MENINGOCOCCAL MULTIVALENT NATIVE OUTER MEMBRANE VESICLE VACCINE, METHODS OF MAKING AND USE THEREOF

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ABSTRACT

The present technology provides vaccine compositions comprising native outer membrane vesicles (NOMVs) from at least one genetically modified strain of Neisseria which provides protective immunity to meningococcal disease, more preferably subtype B meningococcal disease. The present technology further provides methods of immunizing an animal or human against meningococcal disease comprising administering the vaccine composition of the present invention.
FIGURE 1
Master Cell Bank Preparation

Research cell bank
8570 HOPS-G

Set up and grow overnight on GC/DS agar plates. Use growth of one plate to inoculate 100 ml of Catlins Modified liquid medium in a 500 ml Erlenmeyer flask.

Grow for 1-2 hr and transfer culture to 1.0 L Catlins Modified medium in a 2.8 L Fernbach flask. Incubate culture at 37°C with rotary shaking at 160 rpm until culture reaches OD 600nm of 0.7 to 1.0.

Harvest cells by centrifugation, discard supernatant and suspend cells in 100 ml of cryopreservative medium.

Dispense cell suspension in labeled vials @ 1 ml/vial and freeze in liquid Nitrogen bath. 90 vials filled. Store at -80±10°C.

This is the master cell bank for Strain 8570 HOPS-G

Streak on GC/DS and TSA agar plates to verify culture purity

Gram stain sample of culture to test for purity

Test sample for:
1. Contamination on GC/DS and LB plates
2. Viable cell count by plating serial dilutions

Test final frozen product after 1 week for:
1. Viable cell count
2. Correct species by sugar fermentation, oxidase test, gram stain
3. Correct phenotype and antigen expression by colony blotting with panel of monoclonal antibodies.
FIGURE 2
Production Cell Bank Preparation

Master Cell Bank

Set up and grow overnight on GC/DS agar plates. Use growth of one plate to inoculate 100 ml of Catlins Modified liquid medium in a 500 ml Erlenmeyer flask.

Grow for 1-2 hr and transfer culture to 1.0 L Catlins Modified medium in a 2.8 L Fernbach flask. Incubate culture at 37°C with rotary shaking at 160 rpm until culture reaches OD 600nm of 0.7 to 1.0.

Harvest cells by centrifugation, discard supernatant and suspend cells in 100 ml of cryopreservative medium.

Dispense cell suspension in labeled vials @1 ml/vial and freeze in liquid Nitrogen bath. 90 vials filled. Store at -80±10°C. This is the production cell bank for Strain 8570 HOPS-G.

Streak on GC/DS and TSA agar plates to verify culture purity.

Gram stain sample of culture to test for purity.

Test sample for:
1. Contamination on GC/DS and TSA plates
2. Viable cell count by plating serial dilutions

Test final frozen product for:
1. Correct species by sugar fermentation, oxidase test, gram stain
2. Correct phenotype and antigen expression by colony blotting with panel of monoclonal antibodies.
FIGURE 3

Production Cell Bank

Inoculate 6 GC/DS agar plates with each of two vials of production CB and grow 10-14 h at 37°C in 5% CO₂ atmosphere.

Plate out samples of cultures onto TSA agar plates to check for contamination at the time of inoculation of the agar plates.

Use the growth from four agar plates to inoculate 1 L of Modified Catlin's medium in each of three 2.8 L Fernbach flasks. Grow for 4 to 6 h to a final OD 600nm of about 3.0 ± 0.5.

Measure the OD 600nm of cultures after inoculation and at the time of inoculation of the 40 L fermenter.

Plate out samples of cultures onto TSA agar plates to check for contamination at time of 40 L fermenter inoculation.

Use contents of the three flasks (3 L) to inoculate 27 L of Modified Catlin's medium in 40 L fermenter. Continue fermentation at 37±1°C, 200±10 rpm, 30±10 L air/min, pH 7.5±0.1 until the culture OD 600nm reaches 2.0±0.5.

Monitor OD of culture every 30 to 60 min.

Plate out a sample of 400 L fermenter medium before inoculation to verify sterility of medium.

Plate out on TSA plates for purity and plate serial dilutions on GC/DS agar to determine viable CFUs; Gram stain sample of culture to crosscheck purity.

Inactivate culture with phenol to 0.5% for 2 h.

Verify Inactivation: Plate out 100 μl samples on 2 plates each of GC/DS and TSA agar. Incubate GS/DS plates for 18-24 h at 37°C and TSA plates for minimum of 48 h at 30-35°C.

Harvest cells by continuous flow centrifugation using a Sharples Centrifuge. Weigh and freeze cell paste in 500 g portions at -80 ± 10°C. Yield was 2639 g of cell paste.

This is the 8570 IIOPS-G Cell Paste, lot # 1267.
FIGURE 4
Purification of Native Outer Membrane Vesicles

8570 HOPS-G Cell Paste, Lot # 1267, 500 gm

Thaw the cell paste at 2-8°C for 18-24 hr and suspend the cells in 4 volumes of buffer containing 0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA pH 7.5.

First Extraction
Warm the cell suspension at 56 ± 3°C for 30 min; cool; shear in a Waring blender for 3 min at high speed; centrifuge at 23,600 x g for 20 min, 4 ± 2°C; retain supernatant as extract 1; re-extract pellets.

Second Extraction
Suspend pellets in 0.01 M Tris-HCl, pH 7.5, 37 ± 3°C; shear in a Waring blender for 3 min at high speed; centrifuge at 23,600 x g for 20 min, 4 ± 2°C; retain supernatant as extract 2; re-extract pellets.

Centrifuge combined extracts at 23,600 x g for 20 min, 4 ± 2°C; discard pellets and retain supernatant containing extracted membrane vesicles. Store overnight at 2-8°C. Prior to further processing, centrifuge at 4200xg max for 15 min at 2-8°C and discard the small white pellets.

Visual inspection of combined supernatant by PI to determine, based on turbidity, that the yield from the 2 extractions is adequate.

Add 100X MgCl₂ to 15 mM final concentration. Add Benzonase (nuclease) to 100 units per ml and stir at 20 ± 5°C for 60-80 min.
FIGURE 5
Preparation of Native Outer Membrane Vesicles (Continued)
(Room 3, Bldg. 501)

Remove degraded nucleic acids and soluble proteins, etc by ultrafiltration/diafiltration (UFP-750-E-6A cartridge, 750 K MWCO) with 0.01 M Tris-HCl, pH 7.5, buffer and concentrate sample to about 1 liter, recover retentate.

Monitor filtrate for OD at 260 nm and continue diafiltration until OD falls below 0.05.

Perform UV scan to evaluate removal of nucleic acid and to estimate protein concentration.

Ultracentrifuge vesicles at 225,000 x g for 60 min at 2 – 8°C to remove all soluble, non-vesicle material. Retain and resuspend pellets.

Perform UV scan to estimate protein concentration prior to sterile filtration.

Pass suspended vesicles through microfluidizer to reduce vesicle size and insure passage through sterile 0.2 μm filter, 2 cycles at 4-10°C, 19,000 psi.

Samples taken for QC tests of bulk vaccine.

Sterile filter vaccine using 0.2 μm pore size membrane filter (Millipak 60 filtration unit).

Store sterile filtered vaccine at -80 ± 10°C. This is the bulk vaccine Lot # 1277

Vials of final product taken for QC tests and preclinical tests of final product vaccine.

Vialing
Bring to 5% Dextrose, adjust concentration to 200 μg/ml protein and dispense into 10 ml vials. Store at -80 ± 10°C. This is final product vaccine, lot # 1289.
### FIGURE 8

IDENTITY TEST WITH MONOCLONAL ANTIBODIES
Meningococcal 8570 HOPS-G NOMV Vaccine, BPR-779-00, Lot No. 1289

<table>
<thead>
<tr>
<th>Lane</th>
<th>Antibody Specificity</th>
<th>Monoclonal Antibody</th>
<th>Expected Reaction</th>
<th>Test Result</th>
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<tr>
<td>1</td>
<td>Pre-stained Standard</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>L8 LOS</td>
<td>2-1 L8</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>3</td>
<td>L8v LOS</td>
<td>25-1-1LC1</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>L3,7 LOS</td>
<td>9-2-1.379</td>
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<td>JAR 4</td>
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<td>Rmp</td>
<td>9F5</td>
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<td>15-1-P4</td>
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<td>Positive</td>
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<td>PorA PI.14</td>
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<td>MN3C5C</td>
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<td>Positive</td>
</tr>
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<td>2-1-PI.19</td>
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<td>Positive</td>
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<td>476C2G2</td>
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<td>Group B Polysaccharide</td>
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<td>17</td>
<td>Amido Black Stain</td>
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<td></td>
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</tr>
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</table>
FIGURE 9

Mean TNF-α Release from Whole Human Blood Following Incubation with Different Concentrations of NOMV Vaccines (N = 5)

- E. Coli LPS Standard
- Lot 0832 NOMV (WT LOS)
- Lot 1289 NOMV (LpxL1 LOS)
- Lot 1119 NOMV (LpxL2 LOS)

Mean pg/ml TNF-α

0.0001 0.01 1 100 10000

Vaccine Concentration (ng Protein/ml)*
FIGURE 10

IL-6 Release from Whole Human Blood Following Incubation with Different Concentrations of NOMV Vaccines

E. Coli LPS Standard
Lot 6832 NOMV (WT LOS)
Lot 1289 NOMV (LpxL1 LOS)
Lot 1119 NOMV (LpxL2 LOS)
FIGURE 11

TNF-α Release by Whole Human Blood Following Incubation with Different Concentrations of Vaccine Lot 1289 or DOC-extracted OMV from strain 44/76.
FIGURE 12

Dose response of mice to 8570 HOPS-G Vaccine Lot# 1289 with and without adsorption to Rehydragel LV. Sera from the final bleed after three doses were tested for bactericidal activity against four different test strains: 8570 Pi.19:15;L3,7v 8570 Pi.122:4;L37v 8570 Pi.22:14;L3,7v 4475 Pi.17:10;L3,7

Vaccine Groups:
1) 0.1 μg
2) 0.3 μg
3) 1.0 μg
4) 3.0 μg
5) 0.1 μg + Rehydragel LV
6) 0.3 μg + Rehydragel LV
7) 1.0 μg + Rehydragel LV
8) 3.0 μg + Rehydragel LV
9) 1.0 μg + Rehydragel HPA
FIGURE 13

GM Bactericidal Titer of Sera from Mice Vaccinated with 3 μg Dose of B2 (8570 HOPS-G) NOMV Vaccine vs Different Test Strains

[Graph showing the bactericidal titer of sera from mice vaccinated with 3 μg dose of B2 (8570 HOPS-G) NOMV vaccine versus different test strains. The graph compares unadsorbed vs aluminum hydroxide adsorbed conditions.]

Test Strain: 8570 P1.19,15: L(3,7)-5; 8570 P122,14: L(3,7)-5; 44f76 P1.7, 16: L3,7; 44/76 P1.7,16: L3,7.

FIGURE 14

Depletion of Bactericidal Antibody vs 44/76 L3,7 by Serum from Mice Vaccinated with B2 (8570 HOPS-G) NOMV Vaccine

[Graph showing the depletion of bactericidal antibody vs 44/76 L3,7 by serum from mice vaccinated with B2 (8570 HOPS-G) NOMV vaccine. The graph plots antigen concentration (μg/ml) against percent antibody removed.]

Antigen Concentration (μg/ml)

Percent Antibody Removed

- Purified fHbp
- 126L L1 LOS
- 44/76 L3,7 NOMV
- 44/76 L3,7 LOS
- 8532L B8-5 LOS
- Purified Opc
FIGURE 15

Depletion of Bactericidal Activity from Pooled Mouse Serum
Vaccine: 8570 HOS-G1 ΔPorA
Test Strain: WT 8570

FIGURE 16

Depletion of Bactericidal Activity from Pooled Mouse Serum
Vaccine: 8570 HOS-G1 ΔPorA
Test Strain: WT 8570
FIGURE 17

Depletion of Bactericidal Antibodies to Strain 6557(P1.14) from Mouse Serum by Different Antigens
Vaccine = 8570 HOS-G1 (P1.22,14)

<table>
<thead>
<tr>
<th>Antigen Concentration (µg/ml)</th>
<th>Percent Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>100</td>
</tr>
<tr>
<td>0.39</td>
<td>90</td>
</tr>
<tr>
<td>1.68</td>
<td>80</td>
</tr>
<tr>
<td>6.25</td>
<td>70</td>
</tr>
<tr>
<td>25</td>
<td>60</td>
</tr>
</tbody>
</table>

FIGURE 18

Strain B1

Parent
H44/76
B:15:P1.7,16:L3,7
ET-5 Clone
PorB3
TbpB isotype II

Mutations
synX deletion
IpXL1 deletion
Add 2nd PorA (P1.7-1.1)
Over express NadA
Stable expression of L8-3 LOS and Opc

Strain B2

Parent
8570
B:4:P1.19,15:L(3,7)-5
ET-5 Clone
PorB3
TbpB isotype II

Mutations
synX deletion
IpXL1 deletion
Add 2nd PorA (P1.22,14)
Over express fHbp(v.1)
Stable expression of L8-5 LOS and Opc

Strain B3

Parent
B16B6
B:2a:P1.5,2:L2
ET-37 Clone
PorB2
TbpB isotype I

Mutations
synX deletion
IpXL1 deletion
Add 2nd PorA (P1.22-1.4)
Over express fHbp(v.2)
Stable expression of L8-2
FIG. 20A

Strain B1 (L8-3)  
\( \text{Gal} - \text{GlNAc} \)  
\( \text{Alga} \)  
\( \text{Kdo} \)  
\( \text{Lac} \)  
\( \text{Alga} \)  
\( \text{Hep} \)  
\( \text{Kdo} \)  
\( \text{Lipid A} \)  
\( \text{Gal-Glc} - \text{Hep-Kdo-Lipid A} \)  
\( \text{Gal-Glc} - \text{Hep-Kdo-Lipid A} \)  
\( \text{Gal-Glc} - \text{Hep-Kdo-Lipid A} \)  
\( \text{Gal-Glc} - \text{Hep-Kdo-Lipid A} \)  

Strain B2 (L8-5)  
\( \text{Alga} \)  
\( \text{Kdo} \)  
\( \text{Hep} \)  
\( \text{GlcNAc} \)  
\( \text{Alga} \)  
\( \text{Hep} \)  
\( \text{Kdo} \)  
\( \text{Lipid A} \)  
\( \text{Gal-Glc} - \text{Hep-Kdo-Lipid A} \)  
\( \text{Gal-Glc} - \text{Hep-Kdo-Lipid A} \)  
\( \text{Gal-Glc} - \text{Hep-Kdo-Lipid A} \)  
\( \text{Gal-Glc} - \text{Hep-Kdo-Lipid A} \)  

Strain B3 (L8-2)  
\( \text{Alga} \)  
\( \text{Kdo} \)  
\( \text{Hep} \)  
\( \text{GlcNAc} \)  
\( \text{Alga} \)  
\( \text{Hep} \)  
\( \text{Kdo} \)  
\( \text{Lipid A} \)  
\( \text{Gal-Glc} - \text{Hep-Kdo-Lipid A} \)  
\( \text{Gal-Glc} - \text{Hep-Kdo-Lipid A} \)  
\( \text{Gal-Glc} - \text{Hep-Kdo-Lipid A} \)  
\( \text{Gal-Glc} - \text{Hep-Kdo-Lipid A} \)  

Non transformed bacteria of parental Strain B16B6

FIG. 20B

mAb L3, 7, 9  
AlgtA mutants  
Non transformed bacteria of parental strain B16B6  
mAb L8
Knockout of NspA gene by fHbp containing insert

Gent. + IPTG + Anti-fHbp v.1 Ab

IPTG + Anti-fHbp v.1 MAb

Nontransformed Strain B3

Recombinants. No induction

Recombinants. IPTG induction

Non-transformed B15B6
MENINGOCOCCAL MULTIVALENT NATIVE OUTER MEMBRANE VESICLE VACCINE, METHODS OF MAKING AND USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to the U.S. Provisional Application No. 61/057,462 entitled “Meningococcal Multivalent Native Outer Membrane Vesicle Vaccine” filed May 30, 2008. The entire disclosure and contents of the above application is hereby incorporated by reference in its entirety.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] The U.S. Government has rights in this invention.

BACKGROUND OF THE INVENTION

[0003] Neisseria meningitidis is a major cause of meningitis and septicemia world-wide. Meningococcal meningitis is an inflammation of the meninges, the membrane lining the brain and the spinal cord. In both meningococcal septicaemia and meningococcal meningitis, damage is caused by an uncontrolled localized or systemic host inflammatory response. Group B meningococcal disease currently accounts for at least one half of all meningococcal disease in many countries including North and South America, and Europe. The emergence of a new virulent clone of group B Neisseria meningitidis, known as ET5, in Norway in the late 70’s has since been responsible for prolonged epidemics in Norway, Cuba, Brazil, and Chile. These epidemics have created serious public health problems and led to intensive efforts to develop an effective group B vaccine in several of the affected countries. The absence of a U.S.-licensed group B vaccine along with the poor performance of the A and C capsular polysaccharide vaccines in children under 18 months have prevented serious consideration of routine childhood vaccination against meningococcal disease.

[0004] Neisseria meningitidis is divided into 13 serogroups, of which 9 cause invasive disease (A, B, C (C1, C1–), X, Y, W-135, Z, and L). Five of the serotypes are targeted for development of vaccines due to their ability to cause epidemics, including serotypes A, B, C, Y, and W135 which are the target of much vaccine research.

[0005] Vaccines against serogroups A, C, Y and W135 of Neisseria meningitidis that cause nearly all invasive meningococcal disease are available and are routinely used with excellent results. A suitable vaccine against group B strains of Neisseria meningitidis has been more difficult to develop for a variety of reasons. For instance, the capsular polysaccharide which defines the serogroup is ineffective and potentially unsafe for use in a vaccine because it has the same structure as polysialic acid found on certain human cells, specifically blood cells.

[0006] Further adding to the lack of a suitable vaccine is the fact that subcapsular antigens that are surface exposed, such as outer membrane proteins and the lipo polysaccharide (endotoxin), are antigenically variable and/or inconsistently expressed among group B strains. No single antigen has been identified that alone has all the characteristics that are essential for an effective vaccine.

BRIEF SUMMARY OF THE INVENTION

[0007] In one aspect, the present technology provides a vaccine comprising native outer membrane vesicles (NOMV’s) obtained from at least two meningococcal strains that have been genetically modified to provide broad based protection. The native outer membrane vesicles include three different sets of antigens based on PorA, LOS, and conserved outer membrane proteins; and the genetically modified strains have been modified to provide enhanced safety based on inactivation of lpxL1, synX, and lgtA genes. The two meningococcal strains can both express LOS having a different LOS core structure and has an alpha chains consisting of glucose and galactose. Each strain may express at least two different PorA subtype proteins or subtype epitopes which are chosen based on the most prevalent of PorA subtypes among group B case isolates. Further, the vaccine may further include a different conserved surface protein with demonstrated capacity to induce bactericidal antibodies is over-expressed in each strain and are taken from the group consisting of FLBP (GNA1870) variants 1, FLHP variants 2, and FLHP variants 3; NAdA; App; NspA; TbpA and TbpB.

[0008] In a further aspect, the present technology provides a combination of NOMVs from three genetically modified, antigenically diverse Neisseria meningitidis strains. At least one of the strains is selected from (1) H44/76 HOPS-DL which has the following genetic modifications or characteristics: inactivation of the genes synX, lpxL1, and lgtA; insertion of a second porA gene (subtype P1.7-1.1) in the place of opaD; increased expression of NAdA; and stabilized high expression of Opq and PorA; (2) 8570 HOPS-G12 which has the following genetic modifications or characteristics: inactivation of the genes synX, lpxL1, and lgtA; insertion of a second porA gene in place of opaD; increased expression of factor H binding protein variant 1; and stabilized high expression of PorA and Opq; and/or (3) B16B6 HPS-G2A which has the following genetic modifications or characteristics: inactivation of the genes synX, lpxL1, and lgtA; insertion of a second porA gene in place of opaD; increased expression of factor H binding protein variant 2; and stabilized high expression of PorA and Opq. The NOMV are prepared without exposure to detergent or denaturing solvents from packed cells or from spent culture medium. The vaccine may be combined with one or more adjuvants and may be administered intramuscularly and/or intranasally.

[0009] In another aspect, the present technology provides a vaccine composition against meningococcal disease, more preferably group B meningococcal disease, including native outer membrane vesicles (NOMVs) from one or more genetically modified strains of Neisseria meningitidis. The one or more genetically modified strains has been modified by: inactivation of the synX gene, inactivation of the lpxL1 gene, inactivation of the lgtA gene in each strain resulting in expression of a shortened or truncated lipo polysaccharides (LOS) that lacks lacto-N-neotetraose tetrasaccharide, and/or insertion of at least one second antigenically different porA gene in place of the opa gene. In another aspect, the genetically modified strain further comprises increased or stable expression of at least one minor conserved outer membrane protein, and/or stabilized expression of at least one outer membrane protein. The at least one second antigenically different porA gene may express at least one PorA.
In yet another aspect, the present technology provides a genetically modified vaccine strain of Neisseria meningitidis subtype B derived from H44/76 strain comprising the genetic modifications of: i) inactivation of a synX gene, ii) inactivation of the lpxL1 gene, iii) inactivation of the lgtA gene, iv) suppression of the porA gene in place of lppA gene, v) increased expression of factor H binding protein variant 1; and vi) stabilized increased expression of PorA and Opc proteins. In some aspects, the genetically modified strain was derived from the ET-5 wild type strain H44/76 (B:15; P1.7,16; L3.7; P5.5, C).

In yet another aspect, the present technology provides a genetically modified vaccine strain of Neisseria meningitidis subtype B derived from B16B6 comprising the genetic modifications of: i) inactivation of a synX gene, ii) inactivation of the lpxL1 gene, iii) inactivation of the lgtA gene, iv) suppression of a second porA gene (subtype P1.22-1.4) in place of lppA gene, v) increased expression of factor H binding protein variant 2; and vi) stabilized increased expression of PorA and Opc proteins. In some aspects, the genetically modified strain is derived from the ET-37 wild type strain B16B6 (B:2a; P 1.5.2; L2; P5.1.2.5).

In yet another aspect, the present technology provides a genetically modified strain grown in iron deficient medium.

In other aspects, the present technology provides a genetically modified strain wherein inactivation of synX gene, lpxL1 gene, or lgtA gene is by an insertion of a drug resistance gene within the sequence of the inactivated gene.

Yet another aspect provides a vaccine including NOMVs derived from the genetically modified strains of the present technology. The NOMV are prepared from packed cells or spent culture medium without exposure to a detergent or denaturing solvent. The vaccine may further comprise one or more adjuvants. In further aspects, the genetically altered strain is altered to express iron uptake proteins.

In yet another aspect, the present technology provides a vaccine against meningococcal disease comprising a variety of native outer membrane vesicles (NOMVs), wherein at least some of the NOMVs are essentially free of expression or sialylation of lipooligosaccharide (LOS), contain LOS that includes a lipid A with a penta-acetyl structure and contain increased expression levels of at least one minor conserved outer membrane protein, wherein the minor conserved outer membrane protein is selected from proteins that induce bactericidal antibodies. The minor conserved outer membrane protein can be selected from the group consisting of NadA, factor H binding protein (FHBP) variant 1, and FHBP variant 2. In other aspects, at least some of the NOMV comprise shortened or truncated LOS that are essentially free of lacto-N-neotetraose (LNT) tetrasaccharide and/or at least some of the NOMV comprise two or more different PorA proteins.

In another aspect, the present technology provides a method of eliciting an immune response to meningococcal disease in an animal or human comprising administering the composition containing NOMVs from at least one genetically altered strain of N. meningitidis to the animal or human for immunization against meningococcal disease. The vaccine is used for immunization against group B meningococcal disease.

In yet another aspect, the present technology provides a method of preparing a genetically modified strain of N. meningitidis for use in a vaccine against meningococcal disease comprising the steps of: a) selecting a strain of meningococcal type B able to be genetically modified; b) genetically modifying the strain by inactivating the synX gene, c) genetically modifying the strain by inactivating the lpxL1 gene, d) genetically modifying the strain by inactivating the lgtA gene, and e) genetically modifying the strain by increasing expression of one or more minor conserved outer membrane proteins. In further aspects, the method further comprises genetically modifying the strain by inserting at least one second antigenically different porA gene into the open reading frame of the opa gene. In other aspects, the method further comprises the step of genetically modifying the strain to stably express or over express at least one outer membrane protein by replacing the poly-C sequence within the promoter or open reading frame of the at least one outer membrane protein with a sequence containing G and C nucleotides.

Yet another aspect, the present technology provides a method of preparing a vaccine against meningococcal disease comprising the steps of: a) culturing a genetically modified strain of N. meningitidis comprising one or more modulation selected from the group consisting of inactivation of the synX gene, inactivation of the lpxL1 gene, inactivation of the lgtA gene, insertion of at least one second antigenically different porA gene in place of the opa gene, increased or stable expression of at least one minor conserved outer membrane protein, and/or stabilized expression of at least one outer membrane protein; b) expanding the culture by fermentation using the cultured strain of a) to inoculate medium in a fermentor; c) inactivating the fermented culture; d) harvesting N. meningitidis cultured cells by continuous flow centrifugation and collecting cell paste; e) isolating NOMVs from the cell paste; and f) resuspending NOMVs in buffer or carrier suitable for vaccine administration.

**BRIEF DESCRIPTION OF SEVERAL VIEWS OF THE DRAWINGS**

**FIG. 1** is a flow chart depicting the preparation of a master cell bank of cells for the genetically modified strains of *Neisseria* for vaccine production.

**FIG. 2** is a flow chart depicting the production of the cell bank preparation used for making the genetically modified strains of *Neisseria* for vaccine production.
FIG. 3 is a flowchart depicting the fermentation of the Neisseria used for making the genetically modified strains of Neisseria for vaccine production.

FIG. 4 is a flowchart depicting the purification of NOMVs from the genetically modified strains of Neisseria for vaccine production.

FIG. 5 is a continuation of the flowchart from FIG. 4.

FIG. 6 is a picture of a coomassie blue stained gel showing the protein content of standard marker (lane 1), control 8570 HOPS-G NOMV preparation (lane 2), filtered bulk vaccine (lane 3) and final product vaccine (lane 4).

FIG. 7 is a silver stained gel showing lipoooligosaccharide content of the vaccine. Lane 1 is the control ML5 LYS, lane 2 is the filtered bulk vaccine and lane 3 is the final vaccine product. Fifteen µl of a 1:2 dilution of 100 µg/ml of the vaccine were run on the gel (20 µl of 100 µl/ml of 1:2 dilution of control).

FIG. 8 is a picture of an antibody stained western blot showing the identity and composition of the proteins found in the 8570 HOPS-G NOMV vaccine.

FIG. 9 is a graph depicting the TNF-α release from human blood after incubation with different concentrations of the vaccine.

FIG. 10 is a graph depicting IL-6 release from human blood following incubation with different concentrations of the genetically modified NOMV vaccine.

FIG. 11 is a graph depicting the TNF-α release from human blood after incubation with different concentrations of the genetically modified vaccine as compared with DOC-extracted OMV from strain 46/76.

FIG. 12 is a bar graph depicting the bactericidal titers of mice vaccinated with different concentrations of the 8570 HOPS-G Vaccine with or without an adjuvant.

FIG. 13 is a bar graph depicting the bactericidal titer of mice vaccinated with 8570 HOPS-G Vaccine against different test strains.

FIG. 14 is a graph depicting the results of the bactericidal antibody depletion assay for LOS, GNA1870, NOMV and Opc antigens.

FIG. 15 depicts the antibody response of rabbits vaccinated with the 8570 HOPS-G NOMV vaccine with and without adjuvant.

FIG. 16 is a graph depicting the results of an bactericidal depletion assay for test strains against the 8570 HOPS-G1 NOMV vaccine.

FIG. 17 is a graph depicting the results of the bactericidal depletion assay for LOS and FHBP antigens for the 8570 HOPS-G1 PorA knockout strain.

FIG. 18 is a representation of phenotype of the three genetically modified strains of Neisseria (A-B1, B-B2, and C-B3) of the present technology.

FIG. 19 is a representation of the plasmids used to construct the genetically modified strains of Neisseria: a) plasmid constructed to knockout IgA, b) plasmid to express second PorA, c) plasmid to overexpress FHBP driven by orthologous (PtaC if E. coli) promoter, and d) plasmid to overexpress NadA driven by a homologous promoter (PorA promoter of N. meningitidis) and the e) representational scheme of transformation of N. meningitidis with FHBP (variant 1 and 2) and NadA overexpression plasmid via homologous recombination replacing NadA gene.

FIG. 20a is a depiction of the stabilization of the truncated LOS immunotype of NOMV vaccine strain by knockout of the IgA gene of the three genetically modified strains. FIG. 20b is a picture of an immunoblott of the expression of LOS alpha chain by the genetically altered strain B2 and the parental strain (B1686) with monoclonal antibodies against 1,3, 7,9 (left) and 1,8 (right).

FIG. 21 is a picture representation showing the expression of FHBP variant 2 in the genetically modified strain B3. FIG. 21a shows selection of the strain containing the gentamicin resistance recombinant containing the overexpressed FHBP by immunoblotting and FIG. 21b is a Western Blot using JAR4 monoclonal antibody to FHBP showing increased expression of FHBP.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a broadly protective vaccine composition for use in immunization against meningococcal disease, more preferably Neisseria meningitidis subgroup type B. One embodiment of the present technology provides a vaccine composition including native outer membrane vesicles (NOMVs) from at least one, preferably at least two, more preferably at least three genetically modified strains of Neisseria meningitidis. Native outer membrane vesicles, also known as blebs, are vesicles formed or derived from fragments of the outer membrane of gram negative bacterium naturally given off during growth and may be obtained from culture medium or from the cells by mild methods that do not use detergents or denaturing solvents. These NOMV typically comprise outer membrane proteins (OMP)s, lipids, phospholipids, periplasmic material and lipopolysaccharide (LPS) including lipooligosaccharides. Gram negative bacteria, especially pathogens like N. meningitidis, often shed NOMVs during virulent infections in a process known as bleeding. In the present technology, NOMV are vesicles produced from the outer membrane of bacteria without the use of chemical denaturation processes and are produced from the genetically modified strains which are antigenically diverse and have each been genetically modified to improve safety, antigenic stability, and the breadth of the protective immune response.

One embodiment of the present invention provides a vaccine composition comprising native outer membrane vesicles (NOMVs) derived from at least two or more genetically modified strains of N. meningitidis, preferably at least three different genetically modified strains.

Some embodiments of the present technology provide antigenically diverse strains of N. meningitidis, preferably subtypes which include at least three genetic modifications within the genome of the bacteria, more preferably at least five genetic modifications, more suitable at least six genetic modifications. The genetic modifications can include one or more of the following: 1) inactivation of the synX gene, which is essential for sialic acid biosynthesis and results in no capsule expression or sialylation of lipooligosaccharide (LOS); 2) inactivation of the lpxL1 gene which results in a significantly less toxic LOS having lipid A with a penta-acetyl structure; 3) insertion of a second, antigenically different porA gene in place of one of the opa genes (OpaC or OpaD); 4) increased expression of at least one minor conserved outer membrane protein, the minor conserved outer membrane protein demonstrating the ability to induce bactericidal antibodies (for example, but not limited to, NadA, factor H binding protein (FHBP) variant 1, and FHBP variant 2); 5) inactivation of the IgA gene in each
strain which results in the expression of a shortened or truncated LOS that lacks the lacto-N-neotetraose (LNnT) tetrasaccharide; and/or 6) stabilized expression of certain outer membrane proteins, such as Opc and PorA that are susceptible to phase variation in wild type strains.

[0045] The present technology provides genetically modified strains that provide both increased safety of use and increase the breadth of the protective antibody response to meningococcal disease. In one embodiment, the genetically modified strains provide increased safety by incorporating at least one of the following mutations into the bacterial genome: deletion of the synX gene which blocks sialic acid synthesis of capsule and results in the formation of capsule-negative phenotype NOMVs, deletion of the lpxL.1 gene which reduces the endotoxin activity by resulting in a penta-acyl lipid A structure, and/or deletion of the lgtA gene which blocks lacto-N-neotetraose biosynthesis on the lipooligosaccharide (LOS) which stabilized the truncated LOS structure; more preferably the genetically modified strains provide two of these mutations, most preferably the genetically modified strains provide all three of these mutations. In another embodiment of the present technology, the genetically modified strains have an increased breadth of protective antibody response by targeting at least one of three sets of possible protective antigens contained within the NOMVs. The three possible antigens targeted include at least one of the following: PorA protein, at least one conserved minor protein, and/or the LOS core structure, and include any combination thereof. In more preferred embodiments, the genetically modified strain targets at least two of the possible protective antigens, most preferably targeting all three of the possible protective antigens.

[0046] In some embodiments of the present technology, the synX- mutation (inactivation of the synX gene) was inserted into the genetically modified strain by a method as described in U.S. Pat. No. 6,558,677, incorporated by reference herein in its entirety. In brief summary, a pUC19-based plasmid containing the synX gene in which 200 bp sequence was replaced by a kanamycin resistance gene is used to transform the genetically modified strain. Kan resistant transformants were selected and PCR for the presence of the disrupted synX gene and for the capsule negative phenotype. This synX- mutant was constructed based on results and sequence information reported by Swartley and Stephens (Swartley and Stephens (1994) J. Bacteriol. 176: 1530-1534) who showed that insertion of a transposon into the synX gene led to a capsule negative phenotype. The same or an equivalent mutation can be introduced into any transformable N. meningitidis strain. A suitable plasmid for use in transforming meningococci was constructed using the following procedure. Three DNA sequences were pieced together using the splicing by overlap extension (SOE) polymerase chain reaction (PCR) technique (Horton et al. (1989) Gene 77: 61-65). The three DNA sequences included, in order beginning at the 5' end, synXB bases 67 to 681; the kanamycin resistance gene from pUC4K (Pharmacia LKB Biotech Co.) 671 to 1625; and synXB bases 886 to 1589. In addition, at the 5' end, a putative uptake sequence, ACCGTCTGAA, was added by including it at the end of the PCR primer used to amplify the synXB 67 to 691 base sequence. The complete construct was amplified by PCR, purified and blunt ligated into pUC19. pUC19 was used to transform Escherichia coli DH5α and selected on LB agar with 50 μg kanamycin. A kanamycin resistant colony was selected, the DNA extracted, purified, and cut with XbaI. Another copy of the presumptive uptake sequence was ligated into this multiple cloning region site and the resulting plasmid again used to transform E. coli DH5α and kanamycin resistant colonies screened by PCR for presence of the additional uptake sequence. Plasmid DNA was isolated from a selected colony and used as a template for PCR using primers that amplified only the insert part of the plasmid excluding the ampicillin resistance gene which should not be introduced into N. meningitidis. The amplified DNA was then purified and used to transform the genetically modified N. meningitidis strain. The synX(−) mutant of N. meningitidis was selected by kanamycin resistance and confirmed by PCR amplification of the modified region.

[0047] In some embodiments of the present invention, the lpxL.1 gene was inactivated in the genetically modified strains to produce a reduced endotoxic LOS expressed on the NOMVs in the vaccine compositions. The lipid A of N. meningitidis LOS is normally a hexa-acyl structure and is responsible for the endotoxic properties of the LOS. Two acyl-oxo-acyl linked secondary fatty acids present in the lipid A are important for endotoxic activity. The genetically modified strain includes the lpxL.1 mutant as described by van der Ley and co-workers (van der Ley, P., Steeghs, L., Hamstra, H. J., Van Hove, J., Zomer, B., and van Alphen, L. Modification of lipid A biosynthesis in Neisseria meningitidis lpxL mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity. Infection and Immunology 69(10), 5981-5990, 2001.) Deletion of the lpxL.1 gene resulted in expression of normal levels of penta-acyl LOS with greatly reduced endotoxicity as tested by both rabbit pyrogen test and by cytokine release assay using human monocytes from whole blood. Other methods for disrupting the lpxL.1 gene are contemplated in further embodiments of the present technology for use in developing the genetically modified strains.

[0048] In some embodiments, the genetically modified strain contains an insertion of a second, antigenically different porA gene in place of one of the opa gene (Opac or Opad). The major outer-membrane protein, Porin A or PorA of Neisseria meningitidis, is the product of the porA gene. PorA has wide antigenic variation and is subject to phase variations to evade immune selective pressure; therefore it is not always cross-protective to other subtypes. To increase the reactivity of the vaccine compositions against different subtypes of PorA, at least one additional porA gene is inserted into the opacC or opad gene of the genetically altered strain. The PorA serotype selected for insertion is selected based on the most prevalent forms of the PorA found in cases of subtype B meningococcal disease. Suitable PorA serotypes include, but are not limited to: P1.7-1, (from strain M1080); P1.22,14 (from strain M4410); P1.22,1,4; or other suitable PorA serotypes as to be understood by one skilled in the art or described in the current literature, for example, as described by Sacchi et al., Diversity and prevalence of PorA types in Neisseria meningitidis serogroup B in the United States, 1992-1998, J Infect Dis. 2000 October; 182 (4):1169-76. The second PorA genes may be under control of any suitable strong promoter that provided expression of the PorA protein, for example the PorA promoter from suitable strains, e.g., H44/76 strain. Suitable methods of cloning the porA gene into the genetically altered strain would be known to a person skilled in the art, and can
include, but is not limited to homologous recombination. For example, the porA gene may be PCR amplified from bacterial chromosomal DNA, cloned into a cloning vector and recombined into an appropriately constructed plasmid, for example pUC19, using gene splicing by a modification of the overlap extension PCR technique. This construction plasmid can be introduced into the bacterial genome via homologous recombination such as to replace the opa gene. Transformants may be selected by colony blotting with monoclonal antibodies to the Porin. These methods are known to one skilled in the art.

[0049] In further embodiments of the present technology, the modified strains have stable and/or increased expression of at least one minor outer membrane protein. Suitable minor outer membrane proteins demonstrate the ability to induce bactericidal antibodies (for example, but not limited to, NadA, factor H binding protein (FHBP) variant 1, and FHBP variant 2). Not to be bound by any theory, stabilization and/or increased expression of highly conserved surface exposed minor outer membrane proteins identified through genomic analysis as having potential to induce protective antibodies may lead to an increase in the cross-protective immune response. Suitable conserved minor proteins include, but are not limited to, NadA, FHBP variant 1 and 2, and Opc. Methods of stabilizing and/or overexpression of the minor outer membrane protein (OMP) include use of expression plasmids and homologous recombination, or other suitable methods that are known to one skilled in the art. The minor OMPs can be under a strong promoter, for example, but not limited to the N. meningitidis PorA promoter or IPTG-inducible E. coli Ptc promoter.

[0050] As described in the examples below, construct plasmids were used to establish increased expression of FHBp 1 and FHBp 2 in the genetically modified strains, where the overexpressed protein appeared properly processed, lipidated, and translated to the surface of the outer membrane. For example, expression of v.1 under the control of IPTG-inducible E. coli Ptc promoter in strain 8570 HOPS-G (H2) was about 4-fold higher than in the parental strain 8570 and expression of v.2 in strain B16B2 HPS-G, A (B3) was 32-64 fold higher than in the parental strain B16B6 (See FIG. 20). Alternatively, an expression system that utilized the PorA promoter could be used to stabilize/overexpress the minor conserved proteins.

[0051] In further embodiments of the present technology, the genetically modified strains include inactivation of the lgtA gene which results in the expression of a shortened or truncated LOS that lacks the lacto-N-neotetraoside (LNT) tetrasaccharide.

[0052] An important characteristic of meningococcal LOS is phase-variation, which occurs due to high-frequency mutations in homopolymeric tracts of nucleotide residues in the lgtA and other noniserial genes. These mutations switch on or off the expression of the LgtA transferase which mediates the assembly of the LOS α-chain (altering the configuration of substrates on heptose two). This phase-variable activation of the lgtA gene may lead to undesirable elongation of the LOS α-chain resulting in lacto N-neotetraoside which has structural similarity to human blood cell antigens. The genetically modified strains of the present technology have the lgtA gene knocked out by disrupting the native gene with a antibiotic marker or other suitable marker (for use in screening for alternations in the gene), for example, but not limited to the zeomycin resistance gene. Methods of knocking out the lgtA gene are known to one skilled in the art including, but not limited to construct plasmids and homologous recombination or transformation. The mutated AlgA gene was inactive in all modified strains during at least 22 observed passages and this was a stabilized truncated form of the LOS core structure. The deletion of the lgtA gene stabilizes the truncated α-core LOS structure, for example, providing the truncated core structures as depicted in FIG. 19, wherein three exemplary modified strains are shown, for example the B3 strain contains the L8 alpha chain with the L3 core structure. The genetically modified strains of the present technology contain specific LOS core structures corresponding to immunotypes I3, L5 and L2 providing stabilized core structures in which an immune response can be mounted, and in the examples below demonstrate truncated L8-like LOS (L8-3, L8-5, and L8-2) which are able to kill wild type strains expressing full length LOS. AlgA strains are able to elicit antibodies that recognize both the truncated and full-length forms of the LOS structure, without cross-reacting with the lacto-N-neotetraoside oligosaccharides found on human blood cells.

[0053] In further embodiments of the present technology, the genetically modified strains of N. meningitidis have stabilized expression of outer membrane proteins that are normally susceptible to phase variation in wildtype strains, for example, but not limited to, Opc and PorA. The expression of these proteins can be stabilized by methods known in the art, and include the method of replacing the polymeric repeat sequence in either the promoters or within the reading frame of the gene being stabilized with a non-repeating sequence of optimal length form maximal expression. For example, part of the poly-C or poly-G sequence in the promoter of these genes can be replaced with a sequence of the same length containing both C and G nucleotides, for example, 12 bp poly-G sequence of the promoter of opcA (see SEQ. ID. No. 1) was replaced with a new sequence of the same length containing both C and G nucleotides and a Not I site (See SEQ. ID No. 2, Not I site underlined). In other suitable embodiments, the poly-G sequence in the PorA promoter (for example, see SEQ. ID No. 3) can be replaced with a new sequence containing both C and G nucleotides.

[0054] Further embodiments of the present technology provide growth of the vaccine strains in liquid medium containing a low level of iron in order to induce protein expression of proteins involved in uptake of iron, for example transferring binding protein A and B. In some embodiments, the medium used did not contain specific addition of iron chelators such as desferal. One suitable medium is modified from that published by B W Catlin (Catlin B W. (1973) J. Infec. Dis. 128: 178-194) by replacing seven individual amino acids with 1% casamino acids (certified, Difco Laboratories). The medium contained per liter: 0.4 g NH₄Cl, 0.168 g KCl, 5.85 g NaCl, 1.065 g Na₂HPO₄, 0.17 g KH₂PO₄, 0.647 g sodium citrate, 6.25 g sodium lactate (60% syrup), 0.037 g CaCl₂, 21H₂O, 0.0013 g MnSO₄, H₂O, 5 g glycerol, 0.02 g cysteine, 10 g casamino acids, 0.616 g MgSO₄, and distilled water to one liter. The same iron deficient medium was used for the starter flasks and the final culture flasks or fermenters.

[0055] The vaccine composition of the present technology which include NOMVs from at least three different genetically modified strains of subgroup B can provide three potential levels of protection or three types of antigens that each potentially induce a protective antibody response. The
three antigens are the PorA protein (six different PorA subtypes are present in the vaccine, two on each of the three vaccine strains); the lipooligosaccharides (three different LOS core structures are present in the vaccine, one from each strain); and the conserved minor proteins NadA, FHBP variants 1 and 2, and Opc, which have been over expressed in the vaccine strains. Although PorA has a relatively high level of antigenic variation with several hundred different sequence variations having been identified, certain PorA serosubtypes are much more frequently encountered than others and a modest number of different serosubtypes may potentially protect against more than half of group B disease. Having more than one antigen capable of inducing bactericidal antibodies in the vaccine is important because it has been shown that when the surface density of an antigen is low, antibodies to it may not be able to initiate a complement mediated lytic event. But if antibodies to two or more such antigens are present the antibodies can together initiate complement mediated lysis. Genetically modified strains of the present invention include, but are not limited to, the three strains depicted in FIG. 18, including B1 (44/76 HOPS-D), B2 (8570 HOPS-G1) and B3 (B16B86 HOPS-G2) strains.

The present technology provides a vaccine that provides broad spectrum protection against meningococcal disease, specifically meningococcal disease caused by Neisseria meningitidis subgroup B. The vaccine composition of the present invention can be combined with the existing tetavalent A, C, Y, and W-135 vaccine to provide protection against a majority of pathogenic serogroups of N. meningitidis. Not to be bound by any particular theory, the vaccine of the present technology may also provide back up protection against the other pathogenic serogroups as well as the minor serogroups of meningococci since the subcapsular antigens on which it is based are shared across all serogroups of meningococci.

In preferred embodiments of the present technology, the genes of interest or DNA of interest is delivered and integrated into the bacterial chromosome by means of homologous and/or site specific recombination. Integrative vectors used to deliver such genes and/or operons can be conditionally replicative or suicide plasmids, bacteriophages, transposons, or linear DNA fragments obtained by restriction hydrolysis or PCR amplification as known by one skilled in the art. In some embodiments, integration is targeted to chromosomal regions dispensable for growth in vitro. In other embodiments, the gene of interest or DNA of interest can be delivered to the bacterium by means of episomal vectors such as circular-linear replicative plasmids, cosmids, plasmids, lysogenic bacteriophages, or bacterial artificial chromosomes. Selection of recombination events can be selected by means of selectable genetic markers such as genes conferring resistance to antibiotics (e.g., kanamycin, zeomycin, erythromycin, chloramphenicol, gentamycin, etc.), genes conferring resistance to heavy metal and/or toxic compounds or genes complementing auxotrophic mutations. Alternatively, recombination can be screened by PCR amplification, sequencing, restriction digestion or other methods known to one skilled in the art.

A "vaccine" as referred herein is defined as a pharmaceutical or therapeutic composition used to inoculate an animal in order to immunize the animal against infection by an organism, preferably a pathogenic organism. Vaccines typically comprise one or more antigens derived from one or more organisms which on administration to an animal will stimulate active immunity and protect that animal against infection with these or related pathogenic organisms.

The purified NOMVs are prepared for administration to mammals, suitably humans, mice, rats or rabbits, by methods known in the art, which include filtering to sterilize the solution, diluting the solution, adding an adjuvant and stabilizing the solution.

Vaccines of the present invention may be administered to a human or animal by a number of routes, including but not limited to, for example, parenterally (e.g. intramuscularly, transdermally), intranasally, orally, topically, or other routes know by one skilled in the art. The term "parenteral" as used herein includes intravenous, subcutaneous, intradermal, intramuscular, intraarterial injection, or infusion techniques. The vaccine may be in the form of a single dose preparation or in multi-dose vials which can be used for mass vaccination programs. Suitable methods of preparing and using vaccines can be found in Remington’s Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., Osol (ed.) (1980) and New Trends in Developments in Vaccines, Voller et al. (eds.), University Park Press, Baltimore, Md. (1978), incorporated by reference.

A vaccine composition of the present technology is typically administered parenterally in dosage unit formulations containing standard, well-known nontoxic physiologically acceptable carriers, adjuvants, and/or vehicles.

The vaccine compositions of the present technology may further comprise one or more adjuvants. An “adjuvant” is a substance that serves to enhance, accelerate, or prolong the antigen-specific immune response of an antigen when used in combination with specific vaccine antigens but do not stimulate an immune response when used alone. Suitable adjuvants include inorganic or organic adjuvants. Suitable inorganic adjuvants include, but are not limited to, for example, an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate (preferably aluminium hydroxide), 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 derivised polysaccharides or polypephazenes. Other suitable adjuvants are known to one skilled in the art. Suitable Th1 adjuvant systems may also be used, and include, but are not limited to, for example, Monophosphoryl lipid A, other non-toxic derivatives of LPS, and combination of monophosphoryl lipid A, such as 3-de-O-acetylated monophosphoryl lipid A (i.e. MPL) together with an aluminium salt.

Other suitable examples of adjuvants include, but are not limited to, MF59, MPLA, Mycobacterium tuberculos, Bordetella pertussis, bacterial lipopolysaccharides, aminoalkyl glucosamine phosphate compounds (AGP), or derivatives or analogs thereof, which are available from Corixa (Hamilton, Mont.), and which are described in U.S. Pat. No. 6,113,918; e.g., 2-[R]-3-Tetradecanoyl oxytetradecanoylaminojethyl, 2-Deoxy-4-O-phosphono-3-O-[R)-3-tetradecanoyloxytetradecanoyl]-2-[R]-3-tetradecanoyl oxytetradecanoyl]-b-D-glucopyranoside, MPL™ (3-O-deacetylated monophosphoryl lipid A) (available from Corixa) described in U.S. Pat. No. 4,912,094, synthetic polynucleotides such as oligonucleotides containing a CpG motif (U.S. Pat. No. 6,207,646), COG-ODN (CpG oligodeoxynucleotides), polypeptides, saponins such as Quil A or STIMULON™ QS-21 (Antigenics, Framingham, Mass.), described in U.S. Pat. No. 5,057,540, a per-
tussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63, LT-R72, CT-S109, PT-K9/G129; see, e.g., International Patent Publication Nos. WO 95/13302 and WO 92/19265, cholera toxin (either in a wild-type or mutant form). Alternatively, various oil formulations such as stearyl tyrosine (ST, see U.S. Pat. No. 4,258,029), the dipeptide known as MDP, saponin, cholera toxin B subunit (CTB), a heat labile enterotoxin (LT) from *E. coli* (a genetically toxoided mutant LT has been developed), and Emulsomes (Pharmos, LTD., Rehovot, Israel). Various cytokines and lymphokines are suitable for use as adjuvants. One such adjuvant is granulocyte-macrophage colony stimulating factor (GM-CSF), which has a nucleotide sequence as described in U.S. Pat. No. 5,078,996. The cytokine Interleukin-12 (IL-12) is another adjuvant which is described in U.S. Pat. No. 5,723,127. Other cytokines or lymphokines have been shown to have immune modulating activity, including, but not limited to, the interleukins 1-α, 1-β, 2, 4, 5, 6, 7, 8, 10, 13, 14, 15, 16, 17 and 18, the interferons-α, β and γ, granulocyte colony stimulating factor, and the tumor necrosis factors α and β, and are suitable for use as adjuvants.

**[0064]** The vaccine compositions can be lyophilized to produce a vaccine against *N. meningitidis* in a dried form for ease in transportation and storage. Further, the vaccine may be prepared in the form of a mixed vaccine which contains the NOMVs containing the proteins from the genetically altered strains described above and at least one other antigen as long as the added antigen does not interfere with the effectiveness of the vaccine and the side effects and adverse reactions are not increased additively or synergistically. The vaccine can be associated with chemical moieties which may improve the vaccine’s solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the vaccine, eliminate or attenuate any undesirable side effect of the vaccine, etc. Moieties capable of mediating such effects are disclosed in *Remington’s Pharmaceutical Sciences* (1980). Procedures for coupling such moieties to a molecule are well known in the art.

**[0065]** The vaccine may be stored in a sealed vial, ampule or the like. The present vaccine can generally be administered in the form of a spray for intranasal administration, or by nose drops, inhalants, swabs on tonsils, or a capsule, liquid, suspension or elixirs for oral administration. In the case where the vaccine is in a dried form, the vaccine is dissolved or suspended in sterilized distilled water before administration. Any inert carrier is preferably used, such as saline, phosphate buffered saline, or any such carrier in which the NOMV vaccine has suitable solubility.

**[0066]** Vaccine compositions of the present technology may include a carrier. If in a solution or a liquid aerosol suspension, suitable carriers can include, but are not limited to, salt solution, sucrose solution, or other pharmaceutically acceptable buffer solutions. Aerosol solutions may further comprise a surfactant.

**[0067]** Among the acceptable vehicles and solvents that may be used include water, Ringer’s solution, and isotonic sodium chloride solution, including saline solutions buffered with phosphate, lactate, Tris and the like. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium, including, but not limited to, for example, synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

**[0068]** Injectable preparations, for example sterile injectable aqueous or oleaginous suspensions, are formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation are also a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol.

**[0069]** The presently described technology and its advantages will be better understood by reference to the following examples. By providing these specific examples, the applicants do not limit the scope and spirit of the present technology. It will be understood by those skilled in the art that the full scope of the presently described technology encompasses the subject matter defined by the claims appended this specification, and any alterations, modifications, or equivalents of those claims.

**EXAMPLES**

Example 1: Derivation of the Genetically Modified Vaccine Strain of *N. meningitidis* and Production of NOMVs Containing the Outer Membrane Proteins of the Genetically Modified Vaccine Strain

**[0070]** The genetically modified strain 8570 HOPS-G1 was modified by five genetic modifications from a parental strain 8570 which had been analyzed by multilocus enzyme electrophoresis by the laboratory from whom the strain was obtained and determined to belong to the ET-5 clonal complex (Caugant, et al.) The PorA variable regions were sequenced typed and the LOS immunotype was verified before the genetic modifications were made. Strain 8570 was and ET-5 clone 4: Pl19, 15:1.7c, ProB3 (ST4) Tbp2 type II. A series of five sequential genetic modifications were made to the strain as described below:

**[0071]** 1) A second, different porA gene was inserted at the oprD locus knockout the oprD gene. pUC 19-based plasmid pA 18.4 has no antibiotic resistance marker in the insert, was used to insert a second porA gene into the chromosome at the oprD locus, disabling oprD by replacing a 100 bp sequence in the middle of the gene with the insert. The insert contained the new porA gene taken from strain M4410 (B:15: Pl22,14) and placed behind a porA promoter taken from strain H4476. The resulting porA type was Pl19,15: Pl22,14 containing the two porin A genes.

**[0072]** 2) Starting with the strain resulting from 1, with a second PorA expressed, the expression of the outer membrane protein OpcA was stabilized by replacing a 12 bp poly-C sequence in the promoter of opcA with a new sequence of the same length containing both C and G nucleotides. Original promoter sequence (Seq ID No. 1) (poly-G sequence italicized and bold) 5’S’ . . . CATATG-TAAAACCCTCTAAAATTGATGTAGTGATCGGATATGGTAATATGTAAC ATAACGTAAATAATCGTTACGCTTACAATTATATTCTTAAGCTTTCCGC MITTTT . 3’S’ was replaced with a modified promoter sequence (Seq ID No. 2) containing both G and C nucleotides with a Nof I site (underlined)

5’S’ . . . CATATGTTAAAACCCTCTAAAATTGATGTAGTGATCGGATATGGTAATATGTAAC ATTAACGTAAATAATCGTTACGCTTACAATTATATTCTTAAGCTTTCCGC ATTTTT TM TT 3’S’
The replacement sequence was chosen to contain a restriction site for NotI to enable verification of the presence of the replacement sequence. The plasmid used for the transformation was pOpC79 (Seq. ID No. 4). The plasmid insert does not contain an antibiotic marker. Selection of transformants was based on colony blotting with monoclonal antibody to OpcA. The strain to be transformed was chosen to be an OpcA negative phase variant, and strong OpcA positive clones were identified by colony blotting. True transformants were distinguished from OpcA positive phase variants by PCR and restriction enzyme (NotI) analysis.

Starting with the strain resulting from 2, the gene lpxL1, which is an acyl transferase responsible for linking one of two acyl-oxy-acyl linked fatty acids to the lipid A of the LOS, was disabled by replacing a 260 bp sequence in the middle of the lpxL1 gene with an insert containing the tetM antibiotic resistance gene. The tetM gene was obtained from a plasmid pS1954, which was derived from the transposon Tn916 (Swartley, et al. 1993. Mol. Microbiol. 10:299-310). The plasmid used to disable the lpxL1 gene was pMn5 (Seq. ID No. 5). The presence of the insert in the lpxL1 gene was verified by PCR which produced a 3.3 kbp amplicon using primers at the beginning and end of the lpxL1 gene.

Starting with the strain resulting from step 3, expression of the conserved outer membrane protein GNA 1870 (variant 1) (FHBP v.1) was increased by inserting a second copy of the GNA 1870 variant 1 gene in the nspA locus, knocking out expression of NspA. The newly inserted gene was part of an insert that contained a gentamicin antibiotic resistance gene, the E. coli lac operon with the IPTG-inducible Ptae promoter, the GNA1870 variant 1 gene and the rmb terminator; the plasmid used is depicted in FIG. 19c and Seq. ID No. 6. The pUC19 based plasmid, pBE/GNA1870/101, was used in the transformation and homologous recombination to insert the GNA 1870 variant 1 gene into the modified strain. pBE/GNA1870/101 plasmid (7687 b.p.) was constructed with the features as described in Table 1 (sequence can be found in Seq. ID No. 6).

<table>
<thead>
<tr>
<th>Feature</th>
<th>Coordinates (nt #)</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>pUC19***</td>
<td>1-191</td>
<td>New England Biolabs (NEB)</td>
</tr>
<tr>
<td>Sac I site (unique)</td>
<td>192-197</td>
<td>pUC19 cloning site</td>
</tr>
<tr>
<td>Uptake Sequence</td>
<td>198-212</td>
<td>PCR construct</td>
</tr>
<tr>
<td>5' NspA non coding region (NCR)</td>
<td>213-1248</td>
<td>N. mening., 44-76, PCR construct</td>
</tr>
<tr>
<td>Bam H1 site</td>
<td>1249-1254</td>
<td>GentR gene cloning site</td>
</tr>
<tr>
<td>GentR gene</td>
<td>1255-2104</td>
<td>PCR construct of GentR gene</td>
</tr>
<tr>
<td>Sac II (unique)</td>
<td>2105-2110</td>
<td>PCR construct</td>
</tr>
<tr>
<td>Rnp promoter 5' fragment (rest)</td>
<td>2111-2230</td>
<td>Previous plasmid for NspA expression</td>
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<tr>
<td>Mfe I site (unique)</td>
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<td>PCR construct</td>
</tr>
<tr>
<td>Lac promoter</td>
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<td>pMAL-p2X (New England Biolabs)</td>
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<tr>
<td>Ptae promoter</td>
<td>3642-3673</td>
<td>pMAL-p2X, PCR construct</td>
</tr>
<tr>
<td>Lac operator</td>
<td>3674-3702</td>
<td>pMAL-p2X, PCR construct</td>
</tr>
<tr>
<td>RBS</td>
<td>3750-3755</td>
<td>pMAL-p2X, PCR construct</td>
</tr>
<tr>
<td>Nde I site (unique)</td>
<td>3761-3766</td>
<td>PCR construct</td>
</tr>
<tr>
<td>FHBP (variant 1) leader peptide</td>
<td>3764-3823</td>
<td>N. mening., 44-76, PCR construct</td>
</tr>
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<td>FHBP (variant 1) ORF with stop codon</td>
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<tr>
<td>3' NspA and 3'NspA NCR</td>
<td>4597-4638</td>
<td>Previous plasmid for NspA expression</td>
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<td>rmb transcription terminators</td>
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<td>pBAD/His-E (Invitrogen), PCR</td>
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<td>Uptake Sequence</td>
<td>5433-5447</td>
<td>PCR construct</td>
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<tr>
<td>Hind III</td>
<td>5448-5412</td>
<td>pUC19 cloning site</td>
</tr>
<tr>
<td>pUC 19</td>
<td>5454-7887 end</td>
<td>N. mening.</td>
</tr>
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</table>

***Start from nt 1 of pUC 19. The plasmid was modified to remove Nde I site for further convenient cloning as follows. It was deleted by Nde I-EcoRV 1 and 213 bp fragment was removed. Nahk ends were filled as and ligated to restore the plasmid. As a result sited Nde I (183) and EcoRV (385) were destroyed. For cloning of constructs for the expression of target protein we used Sac I and Hind III cloning sites of pUC 19.

The strain resulting from step 4 was transformed with a pUC19-based plasmid containing the synX gene in which a 200 bp sequence was replaced by a kanamycin resistance gene. Kan resistant transformants were selected and tested by PCR for the presence of the disrupted synX gene and for the capsule negative phenotype. The results verified the knockout of the synX gene.

The strain resulting from step 4 was transformed with a plasmid pBE-501 containing zeoancycin gene knocking out the lgtA gene (Seq. ID No. 9). Plasmid pBE-501 contained the features found in Table 2. Knock-out of the lgtA gene produced expression of a shortened or truncated LOS that lacks the ltae-N-neotetraose (LNaT) tetrasaccharide (see FIG. 20).

<table>
<thead>
<tr>
<th>Feature</th>
<th>Coordinates (nt #)</th>
<th>Source</th>
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<tbody>
<tr>
<td>pCR 4-TOPO TA cloning Vector</td>
<td>1-3667</td>
<td>Invitrogen. Type: pUC ori</td>
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<tr>
<td>Uptake Sequence</td>
<td>3668-3682</td>
<td>PCR construct</td>
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**TABLE 2-continued**

<table>
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<th>Feature</th>
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<tr>
<td>LgtA 5' segment</td>
<td>3683-4037</td>
<td>N. mening., 2996, PCR construct</td>
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<tr>
<td>pEM7/Zeo</td>
<td>4038-4122</td>
<td>Invitrogen. Cloning site and EM7 promoter Zeocin</td>
</tr>
<tr>
<td>4123-4497</td>
<td>Zeocin gene provided</td>
<td>LgtA pEM7/Zeo</td>
</tr>
<tr>
<td>4496-4533</td>
<td>Fragment of pEM7/Zeo cloning site LgtA 3'</td>
<td></td>
</tr>
<tr>
<td>segment</td>
<td>4636-5448</td>
<td>N. mening., 2996, PCR construct</td>
</tr>
<tr>
<td>Uptake Sequence</td>
<td>5440-5463</td>
<td>Uptake sequence</td>
</tr>
<tr>
<td>pCR 4-TOPO TA cloning Vector</td>
<td>5464-5759</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

*) LgtA cDNA was digested by BsiHI II. Resulting 3' and 5' sticky ends were filled and Zeocin gene was inserted in this blunt-ended DNA. In case of excision of disruptive Zeocin gene which may occur during the repairation of bacterial DNA, religated 3' and 5' of LgtA fragments will bearing untranslated sequence.

[0077] This genetically modified strain was tested to ensure retention of all five mutations and expression of all expected antigens.

**Example 2: Production of Vaccine Amounts of the Genetically Modified Strain**

[0078] The genetically modified strains were then used for production of master and production cell banks for use in vaccine manufacture as detailed in the flow-charts in FIGS. 1-5 to produce a composition of NOMVs. The NOMVs culture is tested for the expression of the outer membrane proteins and LOS.

**Example 3: Characterization of Vaccines**

[0079] The final product obtained from Example 2 was subjected to quality control testing and preclinical safety and immunogenicity testing in mice and rabbits.

[0080] The composition of the final product vaccine was:

- **[0081]** Protein 2001 ag/ml
- **[0082]** Lipooligosaccharide 36 mg/ml
- **[0083]** Nucleic Acid 2.5 mg/ml
- **[0084]** Sodium Chloride 0.9%
- **[0085]** Tris-HCl Buffer 0.01 M pH 7.6

[0086] The vaccine composition was further analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting. FIG. 6 depicts coomassie blue stained gel showing protein content in the vaccine (lane 4) as compared to control (lane 2) and filtered bulk lot (lane 3). FIG. 7 depicts silver stain gel showing the lipopolysaccharide component of the vaccine (lane 3) as compared control (ML5 LPS, lane 1) and filtered bulk vaccine lane (lane 2). FIG. 8 depicts the results of identity testing of the vaccine for the major component of the NOMV’s vaccine according to the antibodies as listed in Table 3.

**TABLE 3-continued**

<table>
<thead>
<tr>
<th>Lane</th>
<th>Antibody Specificity</th>
<th>Monoclonal Antibody</th>
<th>Expected Reaction</th>
<th>Test Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>TBP2</td>
<td>476/2G2</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>16</td>
<td>Gp B Polysaccharide</td>
<td>2-2-2</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>17</td>
<td>Amido Black Stain</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[0087] The results are found in FIGS. 6, 7 and 8 showing proteins found in the NOMV of the vaccine from the genetically modified strain 8570 HOPS-G NOMV contain the proteins and LOS as described.

**Example 4: General Safety Test of the Vaccine**

[0088] The vaccine was tested in the General Safety Test as prescribed in 21 CFR 510.11. The results for the vaccine are given in Table 4.

**TABLE 4**

<table>
<thead>
<tr>
<th>Test Article</th>
<th>Test Result</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>8570 HOPS-G NOMV Vaccine Lot # 1289</td>
<td>Passed</td>
<td>All animals remained normal and healthy and gained weight</td>
</tr>
</tbody>
</table>

**Example 5: Rabbit Pyrogenicity Test**

[0089] The results of the rabbit pyrogen test for endotoxin activity are given in Table 3 for the genetically modified vaccine 8570 HOPS-G NOMV alone and the vaccine adsorbed to aluminum hydroxide adjuvant. The values given are the highest amount tested that did not induce a fever in the rabbits (temperature increase of >0.5°C). Results of which are found in Table 5.

**TABLE 5**

<table>
<thead>
<tr>
<th>Test Article</th>
<th>Concentration (μg/kg)</th>
<th>Temperature Rise (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8570 HOPS-G NOMV Vaccine Lot # 1289</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>8570 HOPS-G NOMV Vaccine Lot # 1289 adsorbed to aluminum hydroxide (HFA) lot # 1347</td>
<td>0.5</td>
<td>0</td>
</tr>
</tbody>
</table>

*) These tests were performed by BioReliance, Inc. under GLP following the protocol specified in the CFR. ** Amount of aluminum hydroxide/kg that will be used in the human study for all formulations (doses).

[0090] In summary, the vaccine alone passed at 0.4 μg/kg, the aluminum hydroxide adjuvant passed at 15 μg/kg (the largest amount per kg to be used in the clinical study), and the vaccine adsorbed to aluminum hydroxide passed at 0.5 μg/kg but failed at 1.0 μg/kg. Extrapolation of these results on a μg/kg basis suggest the adsorbed vaccine would be non-pyrogenic in humans up to a dose in the range of 25-50 μg.
Example 6: Cytokine Release from Whole Human Blood

[0091] The vaccine was tested for endotoxin content by measuring its ability to induce proinflammatory cytokines TNF-alpha and IL-6, from fresh whole human blood. The results are shown in FIGS. 9 and 10. The data are the mean of 3 (E. Coli LPS Standard and NOMV vaccine lot 1119 with lpxL2 LOS) or 5 (Lot 0832 NOMV with wild type LOS, and Lot 1289 NOMV vaccine with lpxL1 LOS) tests. The error bars are the standard error of the mean. The concentration of NOMV is based on protein but the E. Coli standard LPS is based on LPS by weight. Not to be bound by any particular theory, these results suggest that the current vaccine may have a similar safety profile in human volunteers as was seen with the lpxL2 LOS containing vaccine (Meningococcal 44/76 MOS 5D NOMV vaccine, Lot #1119, BB-IND 12687).

[0092] The activity of the 8570 HOPS-G NOMV Vaccine Lot #1289 was compared to the activity of deoxycholate extracted outer membrane vesicles (OMV). The vesicles were prepared using the basic method described by Fredrikson J J, et al. NIH Annals, 14:67-80, 1991, except 0.5% deoxycholate (DOC) was used throughout the procedure rather than using 1.2% DOC to resuspend the ultra centrifuge pellets. The results of this comparison are shown in FIG. 11.

Example 7: Immunogenicity in Mice and Bacterial Antibiotic Response

[0093] Mice were given three doses of genetically modified vaccine strain 8570 HOPS-G at four week intervals with or without adsorption to aluminum hydroxide adjuvant (Rehydragel LV). Groups of 10 mice were vaccinated intraperitoneally at 0, 4 and 8 weeks with 0.1, 0.3, 1.0 or 3.0 μg of NOMV, the vaccine groups are listed in Table 6. Serum was taken at 0, 7 and 10 weeks. The sera were tested for bactericidal antibodies against four different strains, the parent of the vaccine strain and several related strains using normal human serum as a source of complement. Pre-vaccination sera were uniformly lacking in bactericidal activity.

| TABLE 6 |
|---|---|
| Vaccine Group | Vaccine amount injected |
| 1 | 0.1 μg |
| 2 | 0.3 μg |
| 3 | 1.0 μg |
| 4 | 3.0 μg |
| 5 | 0.1 μg + Rehydragel LV |
| 6 | 0.3 μg + Rehydragel LV |
| 7 | 1.0 μg + Rehydragel LV |
| 8 | 3.0 μg + Rehydragel LV |
| 9 | 1.0 μg + Rehydragel JHA |

[0094] The results obtained with the 10-week sera (three doses of vaccine) are shown in FIG. 12 showing the bactericidal titer of the different vaccine groups for the genetically modified strains. Two of the test strains were isogenic with the parent of the vaccine strain. They were derived from the parent strain by replacing the porA gene with an alternate porA having a different serosubtype specificity. Two of the PorA proteins expressed in these test strains are present in the vaccine (P1.19,15 and P1.22,14), but the third (P1.22-14) is not. The fourth strain, 44/76, has a different PorA, a different PorB, and a different LOS core structure as compared to the vaccine strain. Surprisingly different to published studies in which deoxycholate extracted vesicle vaccines show the PorA antigen as typically the dominant antigen, the results of the vaccine of the present technology demonstrates that the majority of the bactericidal activity was not dependent on the serosubtype of the target strain and hence not against PorA.

[0095] Bacterial antibodies induced in mice by the 8570 HOPS-G NOMV vaccine do not show serosubtype specificity, but appear mostly independent of serosubtype and serotype (FIG. 13). The antibodies killing strain 44/76 were found to be mainly directed against the LOS. Bars are standard error of the mean. The vaccine was administered with and without adsorption to Rehydragel LV aluminum hydroxide adjuvant.

[0096] Analysis of the specificity of the bactericidal antibody response against the heterologous strain 44/76 was undertaken by depletion of bactericidal activity with different isolated antigens. Post-vaccination mouse serum was diluted to the bactericidal endpoint (~50% killing) and incubated in 96-well microplate wells coated with different concentrations of several antigens. LOS and strain 44/76 the serum was tested for bactericidal activity and the percent removal of bactericidal antibody determined. Purified LOS prepared from the target strain (immunotype L3.7) was able to remove nearly all the antibody. Purified LOS (immunotype L8v) prepared from the vaccine strain was able to remove about 70% of the antibody. The conserved protein GNA1870 (purified, recombinant protein) appeared to remove about 20% of the bactericidal activity, which, not to be bound by any particular theory, may indicate some cooperative killing involving both anti-LOS antibody and anti-GNA1870 antibody as shown in FIG. 14.

Example 8: Immunogenicity in Rabbits

[0097] The vaccine was also tested for immunogenicity in rabbits. Groups of four rabbits were vaccinated intramuscularly with different doses of vaccine, with or without adsorption to aluminum hydroxide adjuvant. Three doses were given at six week intervals and blood was drawn two weeks after the last injection. The bactericidal antibody response of the rabbits to four test strains was determined. The test strains included 5 isogenic variants of 8570 expressing different PorA proteins and L3.7v LOS and which has a heterologous PorA and LOS with a different core structure. PorA proteins P1.19,15 and P1.22,14 were present in the vaccine, but P1.22-1,4 was not. The results of the bactericidal tests are given in FIG. 15. Analysis of the cross reactive bactericidal activity toward strain 44/76 was analyzed in the same manner as for the mouse sera and the results were essentially the same. Most of the cross-reactive bactericidal antibodies could be removed by purified LOS homologous to the test strain.

Example 9: Preparation and Animal Testing of a Laboratory Lot of the Complete Multivalent NOMV Vaccine

[0098] In addition to strain 8570 HOPS-G1 which was described in the Examples above, two additional vaccine strains were selected and genetically modified. The first was strain B168B (B:2-n,P1.05.2,L1.2). This strain belongs to the genetic group ET-37 and has a class 2 PorB protein and type I transferrin binding protein B. The second was strain 44/76 (B:15,P1.7,16,L3.7), which belongs to the genetic group E1-5 and is representative of the epidemic strain responsible for the group B meningococcal epidemic in Norway in the 1950’s and 1980’s. It expresses class 3 PorB protein and type II transferrin binding protein B.

[0099] Strain B168B was genetically modified in much the same manner as described for strain 8570 HOPS-G1. Two genes were disabled, synX and lpxL1, to prevent capsule synthesis and sialylation of LOS and to reduce the toxicity of the LOS. A second porA gene (subtype P1.22-4) was inserted in place of the opaD gene. Variant 2 of GNA
1870 (FHBP) with the IPTG inducible E. coli Ptac promoter, was inserted in place of the nspA gene as a second copy using plasmid pBE-201 (Seq. ID No. 7). Plasmid pBE-201 (7687 b.p. for additional expression of FHBP (variant 2)) was constructed with the features as described in Table 7.

TABLE 7

<table>
<thead>
<tr>
<th>Feature</th>
<th>Coordinates (at #)</th>
<th>Source</th>
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<tbody>
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<td>pUC19**</td>
<td>1-191</td>
<td>New England Biolabs (NEB)</td>
</tr>
<tr>
<td>Sac I site (unique)</td>
<td>192-197</td>
<td>pUC19</td>
</tr>
<tr>
<td>Uptake Sequence</td>
<td>198-212</td>
<td>PCR construct</td>
</tr>
<tr>
<td>5' NspA NCR</td>
<td>213-1248</td>
<td>N. mening., 44-76, PCR construct</td>
</tr>
<tr>
<td>Bam H I site</td>
<td>1240-1254</td>
<td>Gent* gene cloning site</td>
</tr>
<tr>
<td>Gent* gene</td>
<td>1255-2104</td>
<td>PCR construct of GentR gene</td>
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<tr>
<td>Sac II (unique)</td>
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<td>PCR construct</td>
</tr>
<tr>
<td>Rmp promoter 5' fragment (rest)</td>
<td>2111-2230</td>
<td>N. mening., PCR construct</td>
</tr>
<tr>
<td>Mfe I site (unique)</td>
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<td>PCR construct</td>
</tr>
<tr>
<td>Lac* operon</td>
<td>2237-3641</td>
<td>pMAL-p2X (New England Biolabs)</td>
</tr>
<tr>
<td>Puc promoter</td>
<td>3642-3673</td>
<td>pMAL-p2X, PCR construct</td>
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<td>Lac operator</td>
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<tr>
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</tr>
<tr>
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<td>Previous plasmid for NspA expression</td>
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<td>Uptake Sequence</td>
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</tr>
<tr>
<td>pUC 19</td>
<td>5454-7687 end</td>
<td>NEB, (AmpR)</td>
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</tbody>
</table>

*Start from nt. 1 of pUC 19. The plasmid was modified to remove Nde I site for further convenient cloning as follows: It was digested by Nde I-EcoRI and 213 b.p. fragment was removed. Sticky ends were filled in and ligated to restore the plasmid. As a result sites Nde I (183) and EcoRI (795) were destroyed. For cloning of constructs for the expression of target protein we used Sac I and Hind III cloning sites of pUC 19.

A phase variant of the resulting strain expressing a truncated alpha chain consisting of glucose and galactose, L2 LOS was selected by colony blotting. The resulting genetically modified strain was designated B16B6 HPS-G2, see FIG. 18.

[0100] Strain 44/76 was also modified genetically in the same pattern as described for strain 8570 HOPS-G1. The two genes, synX and lpxL1, were disabled by insertion mutagenesis, a second porA gene (subtype P1.7-1, 1) was inserted along with its promoter in place of the opu32 gene, and a second copy of nadA was inserted behind a porA promoter in place of the nspA gene. Plasmid pBE-311 was used for homologous recombination to insert the NadA gene, the plasmid 3-11 was constructed with the features as described in Table 8 and the sequence can be found in Seq. ID No. 8.

TABLE 8

<table>
<thead>
<tr>
<th>Feature</th>
<th>Coordinates (at #)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
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<td>1-191</td>
<td>New England Biolabs (NEB)</td>
</tr>
<tr>
<td>Sac I site (unique)</td>
<td>192-197</td>
<td>pUC19 cloning site</td>
</tr>
<tr>
<td>Uptake Sequence</td>
<td>198-212</td>
<td>PCR construct</td>
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<tr>
<td>5' NspA NCR</td>
<td>213-1248</td>
<td>N. mening., 44-76, PCR construct</td>
</tr>
<tr>
<td>Bam H I site</td>
<td>1240-1254</td>
<td>Gent* gene cloning site</td>
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<tr>
<td>Gent* gene</td>
<td>1255-2104</td>
<td>PCR construct of GentR gene</td>
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<tr>
<td>Sac II (unique)</td>
<td>2105-2110</td>
<td>PCR construct</td>
</tr>
<tr>
<td>PscA promoter (44-76) (modified)**</td>
<td>2111-3266</td>
<td>N. mening., 44-76, PCR construct</td>
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<td>NadA (allele 3) leader peptide</td>
<td>3267-3272</td>
<td>N. mening., 2996, PCR construct</td>
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<tr>
<td>NadA (allele 3) ORF with stop codon</td>
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<td>PscA terminator (44-76)</td>
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<td>Bam I</td>
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<td>Uptake Sequence</td>
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<tr>
<td>Hind III</td>
<td>5345-5350</td>
<td>pUC19 cloning site</td>
</tr>
<tr>
<td>pUC 19</td>
<td>5351-7584 end</td>
<td>NEB, (AmpR)</td>
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</tbody>
</table>

*Start from nt. 1 of pUC 19. The plasmid was modified to remove Nde I site for further convenient cloning as follows: It was digested by Nde I-EcoRI and 213 b.p. fragment was removed. Sticky ends were filled in and ligated to restore the plasmid. As a result sites Nde I (183) and EcoRI (785) were destroyed. For cloning of constructs for the expression of target protein we used Sac I and Hind III cloning sites of pUC 19.

**The 14Gs Poly G tract of the 44-76 promoter was modified by replacing with optimal for the expression of 11 Gs.
In addition, expression of OpcA was stabilized by curing the phase variation associated with its gene. This was done as described for strain 8570 HOPS-G1 by breaking up the poly-G string in its promoter in Example 1. The IgA gene was interrupted as in Example 1 producing a truncated LOS. A phase variant of the resulting strain expressing the L8 immunotype was selected by colony blotting with an L8 specific monoclonal antibody. This genetically modified strain was designated 44/76 HOPS-D as shown in Fig. 18. The two additional strains were characterized to confirm stability of all the genetic modifications and stocks of each were frozen down.

Example 10: Preparation of NOMV Vaccine from Strains B16B6 HIPS-G2 and 44/76 HOPS-D

The three genetically modified strains were used to prepare laboratory lots of NOMV vaccine compositions. The doses were given at 0, 4, and 8 weeks. Blood was drawn pre-vaccination and 2 weeks following the last vaccination (at 10 weeks).

Sera from individual mice were tested for bactericidal antibodies against the homologous strains, and pooled serum from each group of 10 mice was tested against a panel of 14 heterologous group B strains and 1 group C strain expressing a broad range of different subcapsular antigens.

The combined multivalent vaccine induced a geometric mean 1:256 titer against each of the three vaccine strains and a 4-fold or greater increase in bactericidal antibodies against 13 of the 15 heterologous strains. Two of the test strains were not killed in spite of having an antigen shared with one of the vaccine strains. The bactericidal titers observed against the panel of strains are given in the Table 9.

### Table 9

<table>
<thead>
<tr>
<th>Bactericidal</th>
<th>Titer of Pooled Serum from Mice Vaccinated with Indicated Vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Strain</td>
<td>B1 + B2 + B3</td>
</tr>
<tr>
<td>44/76</td>
<td>256 256 256 256 1</td>
</tr>
<tr>
<td>8570</td>
<td>256 256 256 256 2</td>
</tr>
<tr>
<td>816136</td>
<td>256 256 256 256 2</td>
</tr>
<tr>
<td>9162</td>
<td>16 16 8 2 1</td>
</tr>
<tr>
<td>M1080</td>
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</tr>
<tr>
<td>3576</td>
<td>128 128 4 2 16</td>
</tr>
<tr>
<td>8047</td>
<td>64 128 1 1 128</td>
</tr>
<tr>
<td>9547</td>
<td>256 256 128 2 64</td>
</tr>
<tr>
<td>531</td>
<td>256 256 256 1 256</td>
</tr>
<tr>
<td>7698</td>
<td>256 128 1 1 128</td>
</tr>
<tr>
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<td>1901</td>
<td>256 32 128 1 8</td>
</tr>
<tr>
<td>9889</td>
<td>512 512 16 8 256</td>
</tr>
<tr>
<td>6275</td>
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<tr>
<td>126E</td>
<td>256 256 4 2 64</td>
</tr>
<tr>
<td>2981</td>
<td>32 8 4 64 2</td>
</tr>
<tr>
<td>46720</td>
<td>1 1 1 1 1</td>
</tr>
<tr>
<td>5557</td>
<td>32 16 1 32 16</td>
</tr>
</tbody>
</table>

strains were grown in Catlin’s modified medium as one liter cultures in Fernbach flasks on a rotary shaker. The cells were harvested by centrifugation, weighed and the cell paste frozen. The cell paste was thawed and used to prepare NOMV following essentially the same procedure as described for the clinical lot of vaccine from strain 8570 HOPS-G1 as described in Example 2. The process was scaled down and ultracentrifugation twice at 225,000g for 60 min at 2-8°C. to remove nucleic acids and all soluble, non-vesicle material.

Example 11: Immunization of Mice with Complete Multivalent Vaccine

Groups of ten CD-1 mice were vaccinated intraperitoneally with two pg of NOMV vaccine from each genetically modified vaccine strain (6 pg total for the combined vaccine with NOMV from three strains). Three

**Vaccine Code:**

- **B1** = 44/76 HOPS-D NOMV
- **B2** = 8570 HOPS-G1 NOMV
- **B3** = B16B6 HOPS-G2 NOMV

These results demonstrate the ability of the combined vaccine to induce bactericidal (protective) antibodies against a broad range of group B strains and potentially strains of other serogroups as well.

Analysis of the bactericidal antibodies using a bactericidal depletion test demonstrated that antibodies to all three sets of antigens were involved in killing at least some of the test strains. In some cases, it appeared that antibodies to more than one antigen were involved and acted together to produce bactericidal activity against a given strain.

Additional groups of mice were vaccinated with NOMV vaccine prepared from isogenic mutants of strain 8570 HOPS-G1. The mutant strains differed in their expression of PorA. Two mutants expressed a single PorA (one or the other of the two in the multivalent vaccine strain) and the third was a PorA knockout mutant expressing no PorA protein. Bactericidal titers induced by each of the four strains against several different test strains are shown in Table 10.
TABLE 10

| Mutant of 8570 HOPS-G1 from Which NOMV Vaccine Was Prepared |
|----------------------------------|------------------|------------------|------------------|
| Test Strain | 8570 (P1.19,15) | 8570 (P1.22,141) | 8570 (P1.19,15) |
| 8570        | 256             | 256             | 256             |
| 4476        | 256             | 256             | 256             |
| B16B6       | 1               | 1               | 1               |
| 9547        | 9               | 4               | 4               |
| 2981        | 64              | 1               | 16              |
| 6557        | 32              | 1               | 128             |
| APorA       |                 |                 |                 |

For the first five test strains in Table 10, the PorA expression had no effect on the titer of bactericidal antibodies induced by the vaccine. For the last two strains, which both express P1.14, the presence of the P1.14 epitope in the vaccine correlated with the capacity of the respective serum to kill the strain. This demonstrates that antibodies to PorA are involved in the observed killing for some strains. For other strains such as the homologous strain and strain 44/76, other antigens are responsible for most of the bactericidal activity. This was demonstrated by analysis with the bactericidal depletion assay. Results of one such assay are given in FIG. 16. The results shown in FIG. 17 demonstrate that antibodies to LOS and FHBP (GNA1870) were involved in the killing of strain 8570 by antiserum to PorA knockout mutant of 8570 HOPS-G1.

1. A vaccine comprising native outer membrane vesicles obtained from at least two meningococcal strains that have been genetically modified to provide broad based protection, wherein the native outer membrane vesicles include three different sets of antigens based on PorA, LOS, and conserved outer membrane proteins; wherein the genetically modified strains have been modified to provide enhanced safety based on inactivation of lpxL1, synX, and IgA genes; and wherein at least one of the genetically modified strains expresses at least two different PorA subtype proteins or subtype epitopes.

2. The vaccine of claim 1 wherein the LOS expressed by each strain has a different LOS core structure and has an alpha chain consisting of glucose and galactose.

3. The vaccine of claim 1 where each strain expresses at least two different PorA subtype proteins or subtype epitopes which are chosen based on the most prevalent of PorA subtypes among group B case isolates.

4. The vaccine of claim 1 wherein a different conserved surface protein with demonstrated capacity to induce bactericidal antibodies is over-expressed in each strain and are taken from the group consisting of FHBP (GNA1870) variants 1, FHBP variants 2, and FHBP variants 3; NadA; App; NspA; TbpA and TbpB.

5. A combination of NOMVs from three genetically modified, antigenically diverse Neisseria meningitidis strains, wherein at least one of the strains is selected from:

(1) H44/76 HOPS-DL which has the following genetic modifications or characteristics:
   - inactivation of the genes synX, lpxL1, and IgA;
   - insertion of a second porA gene in place of opaD;
   - increased expression of factor H binding protein variant 1;
   - and stabilized high expression of PorA and Opc;
   - (2) 8570 HOPS-G4,L which has the following genetic modifications or characteristics:
     - inactivation of the genes synX, lpxL1, and IgA;
     - insertion of a second porA gene in place of opaD;
     - increased expression of factor H binding protein variant 1;
     - and stabilized high expression of PorA and Opc;
   - (3) B16B6 HOPS-G2,A which has the following genetic modifications or characteristics:
     - inactivation of the genes synX, lpxL1, and IgA;
     - insertion of a second porA gene in place of opaD;
     - increased expression of factor H binding protein variant 2;
     - and stabilized high expression of PorA and Opc.

6. The combination of vaccine strains of claim 5 wherein strain H44/76 HOPS-DL was derived from the ET-5 wild type strain H44/76 (B:15: P1.7,16: L3,7:P5.5,C).

7. The combination of vaccine strains of claim 5 wherein strain 8570 HOPS-G4,L was derived from the ET-5 wild type strain 8570 (B:4: P1.19,15: L3,7:P5.5,11,C).

8. The combination of vaccine strains of claim 5 wherein strain B16B6 HOPS-G2,A is derived from the ET-37 wild type strain B16B6 (B:2a; P1.5,2: L2:P5.1,2,5).

9-13. (canceled)

14. A vaccine composition against meningococcal disease comprising native outer membrane vesicles (NOMVs) from one or more genetically modified strains of Neisseria meningitidis, wherein the one or more genetically modified strains has been modified by:
   - a. inactivation of the synX gene,
   - b. inactivation of the lpxL1 gene,
   - c. inactivation of the IgA gene in each strain resulting in expression of a shortened or truncated lipooligosaccharides (LOS) that lacks N-neotetraose tetrascarbohydrate, and
   - d. insertion of at least one second antigenically different porA gene in place of the opa gene.

15. (canceled)

16. The vaccine composition of any one of claim 14 wherein the genetically modified strain further comprises stabilized expression of at least one outer membrane protein, wherein the outer membrane protein is selected from the group comprising Opc and PorA.

17. (canceled)

18. (canceled)

19. (canceled)

20. A genetically modified vaccine strain of Neisseria meningitidis subtype B derived from H44/76 strain comprising the genetic modifications of
   - i. inactivation of a synX gene,
   - ii. inactivation of the lpxL1 gene,
   - iii. inactivation of the IgA gene,
   - iv. insertion of a second porA gene in place of the opaD gene,
   - v. increased expression of NadA compared with the native strain, and
   - vi. stabilized increased expression of Opc and PorA proteins.

21. The genetically modified strain of claim 20 wherein strain H44/76 HOPS-DL was derived from the ET-5 wild type strain H44/76 (B:15: P1.7,16: L3,7:P5.5,C).

22. (canceled)
23. A genetically modified vaccine strain of *Neisseria meningitidis* subtype B strain: derived from 8570 comprising the genetic modifications of:
i) inactivation of a synX gene,
ii) inactivation of the lpXl1 gene,
iii) inactivation of the lgtA gene,
iv) insertion of a second porA gene in place of opaD;
v) increased expression of factor H binding protein variant 1; and
vi) stabilized increased expression of PorA and Opc proteins.
24. The genetically modified strain of claim 23, wherein the genetically modified strain was derived from the ET-5 wild type strain 85 70/B:4: P 1.19,15: L3,7,v: P5.5,11,C).
25. (canceled)
26. A genetically modified vaccine strain of *Neisseria meningitidis* subtype B derived from B16B6 comprising the genetic modifications of:
i) inactivation of a synX gene,
ii) inactivation of the lpXl1 gene,
iii) inactivation of the lgtA gene,
iv) insertion of a second porA gene (subtype P1.22-1.4) in place of opaD;
v) increased expression of factor H binding protein variant 2; and
vi) stabilized increased expression of PorA and Opc proteins.
27. The genetically modified strain of claim 26, wherein the genetically modified strain is derived from the ET-37 wild type strain B16B6 (B:2a:P 1.5,2; L2:P5.1,2,5).
28.-29. (canceled)
30. A vaccine composition comprising NOMV from one or more genetically modified strain of *Neisseria meningitidis* subtype B selected from the group consisting of:
a) a genetically modified vaccine strain of *Neisseria meningitidis* subtype B derived from: H44/76 strain comprising the genetic modifications of
i) inactivation of a synX gene,
ii) inactivation of the lpXl1 gene,
iii) inactivation of the lgtA gene,
iv) insertion of a second porA gene in the place of a opaD gene,
v) increased expression of NadA compared with the native strain, and
vi) stabilized increased expression of Opc and PorA proteins;
b) a genetically modified vaccine strain of *Neisseria meningitidis* subtype B strain derived from 8570 comprising the genetic modifications of:
i) inactivation of a synX gene,
ii) inactivation of the lpXl1 gene,
iii) inactivation of the lgtA gene,
iv) insertion of a second porA gene in place of opaD;
v) increased expression of factor H binding protein variant 1; and
vi) stabilized increased expression of PorA and Opc proteins; and
c) a genetically modified vaccine strain of *Neisseria meningitidis* subtype B derived from B16B6 comprising the genetic modifications of:
i) inactivation of a synX gene,
ii) inactivation of the lpXl1 gene,
iii) inactivation of the lgtA gene,
iv) insertion of a second porA gene (subtype P1.22-1.4) in place of opaD;
v) increased expression of factor H binding protein variant 2; and
vi) stabilized increased expression of PorA and Opc proteins.
31. The vaccine composition of claim 30, wherein the vaccine composition comprises NOMVs from two or more genetically modified strains.
32. (canceled)
33. The vaccine composition of claim 30, wherein the vaccine composition comprises NOMVs from three or more genetically modified strains.
34.-37. (canceled)
38. A vaccine against meningococcal disease comprising a variety of native outer membrane vesicles (NOMVs), wherein at least some of the NOMVs are essentially free of expression or siaylation of lipooligosaccharide (LOS), contain LOS that includes a lipid A with a penta-acyle structure and contain increased expression levels of at least one minor conserved outer membrane protein, wherein the minor conserved outer membrane protein is selected from proteins that induce bactericidal antibodies.
39. The vaccine of claim 38, wherein the minor conserved outer membrane protein is selected from the group consisting of NadA, factor H binding protein (FHBP) variant 1, and FHBP variant 2.
40. The vaccine of claim 38, wherein at least some of the NOMV comprise shortened or truncated LOS that are essentially free of lacto-N-neotetraose (Lnt) tetrasaccharide.
41. The vaccine of claim 38, wherein at least some of the NOMV comprise two or more different PorA proteins.
42.-44. (canceled)
45. A method of preparing a genetically modified strain of *N. meningitidis* comprising:
a) selecting a strain of meningococcal type B able to be genetically modified;
b) genetically modifying the strain by inactivating the synX gene,
c) genetically modifying the strain by inactivating the lpXl1 gene,
d) genetically modifying the strain by inactivating the lgtA gene, and
e) genetically modifying the strain by increasing expression of one or more minor conserved outer membrane proteins.
46. The method of claim 45, further comprising:
.genetically modifying the strain by inserting at least one second antigenically different porA gene into the open reading frame of the opa gene.
47. The method of claim 45, further comprising:
.genetically modifying the strain to stably express or overexpress at least one outer membrane protein by replacing the poly-C sequence within the promoter or open reading frame of the at least one outer membrane protein with a sequence containing G and C nucleotides.
48. A method of preparing a vaccine against meningococcal disease comprising the steps of:
a) culturing a genetically modified strain of *N. meningitidis* comprising one or more modification selected from the group consisting of:
a. inactivation of the synX gene,
b. inactivation of the lpXl1 gene,
c. inactivation of the IgtA gene,
d. insertion of at least one second antigenically different porA gene in place of the opa gene,
e. increased or stable expression of at least one minor conserved outer membrane protein, and
f. stabilized expression of at least one outer membrane protein;
b) expanding the culture by fermentation using the cultured strain of a) to inoculate medium in a fermentor;
c) inactivating the fermented culture;
d) harvesting *N. meningitidis* cultured cells by continuous flow centrifugation and collecting cell paste;
e) isolating NOMVs from the cell paste; and
f) resuspending NOMVs in buffer or carrier suitable for vaccine administration.