleted.

These data suggest that FrpB drives the immune response, but since this outer membrane protein is highly variable, antibodies against this protein are only able to induce killing of the homologous strain.

Claims

1. An immunogenic composition comprising an isolated transferrin binding protein (Tbp) or antigenic fragment thereof and an isolated Hsf like protein or antigenic fragment thereof from the same or different Gram negative bacteria.

2. The immunogenic composition of claim 1 in which the transferrin binding protein or fragment thereof and Hsf like protein or fragment thereof are from Neisseria.

3. The immunogenic composition of claims 1-2 in which the transferrin binding protein or fragment thereof is derived from *N. meningitidis*.

4. The immunogenic composition of claims 1-3 in which the Hsf like protein or fragment thereof is derived from *N. meningitidis*.

5. The immunogenic composition of claims 1-4 in which the transferrin binding protein or fragment thereof is derived from *N. meningitidis* serogroup B.

6. The immunogenic composition of claims 1-5 in which the Hsf like protein or fragment thereof is derived from *N. meningitidis* serogroup B.

7. The immunogenic composition of claims 1-6 in which the transferrin binding protein or fragment thereof is derived from *N. gonorrhoeae*.

8. The immunogenic composition of claims 1-7 in which the Hsf like protein or antigenic fragment thereof is derived from *N. gonorrhoeae*.

9. The immunogenic composition of claim 1-8 in which the transferrin binding protein or antigenic fragment thereof is derived from *Moraxella catarrhalis*.

10. The immunogenic composition of claim 1-9 in which the Hsf like protein or antigenic fragment thereof is derived from *Moraxella catarrhalis*.

11. The immunogenic composition of claim 1-10 in which the transferrin binding protein or antigenic fragment thereof is derived from *Haemophilus influenzae*.

12. The immunogenic composition of claim 1-11 in which the Hsf like protein or antigenic fragment thereof is derived from *Haemophilus influenzae*.

13. The immunogenic composition of claims 1-12 in which the transferrin binding protein is TbpA or an antigenic fragment thereof.

14. The immunogenic composition of claim 13 comprising high molecular weight form TbpA or low molecular weight form TbpA or both high molecular weight form TbpA and low molecular weight form TbpA.

15. The immunogenic composition of claims 1-14 in which the Hsf like protein is Hsf or an antigenic fragment thereof.

16. The immunogenic composition of claims 1-15 comprising antigenic fragments of Tbp and/or Hsf like protein capable of generating a protective response against Neisserial, *Moraxella catarrhalis* or *Haemophilus influenzae* infection.

17. The immunogenic composition of claim 16 comprising antigenic fragments of TbpA and/or Hsf.

18. The immunogenic composition of claims 1-17 comprising a fusion protein of Tbp and Hsf like protein or antigenic fragments thereof.

19. The immunogenic composition of claim 18 comprising a fusion protein comprising TbpA and Hsf or antigenic fragments thereof capable of generating a protective response against Neisserial infection.

20. An isolated immunogenic composition comprising an outer membrane vesicle preparation derived from Gram negative bacteria, in which expression of both transferrin binding protein and Hsf like protein are at least 1.5 fold higher than naturally occurring in the unmodified Gram negative bacteria.

21. The immunogenic composition of claim 20 in which the expression of transferrin binding protein is upregulated by growth under iron limitation conditions.

22. The immunogenic composition of claim 20-21 in which at least a part of the outer membrane vesicle preparation is derived from Neisseria.

23. The immunogenic composition of claims 20-22 in which at least a part of the outer membrane vesicle preparation is derived from *Neisseria meningitidis*.

24. The immunogenic composition of claims 20-23 in which at least a part of the outer membrane vesicle preparation is derived from *Neisseria meningitidis* serogroup B.

25. The immunogenic composition of claims 20-22 in which at least a part of the outer membrane vesicle preparation is derived from *Neisseria gonorrhoeae.*

26. The immunogenic composition of claims 20-25 wherein a host cell from which the outer membrane vesicle preparation is derived has been engineered so as to down-regulate the expression of one or more of LgtB and LgtE, preferably the former.

27. The immunogenic composition of claims 20-26 wherein a host cell from which the outer membrane vesicle preparation is derived is unable to synthesise capsular polysaccharides and has preferably been engineered so as to down-regulate the expression of and preferably to delete one or more of siaD, ctrA, ctrB, ctrC, ctrD, synA (equivalent to synX and siaA), synB (equivalent to siaB and synC (equivalent to siaC), most preferably siaD.

28. The immunogenic composition of claims 20-27 wherein a host cell from which the outer membrane vesicle preparation is derived has been engineered so as to

down-regulate the expression of and preferably delete one or more of OpC, OpA and PorA, preferably PorA.

29. The immunogenic composition of claims 20-28 wherein a host cell from which the outer membrane vesicle preparation is derived has been engineered so as to down-regulate the expression of and preferably delete FrpB.

30. The immunogenic composition of claims 20-29 wherein a host cell from which the outer membrane vesicle preparation is derived has been engineered so as to down-regulate the expression of and preferably delete msbB and/or HtrB, preferably msbB.

31. The immunogenic composition of claims 20-30 wherein the outer membrane vesicle preparation contains LPS which is conjugated to an outer membrane protein (OMP).

32. The immunogenic composition of claim 31 wherein LPS is conjugated (preferably intra-bleb) to OMP in situ in the outer membrane vesicle preparation.

33. The immunogenic composition of claims 20-32 in which at least a part of the outer membrane vesicle preparation is derived from *Moraxella catarrhalis*.

34. The immunogenic composition of claims 20-33 in which at least a part of the outer membrane vesicle preparation is derived from *Haemophilus influenzae*.

35. The immunogenic composition of claims 20-34 comprising an outer membrane vesicle preparation isolated from two or more strains of Gram negative bacteria.

36. The immunogenic composition of claim 35 in which transferrin binding protein and Hsf like protein are upregulated on different vesicles originating from different bacterial strains or on the same vesicles originating from the same bacterial strain.

37. The immunogenic preparation of claims 20-36 comprising an outer membrane vesicle preparation in which enhanced transferrin binding protein expression is derived from a polynucleic acid introduced into the Gram negative bacteria.

38. The immunogenic composition of claims 20-37 comprising an outer membrane vesicle preparation in which enhanced Hsf like protein expression is derived from a polynucleic acid introduced into the Gram negative bacteria.

39. The immunogenic composition of claims 20-38 comprising an outer membrane vesicle preparation in which enhanced transferrin binding protein and Hsf like protein expression is derived from a polynucleic acid encoding both proteins which was introduced into the Gram negative bacteria.

40. The immunogenic composition of claims 20-39 in which a bacterial strain has been genetically engineered so as to introduce a stronger promoter sequence upstream of a gene encoding transferrin binding protein.

41. The immunogenic composition of claims 20-40 in which a bacterial strain has been genetically engineered so as to introduce a stronger promoter sequence upstream of a gene encoding Hsf like protein.

42. The immunogenic composition of claims 20-41 in which a bacterial strain has been genetically engineered so as to introduce a stronger promoter sequence upstream of genes encoding transferrin binding protein and Hsf like protein.

43. The immunogenic composition of claims 20-42 in which the transferrin binding protein is TbpA which is preferably high molecular weight TbpA, low molecular weight TbpA or both high molecular weight TbpA and low molecular weight TbpA, most preferably from *N. meningitidis*.

44. The immunogenic composition of claims 20-43 in which the Hsf like protein is Hsf from *Neisseria meningitidis*.

45. The immunogenic composition of claims 1-44 further comprising plain or conjugated bacterial capsular polysaccharide or oligosaccharide.

46. The immunogenic composition of claim 1-45 comprising two or more bacterial capsular polysaccharides or oligosaccharides conjugated to transferrin binding protein or Hsf like proteins or both.

47. The immunogenic composition of claim 45-46 wherein the capsular polysaccharide or oligosaccharide is derived from one or more bacteria selected from the group consisting of *Neisseria meningitidis* serogroup A, *Neisseria meningitidis* serogroup C, *Neisseria meningitidis* serogroup Y, *Neisseria meningitidis* serogroup W-135, *Haemophilus influenzae* b, *Streptococcus pneumoniae*, Group A Streptococci, Group B Streptococci, *Staphylococcus aureus* and *Staphylococcus epidermidis*.

48. An immunogenic composition comprising one or more polynucleotide(s) encoding a transferrin binding protein or antigenic fragment thereof and a Hsf like proteinor antigenic fragment thereof whose expression is driven by a eukaryotic promoter.

49. The immunogenic composition of claim 48 wherein TbpA and Hsf of Neisseria are encoded.

50. The immunogenic composition of claims 48-49 wherein TbpA and Hsf of *Neisseria meningitidis* are encoded.

51. The immunogenic composition of claims 1-50 comprising an adjuvant.

52. The immunogenic composition of claim 51 comprising aluminium salts.

53. The immunogenic composition of claim 51-52 comprising 3D-MPL.

54. The immunogenic composition of claim 51 comprising an adjuvant containing CpG.

55. A vaccine comprising the immunogenic composition of claims 1-54 and a pharmaceutically acceptable excipient.

56. A method for treatment or prevention of Gram negative bacterial disease comprising administering a protective dose or an effective amount of the vaccine of claim 55.

57. The method of claim 56 in which Neisserial infection is prevented or treated.

58. A use of the vaccine of claim 55 in the preparation of a medicament for treatment or prevention of Gram negative bacterial infection.

59. The use of claim 58 in the preparation of a medicament for treatment or prevention of Neisserial infection.

60. A genetically engineered Gram negative bacterial strain from which the outer membrane vesicles within the immunogenic composition of claims 20-44 can be derived.

61. A method of making the immunogenic composition of claims 1-17 comprising a step of mixing together isolated transferrin binding protein and isolated Hsf like protein or antigenic fragments thereof.

62. A method of making the immunogenic composition of claims 20-44 comprising a step of isolating outer membrane vesicles from a Gram negative bacterial culture.

63. The method of claim 62 wherein the step of isolating outer membrane vesicles involves extraction with 0-0.5%, 0.02-0.4%, 0.04-0.3%, 0.06-0.2%, 0.08-0.15% or preferably 0.1% detergent, preferably DOC.

64. A method of making the immunogenic composition of claim 47 comprising the step of conjugating bacterial capsular polysaccharides or oligosaccharides to transferrin binding protein and/or Hsf like protein.

65. A method of making the vaccine of claim 55 comprising a step of combining the immunogenic composition of claims 1-54 with a pharmaceutically acceptable excipient.

66. A method of preparing an immune globulin for use in prevention or treatment of Neisserial infection comprising the steps of immunising a recipient with the vaccine of claim 55 and isolating immune globulin from the recipient.

67. An immune globulin preparation obtainable from the method of claim 66.

68. A pharmaceutical preparation comprising the immune globulin preparation of claim 67 and a pharmaceutically acceptable excipient.

69. A pharmaceutical preparation comprising monoclonal antibodies against TbpA and Hsf of *Neisseria meningitidis* and a pharmaceutically acceptable excipient.

70. A method for treatment or prevention of Gram negative bacterial infection comprising a step of administering to the patient an effective amount of the pharmaceutical preparation of claims 68-69.

71. A use of the pharmaceutical preparation of claims 68-69 in the manufacture of a medicament for the treatment or prevention of Gram negative bacterial disease.