

VACCINE COMPRISING PROTEIN NMB0964 FROM NEISSERIA MENINGITIDIS

FIELD OF THE INVENTION

This invention relates to immunogenic compositions for the prevention of diseases caused by *Neisseria* bacteria, in particular *Neisseria meningitidis*.

BACKGROUND OF THE INVENTION

Neisseria strains of bacteria are the causative agents for a number of human pathologies, against which there is a need for effective vaccines to be developed. In particular *Neisseria gonorrhoeae* and *Neisseria meningitidis* 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 meningitis 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. Achtman 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).

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.

SUMMARY OF THE INVENTION

The present inventors have found that the Neisserial antigen NMB0964 (NMB numbers refer to Neisseria meningitidis group B genome sequences available from www.neisseria.org) [known as NMA1161 in the Neisseria meningitidis group A genome of strain Z2491, and as BASB082 in WO 00/55327, and as ZnuD] is a conserved antigen throughout neisseria and can induce bactericidal antibodies against a range of neisserial strains. The inventors have found this antigen functions as a Zn^{2+} receptor in the bacterium, and its expression is regulated by the level of Zn^{2+} in the medium.

The present invention generally provides methods and compositions for eliciting an immune response against Neisseria spp. bacteria in a subject, particularly against a Neisseria meningitidis serogroup B strain.

In one aspect the present invention provides an immunogenic composition comprising: isolated outer membrane vesicles prepared from a Neisseria species bacterium, wherein the Neisseria species bacterium produces a level of a NMB0964 polypeptide sufficient to provide for production of a vesicle that, when administered to a subject, elicits anti-NMB0964 antibodies; and a pharmaceutically acceptable excipient.

This may be achieved due to the Neisseria species bacterium being genetically modified in NMB0964 polypeptide production by for instance: disrupting the functional expression of the Zur repressor (NMB1266) – a protein which switches off expression of NMB0964 in the presence of Zn^{2+} in the medium; replacing the NMB0964 promoter with one that does not bind Zur, in particular with a stronger promoter than the endogenous NMB0964 promoter such as a lac promoter; or through using a medium low in Zn^{2+} concentration – i.e. under 5, 4, 3, 2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05 or 0.01 μM free Zn^{2+} – (such as Roswell Park Memorial Institute medium 1640 (RPMI) which has around 1.69 μM Zn^{2+} by ICP-MS), or removing Zn^{2+} in the medium, for instance using a known zinc chelator such as TPEN (N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine) – enough should be added to the medium such that the expression of the NMB0964 is maximised.

The Neisseria species bacterium may be deficient in capsular polysaccharide, for instance through disruption of functional expression of the *siaD* gene. It may be disrupted in the functional expression of the *msbB* and/or *htrB* genes to detoxify the LOS in the outer membrane vesicle. It may be disrupted in the expression of one or more the following genes: *PorA*, *PorB*, *OpA*, *OpC*, *PilC*, or *FrpB*. It may be disrupted in the functional expression of the *IgtB* gene. Such disruption methods are described in WO 01/09350 and WO2004/014417. The Neisseria species bacterium may be of immunotype L2 or L3.

Methods for the preparation or isolation of outer membrane vesicles (also known as microvesicles or blebs) from Neisserial strains are well known in the art, and are described in WO 01/09350 and WO2004/014417. Typically outer membrane vesicles are isolated by extracting either without a detergent, or with 0-0.5, 0.02-0.4, 0.04-0.3, 0.06-0.2, or 0.08-0.15 % detergent, for instance deoxycholate, e.g. with around or exactly 0.1% deoxycholate.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1. Detection of Tdfl on Western blot. (A) HB-1 grown in TSB (lane 1), RPMI (lane 2) and the *tdfl* knockout strain grown in RPMI (lane 3). (B) HB-1 grown in RPMI with increasing amounts of TSB added. (C) HB-1 grown in RPMI (lane 1), supplemented with 0.5 μM zinc (lane 2) or 1 μM zinc (lane 4). (D) HB-1 grown in RPMI (lane 1), with increasing concentrations of TPEN (0.1, 0.5 and 1 μM in lanes 2-4, respectively)

Figure 2. Tdfl expression in wild type and *zur* mutant strains. The presence of Tdfl in cell lysates of HB-1 and the *zur* mutant grown in RPMI, RPMI with 600 nM zinc or TSB was assessed by Western blot analysis.

Figure 3. Topology model of Tdfl. The plug domain is colored dark grey, the beta strands light gray and the extracellular loops white. The histidine/aspartic acid stretches are boxed.

Figure 4. Zinc binding and transport by Tdfl. (A) Zinc binding to outer membrane vesicles either containing or not Tdfl was measured by a PAR competition assay (B) Intracellular zinc concentrations as measured by ICP-MS of the wild-type strain, the *tdfl* mutant and the *tonB* mutant.

Figure 5. Zinc regulation of Tdfl is highly conserved in meningococci. Western blot of cell lysates of the indicated strains grown in RPMI with or without added zinc. ^a Clonal group designations taken from (36); – indicates that the strain was typed by Multi-Locus Enzyme Electrophoresis but could not be assigned to a specific clone.

Figure 6. Protein profile of the Tdfl vaccine. Outer membrane vesicles used to immunize mice for antiserum production were separated by SDS-PAGE and stained with Coomassie brilliant blue.

Figure 7. Impact of IPTG on expression of Tdfl on cells used in SBA. See Example 1.

Supplementary Figure 1. Amino acid sequence alignment of Tdfl of *N. meningitidis* strains MC58 with those of 053422, FAM18 and Z2491, the carrier strains α 14, α 153 and α 275 The TonB box (Tb), the plug domain, the loops and the transmembrane domains (Tm) are marked above the sequence and the His- and Asp-rich stretches are underlined.

Supplementary Figure 2. Amino acid sequence alignment of the Tdfl homologues. The histidine aspartic acid rich stretches are highlighted in grey.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that an OMV vaccine prepared either in specific culture conditions low in Zn²⁺, or from a mutant *N. meningitidis* strain engineered to either over-express NMB0964 or to remove the Zinc repression mechanism mediated through Zur, is enriched in NMB0964, and such OMVs may elicit good bactericidal antibody responses compared to OMVs which have not been prepared with these methods.

By the term NMB0964 polypeptide herein it includes the neisserial Tdfl polypeptide (encoded by the *tdfl* gene) in general from any neisserial strain (the protein is so well conserved amongst neisserial strains its identity in any particular neisserial strain is readily ascertainable by persons skilled in the art). The term therefore includes the NMA1161 sequence, and the BASB082 polypeptide sequence (and all the Polypeptides of the Invention concerning the BASB082 polypeptide) of WO 00/55327. For instance the NMB0964 polypeptide of the invention will cover SEQ ID NO: 2 of WO00/55327 or polypeptides with more than 70, 80, 90 or 95% sequence identity with said SEQ ID NO:2, or polypeptides comprising immunogenic fragments of 7, 10, 12, 15 or 20 (or more) contiguous amino acids from said SEQ ID NO: 2 (in particular said immunogenic fragments being capable of eliciting – if necessary when coupled to a protein carrier – an immune response which can recognise said SEQ ID NO: 2). Particularly preferred NMB0964 immunogenic fragment embodiments are those extracellular loop sequences shown in the topology diagram of Figure 3 as applied to any given NMB0964 sequence. In particular the third extracellular loop is provided (wherein the 2 Cys residues are optionally disulphide linked or not). Said NMB0964 immunogenic fragment polypeptide sequences may have more than 70, 80, 90 or 95% sequence identity with said extracellular loop sequences (as defined in Figure 3) from SEQ ID NO:2 of WO 00/55327, or may be polypeptides comprising immunogenic fragments of 7, 10, 12, 15 or 20 (or more) contiguous amino acids from said extracellular loop sequences (as defined in Figure 3) from SEQ ID NO: 2 (in particular said immunogenic fragments being capable of eliciting – if necessary when coupled to a protein carrier – an immune response which can recognise said SEQ ID NO: 2) and are provided as NMB0964 polypeptides of the invention. Said NMB0964 immunogenic fragment polypeptide sequences may have more than 70, 80, 90, 95, 99 or 100% sequence identity with the sequence from the third extracellular loop sequence given in Figure 3 (wherein optionally the 2 Cys residues should be conserved, and may or may not be disulphide linked), or may be polypeptides comprising immunogenic fragments of 7, 10, 12, 15 or 20 (or more) contiguous amino acids from said extracellular loop sequence (in particular said immunogenic fragments being capable of eliciting – if necessary when coupled to a protein carrier – an immune response which can recognise SEQ ID NO: 2 of WO00/55327) and are provided as NMB0964 polypeptides of the invention. In one embodiment the NMB0964 immunogenic fragment polypeptides are not full-length NMB0964 (mature sequence or with signal sequence) polypeptides. Thus a further aspect of the invention is a immunogenic composition

comprising such NMB0964 immunogenic fragment polypeptide sequences of the invention and a pharmaceutically acceptable excipient.

The term "a level of a NMB0964 polypeptide sufficient to provide for production of a vesicle that, when administered to a subject, elicits anti-NMB0964 antibodies" in one embodiment indicates that the level is sufficient to induce detectable bactericidal antibodies, for instance SBA titres of 100 or more, for instance it indicates that 5µg total protein content outer membrane vesicles of the invention when intramuscularly injected into mice at days 0, 21 and 28 produces serum on day 42 which generates an SBA titre of over 100 (for instance greater than 150, 200, 250, 300, 350, 400, 500, 700, 900 or 1000) using the SBA assay in the "Serum Bactericidal Assay" section of Example 2.

The heterologous promoter associated with the polypeptide of the invention being "stronger" than the non-repressed endogenous promoter of the polypeptide of the invention means that its use results in the expression of more polypeptide of the invention than when a non-repressed endogenous promoter of the polypeptide of the invention is utilised.

The term "protective immunity" means that a vaccine or immunization schedule that is administered to a mammal induces an immune response that prevents, retards the development of, or reduces the severity of a disease that is caused by *Neisseria meningitidis*, or diminishes or altogether eliminates the symptoms of the disease.

The phrase "a disease caused by a strain of serogroup B of *Neisseria meningitidis*" encompasses any clinical symptom or combination of clinical symptoms that are present in an infection with a member of serogroup B of *Neisseria meningitidis*. These symptoms include but are not limited to: colonization of the upper respiratory tract (e.g. mucosa of the nasopharynx and tonsils) by a pathogenic strain of serogroup B of *Neisseria meningitidis*, penetration of the bacteria into the mucosa and the submucosal vascular bed, septicemia, septic shock, inflammation, haemorrhagic skin lesions, activation of fibrinolysis and of blood coagulation, organ dysfunction such as kidney, lung, and cardiac failure, adrenal hemorrhaging and muscular infarction, capillary leakage, edema, peripheral limb ischaemia, respiratory distress syndrome, pericarditis and meningitis.

"Serogroup" as used herein refers to classification of *Neisseria meningitidis* by virtue of immunologically detectable variations in the capsular polysaccharide. About 12 serogroups are known: A, B, C, X, Y, Z, 29-E, W-135, H, I, K and L. Any one serogroup can encompass multiple serotypes and multiple serosubtypes.

"Enriched" means that an antigen in an antigen composition is manipulated by an experimentalist or a clinician so that it is present in at least a three-fold greater concentration by total weight, usually at least 5-fold greater concentration, more preferably at least 10-fold greater concentration, or at least 100-fold greater concentration than the concentration of that antigen in the strain from which the antigen composition was obtained. Thus, if the concentration of a particular antigen is 1 microgram per gram of total bacterial preparation (or of total bacterial protein), an enriched preparation would contain at least 3 micrograms per gram of total bacterial preparation (or of total bacterial protein).

The NMB0964 polypeptide of the invention may be enriched in the outer membrane vesicles of the invention through the methods discussed herein (for instance the culture conditions, or the overexpression of the polypeptide through recombinant means).

The term "heterologous" refers to two biological components that are not found together in nature. The components may be host cells, genes, or regulatory regions, such as promoters. Although the heterologous components are not found together in nature, they can function together, as when a promoter heterologous to a gene is operably linked to the gene. Another example is where a *Neisseria* sequence is heterologous to a *Neisseria* host of a different strain. "Heterologous" as used herein in the context of proteins expressed in two different bacterial strains, indicates that the proteins in question differ in amino acid sequence.

The production strain can be a capsule deficient strain. Capsule deficient strains can provide vesicle-based vaccines that provide for a reduced risk of eliciting a significant autoantibody response in a subject to whom the vaccine is administered (e. g., due to production of antibodies that cross-react with sialic acid on host cell surfaces). "Capsule deficient" or "deficient in capsular polysaccharide" as used herein refers to a level

of capsular polysaccharide on the bacterial surface that is lower than that of a naturally-occurring strain or, where the strain is genetically modified, is lower than that of a parental strain from which the capsule deficient strain is derived. A capsule deficient strain includes strains that are decreased in surface capsular polysaccharide production by at least 10%, 20%, 25%, 30%, 40%, 50%, 60%, 75%, 80%, 85%, 90% or more, and includes strains in which capsular polysaccharide is not detectable on the bacterial surface (e.g., by whole cell ELISA using an anti-capsular polysaccharide antibody).

Capsule deficient strains include those that are capsule deficient due to a naturally-occurring or recombinantly-generated genetic modification. Naturally-occurring capsule deficient strains (see, e.g., Dolan-Livengood et al. *J. Infect. Dis.* (2003) 187(10): 1616-28), as well as methods of identifying and/or generating capsule-deficient strains (see, e.g., Fisseha et al. (2005) *Infect. Immun.* 73(7):4070-4080; Stephens et al. (1991) *Infect Immun* 59(11):4097-102; Frosch et al. (1990) *Mol Microbiol.* 1990 4(7):1215-1218) are known in the art.

Modification of a Neisserial host cell to provide for decreased production of capsular polysaccharide may include modification of one or more genes involved in capsule synthesis, where the modification provides for, for example, decreased levels of capsular polysaccharide relative to a parent cell prior to modification. Such genetic modifications can include changes in nucleotide and/or amino acid sequences in one or more capsule biosynthesis genes rendering the strain capsule deficient (e.g., due to one or more insertions, deletions, substitutions, and the like in one or more capsule biosynthesis genes). Capsule deficient strains can lack or be non-functional for one or more capsule genes. Of particular interest are strains that are deficient in sialic acid biosynthesis.

Such strains can provide for production of vesicles that have reduced risk of eliciting anti-sialic acid antibodies that cross-react with human sialic acid antigens, and can further provide for improved manufacturing safety. Strains having a defect in sialic acid biosynthesis (due to either a naturally occurring modification or an engineered modification) can be defective in any of a number of different genes in the sialic acid biosynthetic pathway. Of particular interest are strains that are defective in a gene product encoded by the N-acetylglucosamine-6-phosphate 2-epimerase gene (known as synX AAF40537. 1 or siaA AAA20475), with strains having this gene inactivated being of especial interest. For example, in one embodiment, a capsule deficient strain is generated by disrupting production of a functional synX gene product (see, e.g., Swartley et al. (1994) *J Bacteriol.* 176(5):1530-4).

Capsular deficient strains can also be generated from naturally-occurring strains using non-recombinant techniques, e.g., by use of bactericidal anti-capsular antibodies to select for strains that reduced in capsular polysaccharide.

In general as noted above, vesicles can be produced according to the invention using a naturally-occurring or modified non-naturally-occurring Neisserial strain that produces vesicles with sufficient NMB0964 protein that, when administered to a subject, provide for production of anti- NMB0964 antibodies.

In one embodiment, the Neisserial strain used to produce vesicles according to the invention can be naturally occurring strains that express a higher level of NMB0964 relative to strains that express no detectable or a low level of NMB0964.

In another embodiment, the Neisserial strain is modified by recombinant or non-recombinant techniques to provide for a sufficiently high level of NMB0964 production.

Such modified strains generally are produced so as to provide for an increase in NMB0964 production that is 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, or 10-fold or greater over NMB0964 production in the unmodified parental cell or over NMB0964 production of the strain RM1090 or H44/76. Any suitable strain can be used in this embodiment, including strains that produce low or undetectable levels of NMB0964 prior to modification and strains that naturally produce high levels of NMB0964 relative to strains that express no detectable or a low level of NMB0964.

Modified strains may be produced using recombinant techniques, usually by introduction of nucleic acid encoding a NMB0964 polypeptide or manipulation of an endogenous NMB0964 gene to provide for increased expression of endogenous NMB0964.

As noted above, this may be done by introduction of nucleic acid encoding a NMB0964 polypeptide or manipulation of an endogenous NMB0964 gene to provide for increased expression of endogenous NMB0964.

Endogenous NMB0964 expression can be increased by altering in situ the regulatory region controlling the expression of NMB0964. Methods for providing for increased expression of an endogenous Neisserial gene are known in the art (see, e. g., WO 02/09746).

Modification of a Neisserial host cell to provide for increased production of endogenous NMB0964 may include partial or total replacement of all or a portion of the endogenous gene controlling NMB0964 expression, where the modification provides for, for example, enhanced transcriptional activity relative to the unmodified parental strain.

Increased transcriptional activity may be conferred by variants (point mutations, deletions and/or insertions) of the endogenous control regions, by naturally occurring or modified heterologous promoters or by a combination of both. In general the genetic modification confers a transcriptional activity greater than that of the unmodified endogenous transcriptional activity (e.g., by introduction of a strong promoter), resulting in an enhanced expression of NMB0964.

Typical strong promoters that may be useful in increasing NMB0964 transcription production can include, for example, the promoters of *porA*, *porB*, *lbpB*, *tbpB*, *p110*, *hpuAB*, *lgtF*, *Opa*, *p110*, *lst*, and *hpuAB*. *PorA*, *RmpM* and *PorB* are of particular interest as constitutive, strong promoters. *PorB* promoter activity is contained in a fragment corresponding to nucleotides -1 to -250 upstream of the initiation codon of *porB*.

Methods are available in the art to accomplish introduction of a promoter into a host cell genome so as to operably link the promoter to an endogenous NMB0964 -encoding nucleic acid. For example, double cross-over homologous recombination technology to introduce a promoter in a region upstream of the coding sequence, e.g., about 1000 bp, from about 30-970 bp, about 200-600 bp, about 300-500 bp, or about 400 bp upstream (5') of the initiation ATG codon of the NMB0964 -encoding nucleic acid sequence to provide for up-regulation. Optimal placement of the promoter can be determined through routine use of methods available in the art.

For example, a highly active promoter (e.g., *PorA*, *PorB* or *RmpM* promoters) upstream of the targeted gene. As an example, the *PorA* promoter can be optimized for expression as described by van der Ende et al. *Infect Immun* 2000;68:6685-90. Insertion of the promoter can be accomplished by, for example, PCR amplification of the upstream segment of the targeted NMB0964 gene, cloning the upstream segment in a vector, and either inserting appropriate restriction sites during PCR amplification, or using naturally occurring restriction sites to insert the *PorA* promoter segment. For example, an about 700 bp upstream segment of the NMB0964 gene can be cloned. Using naturally occurring restriction enzyme sites located at an appropriate distance (e.g., about 400 bp) upstream of the NMB0964 promoter within this cloned segment a *PorA* promoter segment is inserted. An antibiotic (e.g., erythromycin) resistance cassette can be inserted within the segment further upstream of the *PorA* promoter and the construct may be used to replace the wild-type upstream NMB0964 segment by homologous recombination.

Another approach involves introducing a NMB0964 polypeptide-encoding sequence downstream of an endogenous promoter that exhibits strong transcriptional activity in the host cell genome. For example, the coding region of the *RmpM* gene can be replaced with a coding sequence for a NMB0964 polypeptide. This approach takes advantage of the highly active constitutive *RmpM* promoter to drive expression.

Neisserial strains can be genetically modified to over-express NMB0964 by introduction of a construct encoding a NMB0964 polypeptide into a Neisserial host cell. The NMB0964 introduced for expression is referred to herein as an "exogenous" NMB0964. The host cell produces an endogenous NMB0964, the exogenous NMB0964 may have the same or different amino acid sequence compared to the endogenous NMB0964.

The NMB0964 polypeptides useful in the invention also include fusion proteins, where the fusion protein comprises a NMB0964 polypeptide having a fusion partner at its N-terminus or C-terminus. Fusion partners

of interest include, for example, glutathione S transferase (GST), maltose binding protein (MBP), His-tag, and the like, as well as leader peptides from other proteins.

Sequence identity can be determined using methods for alignment and comparison of nucleic acid or amino acid sequences, which methods are well known in the art. Comparison of longer sequences may require more sophisticated methods to achieve optimal alignment of two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2:482, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. (USA)* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the best alignment (i.e. resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected.

Optimal alignment of sequences for comparison can be conducted, e. g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BBSTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (see generally, *Current Protocols in Molecular Biology*, F.M. Ausubel et al., eds., *Current Protocols*, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1995 Supplement) (Ausubel)).

Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1990) *J. Mol. Biol.* 215: 403-410 and Altschuel et al. (1977) *Nucleic Acids Res.* 25: 33 89-3402, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al, *supra*).

These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W , T , and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences), uses as defaults a wordlength (W) of 11, an expectation (E) of 10, $M=5$, $N=-4$, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)).

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873-5877 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability ($P(N)$), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

A further indication that two nucleic acid sequences or polypeptides share sequence identity is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below.

Thus, a polypeptide typically share sequence identity with a second polypeptide, for example, where the two polypeptides differ only by conservative substitutions. Another indication that two nucleic acid sequences share sequence identity is that the two molecules hybridize to each other under stringent conditions. The selection of a particular set of hybridization conditions is selected following standard methods in the art (see, for example, Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (1989) Cold Spring Harbor, N.Y.). An example of stringent hybridization conditions is hybridization at 50°C or higher and 0.1 x SSC (15 mM sodium chloride/1.5 mM sodium citrate). Another example of stringent hybridization conditions is overnight incubation at 42°C in a solution: % formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 mg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1 x SSC at about 65°C.

Stringent hybridization conditions are hybridization conditions that are at least as stringent as the above representative conditions, where conditions are considered to be at least as stringent if they are at least about 80% as stringent, typically at least about 90% as stringent as the above specific stringent conditions. Other stringent hybridization conditions are known in the art and may also be employed to identify nucleic acids of this particular embodiment of the invention.

Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

Methods and compositions which can be readily adapted to provide for genetic modification of a *Neisserial* host cell to express an exogenous NMB0964 polypeptide are known in the art. Exemplary vectors and methods are provided in WO 02/09746 and O'Dwyer et al. *Infect Immun* 2004;72:651 1-80.

Methods for transfer of genetic material into a *Neisserial* host include, for example, conjugation, transformation, electroporation, calcium phosphate methods and the like. The method for transfer should provide for stable expression of the introduced NMB0964 - encoding nucleic acid. The NMB0964 -encoding nucleic acid can be provided as a inheritable episomal element (e.g., plasmid) or can be genomically integrated.

Suitable vectors will vary in composition depending what type of recombination event is to be performed. Integrative vectors can be conditionally replicative or suicide plasmids, bacteriophages, transposons or linear DNA fragments obtained by restriction hydrolysis or PCR amplification. Selection of the recombination event can be accomplished by means of selectable genetic marker such as genes conferring resistance to antibiotics (for instance kanamycin, erythromycin, chloramphenicol, or gentamycin), genes conferring resistance to heavy metals and/or toxic compounds or genes complementing auxotrophic mutations (for instance pur, leu, met, aro).

In one embodiment, the vector is an expression vector based on episomal plasmids containing selectable drug resistance markers that autonomously replicate in both *E. coli* and *N. meningitidis*. One example of such a "shuttle vector" is the plasmid pFP10 (Pagotto et al. *Gene* 2000 244:13-19).

IMMUNIZATION

In general, the methods of the invention provide for administration of one or more antigenic compositions of the invention to a mammalian subject (e.g., a human) so as to elicit a protective immune response against more than one strain of *Neisseria* species bacteria, and thus protection against disease caused by such bacteria. In particular, the methods of the invention can provide for an immunoprotective immune response against a 1, 2, 3, 4, or more strains of *Neisseria meningitidis* species, where the strains differ in at least one of serogroup, serotype, serosubtype, or NMB0964 polypeptide. Of particular interest is induction of a

protective immune response against multiple strains of *Neisseria meningitidis* of serogroup B, particularly where the strains differ in serosubtype (e.g., have heterologous PorAs). Also of particular interest is induction of a protective immune response against strains that are heterologous to one other in terms of PorA and/or NMB0964.

The antigenic compositions of the invention can be administered orally, nasally, nasopharyngeally, parenterally, enterically, gastrically, topically, transdermally, subcutaneously, intramuscularly, in tablet, solid, powdered, liquid, aerosol form, locally or systemically, with or without added excipients. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980).

It is recognized that oral administration can require protection of the compositions from digestion. This is typically accomplished either by association of the composition with an agent that renders it resistant to acidic and enzymatic hydrolysis or by packaging the composition in an appropriately resistant carrier. Means of protecting from digestion are well known in the art.

The compositions are administered to an animal that is at risk from acquiring a *Neisseria* disease to prevent or at least partially arrest the development of disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for therapeutic use will depend on, e. g., the antigenic composition, the manner of administration, the weight and general state of health of the patient, and the judgment of the prescribing physician. Single or multiple doses of the antigenic compositions may be administered depending on the dosage and frequency required and tolerated by the patient, and route of administration.

The antigenic compositions (herein also known as immunogenic compositions) described herein can comprise a mixture of vesicles which vesicles can be from the same or different strains. In another embodiment, the antigenic compositions can comprise a mixture of vesicles from 2, 3, 4, 5 or more strains.

The antigenic compositions are administered in an amount effective to elicit an immune response, particularly a humoral immune response, in the host. Amounts for the immunization of the mixture generally range from about 0.001 mg to about 1.0 mg per 70 kilogram patient, more commonly from about 0.001 mg to about 0.2 mg per 70 kilogram patient. Dosages from 0.001 up to about 10 mg per patient per day may be used, particularly when the antigen is administered to a secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ. Substantially higher dosages (e.g. 10 to 100 mg or more) are possible in oral, nasal, or topical administration. The initial administration of the mixture can be followed by booster immunization of the same or different mixture, with at least one booster, more usually two boosters, being preferred.

The antigen compositions are typically administered to a mammal that is immunologically naïve with respect to *Neisseria*, particularly with respect to *Neisseria meningitidis*. In a particular embodiment, the mammal is a human child about five years or younger, and preferably about two years old or younger, and the antigen compositions are administered at any one or more of the following times: two weeks, one month, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 months, or one year or 15, 18, or 21 months after birth, or at 2, 3, 4, or 5 years of age.

In general, administration to any mammal is preferably initiated prior to the first sign of disease symptoms, or at the first sign of possible or actual exposure to *Neisseria*.

EXAMPLES

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

Example 1

Immunogenicity of OMVs with up-regulation of Tdfl

Tdfl is a gene which is thought to be expressed when *N. meningitidis* is within the blood. It is therefore not normally expressed when strains are grown in conventional culture media, but wild-type strain H44/76, for example, can be made to express the protein in special culture conditions (RPMI culture media supplemented with hemin). The following experiment details the use of an H44/76 strain where Tdfl expression has been recombinantly made inducible (through the use of IPTG). This allows the over-expression of Tdfl on the surface of OMV vaccines made from the strain, and provides an easy way of culturing a strain expressing the antigen to establish whether antibodies generated against Tdfl are capable of killing such a modified strain which expresses Tdfl under normal culture conditions (+IPTG). The impact of IPTG on expression of Tdfl on cells used in the SBA is shown in Figure 7.

Groups of 10 mice were immunized three times with OMV by the intramuscular route on day 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 *Neisseria meningitidis* strain H44/76, engineered so that capsular polysaccharides and PorA were down regulated and LOS immunotype was *galE* type. A comparison was made of OMVs in which Tdfl was or was not up-regulated (up-regulation under the control of IPTG inducible promoter). On day 42, blood samples were taken for analysis by serum bactericidal assay using either the homologous strain H44/76 (B:15:P1.7,16) expressing or not Tdfl (after addition or not of IPTG in the culture media).

N. meningitidis strains were cultivated overnight on GC-agar with 10 µg/ml chloramphenicol Petri Dishes at 37°C + 5% CO₂. They were sub-cultured for 3 hours in a liquid TSB medium supplemented or not with IPTG 1000 µM. Individual sera were inactivated for 30 min at 56°C. Serum samples were diluted in HBSS-BSA 0.5% and then twofold diluted (8 dilutions) in a volume of 25 µl in flat bottom microplates. Bacteria were diluted in HBSS-BSA 0.5% to yield 8.10³ CFU/ml and 12.5µl of this dilution was added to the serum dilution. Rabbit complement (12.5µl) was also added to each well. After 75 min of incubation at 37°C under shaking, 15 µl of the mixture was spread onto pre-warmed GC-agar plates incubated overnight at 37°C +CO₂.

The CFU's were counted and the percentage of killing was calculated. The SBA titer is the dilution giving 50% of killing.

SBA titers: impact of expression of Tdfl by target cells

	SBA titers
H44/76 without IPTG	< 50
H44/76 with IPTG	400; 400; 800

Without IPTG, Tdfl is not expressed on target cells which are not killed by sera from mice immunized with up-regulated Tdfl OMVs. When the expression of Tdfl is specifically induced by IPTG the target cells express Tdfl and are killed by anti-Tdfl-OMVs mice sera.

Example 2: A novel zinc-regulated outer membrane protein in *Neisseria meningitidis* with vaccine potential

ABSTRACT

Since the concentration of free iron in the human host is low, efficient iron-acquisition mechanisms constitute important virulence factors for pathogenic bacteria. In the Gram-negative bacteria, TonB-dependent outer membrane receptors are implicated in iron acquisition. However, transport across the bacterial outer membrane of other metals that are also scarce in the human host is far less clear. In this study we characterized a novel TonB-dependent receptor in *Neisseria meningitidis*. We show that the bacteria produce this protein under zinc limitation and that it is involved in zinc uptake. Furthermore, since the protein is highly conserved among isolates and is capable of inducing bactericidal antibodies, it constitutes a novel candidate for the development of a vaccine against *N. meningitidis* for which no effective universal vaccine is available so far. Homologues of the protein, designated Tdfl, are found in many other pathogens residing in the respiratory tract, suggesting that receptor-mediated zinc uptake is particularly important for survival in this niche.

INTRODUCTION

The cell envelope of Gram-negative bacteria consists of two membranes, the inner and the outer membrane, which are separated by the periplasm containing the peptidoglycan layer. The outer membrane forms a barrier for harmful compounds from the environment. Most nutrients can pass the outer membrane by passive diffusion via abundant channel-forming outer membrane proteins, collectively called porins. However, diffusion is not an option when the extracellular concentration of a nutrient is low. This is the case, for example, for iron. Pathogens are confronted with low concentrations of free iron within the human host, where iron is bound by iron-transport and -storage proteins, such as lactoferrin and transferrin. Hence, efficient iron acquisition mechanisms constitute important virulence factors and have been studied extensively in many pathogens (1, 2).

When grown under iron-limiting conditions, Gram-negative bacteria induce the synthesis of outer membrane proteins that function as receptors for the iron-binding proteins of the host, for heme, or for siderophores, which are small iron-chelating compounds produced and secreted by the bacteria under iron limitation. The resolved crystal structures of such receptors revealed 22-stranded β -barrels, which do not form open channels but are closed by an N-terminal plug domain (3). After binding of the ligand to the receptor, the subsequent uptake is an active process that requires the energy of the proton gradient across the inner membrane, which is coupled to the receptors in the outer membrane via a complex of three proteins, the TonB complex (4, 5).

While iron-acquisition mechanisms have been studied extensively in many Gram-negative bacteria, little is known yet about the transport of other essential heavy metals, such as zinc and manganese, across the bacterial outer membrane. The concentration of these trace elements also is low in the human host, which, for example, responds to infections by the production of metallothioneins and calprotectin thereby reducing the availability of metals to the invading pathogens (6, 7). Therefore, Gram-negative pathogens likely possess effective acquisition mechanisms for these metals, which may or may not resemble the iron-acquisition systems.

Neisseria meningitidis is an obligate human pathogen that can colonize the nasopharyngeal mucosa asymptotically. Occasionally the bacterium enters the bloodstream and can cause meningitis and sepsis with a high mortality rate (8). While vaccines are available for most pathogenic serogroups of *N. meningitidis* based on the capsular polysaccharides, a vaccine against serogroup B meningococci is lacking. The polysaccharide capsule of the serogroup B strains is poorly immunogenic due to its resemblance to human glycoproteins (9). Thus, subcapsular antigens are being studied as alternative vaccine components; however, these studies are frustrated by the high antigenic variability of the major outer membrane proteins. Therefore, attention has shifted to minor antigens, including the TonB-dependent receptors.

When grown under iron limitation, *N. meningitidis* produces TonB-dependent receptors for lactoferrin (10), transferrin (11), hemoglobin (12, 13) and enterobactin (14), all involved in the uptake of iron. Based on homology searches, Turner *et al* (15) identified seven additional genes for putative TonB-dependent family (Tdf) members in the available genome sequences of three *Neisseria* strains. Interestingly, the expression of some of these *tdf* genes appeared unaffected by iron availability in various microarray studies (16, 17), indicating that their products might be implicated in the transport of metals other than iron. Here we studied the regulation of the synthesis, the function and the vaccine potential of one of these receptors and show that this receptor is involved in the uptake of zinc.

RESULTS

Tdfl is not a heme receptor

Tdfl (locus tags NMA1161 and NMB0964 in the sequenced genomes of *N. meningitidis* serogroup A strain Z2491 and serogroup B strain MC58, respectively) was previously identified as one of seven novel putative TonB-dependent receptors present in the *Neisseria* genomes (15) and was found to be up-regulated in the presence of naïve human serum (18). Since almost all TonB-dependent receptors studied to date are involved in iron acquisition we assumed that Tdfl transports an iron complex. This idea was strengthened by the fact that blast searches (19) with the amino-acid sequence of NMA1161 revealed high sequence similarity to outer membrane receptors for the uptake of heme, such as HumA of *Moraxella catarrhalis* (20) with 41% identity and 58% similarity.

To assess the function of Tdfl, we constructed a *tdfl* deletion mutant of a non-encapsulated derivative of serogroup B strain H44/76 called HB-1. We found similar binding of heme to HB-1 and the *tdfl* mutant as assessed by dot blot analysis and the *tdfl* mutant strain could still grow on plates with heme as the sole iron source. We could also not find increased heme binding by *Escherichia coli* cells expressing Tdfl. Also we were unable to complement an *E. coli* heme auxotroph (data not shown). Therefore, we hypothesized that Tdfl, although homologous to heme receptors, does not function as a heme receptor.

Regulation of *tdfl* by zinc

Since Tdfl is not a heme receptor and is not found to be regulated by iron, we sought conditions where we could detect *tdfl* expression in the capsule deficient H44/76 *Neisseria*

meningitidis HB-1. We could never detect Tdfl on Western blots when the bacteria were grown in tryptic soy broth (TSB), a complex rich medium (Fig. 1 A, lane 1). However, when the bacteria were grown in the chemically defined RPMI medium, Tdfl was detectable in bacterial lysates (Fig. 1 A, lane 2). The specificity of the signal detected was demonstrated by its absence in the *tdfl* knockout strain grown in RPMI (Fig. 1 A, lane 3). We noted that the presence of even small amounts of TSB added to RPMI negatively affected Tdfl synthesis (Fig. 1 B); apparently TSB contains a compound that represses the transcription of *tdfl*. Since we noticed that RPMI does not contain a source of trace metals, we decided to test whether addition of a cocktail of trace metals, containing cobalt, molybdenum, manganese, copper and zinc, would repress *tdfl* expression, which indeed appeared to be the case. We then tested all these metals separately and found that specifically zinc, even at sub- μ M concentrations, caused repression of *tdfl* expression (Fig. 1 C). Since standard RPMI is not supplemented with a specific zinc source, the available zinc required for bacterial growth presumably comes from the water and/or traces in the salts used to make the medium. We measured the zinc concentration in RPMI medium by inductively coupled plasma mass spectrometry (ICP-MS) and found it to be ~ 110 parts per billion ($\sim 1.69 \mu\text{M}$). The zinc regulation of *tdfl* became even more evident when we supplemented the RPMI medium with the specific zinc chelator N,N,N',N'-Tetrakis-(2-pyridylmethyl)-Ethylenediamine (TPEN). Addition of TPEN to the medium resulted in a dose-dependent increase in Tdfl synthesis (Fig. 1 D). However, concentrations above $1 \mu\text{M}$ TPEN totally inhibited cell growth presumably due to total zinc depletion from the medium. Growth could be restored by the addition of zinc (data not shown). The zinc regulation of *tdfl* was confirmed by real-time quantitative PCR (RT-qPCR) using total RNA obtained from cultures grown in RPMI supplemented or not with 500 nM zinc or $0.5 \mu\text{M}$ TPEN. The data showed a 13.8-fold repression in the presence of zinc and a 3.8-fold up regulation in the presence of TPEN. The fold difference between added TPEN and zinc was 52.6-fold.

Role of the transcriptional regulator Zur in *tdfl* expression

In *E. coli*, the zinc uptake regulator (Zur) has been shown to regulate the expression of the *znuACB* genes, which encode the periplasmic binding protein, the ATPase and the integral inner membrane component required for zinc transport from the periplasm to the cytoplasm (23). In the presence of zinc, Zur binds a Zur-binding element (consensus GAAATGTTATANTATAACATTTC) in the promoter of the *znuACB* operon and thereby blocks transcription.

In the genome sequence of *N. meningitidis* strain MC58, we identified homologues of the *E. coli zur* gene, i.e. NMB1266, and of *znuCBA*, i.e. NMB0588, NMB0587, and NMB0586. In addition, we found sequences resembling the *E. coli* Zur binding consensus in the regions upstream of the neisserial *tdfl* (GtAATGTTATATaATAACAaact) and *znuC* (cAAAcGTTATACagTAtCATaTC) (identical nucleotides to the *E. coli* consensus are in capital case). To confirm the involvement of Zur in the regulation of *tdfl* expression, we generated a *zur* mutant of strain HB-1, which, indeed, produced Tdfl constitutively (Fig. 2). Also, RT-qPCR demonstrated the involvement of Zur in the expression of *znuA* and *tdfl* as *znuA* and *tdfl* expression levels increased 5- and 34-fold, respectively, in the *zur* mutant compared to its parent strain both grown in the presence of zinc.

Tdfl facilitates zinc acquisition

Since the expression of *tdfl* is regulated by the availability of zinc, it is likely that Tdfl acts as a receptor for zinc or a zinc-containing complex. We first analyzed the amino acid sequence and constructed a topology model of Tdfl using the PROFtmb program at www.rostlab.org, (Fig. 3).

Tdfl contains two cysteine residues in the putative extracellular loop L3. If these cysteines form a disulfide bond (supported by our analysis of the membrane fraction of bacteria by SDS-PAGE with and without DTT where incubation of the sample with the reducing agent resulted in a shift in electrophoretic mobility, presumably due to the disruption of the disulfide bond), they bring two stretches of amino acid residues, both rich in histidine and aspartic acid residues, in close proximity (Fig. 3), which could be of functional importance, since also in the periplasmic ZnuA protein of *E. coli*, a stretch of His and Asp residues is involved in binding zinc (25). Thus, we considered the possibility that Tdfl binds free zinc and transports it to the periplasm. To test this hypothesis we first determined whether Tdfl could bind zinc. We compared outer membrane vesicles with and without Tdfl for their ability to compete with 4-(2-pyridylazo)resorcinol (PAR) for zinc. The outer membrane vesicles containing Tdfl showed ~40% increased binding of zinc compared to the vesicles without Tdfl (Fig. 4A). To test transport of zinc we compared the *tdfl* knockout, a *tonB* knockout and their parent strain for the accumulation of intracellular zinc using ICP-MS. HB-1 accumulated ~33% more zinc than the *tdfl* mutant or the *tonB* mutant, indicating that Tdfl transports free zinc and that this transport needs the TonB system (Fig. 4B).

If indeed Tdfl is involved in the uptake of free zinc, than one would expect derepression of *znu* gene expression to occur at higher external zinc concentrations in the *tdfl* mutant as compared with the wild-type strain. To test this idea, we grew the *tdfl* mutant and the parent strain in RPMI medium with 500 nM additional zinc, which largely, but not completely represses *tdfl* expression in the wild-type strain (Fig. 1 C). We subsequently measured the relative levels of *tdfl* and *znuA* mRNA by RT-qPCR. The *tdfl* mutant still contains the first 437 nucleotides of the *tdfl* gene that were used for the detection of gene expression. In the *tdfl* mutant, there was 18.6-fold more *tdfl* and 7.4-fold more *znuA* expressed, showing that indeed the intracellular zinc concentration in the *tdfl* mutant is lower than that in the parent strain under the applied growth conditions. Also a *znuA* knockout strain expressed high levels of Tdfl in the presence of zinc, confirming that ZnuA is required to sustain sufficient zinc levels in the cell (Fig. 4C). Thus, both Tdfl and ZnuA are involved in the transport of zinc.

Conservation of Tdfl

Besides the function of Tdfl we also want to investigate whether Tdfl is a vaccine candidate for a universal *N. meningitidis* vaccine. One of the criteria is that the antigen has to be conserved. We first looked at the available *N. meningitidis* genomes and found that Tdfl has a striking 97-99% amino acid identity of the mature protein (Figure S1). The sequence differences are scattered throughout the protein and are not clustered in predicted extracellular loop regions, which are often antigenically variable in *Neisseria* outer membrane proteins (Figure S1). We subsequently analyzed the presence of Tdfl in a panel of 32 different *N. meningitidis* isolates from different serogroups and different clonal lineages. Each strain was grown in RPMI medium supplemented or not with 500 nM zinc and analyzed by Western blotting with the antiserum raised against Tdfl of H44/76. All strains showed a repression of Tdfl in the presence of zinc (Fig. 5).

We then wanted to know the homology of Tdfl to other pathogenic bacteria. We first compared Tdfl with *N. gonorrhea* and found a 96% identity and a 97% similarity between these two *Neisseria* strains. Next, we used the blast program at NCBI with a cutoff of 40% identity at the amino acid level to search for homologs of Tdfl in other pathogenic bacteria. We identified homologs in other pathogenic bacteria, including *M. catarrhalis*, *Haemophilus parasuis*, *Mannheimia haemolytica*, *Acinetobacter baumannii*, *Pasteurella multocida*, *Bordetella pertussis* and *Actinobacillus pleuropneumoniae*, averaging a 41% identity and 59% similarity at the amino acid level and all Tdfl homologs have the His/Asp region (Figure S2). Interestingly, in *B. pertussis* the *tdfl* homologue is located adjacent to homologues of the *znuABC* and *zur* genes, again

indicating a functional relationship between these genes. Furthermore, all these TdfI homologs contain His- and Asp-rich stretches (Figure S2).

TdfI induces bactericidal antibodies

To investigate the vaccine potential of TdfI, we immunized mice with Neisserial outer membrane vesicles containing overexpression levels of this protein (Fig. 6A) and tested the resultant sera for the presence of bactericidal antibodies. Routinely, we perform serum bactericidal assays on bacteria grown in TSB medium; however, under these conditions *tdfI* is not expressed. Therefore, we tested the sera for bactericidal activity on a strain that expressed TdfI from an isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible promoter and compared cultures grown with and without IPTG. The bactericidal titers of the sera were $<1:100$ when IPTG was absent, but $1:1042$ when IPTG was present during growth of the bacteria. Titers in pre-immune sera were also $<1:100$. These data clearly show that TdfI is able to elicit bactericidal antibodies. We also wanted to investigate whether normal chromosome-encoded *tdfI* expression levels are sufficient to mediate complement-mediated killing. For this we employed the *zur* knockout strain that produces TdfI constitutively in the TSB medium and grows comparable to the wild-type strain in this medium.

DISCUSSION

The high-affinity ZnuABC uptake system for zinc has previously been identified in *N. gonorrhoeae* (30). Homologues can be found in the meningococcal genome, as described above, and in the genomes of many other bacteria. In *Salmonella enterica* this ABC transporter has been associated with virulence (31). In no case, an outer membrane receptor involved in zinc acquisition has been identified and it is thought that zinc diffuses through the porins.

In the human host, however, the free zinc levels are most likely too low to sustain bacterial growth by passive diffusion. The total amount of zinc in human serum is approximately $19\ \mu\text{M}$, but the vast majority is bound by serum proteins such as albumin (32). Here we have identified an outer membrane receptor, TdfI that is regulated by zinc. The addition of $700\ \text{nM}$ zinc to the growth medium completely repressed TdfI expression. The function of TdfI is to bind and transport of unbound (free) zinc. We predict that the zinc is bound initially by the His/Asp stretch in the external loop and then internalized via two histidines that are on top of the plug domain (Fig 3b). A possible role for the TonB system in zinc uptake is that it pulls the plug out of the barrel and with this movement the zinc bound to the two His residues is transported into the periplasm where it is picked up by the periplasmic binding protein ZnuA.

Interestingly, similar regulation of *tdfI* and *znuA* expression was reported in a microarray study using *N. gonorrhoeae* (33). The *tdfI* homolog NGO1205 and the *znuA* homolog NGO0168 were upregulated in a mutant lacking the NGO0542 gene. This gene was annotated in that study as *perR* because of its homology to a manganese-dependent peroxide-responsive regulator found in gram-positive organisms (34). However, this is the same gene we have annotated as *zur*. The *zur* annotation is clearly more accurate, because we show an identical regulation by the absence of *zur* or the absence of zinc. More evidence for the annotation *zur* rather than *perR* comes from the same study in *N. gonorrhoeae*. Microarrays performed with the gonococcal *perR* mutant showed upregulation also of the ribosomal proteins L31 and L36. The Neisserial genomes contain two copies for each of the genes encoding these proteins with one form of each protein containing a zinc ribbon motif. Zinc availability was found to be the key factor controlling the type of L31/L36 protein expressed in *B. subtilis* (34). In the gonococcal *perR* mutant, expression specifically of L31 and L36 paralogs lacking the zinc ribbons is induced, highly indicative of a disturbed zinc regulation in a *perR* mutant. Moreover in another study (17) a microarray was performed to identify the response to oxidative stress and neither *perR* nor any of the genes identified in the

PcrR study (33) were de-repressed and we do not see any regulatory effect of manganese on the expression of *tdfI* and *znuA*.

Previously, *tdfI* expression was reported to be induced in the presence of active complement (18). In this microarray study expression profiles were compared of *N. meningitidis* grown in the presence of serum and heat-inactivated serum, and TdfI was found 23-fold de-repressed in the presence of the untreated serum. The relationship between zinc and complement regulation may not be obvious at first sight. A possible explanation for finding similar regulatory circuits may be that the bacteria in the array study were pre-grown in RPMI with BSA. Albumin is known to chelate zinc, and therefore, pre-growth conditions may have been severely zinc-limited. Heat-treatment of human serum will release zinc from albumin, thereby repressing *tdfI* expression. This explanation is strengthened by the fact that TdfI expression is induced when BSA is added to TSB medium during bacterial growth (data not shown).

A study by Hagen and Cornelissen (35) investigated whether any of the Tdf proteins is essential for intracellular survival of *N. gonorrhoeae* in human epithelial cells. The authors also tested a TdfI homolog knockout (NG1205), but this mutant was not affected in the intracellular survival.

The conservation of TdfI is striking; with an identity of 98.6% among the sequenced *N. meningitidis* strains and a 99.2% similarity at the amino acid level of the mature protein. The TdfI protein was found in all meningococci tested and all strains showed zinc-regulated expression of *tdfI*. Between the TdfI proteins of the sequenced meningococcal and gonococcal strains there is 96.1% identity and 97.3% similarity at the amino acid level. The differences between the sequences of TdfI are scattered throughout the protein and do not cluster in a specific loop. We find an average 41% amino acid identity of TdfI with homologs in other bacteria and in all cases the His/Asp stretch is conserved. Intriguingly, TdfI homologs were particularly found in bacterial species residing in the respiratory tract of humans and animals. Possibly in the mucosal layers of the respiratory tract the unbound zinc concentration is too low to allow sufficient passive diffusion through the porins and therefore TdfI becomes essential for bacterial growth and survival. While TdfI is not essential for intracellular survival (35) it could be essential in the bodily fluids like serum and liquor where the free zinc concentration could also be very low. Also, we cannot rule out that TdfI additionally recognizes a complexed form of zinc which may be available in the respiratory tract, serum and or cerebral fluid.

We have further shown that TdfI can induce bactericidal antibodies in mice and that these antibodies are specifically directed at TdfI. Also when we used bacteria expressing TdfI from the chromosomal locus we could detect bactericidal activity, showing that during infection the antigen concentration is high enough to allow clearing of *N. meningitidis*.

The high level of conservation and the possibility to raise TdfI-specific bactericidal antibodies make TdfI an excellent vaccine candidate.

MATERIALS AND METHODS

Abbreviations used: IPTG, isopropyl β -D-1-thiogalactopyranoside; PAR, 4-(2-pyridylazo)resorcinol; RPMI, Roswell Park Memorial Institute medium 1640; Tdf, TonB-dependent family; TPEN, N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine; TSB, tryptic soy broth; ICP-MS, Inductively coupled plasma mass spectrometry.

Bacterial strains and growth conditions.

Neisserial strains, listed in Fig. 5 are from the laboratory collection. Except when indicated otherwise, experiments were performed with strain HB-1 and mutants thereof. HB-1 is a non-encapsulated derivative of serogroup B strain H44/76 (Bos & Tommassen, 2005). *N. meningitidis*

was grown on GC agar (Oxoid) plates containing Vitox (Oxoid) and antibiotics when appropriate (kanamycin, 100 µg/ml; chloramphenicol, 10 µg/ml) in candle jars at 37°C. Liquid cultures were grown in TSB (Difco) or in RPMI (Sigma) in plastic flasks at 37°C with shaking. IPTG, zinc, and TPEN were added in the concentrations indicated. Metals were added as a cocktail (340 nM ZnSO₄, 160 nM Na₂MoO₄, 800 nM MnCl₂, 80 nM CoCl₂ and 80 nM CuSO₄ final concentrations) or as single compounds in the same concentrations as in the cocktail unless indicated otherwise. Ferric chloride was added as a final concentration of 8 µM. *E. coli* strains DH5α and TOP10F' (Invitrogen) were used for routine cloning and BL21(DE3) (Invitrogen) for expression. An *E. coli hemA* mutant was used to assess the heme transport of Tdfl ((22)). *E. coli* was propagated on Luria-Bertani medium supplemented when appropriate with 100 µg/ml ampicillin, 50 µg/ml kanamycin, or 25 µg/ml chloramphenicol. For the *E. coli* heme-auxotroph C600 *hemA::kan* (22) the medium was supplemented with 5-aminolevulinic acid.

Construction of plasmids and mutants.

All primers were designed on the MC58 genome sequence, using NMB0964 (*tdfl*), NMB1730 (*tonB*), NMN0586 (*znuA*), NMB1266 (*zur*).

For high-level protein production in *E. coli* the *tdfl* gene without the signal sequence-encoding part was amplified from chromosomal DNA of strain H44/76 by PCR using the primers 0964-F-GATCATATGCATGAACTGAGCAATCGGTG- and 0964-R - GATGGATCCTTAAATCTTCACGTTACGCGGCC- that carry the restriction sites NdeI and BamHI, respectively (bold). The resulting product was cloned into pCRII-TOPO according to the manufacturer's recommendation (Invitrogen), yielding pCRII-*tdfl*, and subcloned into pET11a (Novagen) using NdeI/BamHI restriction, resulting in plasmid pET11a-*tdfl*.

To obtain a *tdfl* deletion construct, a kanamycin-resistance gene cassette (36) was amplified by PCR with the primers Kan-R -TGACGCGTCTCGACGCTGAGGTCTGC- and Kan-F - TGTGTACAGTCGACTTCAGACGGCCACG- and cloned after MluI and BsrGI digestion into pCRII-*tdfl* digested with the same enzymes. In the resulting construct, pCRII-*tdfl::kan*, the kanamycin-resistance cassette substitutes for the region between bp 437 and 1344 of *tdfl*. pCRII-*tdfl::kan* was used in a PCR with the 0964-R and 0964-F primers and the resulting product was used to transform HB-1 (37). Kanamycin-resistant colonies were tested for correct gene replacement by PCR.

The entire *tdfl* gene from H44/76 was amplified with primers Tdfl-F - GCATCATATGGCACAACACTACACTCAAACCC- and Tdfl-R - ATGACGTCTTAAACTTCACGTTACGCGGCC- that contain recognition sites for NdeI and AatII (bold), respectively. The resulting PCR product was cloned into pCRII-TOPO and subcloned into pEN11-pldA (36) using NdeI and AatII restriction sites. The resulting plasmid, pEN11-*tdfl*, constitutes a *Neisserial* replicative plasmid, containing a *lacI^Q* gene and a tandem *lac/tac* promoter for controlled expression of *tdfl*.

The construct to generate a *tonB* knockout was made by amplifying DNA fragments upstream and downstream of the *tonB* gene using primers tonB-1 (GTACGATGATTGTGCCGACC), tonB-2 (ACTTTAAACTCCGTGCGACGCAAGTCGACTGCGGGGGTTAA) with AccI restriction sites (bold) for one fragment, and, tonB-3 (TTAACCCCCGCGAGTCGACTTGCGTCGACGGAGTTTAAAGT) with restriction site AccI (bold) and tonB-4 (GCCATACTGTTGCGGATTTGA) for the other fragment. The two fragments were each cloned into pCRII-TOPO and then ligated to each other using the introduced restriction site AccI and the SpeI site in the pCRII-TOPO vector. The AccI site was subsequently used to clone the chloramphenicol transacetylase gene from pKD3 (38) previously cloned into pCRII-TOPO by PCR amplification with primers containing an AccI site. The resulting construct was

amplified by PCR using primers tonB-1 and tonB-4 and this linear fragment was used to transform *N. meningitidis* HB-1.

The *zur* gene was knocked out following the same strategy. Upstream and downstream fragments were amplified in this case with primers: zur-1 (TTCGCCGATGGCGGAATACA), zur-2 (CTTTCAGCGCAAAGTCGACTCCGTCGACGCGTGCCTGTTC) with the restriction site *AccI* in bold, zur-3 (GAACAGGCACGCGTCGACGGAGTCGACTTTGCGCTGAAAG) with the restriction site *AccI* in bold and zur-4 (TCCTATTGCGCAATACCCCC)

A *porA* derivative of *N. meningitidis* strain H44/76, called CE2001 (39) was transformed with pMF121, resulting in deletion of the entire capsule locus and production of lipopolysaccharide with a truncated outer core (36). A pLAFR-derived plasmid containing the *tonB*, *exbB* and *exbD* genes of *N. meningitidis* (13) was described previously.

SDS-PAGE and Western blot analysis.

Cell lysates were prepared from bacteria grown for 6 hours. The cells were diluted to OD_{600nm} 1, pelleted, and boiled in 100 µl SDS-PAGE sample buffer containing 2% SDS and 5% 2-mercaptoethanol. Proteins were separated by standard SDS-PAGE. Gels were either stained with Coomassie brilliant blue or the proteins were transferred to nitrocellulose membranes (Protran) using a wet transfer system (Biorad) in 25 mM Tris-HCl, 192 mM glycine, 20% methanol. Membranes were blocked for 1 h in PBS containing 0.1% Tween 20 and 0.5% Protifar (Nutricia). Blots were incubated with antibodies in blocking buffer. Antibody binding was detected by using goat anti-rabbit IgG peroxidase-conjugated secondary antibodies (Biosource) and enhanced chemiluminescence detection (Pierce).

Immunizations.

BL21(DE3) cells containing pET11a-*tdfI* were grown in LB to an OD A₆₀₀ of 0.6 after which 1 mM IPTG was added and growth was continued for 2 h. The TdfI protein accumulated in inclusion bodies, which were isolated as described (40), and the purified protein was used to immunize rabbits at Eurogentec. The resulting antiserum, SN1042, was used in a 1/5000 dilution.

Outer membrane vesicles of strain CE1523 /pEN11-*tdfI* grown in the presence or absence of 1 mM IPTG, were prepared by deoxycholate extraction (41) and used to immunize mice as described (32). Sera from ten mice per group were collected after 42 days and pooled. The experiments complied with the relevant national guidelines of Belgium and institutional policies of GlaxoSmithKline Biologicals.

RT-qPCR.

RT-qPCR was performed using an Applied Biosystems 7900HT Fast Real-Time PCR System and SYBR green master mix (Applied Biosystems) according to the manufacturer's recommendations. Total RNA was isolated by resuspending approximately 4x10⁹ *Neisseria* cells in 3 ml Trizol (Invitrogen). After the addition of 600 µl chloroform and centrifugation, the upper phase was mixed 1:1 with 75% ethanol. This was loaded on a nucleospin RNA II column (Macherey-Nagel), which was subsequently washed with buffer R3 from the nucleospin RNA II kit and eluted with 100 µl water. The RNA was then treated with Turbo DNA Free (Ambion) to yield DNA-free RNA. To generate the cDNA, 1 µg of total RNA was reverse transcribed from random hexamers using transcripter High fidelity cDNA synthesis kit (Roche) according to the manufacturer's recommendations. As a control, parallel samples were prepared in which the reverse transcriptase was omitted from the reaction mixture. PCRs were performed in triplicate in a 25-µl volume in a 96-well plate (Applied Biosystems) with the following cycle parameters: 95°C for 10 min for enzyme activation followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A melting plot was performed to ensure that the signal originated from the specific amplicon. Data analysis was

performed using the comparative cycle threshold method (Applied Biosystems) to determine relative expression levels. The *rmpM* transcript was used to normalize all data.

ICP-MS.

Total zinc concentrations were measured by ICP-MS at the integrated laboratory of the department of Geochemistry at the Utrecht University. *N. meningitidis* strains were grown in RPMI medium from a 0.1 starting OD A_{550} for 6 h; at this time point a sample was taken and the remaining culture was grown for an additional hour in the presence of 1 μ M zinc. After this hour, a second sample was taken. Both samples (7 ml) were washed in phosphate-buffered saline and resuspended in water, killed for 1 h at 56°C and frozen at -80°C. The samples were then thawed, sonicated and filtered through 0.22- μ m filters (Millipore).

PAR competition assay.

The PAR competition assay is a colorimetric reaction where the orange color of the PAR-zinc complex changes towards yellow in the presence of a protein or chemical that is able to release zinc from PAR. The assay was performed as described (42) with the following modifications: Instead of 50 μ M we added 30 μ M zinc and we first measured the PAR-zinc solution and then added the outer membrane vesicles to the cuvette and re-measured the solution. In this way we avoided the potential color change induced in time by UV. The data was then first normalized to the PAR-zinc measurement and then to the PAR alone sample to obtain the binding values for the outer membrane vesicles. The results shown are the normalized data of the absorption at 500 nm.

Serum bactericidal assay.

Wild-type H44/76 was transformed with pEN11-*tdfI* and inoculated from overnight grown plates in TSB with 125 μ M FeCl₃ with or without 1 mM IPTG in shaking flasks for 3 h at 37°C until an OD A_{550} of 0.5 was reached. Serum to be tested was diluted 1:100 in Hank's balanced salt solution (HBSS) (GIBCO), 0.3% BSA and then serially diluted (two-fold dilution steps, eight dilutions) in a 50- μ l volume in sterile U-bottom 96-well microtiter plates (NUNC). Bacteria were diluted in HBSS, 0.3% BSA to yield ~13,000 CFU per ml and 37.5 μ l samples of the suspension were added to the serum dilutions. The microtiter plates were incubated at 37°C for 15 min while shaking. Subsequently, 12.5 μ l of baby-rabbit complement (Pelfreez) or, as control for toxicity of the sera, heat-inactivated (56°C for 45 min) complement was added to the wells. After 1 h incubation at 37°C while shaking, the microtiter plates were placed on ice to stop the killing. Of each well, 20 μ l was spotted on GC plates while plates were tilted to allow the drop to "run" down the plate. After overnight incubation, colonies were counted and the percentage of killing was calculated. The bactericidal titer was defined as the highest serum dilution yielding >50% killing.

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Table 1. Conservation of the mature TdfI protein sequence in the sequenced *Neisseria* strains.

Similarity (%)	Identity (%)							
	Strain	MC58	Fam18	Z2491	053442	FA1090	NCCP 11945	ST-640
	<i>N. meningitidis</i> MC58		730/734 (99.5)	720/734 (98.1)	720/734 (98.1)	706/734 (96.2)	707/734 (96.3)	712/734 (97.0)
	<i>N. meningitidis</i> Fam18	733/734 (99.9)		722/734 (98.4)	718/734 (97.8)	705/734 (96.0)	706/734 (96.2)	712/734 (97.0)
	<i>N. meningitidis</i> Z2491	725/734 (98.8)	726/734 (98.9)		716/734 (97.5)	707/734 (96.3)	706/734 (96.2)	710/734 (96.7)
	<i>N. meningitidis</i> 053442	726/734 (98.9)	727/734 (99.0)	723/734 (98.5)		706/734 (96.2)	707/734 (96.3)	707/734 (96.3)
	<i>N. gonorrhoeae</i> FA1090	715/734 (97.4)	714/734 (97.3)	714/734 (97.3)	715/734 (97.4)		733/734 (99.9)	702/734 (95.6)
	<i>N. gonorrhoeae</i> NCCP11945	716/734 (97.5)	715/734 (97.4)	713/734 (97.1)	716/734 (97.5)	733/734 (99.9)		701/734 (95.5)
	<i>N. lactamica</i> ST-640	717/734 (97.7)	718/734 (97.8)	718/734 (97.8)	715/734 (97.4)	711/734 (96.9)	710/734 (96.7)	

CLAIMS

WHAT IS CLAIMED IS:

- 5 1. An immunogenic composition comprising: isolated outer membrane vesicles prepared from a *Neisseria* species bacterium, wherein the *Neisseria* species bacterium produces a level of a NMB0964 polypeptide, which in *N. meningitidis* strain MC58 has the sequence shown in Figure S1, sufficient to provide for production of a vesicle that, when administered to a subject, elicits anti-NMB0964 antibodies; and a
10 pharmaceutically acceptable excipient, wherein
 - (i) said *Neisseria* species bacterium is genetically modified through the disruption of functional expression of the Zur repressor (NMB1266); and/or
 - (ii) said *Neisseria* species bacterium is cultured in a medium which has less than 5, 4, 3, 2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05 or 0.01 μM free Zn^{2+} ; or
 - 15 (iii) said *Neisseria* species bacterium is cultured in a medium comprising a Zn^{2+} chelator.
2. The immunogenic composition of claim 1, wherein the level of NMB0964 polypeptide produced by the *Neisseria* species bacterium is:
20 greater than that made by *N. meningitidis* strain H44/76 grown in tryptic soy broth (TSB), or;
is the same or greater than that made by *N. meningitidis* strain H44/76 grown in Roswell Park Memorial Institute medium 1640 (RPMI), or;
is the same or greater than that made by *N. meningitidis* strain H44/76 grown in
25 Roswell Park Memorial Institute medium 1640 (RPMI) with 1 μM TPEN (N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine), or;
is the same or greater than that made by *N. meningitidis* strain H44/76 in a medium which has less than 5, 4, 3, 2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05 or 0.01 μM free Zn^{2+} .
30
3. The immunogenic composition of claim 1 or 2, wherein the *Neisseria* species bacterium is *Neisseria meningitidis*, or *Neisseria meningitidis* serogroup B.
4. The immunogenic composition of claims 1-3, wherein the *Neisseria* species bacterium
35 is deficient in capsular polysaccharide, preferably wherein the *Neisseria* species bacterium is deficient in capsular polysaccharide through disruption of functional expression of the *siaD* gene.
5. The immunogenic composition of claims 1-4, wherein the *Neisseria* species bacterium
40 is disrupted in:
the functional expression of the *msbB* and/or *htrB* genes; and/or

the expression of one or more of the following genes: PorA, PorB, OpA, OpC, PilC, or FrpB; and/or the functional expression of the lgtB gene.

- 5 6. The immunogenic composition of claims 1-5, wherein the *Neisseria* species bacterium is of immunotype L2 or L3.
- 10 7. The immunogenic composition of claims 1-6, wherein the outer membrane vesicles are isolated by extracting with 0-0.5, 0.02-0.4, 0.04-0.3, 0.06-0.2, or 0.08-0.15 % detergent, for instance deoxycholate, e.g. with around or exactly 0.1% deoxycholate.
- 15 8. The immunogenic composition of claim 1, wherein the Zn^{2+} chelator is present in the medium at a concentration of 0.01-100, 0.1-10, 0.3-5, or 0.5-1 μM .
- 20 9. The immunogenic composition of claim 1 or 8, wherein the Zn^{2+} chelator present in the medium is TPEN.
- 25 10. A method of producing an immunogenic composition, the method comprising:
culturing a *Neisseria* species bacterium producing a NMB0964 polypeptide, which in *N. meningitidis* strain MC58 has the sequence shown in Figure S1, wherein the
NMB0964 polypeptide is produced at a level sufficient to provide for production of
outer membrane vesicles that, when administered to a subject, elicit anti-NMB0964
antibodies; preparing outer membrane vesicles from the cultured bacterium; and
combining the outer membrane vesicles with a pharmaceutically acceptable excipient
to produce an immunogenic composition suitable for administration to a subject,
wherein
 (i) said *Neisseria* species bacterium has been genetically modified through the
disruption of functional expression of the Zur repressor (NMB1266); and/or
 (ii) said culturing of the *Neisseria* species bacterium is in a medium which has less
than 5, 4, 3, 2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05 or 0.01 μM free
 Zn^{2+} ; or
 (iii) said culturing of the *Neisseria* species bacterium is in a medium comprising a
 Zn^{2+} chelator.
- 35 11. The method of claim 10, wherein:
the level of NMB0964 polypeptide produced by the *Neisseria* species bacterium is
as defined in claim 2;
the *Neisseria* species bacterium is as defined in any one of claims 3-6; and/or
the step of preparing outer membrane vesicles is carried out as defined in claim 7,
or is carried out without use of a detergent.
- 40

12. The method of claim 10 or claim 11, wherein the Zn^{2+} chelator is present in the medium at a concentration of 0.01-100, 0.1-10, 0.3-5, or 0.5-1 μM .
- 5 13. The method of claims 10-12, wherein the Zn^{2+} chelator present in the medium is TPEN.
14. An immunogenic composition obtainable by the method of claim 10.
- 10 15. A use of the immunogenic composition of claims 1-9 in the manufacture of a vaccine for the prevention of Neisserial disease; or
a vaccine for use in the prevention of Neisserial disease, for instance *N. meningitidis* or *N. meningitidis* serogroup B, comprising the immunogenic composition of claims 1-9, or made by the method of claims 10-13.