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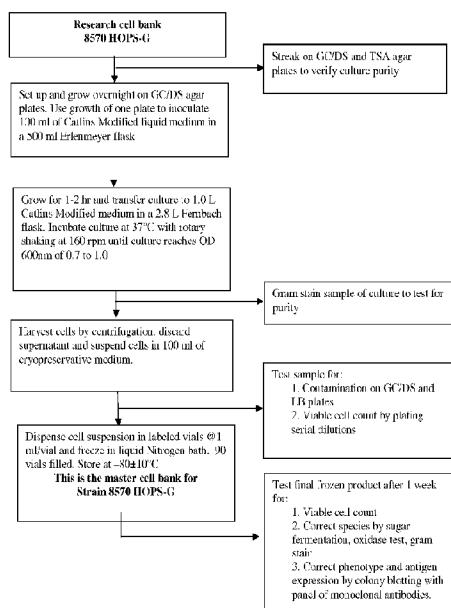
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*[Continued on next page]*

(54) Title: MENINGOCOCCAL MULTIVALENT NATIVE OUTER MEMBRANE VESICLE VACCINE, METHODS OF MAKING AND USE THEREOF

FIGURE 1

Master Cell Bank Preparation



(57) 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.

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TITLE

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 Number 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 septicemia 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 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

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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 lipooligosaccharide (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

**[0006A]** In a first aspect, the present invention provides 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 Porin A (PorA), one or more lipooligosaccharide (LOS), and one or more conserved outer membrane proteins; wherein the genetically modified strains have been modified to provide enhanced safety based on inactivation of *lpxL1*, *synX*, and *lgtA* genes; and wherein at least one of the genetically modified strains expresses at least two different PorA subtype proteins or subtype epitopes from group B *Neisseria meningitidis*.

**[0006B]** In a second aspect, the present invention provides 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 *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 Opc and PorA; (2) 8570 HOPS-G<sub>AL</sub> 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 Opc; and (3) B16B6 HPS-G<sub>2A</sub> 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 Opc.

**[0006C]** In a third aspect, the present invention provides a method of using the vaccine of the first aspect administered intramuscularly and/or intranasally for immunization against meningococcal disease and/or group B meningococcal disease.

**[0006D]** In a fourth aspect, the present invention provides 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: i. inactivation of the *synX* gene, ii. inactivation of the *lpxL1* gene, iii. inactivation of the *lgtA* gene in each strain resulting in expression of a shortened or truncated lipooligosaccharides (LOS) that lacks lacto-N-neotetraose tetrasaccharide, and iv. insertion of at least one second antigenically different *porA* gene in place of the *opa* gene.

**[0006E]** In a fifth aspect, the present invention provides a genetically modified vaccine strain of *Neisseria meningitidis* subtype B strain: comprising H44/76 HOPS-DL strain.

**[0006F]** In a sixth aspect, the present invention 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) 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.

**[0006G]** In a seventh aspect, the present invention provides a genetically modified vaccine strain of *Neisseria meningitidis* subtype B comprising strain: 8570 HOS-G1.

**[0006H]** In an eighth aspect, the present invention provides 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.

**[0006I]** In a ninth aspect, the present invention provides a genetically modified vaccine strain of *Neisseria meningitidis* subtype B comprising B16B6 HPS-G<sub>2</sub>A strain.

**[0006J]** In a tenth aspect, the present invention 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) 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

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PorA and Opc proteins.

**[0006K]** In an eleventh aspect, the present inspection provides a vaccine composition comprising NOMV from one or more genetically modified strain of any one of the sixth to tenth aspects.

**[0006L]** In a twelfth aspect, the present invention provides a method of eliciting an immune response to meningococcal disease in an animal or human comprising administering the composition of any one of the first, second or eleventh aspects to the animal or human for immunization against any one or more of meningococcal disease or group B meningococcal disease.

**[0006M]** In a thirteenth aspect, the present invention 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; and f) genetically modifying the strain by inserting at least one second antigenically different *porA* gene into the open reading frame of the *opa* gene.

**[0006N]** In a fourteenth aspect, the present invention 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 modification selected from the group consisting of: i. inactivation of the *synX* gene, ii. inactivation of the *lpxL1* gene, iii. inactivation of the *IgtA* gene, iv. insertion of at least one second antigenically different *porA* gene in place of the *opa* gene, v. increased or stable expression of at least one minor conserved outer membrane protein, and vi. 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.

**[0007]** In one aspect, the present technology provides a vaccine comprising native outer membrane vesicles (NOMVs) 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

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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 FHPB (GNA1870) variants 1, FHPB variants 2, and FHPB 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 stains 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 Ope and PorA; (2) 8570 HOPS-GAL 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 Ope; 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 Ope. The NOMV are prepared without exposure to detergent

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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 lipooligosaccharides (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 subtype protein or subtype epitope selected from the most prevalent of PorA subtypes of meningitidis group B isolates.

**[0010]** In yet another aspect, the present technology provides a genetically modified vaccine strain of *Neisseria meningitidis* subtype B strain. The genetically modified vaccine strain may include H44/76 HOPS-D strain (B1), 8570 HOS-G1 strain (B2), and/or B16B6 HPS-G<sub>2</sub>A strain (B3).

**[0011]** 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) insertion of a second *porA* gene in the place of a *opaD* gene, v) increased expression of Nad $\Delta$  compared with the native strain, and vi) stabilized increased expression of Opc and PorA 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).

**[0012]** In another aspect, the present technology provides 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. In some aspects, the genetically modified strain was derived from the ET-5 wild type strain 85 70(B:4: P 1.19,15: L3,7v: P5.5,11,C).

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[0013] 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) insertion of a second *porA* gene (subtype P1.22-1,4) in place of *opaD*; v) increased expression of factor II 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).

[0014] In some aspects, the present technology provides a genetically modified strain grown in iron deficient medium.

[0015] 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.

[0016] 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.

[0017] In a further 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-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. The minor conserved outer membrane protein can be selected from the group consisting of NadA, factor H binding protein (FHPB) variant 1, and FHPB variant 2. In other aspects, at least some of the NOMV comprise shortened or truncated LOS that are essentially free of lacto-N-neotetraose (LNnT) tetrasaccharide and/or at least some of the NOMV comprise two or more different PorA proteins.

[0018] 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.

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[0019] In a further 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 *IgtA* 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.

[0020] In 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 modification selected from the group consisting of inactivation of the *synX* gene, inactivation of the *lpxL1* gene, inactivation of the *IgtA* 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

[0021] Figure 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.

[0022] Figure 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.

[0023] Figure 3 is a flow chart depicting the fermentation of the *Neisseria* used for making the genetically modified strains of *Neisseria* for vaccine production.

[0024] Figure 4 is a flow chart depicting the purification of NOMVs from the genetically modified strains of *Neisseria* for vaccine production.

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[0025] Figure 5 is a continuation of the flow chart from Figure 4.

[0026] Figure 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).

[0027] Figure 7 is a silver stained gel showing lipooligosaccharide content of the vaccine. Lane 1 is the control ML5 LPS, lane 2 is the filtered bulk vaccine and lane 3 is the final vaccine product. Fifteen  $\mu$ l of a 1:2 dilution of 100  $\mu$ g/ml of the vaccine were run on the gel (20  $\mu$ l of 100  $\mu$ l/ml of 1:2 dilution of control).

[0028] Figure 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.

[0029] Figure 9 is a graph depicting the TNF- $\alpha$  released from human blood after incubation with different concentrations of the vaccine.

[0030] Figure 10 is a graph depicting IL-6 release from human blood following incubation with different concentrations of the genetically modified NOMV vaccine.

[0031] Figure 11 is a graph depicting the TNF- $\alpha$  released from human blood after incubation with different concentrations of the genetically modified vaccine as compared with DOC-extracted OMV from strain 44/76.

[0032] Figure 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.

[0033] Figure 13 is a bar graph depicting the bactericidal titer of mice vaccinated with 8570 HOPS-G Vaccine against different test strains.

[0034] Figure 14 is a graph depicting the results of the bactericidal antibody depletion assay for LOS, GNA1870, NOMV and Opc antigens.

[0035] Figure 15 depicts the antibody response of rabbits vaccinated with the 8570 HOPS-G NOMV vaccine with and without adjuvant.

[0036] Figure 16 is a graph depicting the results of an bactericidal depletion assay for test strains against the 8570 HOPS-G1 NOMV vaccine.

[0037] Figure 17 is a graph depicting the results of the bactericidal depletion assay for LOS and FHBPs antigens for the the 8570 HOPS-G1 PorA knockout strain.

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[0038] Figure 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.

[0039] Figure 19 is a representation of the plasmids used to construct the genetically modified strains of *Neisseria*: a) plasmid constructed to knockout *lgtA*, b) plasmid to express second PorA, c) plasmid to overexpress fIIbp 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 fIIbp (variant 1 and 2) and NadA overexpression plasmid via homologous recombination replacing *NspA* gene.

[0040] Figure 20a is depiction of the stabilization of the truncated LOS immunotype of NOMV vaccine strain by knockout of the *lgtA* gene of the three genetically modified strains. Figure 20b is a picture of an immunoblot of the expression of LOS alpha chain by the genetically altered strain B2 and the parental strain (B16B6) with monoclonal antibodies against L3, 7,9 (left) and L8 (right).

[0041] Figure 21 is a picture representation showing the expression of fHbp variant 2 in the genetically modified strain B3. Figure 21a) shows selection of the strain containing the gentamicin resistance recombinant containing the overexpressed fHbp by immunoblotting and Figure 21b) is a Western Blot using JAR4 monoclonal antibody to fHbp showing increased expression of fHbp.

#### DETAILED DESCRIPTION OF THE INVENTION

[0042] The present technology 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 (OMPs), 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 blebbing. 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.

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[0043] 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.

[0044] Some embodiments of the present technology provide antigenically diverse strains of *N. meningitidis*, preferably subtype B 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-acyl 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 *lgtA* 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 capsid and results in the formation of capsule-negative phenotype NOMVs, deletion of the *lpxL1* gene which reduces the endotoxin activity by resulting in a penta-acyl lipid A structure, and/or deletion of the *lgtA* gene which block 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 breath 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

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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. Patent 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 tested by 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 1623; and synXB bases 886 to 1589. In addition, at the 5' end, a putative uptake sequence,  $\Delta$ CCGTCTG $\Delta$  $\Delta$ , 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* DH5 $\alpha$  and selected on LB agar with 50  $\mu$ g 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* DH5 $\alpha$  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 *lpxL1* 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-oxy-acyl linked secondary fatty acids

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present in the lipid A are important for endotoxic activity. The genetically modified strain includes the *lpxL1* 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 Immunity* 69(10), 5981-5990, 2001.) Deletion of the *lpxL1* 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 *lpxL1* 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 *opaC* or *opaD* gene of the genetically altered strain. The PorA serotype selected for insertion is selected based on the most prevalent forms of 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 Oct;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 recloned 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.

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**[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, fIIBP 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* ptac 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 translocated to the surface of the outer membrane. For example, expression of v.1 under the control of IPTG-inducible *E. coli* Ptac promoter in strain 8570 HOPS-G (B2) was about 4-fold higher than in the parental strain 8570 and expression of v.2 in strain B16B2 HPS-G<sub>2</sub>Δ (B3) was 32-64 fold higher than in the parental strain B16B6 (See Figure 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-neotetraose (LNnT) 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 *lgtA* and other neisserial 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 substituents 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-neotetraose 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 alterations 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

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homologous recombination or transformation. The mutated  $\Delta lgtA$  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  $\alpha$ -core LOS structure, for example, providing the truncated core structures as depicted in Figure 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 preset technology contain specific LOS core structures corresponding to immunotypes L3, 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.  $\Delta lgtA$  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-neotetraose 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 for 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 desferrol. One suitable medium is modified from that published by BW Catlin (Catlin BW. (1973) *J. Infect. Dis.* 128: 178-194) by replacing several individual amino acids with 1% casamino acids (certified, Difco Laboratories). The medium contained per liter: 0.4 g NH<sub>4</sub>Cl, 0.168 g KCl, 5.85 g NaCl, 1.065 g Na<sub>2</sub>HPO<sub>4</sub>, 0.17 g KH<sub>2</sub>PO<sub>4</sub>, 0.647 g sodium citrate, 6.25 g sodium lactate (60% syrup), 0.037 g CaCl<sub>2</sub>.2H<sub>2</sub>O, 0.0013 g MnSO<sub>4</sub>.H<sub>2</sub>O, 5 g glycerol, 0.02 g cysteine, 10 g casamino acids, 0.616 g

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MgSO<sub>4</sub>, 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, FIIBP 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 Figure 18, including B1 (44/76 HOPS-D), B2 (8570 HOPS-G1) and B3 (B16B6 HPS-G2) strains.

**[0056]** 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 tetravalent 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.

**[0057]** 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

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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.

**[0058]** 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.

**[0059]** The purified NOMVs are prepared for administration to mammals, suitably humans, mice, rats or rabbits, by methods known in the art, which can include filtering to sterilize the solution, diluting the solution, adding an adjuvant and stabilizing the solution.

**[0060]** 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 known by one skilled in the art. The term parenteral as used hereinafter 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 flasks 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.

**[0061]** 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.

**[0062]** 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

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aluminium salt such as aluminum 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 polyphosphazenes. 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, Monophosphhorly lipid A, other non-toxic derivatives of LPS, and combination of monophosphoryl lipid A, such as 3-de-O-acrylated monophosphorly lipid A (#D-MPL) together with an aluminium salt.

[0063] Other suitable examples of adjuvants include, but are not limited to, MF59, MPLA, *Mycobacterium tuberculosis*, *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-Tetradecanoyloxytetradecanoylamino]ethyl, 2-Deoxy-4-O-phosphono-3-O-[(R)-3-tetradecanoyoxytetradecanoyl]2-[(R)-3-tetradecanoyoxytetradecanoylamino]-b-D-glucopyranoside, MPL<sup>TM</sup> (3-O-deacylated 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<sup>TM</sup> QS-21 (Antigenics, Framingham, Mass.), described in U.S. Pat. No. 5,057,540, a pertussis 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 93/13302 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- $\alpha$ , 1- $\beta$ , 2, 4, 5, 6, 7, 8, 10, 13, 14, 15, 16, 17 and 18, the interferons- $\alpha$ ,  $\beta$  and  $\gamma$ , granulocyte colony stimulating factor, and the tumor necrosis factors  $\alpha$  and  $\beta$ , 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

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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. These examples are provided to describe specific embodiments of the present technology. 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

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the presently described technology encompasses the subject matter defined by the claims appending 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:P1.19, 15:L7v, 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 *opaD* locus knocking out the *opaD* 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 *opaD* locus, disabling *opaD* 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:PI.22,14) and placed behind a *porA* promoter taken from strain H44/76. The resulting *porA* type was P1.19,15: P1.22,14 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'..CATAGTAAACCTCTAAAATTGGATTGTAGTCGGATATGGTAACATAACGTAAATA ATCGTTACGCTTACAATTATATTCTTAAGCTTCGGGGGGGGGGGATT..3' was replaced with a modified promoter sequence (Seq ID No. 2) containing both G and C nucleotides with a Not I site (underlined) 5'..CATAGTAAACCTCTAAAATTGGATTGTAGTCGGATATGGTAACATAACGTAAATA ATCGTTACGCTTACAATTATATTCTTAAGCTTCCGCGGCCGCCATT..3'

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

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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 (Not I) analysis.

[0073] 3) Starting with the strain resulting from 2, the gene *lpxLl*, 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 *lpxLl* gene with an insert containing the tetM antibiotic resistance gene. The tetM gene was obtained from a plasmid pJS1934, which was derived from the transposon Tn916 (Swartley, et al. 1993. Mol. Microbiol. 10:299-310). The plasmid used to disable the *lpxLl* gene was pMn5 (Seq. ID No. 5). The presence of the insert in the *lpxLl* gene was verified by PCR which produced a 3.3 kbp amplicon using primers at the beginning and end of the *lpxLl* gene.

[0074] 4) Starting with the strain resulting from step 3, expression of the conserved outer membrane protein GNA 1870 (variant 1) (FHPB 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 Ptac promoter, the GNA1870 variant 1 gene and the rrnB terminator, the plasmid used is depicted in Figure 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 1

Feature	Coordinates (nt ##)	Source
pUC19 <sup>*)</sup>	1-191	New England Biolabs (NEB)
Sac I site (unique)	192-197	pUC19 cloning site
Uptake Sequence	198-212	PCR construct
5' NspA non coding region (NCR)	213-1248	<i>N. mening.</i> , 44-76, PCR construct
Bam H I site	1249-1254	Gent <sup>R</sup> gene cloning site
Gent <sup>R</sup> gene	1255-2104	PCR construct of Gent <sup>R</sup> gene
Sac II (unique)	2105-2110	PCR construct
Rmp promoter 5' fragment (rest)	2111-2230	Previous plasmid for NspA expression
Mfe I site (unique)	2231-2236	PCR construct
<i>Lac<sup>d</sup></i> operon	2237-3641	pMAL-p2X (New England Biolabs)
<b>P<sub>tac</sub> promoter</b>	3642-3673	pMAL-p2X, PCR construct
<i>Lac</i> operator	3674-3702	pMAL-p2X, PCR construct
RBS	3750-3755	pMAL-p2X, PCR construct
Nde I site (unique)	3761-3766	PCR construct
fHBP (variant 1) leader peptide	3764-3823	<i>N. mening.</i> , 44-76, PCR construct
fHBP (variant 1) ORF with stop codon	3824-4588	<i>N. mening.</i> , 44-76, PCR construct
SgrAI site (unique)	4589-4596	<i>N. mening.</i> , 44-76, PCR construct
3'NspA and 3'NspA NCR	4597-4638	Previous plasmid for NspA expression
rrnB transcription terminators	4639-4945	pBAD/Thio-E (Invitrogen), PCR
3' NspA NCR	4946-5432	<i>N. mening.</i> , PCR construct
Uptake Sequence	5433-5447	PCR construct
Hind III	5448-5453	pUC19 cloning site
pUC 19	5454-7687 end	NEB (Amp. <sup>R</sup> )

\*) Start from nt. 1 of pUC 19. The plasmid was modified to remove Nde I site for further convenient cloning as follow: It was digested by Nde I - EcoR I 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 (395) were destroyed. For cloning of constructs for the expression of target protein we used Sac I and Hind III cloning sites of pUC 19.

[0075] 5) 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.

[0076] 6) The strain resulting from step 4 was transformed with a plasmid pBE-501 containing zeomycin 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 lacto-N-neotetraose (LNnT) tetrasaccharide (see Figure 20).

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**Table 2**

<u>Feature</u>	<u>Coordinates (nt #)</u>	<u>Source</u>
pCR 4-TOPO TA cloning Vector	1-3667	Invitrogen. Type: pUC ori
Uptake Sequence	3668-3682	PCR construct
LgtA 5' segment	3683-4037	<i>N. mening.</i> , 2996, PCR construct
pEM7/Zeo	4038-4122	Invitrogen. Cloning site and EM7 promoter Zeocin
	4123-4497	Zeocin <sup>®</sup> gene provided ΔLgtA pEM7/Zeo
	4498-4633	Fragment of pEM7/Zeo cloning site LgtA 3'
segment	4634-5448	<i>N. mening.</i> , 2996, PCR construct
Uptake Sequence	5449-5463	Uptake sequence
pCR 4-TOPO TA cloning Vector	5464-5759 end	Invitrogen

\*) LgtA cDNA was digested by BssH II. Resulting 3' and 5' sticky ends were refilled and Zeocin gene was inserted in this blunt ended cDNA. In case of excising of disruptive Zeocin gene which may occur during of the reparation of bacterial DNA, relegated 5' and 3' of LgtA fragments will bearing unrepairable sequence

**[0077]** This genetically modified strain was tested to unsure 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 Figures 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:

Protein 2001ag/ml  
Lipooligosaccharide 36 tg/ml  
Nucleic Acid 2.5 µg/ml  
Sodium Chloride 0.9%  
Tris-HCl Buffer 0.01 M pH 7.6

**[0081]** The vaccine composition was further analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting. Figure 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). Figure 7 depicts silver stain gel showing the liposaccharide component of the vaccine (lane 3) as compared with control (M1.5 LPS, land 1) and filtered bulk vaccine lot (lane 2). Figure 8 depicts the results of identity testing of the vaccine for the major components of the NOMVs vaccine according to the antibodies as listed in Table 3.

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**Table 3**

Lane	Antibody Specificity	Monoclonal Antibody	Expected Reaction	Test Result
1	Pre-stained standard	NA		
2	L8 LOS	2-1 L8	Trace	Trace
3	L8v LOS	25-1-LC1	Positive	Positive
4	L3,7 LOS	9-2-L379	Trace	Trace
5	Lip (H8)	2-1-CA2	Positive	Positive
6	Opa P5.10	23-1-P5.10	Negative	Negative
7	Opa P5.11	MF7-1-P5.11	Negative	Negative
8	Opc (P5.C)	B306-P5C	Positive	Positive
9	FHBP I (GNA1870)	JAR 4	Positive	Positive
10	Rmp	9F5	Positive	Positive
11	PorB P4	15-1-P4	Positive	Positive
12	PorA P1.14	MN21G3.17	Positive	Positive
13	PorA P1.15	MN3C5C	Positive	Positive
14	PorA P1.19	2-1-P1.19	Positive	Positive
15	TBP2	476C2G2	Positive	Positive
16	Gp B Polysaccharide	2-2-B	Negative	Negative
17	Amido Black Stain	NA		

**[0082]** The results are found in Figures 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**

**[0083]** The vaccine was tested in the General Safety Test as prescribed in 21 CFR 610.11. The results for the vaccine are given in Table 4.

**Table 4**

Test Article	Test Result	Comments
8570 HOPS-G NOMV Vaccine Lot # 1289	Passed	All animals remained normal and healthy and gained weight

**Example 5: Rabbit Pyrogenicity Test\***

**[0084]** 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

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hydroxide adjuvant. The values given are the highest amounts tested that did not induce a fever in the rabbits (temperature increase of  $> 0.5^{\circ}\text{C}$ ), results of which are found in Table 5.

**Table 5**

Test Article	Conc. Tested	Temperature Rise for Indicated Rabbit		
		1	2	3
8570 HOPS-G NOMV Vaccine Lot # 1289	0.4 pg/kg	0.1	0.2	0.2
8570 HOPS-G NOMV Vaccine Lot # 1289 adsorbed to aluminum Rehydragel HPA lot # 1347	0.5 $\mu\text{g}/\text{kg}$	0	0	0

\* 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).

**[0085]** In summary, the vaccine alone passed at 0.4 pg/kg, the aluminum hydroxide adjuvant passed at 15 pg/kg (the largest amount per kg to be used in the clinical study), and the vaccine adsorbed to aluminum hydroxide passed at 0.5  $\mu\text{g}/\text{kg}$  but failed at 1.0 pg/kg. Extrapolation of these results on a pg/kg basis suggest the adsorbed vaccine would be non-pyrogenic in humans up to a dose in the range of 25 - 50  $\mu\text{g}$ .

#### **Example 6: Cytokine Release from Whole Human Blood**

**[0086]** 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 Figures 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).

**[0087]** 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 Fredriksen JH, et al. NIPH Annals, 14:67-80, 1991, except 0.5% deoxycholate

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(DOC) was used through out the procedure rather than using 1.2% DOC to resuspend the ultracentrifuge pellets. The results of this comparison are shown in Figure 11.

**Example 7: Immunogenicity in Mice and Bactericidal antibody response**

**[0088]** 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  $\mu$ g 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 $\mu$ g
2	0.3 $\mu$ g
3	1.0 $\mu$ g
4	3.0 $\mu$ g
5	0.1 $\mu$ g + Rehydragel LV
6	0.3 $\mu$ g + Rehydragel LV
7	1.0 $\mu$ g + Rehydragel LV
8	3.0 $\mu$ g + Rehydragel LV
9	1.0 $\mu$ g + Rehydragel HPA

**[0089]** The results obtained with the 10-week sera (three doses of vaccine) are shown in Figure 12 showing the bactericidal titer of the different vaccine groups for the genetically modified stains. 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 (P1.22-1,4) 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.

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[0090] Bactericidal antibodies induced in mice by the 8570 HOPS-G NOMV vaccine do not show serosubtype specificity, but appear mostly independent of serosubtype and serotype (Figure 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.

[0091] 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. After 4-hrs incubation, 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 Figure 14.

#### **Example 8: Immunogenicity in Rabbits**

[0092] 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 3 isogenic variants of 8570 expressing different PorA proteins and L3,7v LOS and strain 44/76 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 Figure 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.**

[0093] 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 B 16B6 (B:2a:P

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1 .5,2:L2). 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 ET-5 and is representative of the epidemic strain responsible for the group B meningococcal epidemic in Norway in the 1970's and 1980's. It expresses a class 3 PorB protein and type II transferrin binding protein B.

**[0094]** Strain B16B6 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 (FIIBP) 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

Feature	Coordinates (nt ##)	Source
pUC 19*	1-191	New England Biolabs (NEB)
Sac I site (unique)	192-197	pUC19
Uptake Sequence	198-212	PCR construct
5' NspA NCR	213-1248	<i>N. mening.</i> , 44-76, PCR construct
Bam H I site	1249-1254	Gent <sup>r</sup> gene cloning site
Gent <sup>r</sup> gene	1255-2104	PCR construct of Gent <sup>R</sup> gene
Sac II (unique)	2105-2110	PCR construct
Rmp promoter 5' fragment (rest)	2111-2230	<i>N. mening.</i> , PCR construct
Mfe I site (unique)	2231-2236	PCR construct
<i>Lac<sup>q</sup></i> operon	2237-3641	pMAL-p2X (New England Biolabs)
Ptac promoter	3642-3673	pMAL-p2X, PCR construct
<i>Luc</i> operator	3674-3702	pMAL-p2X, PCR construct
RBS	3750-3755	pMAL-p2X, PCR construct
Nde I site (unique)	3761-3766	PCR construct
fHBP (variant 2) leader peptide	3764-3823	<i>N. mening.</i> , 2996, PCR construct
fHBP (variant 2) ORF with stop codon	3824-4588	<i>N. mening.</i> , 2996, PCR construct
SgrAI site (unique)	4589-4596	<i>N. mening.</i> , 44-76, PCR construct
3'NspA and 3'NspA NCR	4597-4638	Previous plasmid for NspA expression
rrnB transcription terminators	4639-4945	pBAD/Thio-E (Invitrogen), PCR
3' NspA NCR	4946-5432	<i>N. mening.</i> , PCR construct
Uptake Sequence	5433-5447	PCR construct
Hind III	5448-5453	pUC19 cloning site
pUC 19	5454-7687 end	NEB, (Amp <sup>R</sup> )

\*) Start from nt. 1 of pUC 19. The plasmid was modified to remove Nde I site for further convenient cloning as follow: It was diges by Nde I - EcoR I and 213 b.p.fragment was removed. Sticky ends were filled in and ligated to restore the plasmid. As a result sited N I (183) and EcoRI (395) were destroyed. For cloning of constructs for the expression of target protein we used Sac I and Hind III clon 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 Figure 18.

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**[0095]** 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 *opaD* 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**

<b>Feature</b>	<b>Coordinates (nt ##)</b>	<b>Source</b>
pUC19 <sup>*)</sup>	1-191	New England Biolabs (NEB)
Sac I site (unique)	192-197	pUC19 cloning site
Uptake Sequence	198-212	PCR construct
5' NspA NCR	213-1248	<i>N. mening.</i> , 44-76, PCR construct
Bam H I site	1249-1254	Gent <sup>r</sup> gene cloning site
Gent <sup>r</sup> gene	1255-2104	PCR construct of Gent <sup>R</sup> gene
Sac II (unique)	2105-2110	PCR construct
PorA promoter (44-76) (modified)**	2111-3266	<i>N. mening.</i> , 44-76, PCR construct
Nde I site (unique)	3267-3272	PCR construct
NadA (allele 3) leader peptide	3270-3338	<i>N. mening.</i> , 2996, PCR construct
NadA (allele 3) ORI <sup>t</sup> with stop codon	3339-4487	<i>N. mening.</i> , 2996, PCR construct
SgrAI site (unique)	4488-4495	<i>N. mening.</i> , 44-76, PCR construct
PorA terminator (44-76)	4496-4910	<i>N. mening.</i> , 44-76, PCR construct
Bsm I	4911-4916	PCR construct
3' NspA NCR	4917-5329	<i>N. mening.</i> , PCR construct
Uptake Sequence	5330-5344	PCR construct
Hind III	5345-5350	pUC19 cloning site
pUC 19	5351-7584 end	NEB, (Amp <sup>R</sup> )

<sup>\*)</sup> Start from nt. 1 of pUC 19. The plasmid was modified to remove Nde I site for further convenient cloning as follow: It was digested by Nde I - EcoR I 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 (395) were destroyed. For cloning of constructs for the expression of target protein we used Sac I and Hind III cloning sites of pUC 19.

<sup>\*\*) The 14Gs Poly G tract of the 44-76 promoter was modified by replacing with optimal for the expression 11Gs.</sup>

**[0096]** 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 *lgtA* 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 Figure 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 HPS-G2 and 44/76 HOPS-D.**

**[0097]** The three genetically modified strains were used to prepare laboratory lots of NOMV vaccine compositions. The 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

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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 225,000 x g 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**

**[0098]** 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 doses were given at 0, 4, and 8 weeks. Blood was drawn pre-vaccination and 2 weeks following the last vaccination (at 10 weeks).

**[0099]** 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.

**[00100]** 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: Bactericidal Titers of Pooled Mouse Sera against a Diverse Panel of Test Strains**

Bactericidal Test Strain	Antigens Expressed	Titer of Pooled Serum from Mice Vaccinated with Indicated Vaccine				
		B1 + B2 + B3	B2 + B3	B1	B2	B3
44/76	B:15:PI.7,16:P5.11,C:L3,7	256	256	256	256	1
8570	B:4:PI.19,15:P5.5:L3,7v	256	256	256	256	2
816136	B:2a:PI.5,2:P5.1a:L2	256	256	1	1	256
9162	B:15:PI.7-2,3:P5.10,11:L3,7*	16	16	8	2	1
M1080	B:1,19:PI.1:P5.3:L1,3,7	2	1	1	1	1
3576	B:NT:PI.22-1:L3,7	128	128	4	2	16
8047	B:2b:PI.5,2:L3,4,7	64	128	1	1	128
9547	B:4:PI.4:L1	256	256	128	2	64
531	B:2a:PI.5,2:P5.1a,1b,4,5:L3,7	256	256	256	1	256
7608	B:2b:PI.5,2:P5.2,c:L4	256	128	1	1	128
6940	B:8,19:PI,NT:L1	4	4	1	1	1
1901	B:8,19:PI,NT:P5.C:L1,3,7	256	32	128	1	8
99M	B:2a:PI.2:P5.1a,1b,5:L3,7	512	512	16	8	256
6275	B:2a:PI.2:P5.1a,4,5:L3,7	—	512	32	1	512
126E	C:8,19:PI.5,2:L1:P5.c	256	256	4	2	64
2981	B:8,19:PI.14:L1	32	8	4	64	2
M4720	B:19:PI.22,14,L2	1	1	1	1	1
6557	B:17:PI.14:L1(3,7)	32	16	1	32	16

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[00101] Vaccine Code:B1 = 44/76 HOPS-D NOMV  
 B2 = 8570 HOS-G1 NOMV  
 B3 = B16B6 HPS-G2 NOMV

[00102] 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.

[00103] 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.

[00104] 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**

Test Strain	Mutant of 8570 HOPS-G1 from Which NOMV Vaccine Was Prepared			
	8570 (P1.19,15 and P1.22,14)	8570 (P1.19,15)	8570 (P1.22,14)	8570 APorA
8570	256	256	256	256
44/76	256	256	256	256
B16B6	1	1	1	1
3576	2	8	8	8
9547	2	4	4	2
2981	64	1	16	1
6557	32	1	128	1

[00105] 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 strain, 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 Figure 16. The results shown in Figure 17 demonstrate

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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.

**CLAIMS:**

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 Porin A (PorA), one or more lipoooligosaccharide (LOS), and one or more conserved outer membrane proteins; wherein the genetically modified strains have been modified to provide enhanced safety based on inactivation of *lpxL1*, *synX*, and *lgtA* genes; and wherein at least one of the genetically modified strains expresses at least two different PorA subtype proteins or subtype epitopes from group B *Neisseria meningitidis*.

2. The vaccine of claim 1 where each strain expresses any one or more of (a) a different LOS core structure and has an alpha chain consisting of glucose and galactose, (b) 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, and/or (c) 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.

3. 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 *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 Opc and PorA;

(2) 8570 HOPS-G<sub>AL</sub> 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 Opc; and

(3) B16B6 HPS-G<sub>2</sub>A which has the following genetic modifications or characteristics:

inactivation of the genes *synX*, *lpxL 1*, 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 Opc.

4. The combination of vaccine strains of claim 3 wherein strain H44/76 HOPS-DL was derived from the ET-5 wild type strain H44/76 (B:15: P1.7,16: L,3,7:P5.5,C); wherein strain 8570 HOPS-G<sub>1</sub>L was derived from the ET-5 wild type strain 8570 (B:4: P 1.19,15: L3,7v: P5.5,11,C); and/or wherein strain B16B6 HPS-G<sub>2</sub>L is derived from the ET-37 wild type strain B16B6 (B:2a:P 1.5,2: L2:P5.1,2,5).

5. The vaccine of claim 1 where the NOMV are prepared without exposure to detergent or denaturing solvents from packed cells or from spent culture medium.

6. The vaccine of claim 1 where the NOMV are combined with one or more adjuvants including aluminum hydroxide or aluminum phosphate, MF 59, CPG-ODN, or MPLA.

7. A method of using the vaccine of claim 1 administered intramuscularly and/or intranasally for immunization against meningococcal disease and/or group B meningococcal disease.

8. 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:

- i. inactivation of the *synX* gene,
- ii. inactivation of the *lpxL1* gene,
- iii. inactivation of the *lgtA* gene in each strain resulting in expression of a shortened or truncated lipooligosaccharides (LOS) that lacks lacto-N-neotetraose tetrasaccharide, and
- iv. insertion of at least one second antigenically different *porA* gene in place of the *opa* gene.

9. The vaccine composition of claim 8, wherein the genetically modified strain further comprises one or more of any of the following (a) increased or stable expression of at least one minor conserved outer membrane protein, (b) stabilized expression of at least one outer membrane protein, wherein the outer membrane protein is selected from the group comprising Opc and PorA, (c) at least one second antigenically different *porA* gene expresses at least one PorA subtype protein or subtype epitope selected from the most prevalent of PorA subtypes of meningitidis group B isolates, and/or (d) the at least one minor conserved outer membrane protein is selected from the group consisting of: FHBP (GNA1870) variants 1, FHBP variant 2, FHBP variant 3; NadA; App, NspA, TbpA and B.

10. A genetically modified vaccine strain of *Neisseria meningitidis* subtype B strain: comprising H44/76 HOPS-DL strain.

11. 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.

12. A genetically modified vaccine strain of *Neisseria meningitidis* subtype B comprising strain: 8570 HOS-G1.

13. 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.

14. A genetically modified vaccine strain of *Neisseria meningitidis* subtype B comprising B16B6 HPS-G<sub>2</sub>A strain.

15. 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.

16. A vaccine composition comprising NOMV from one or more genetically modified strain of any one of claims 11-15.

17. The vaccine composition of claim 16, wherein the vaccine composition comprises NOMVs from two or more genetically modified strains, preferably NOMVs from three or more genetically modified strains.

18. The vaccine composition of any one of claims 1-6, 8, and 16-17, wherein the NOMV are prepared from packed cells or spent culture medium without exposure to a detergent or denaturing solvent.

19. The vaccine composition of any one of claims 1-6, 8, and 16-17, where the NOMV are combined with one or more adjuvants.

20. The vaccine composition of any one of claims 1-6, 8, and 16-17, wherein the genetically altered strain is altered to express iron uptake proteins.

21. A method of eliciting an immune response to meningococcal disease in an animal or human comprising administering the composition of any one of claims 1-6, 8, and 16-17-to the animal or human for immunization against any one or more of meningococcal disease or group B meningococcal disease.

22. 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 *IgtA* gene, and
- e) genetically modifying the strain by increasing expression of one or more minor conserved outer membrane proteins; and
- f) genetically modifying the strain by inserting at least one second antigenically different *porA* gene into the open reading frame of the *opa* gene.

23. The method of claim 22, further comprising the steps 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.

24. 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:
  - i. inactivation of the *synX* gene,
  - ii. inactivation of the *lpxL1* gene,
  - iii. inactivation of the *IgtA* gene,
  - iv. insertion of at least one second antigenically different *porA* gene in place of the *opa* gene,

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-35-

- v. increased or stable expression of at least one minor conserved outer membrane protein, and
- vi. 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.

25. The vaccine of claim 2 or 9, where each most prevalent of PorA subtypes among group B case isolates is selected from the group consisting of P1.7-1,1, P1.22,14, and P1.22-1,4.

26. A vaccine according to claim 1, substantially as hereinbefore described with reference to any one of the Examples of the specification.

**The U.S.A., as represented by The Secretary of the Army, on behalf of Walter Reed Army Institute of Research**

**Patent Attorneys for the Applicant/Nominated Person**

**SPRUSON & FERGUSON**

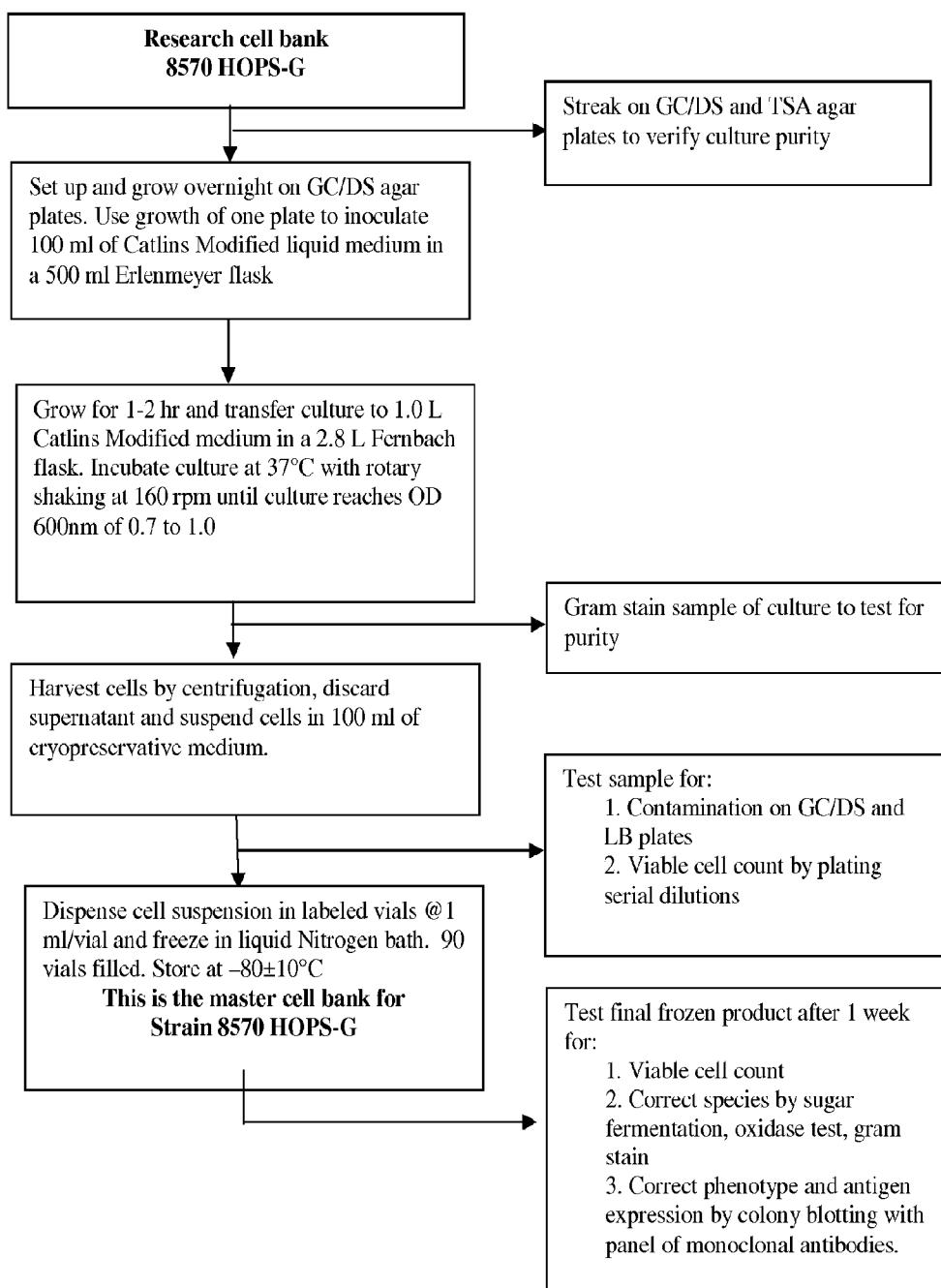
**FIGURE 1****Master Cell Bank Preparation**

FIGURE 2

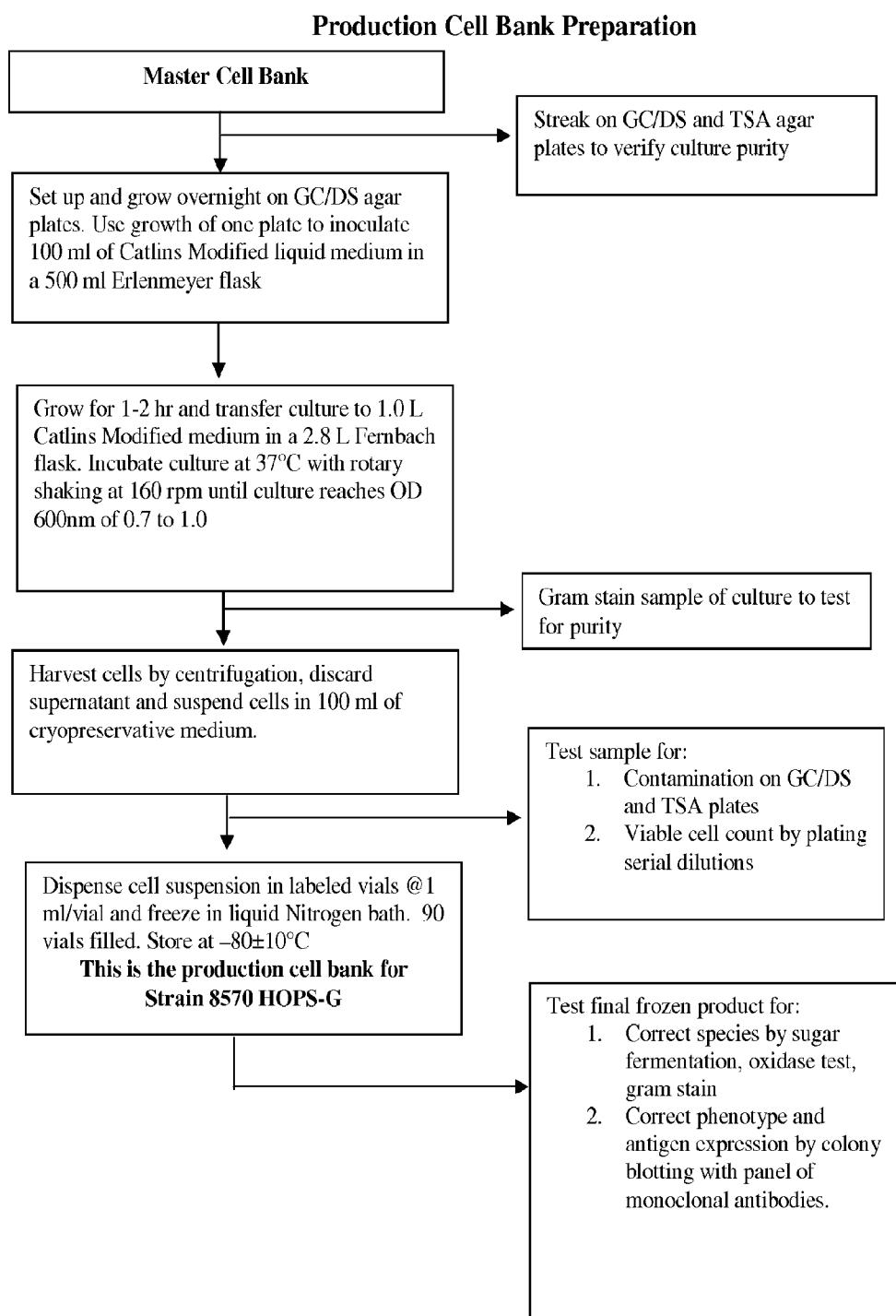


FIGURE 3

## Fermentation

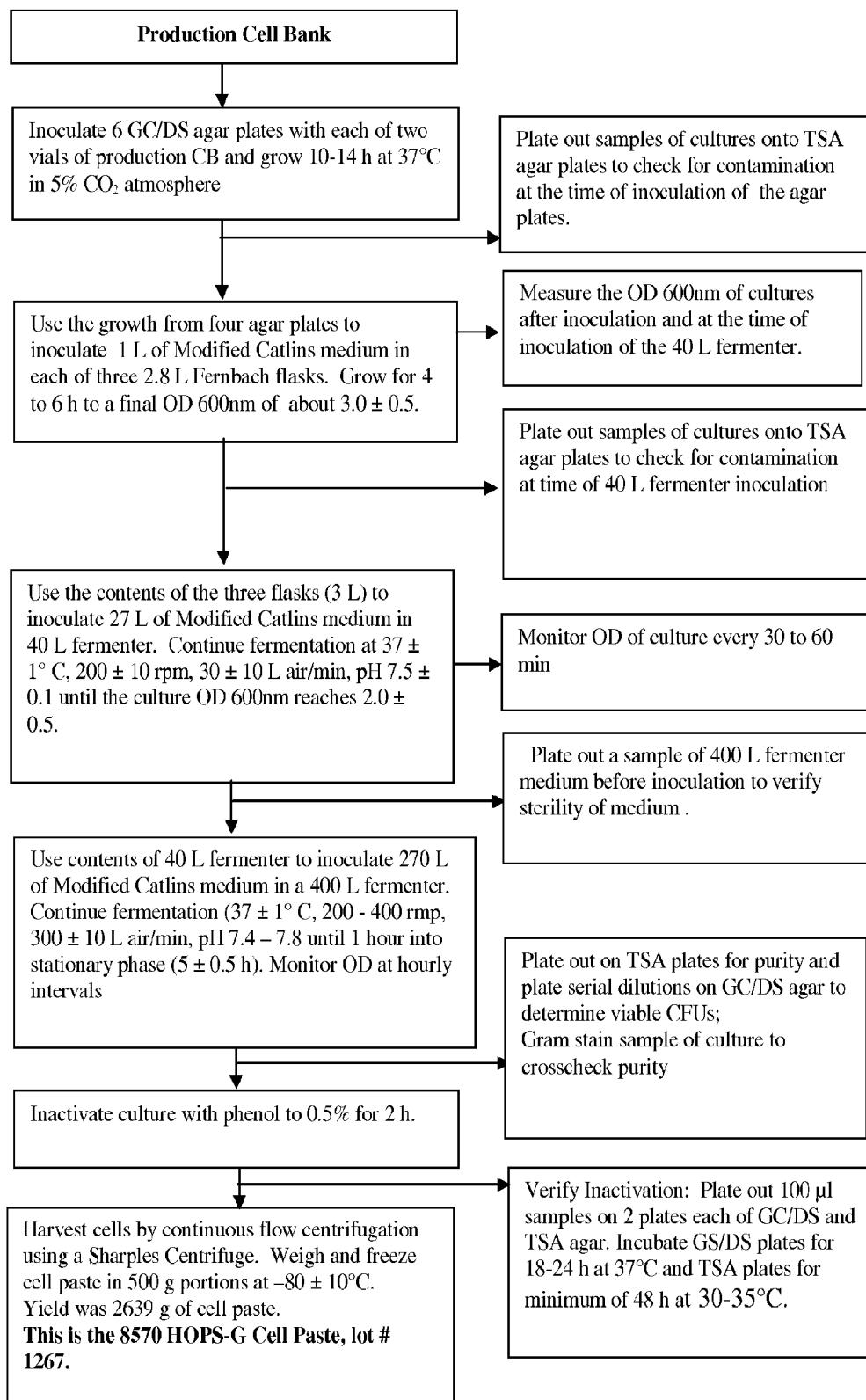
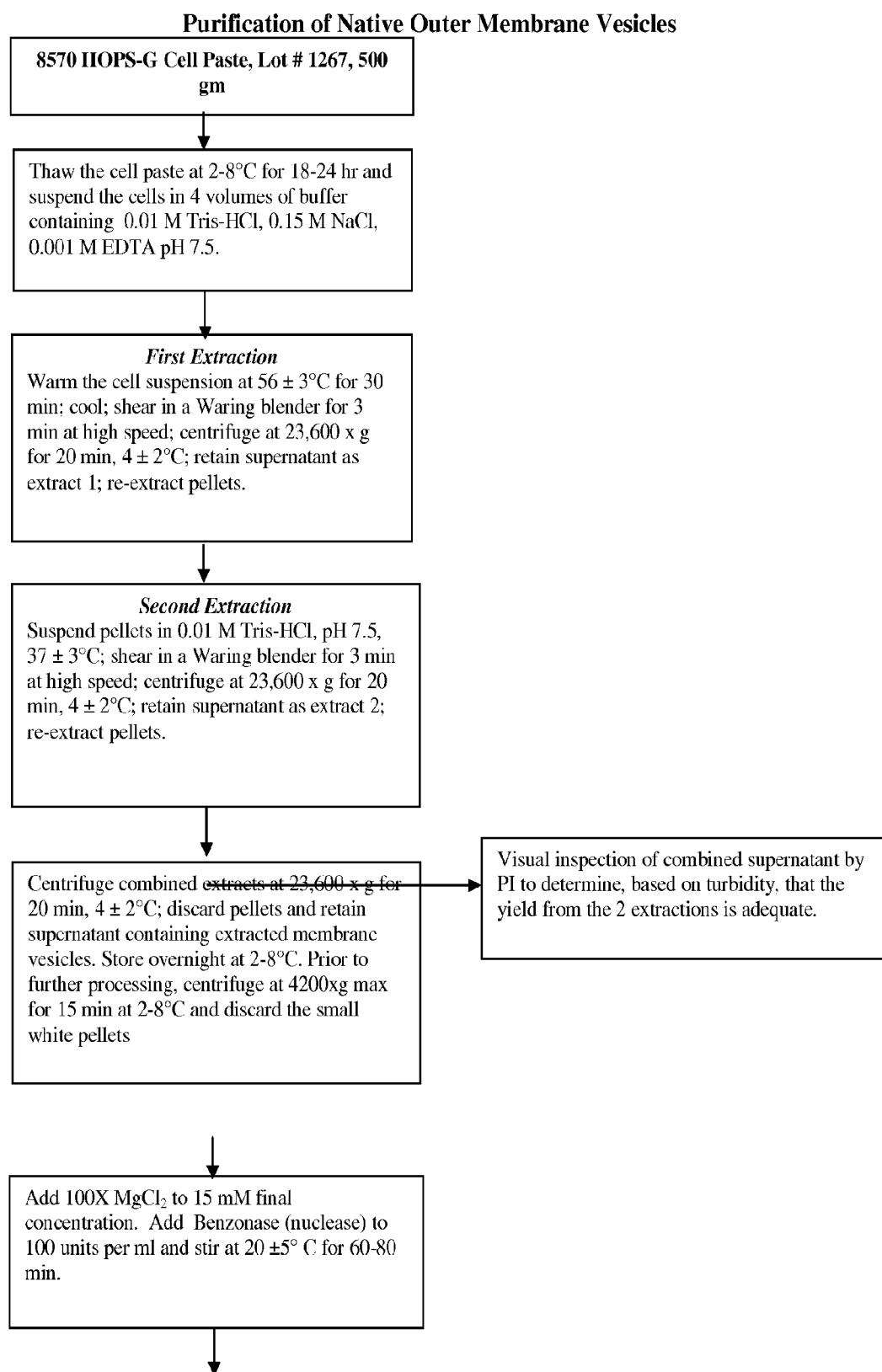
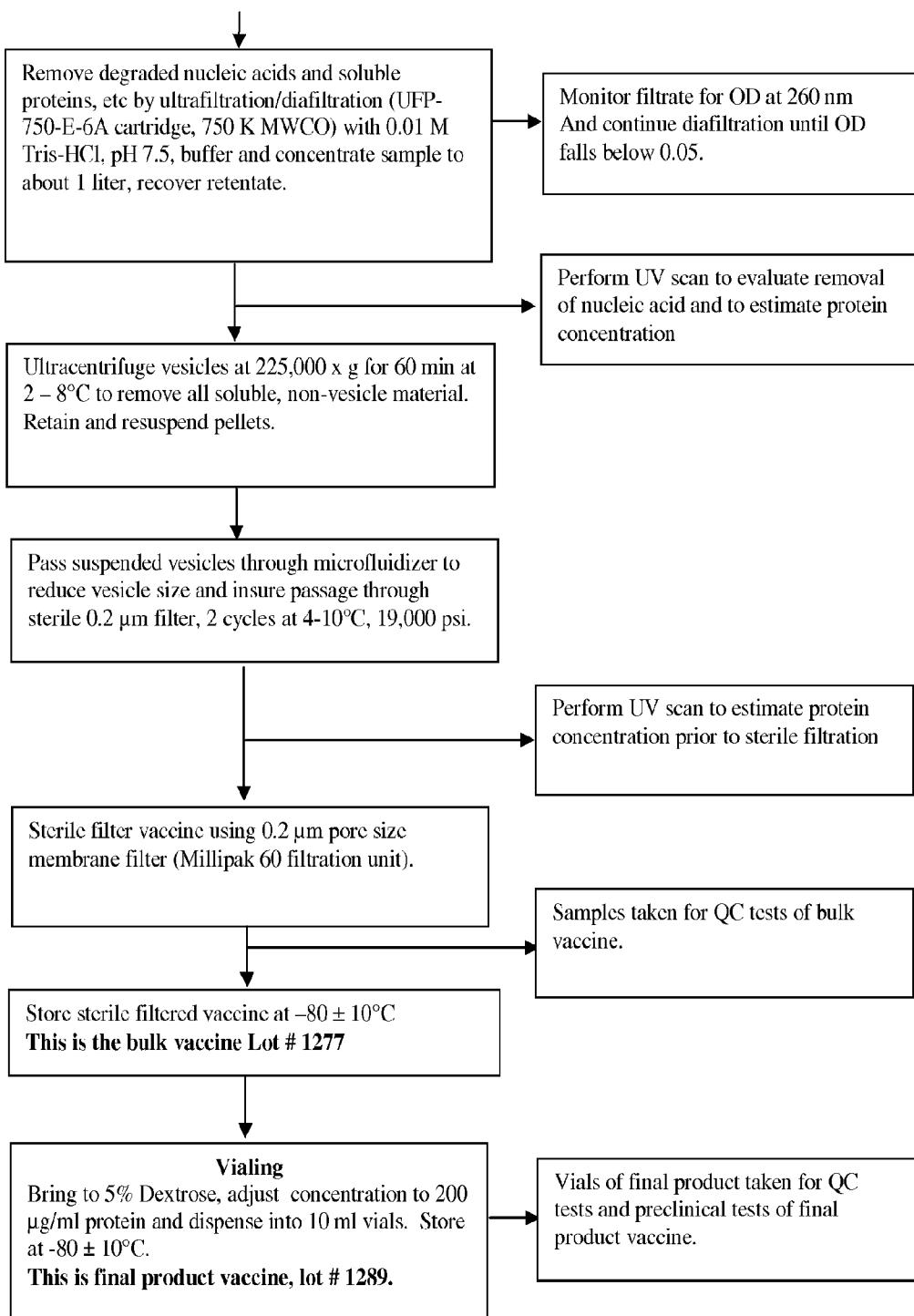


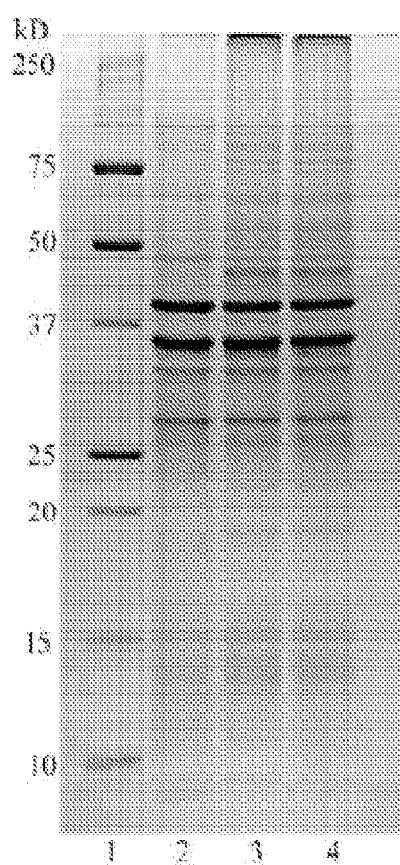
FIGURE 4



**FIGURE 5**

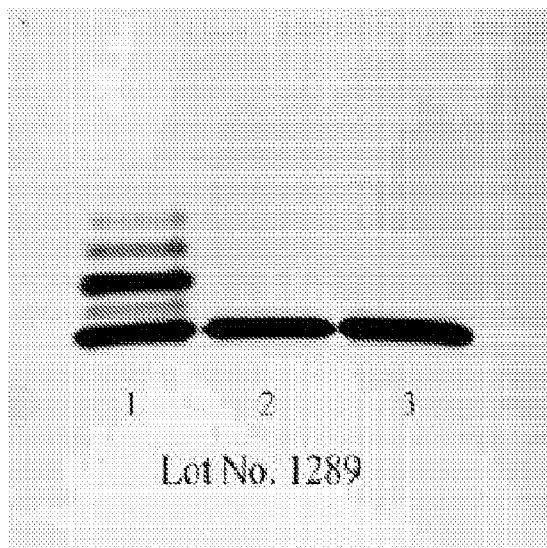
**Preparation of Native Outer Membrane Vesicles (Continued)**  
**( Room 3 , Bldg. 501)**



**FIGURE 6**

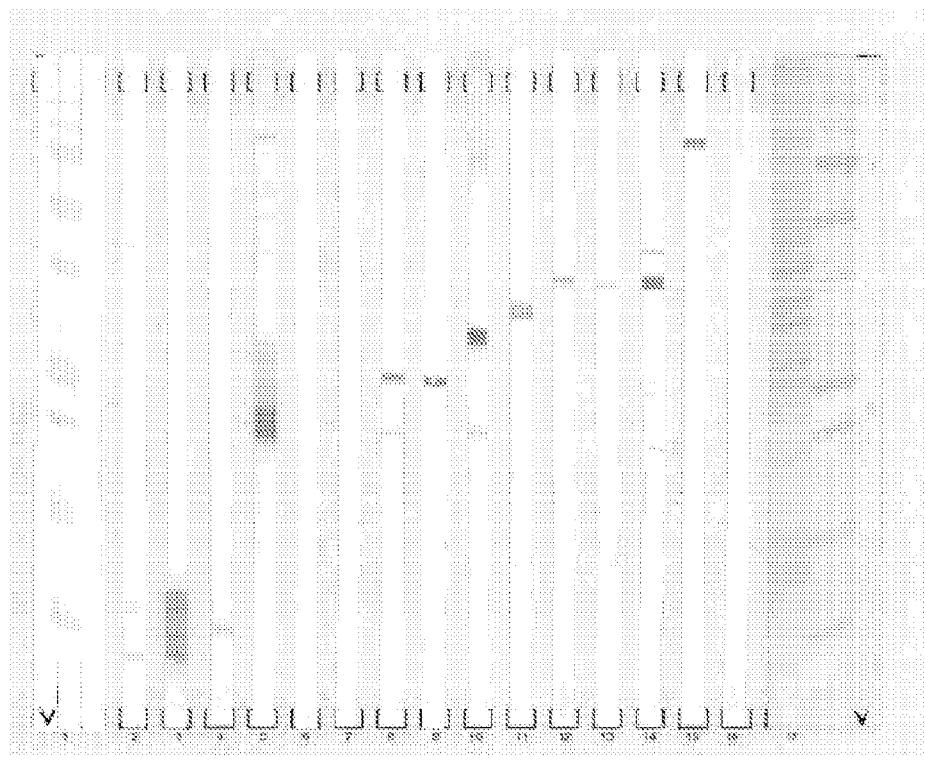
Lot No. 1289

**FIGURE 7**



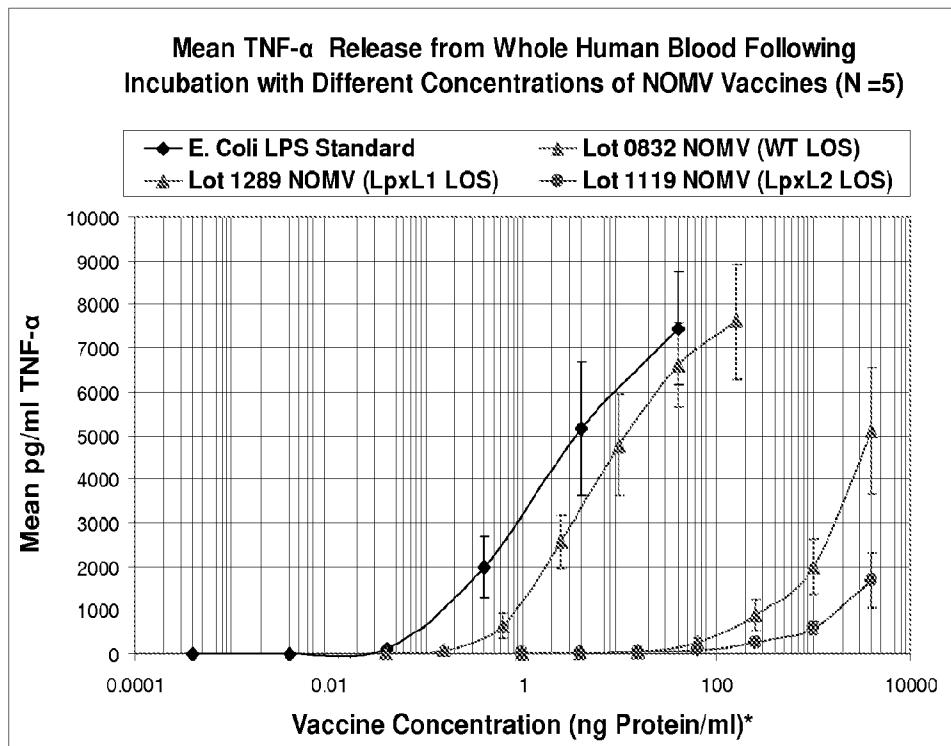
## FIGURE 8

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



Lane	Antibody Specificity	Monoclonal Antibody	Expected Reaction	Test Result
1	Pre-stained Standard	NA		
2	L8 LOS	2-1 L8	Trace	Trace
3	L8v LOS	25-1-LC1	Positive	Positive
4	L3,7 LOS	9-2-L379	Trace	Trace
5	Lip (H8)	2-1-CA2	Positive	Positive
6	Opa P5.10	23-1-P5.10	Negative	Negative
7	Opa P5.11	MF7-1-P5.11	Negative	Negative
8	Opc (P5.C)	B306-P5C	Positive	Positive
9	FHBP 1 (GNA1870)	JAR 4	Positive	Positive
10	Rmp	9F5	Positive	Positive
11	PorB P4	15-1-P4	Positive	Positive
12	PorA P1.14	MN21G3.17	Positive	Positive
13	PorA P1.15	MN3C5C	Positive	Positive
14	PorA P1.19	2-1-P1.19	Positive	Positive
15	TBP2	476C2G2	Positive	Positive
16	Group B Polysaccharide	2-2-B	Negative	Negative
17	Amido Black Stain	NA		

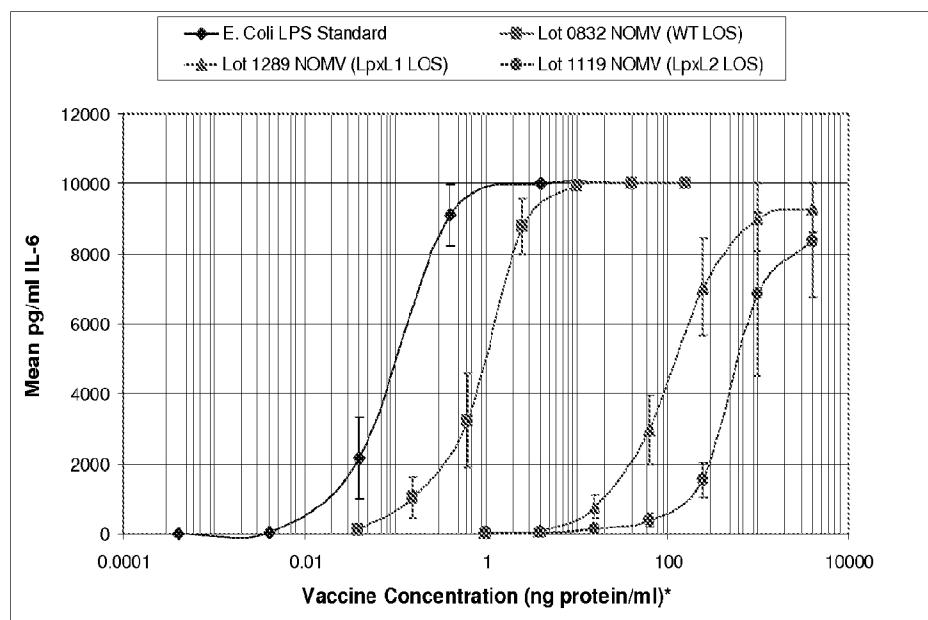
FIGURE 9



10/10

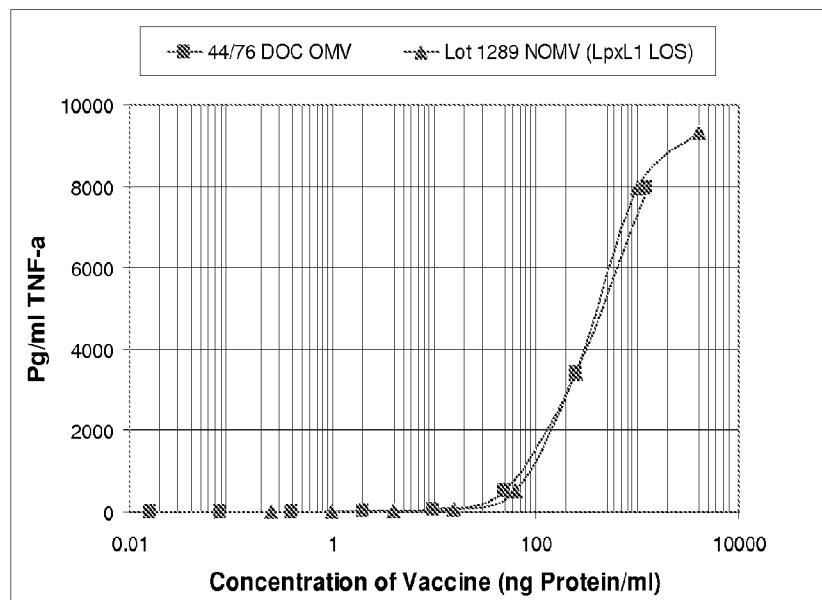
**FIGURE 10**

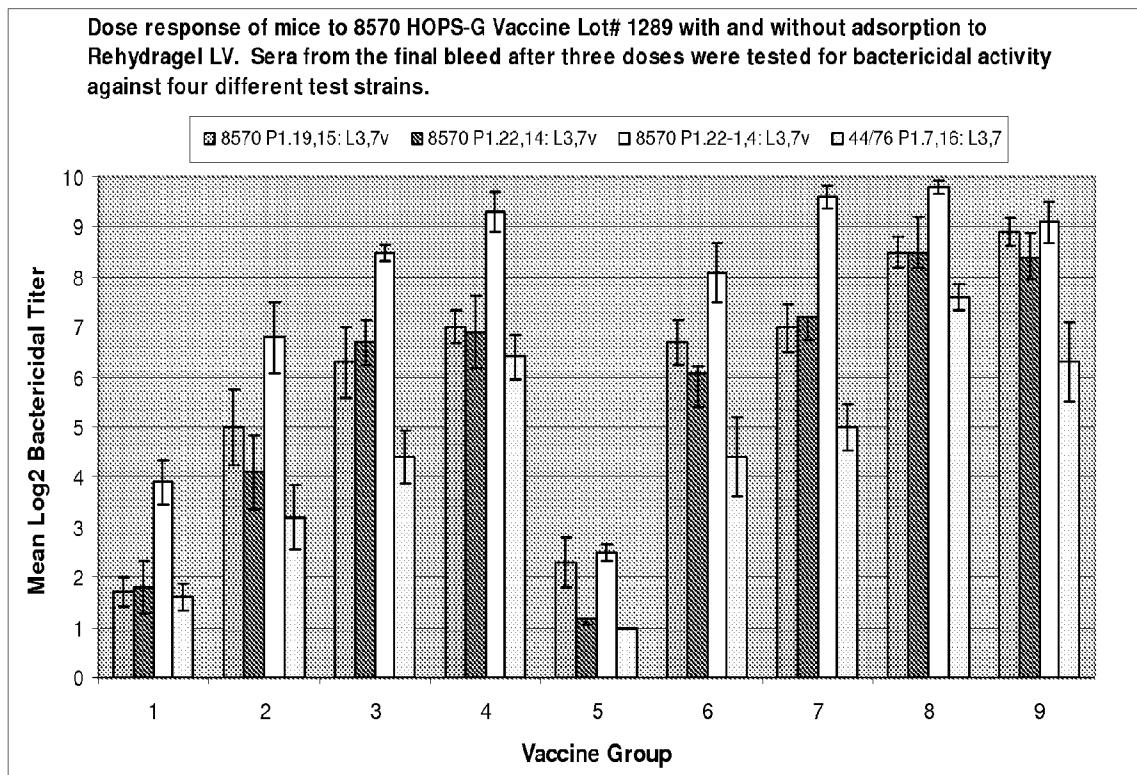
IL-6 Release from Whole Human Blood Following Incubation with Different Concentrations of NOMV Vaccines



**FIGURE 11**

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



**FIGURE 12****Vaccine Groups:**

1) 0.1 $\mu$ g	5) 0.1 $\mu$ g + Rehydragel LV
2) 0.3 $\mu$ g	6) 0.3 $\mu$ g + Rehydragel LV
3) 1.0 $\mu$ g	7) 1.0 $\mu$ g + Rehydragel LV
4) 3.0 $\mu$ g	8) 3.0 $\mu$ g + Rehydragel LV
	9) 1.0 $\mu$ g + Rehydragel HPA

FIGURE 13

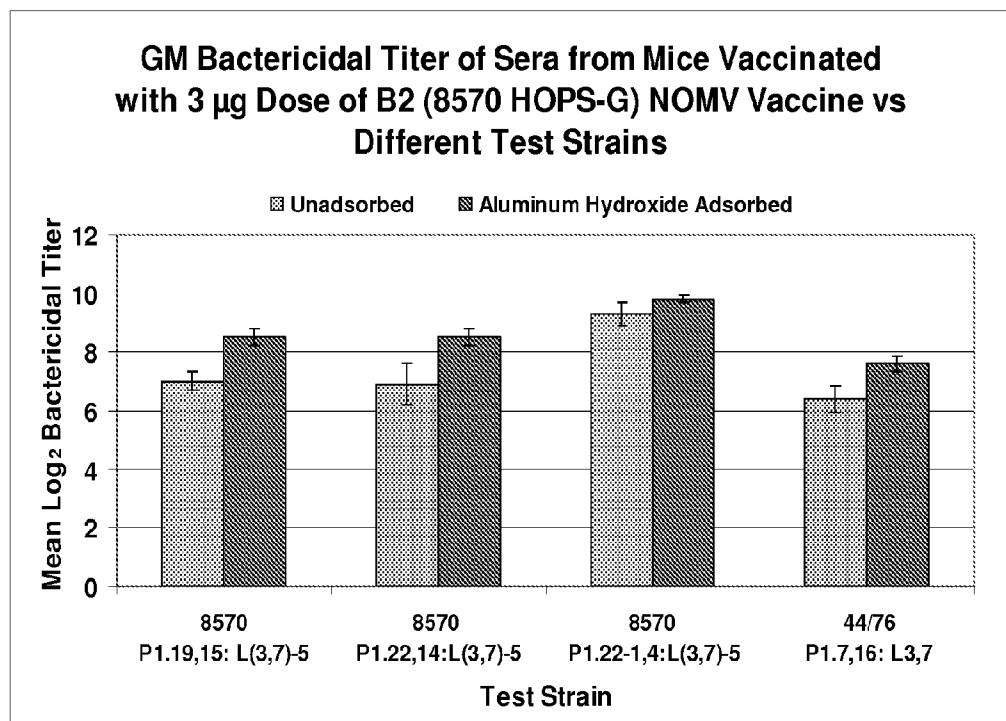


FIGURE 14

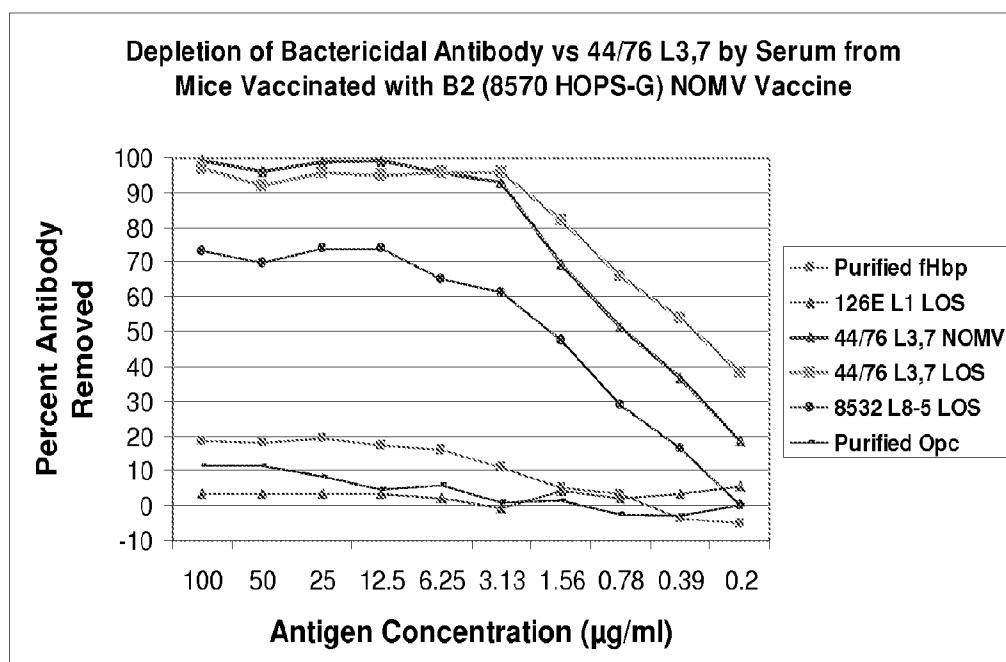


FIGURE 15

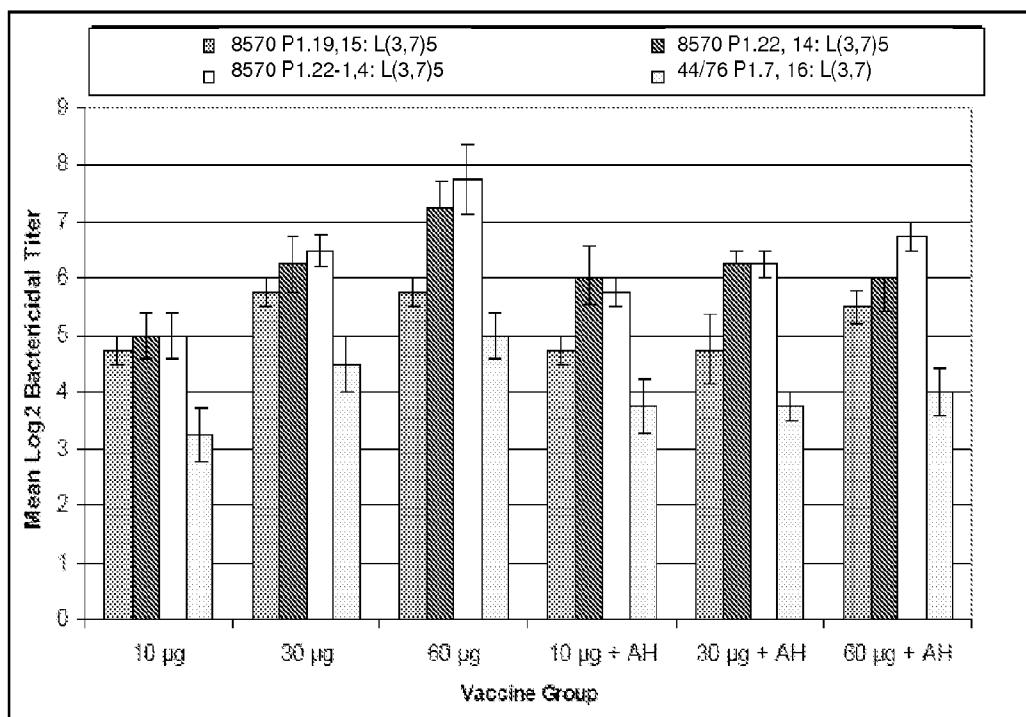


FIGURE 16

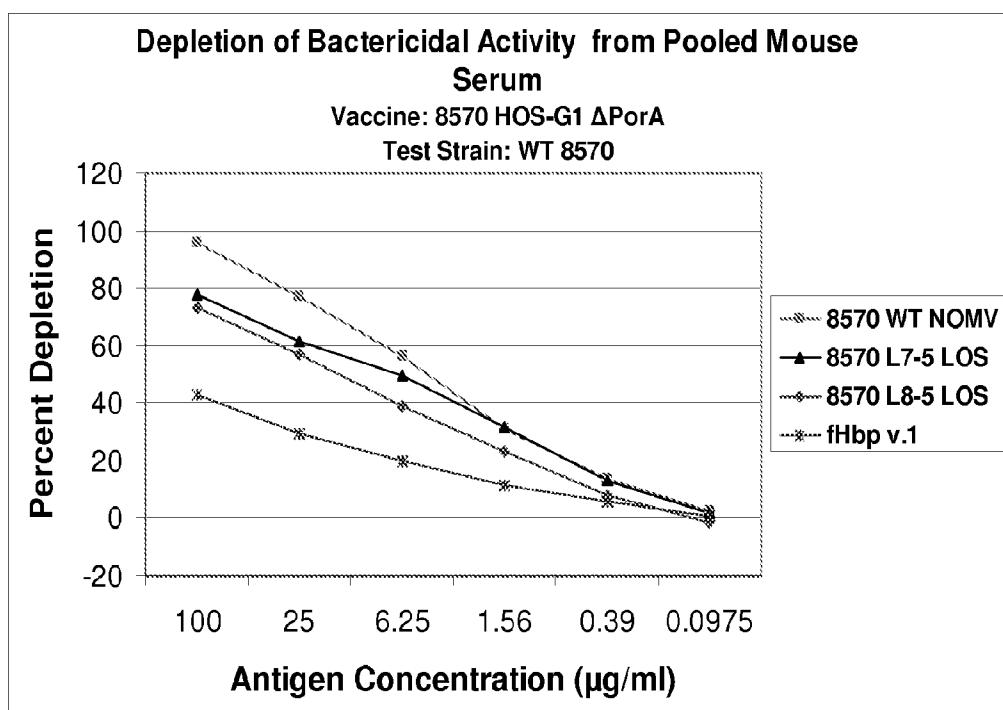


FIGURE 17

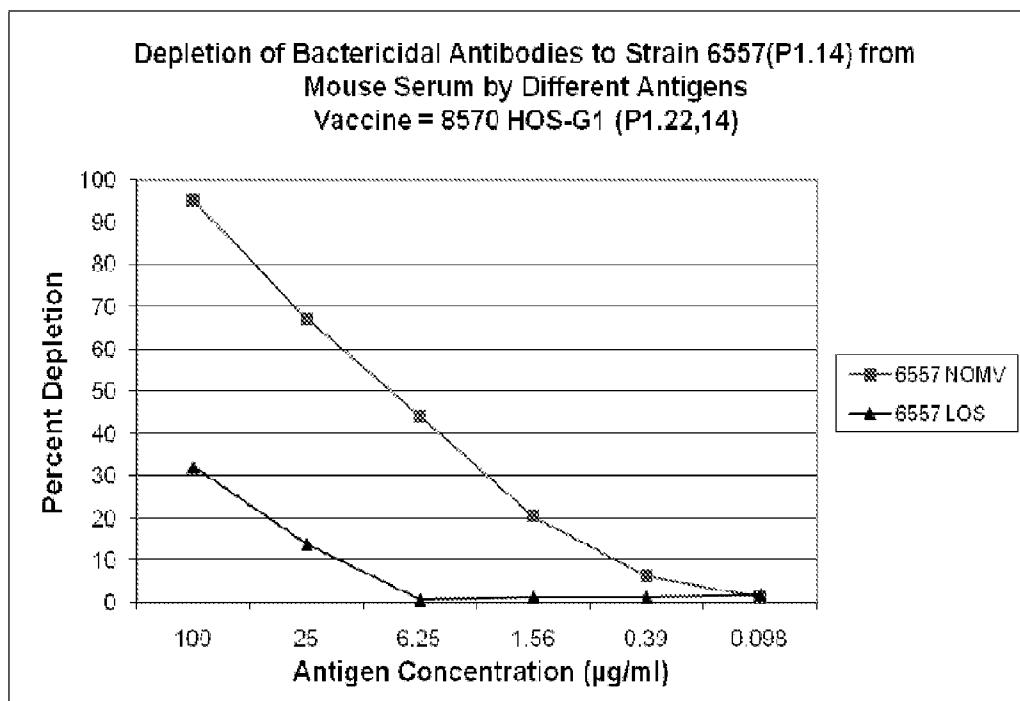


FIGURE 18

**Strain B1**

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

**Strain B2**

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

**Strain B3**

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

*Mutations*

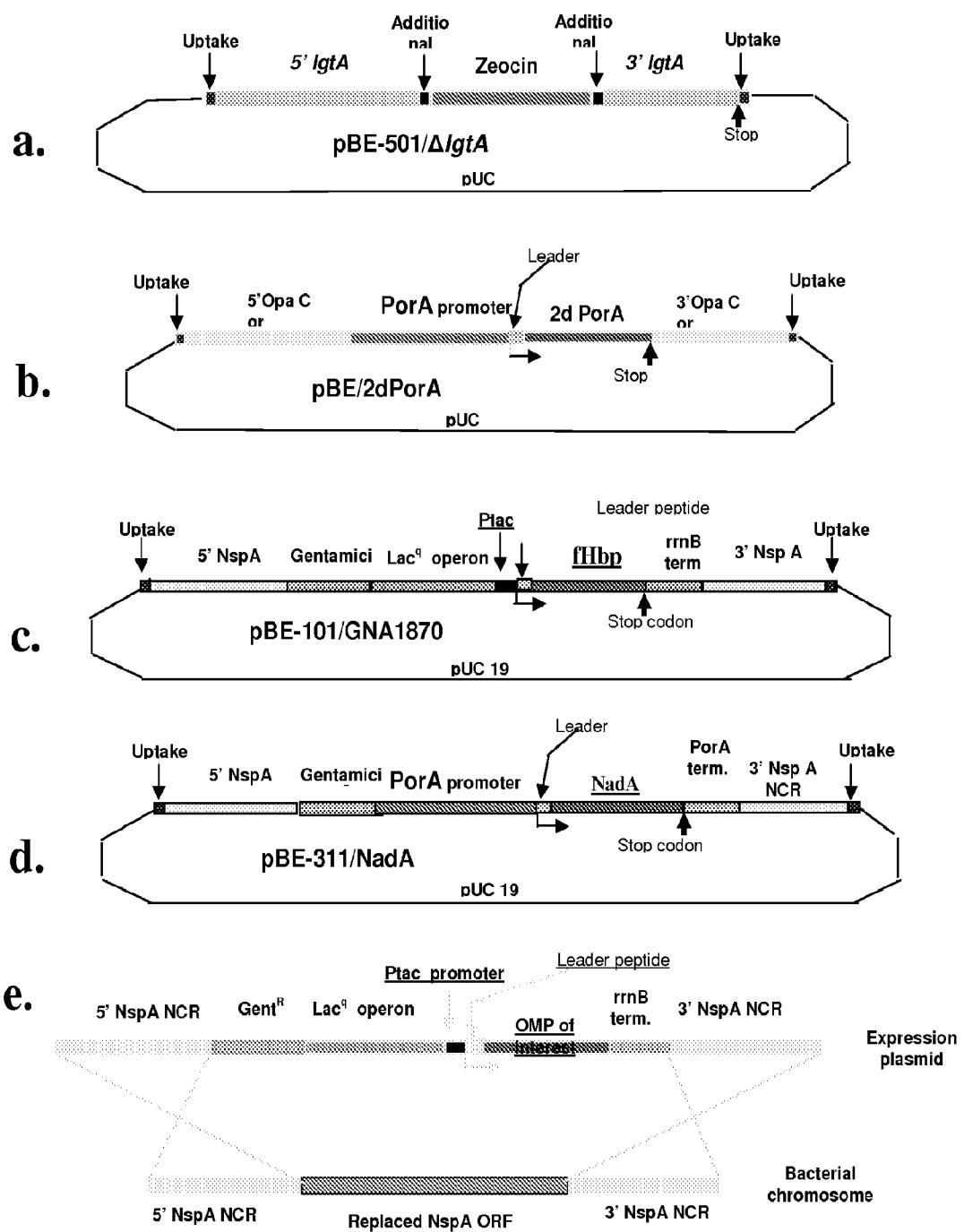
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Over express NadA  
Stable expression of L8-3  
LOS and Opc

*Mutations*

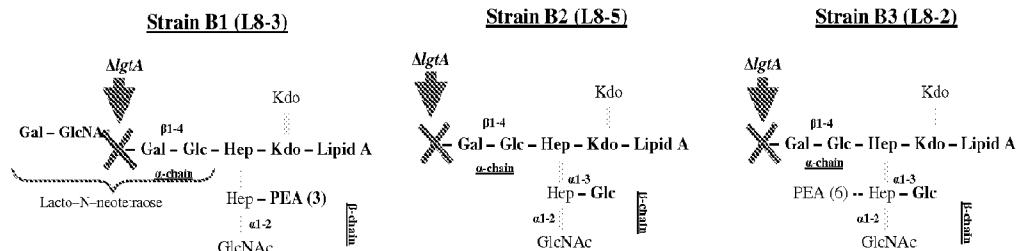
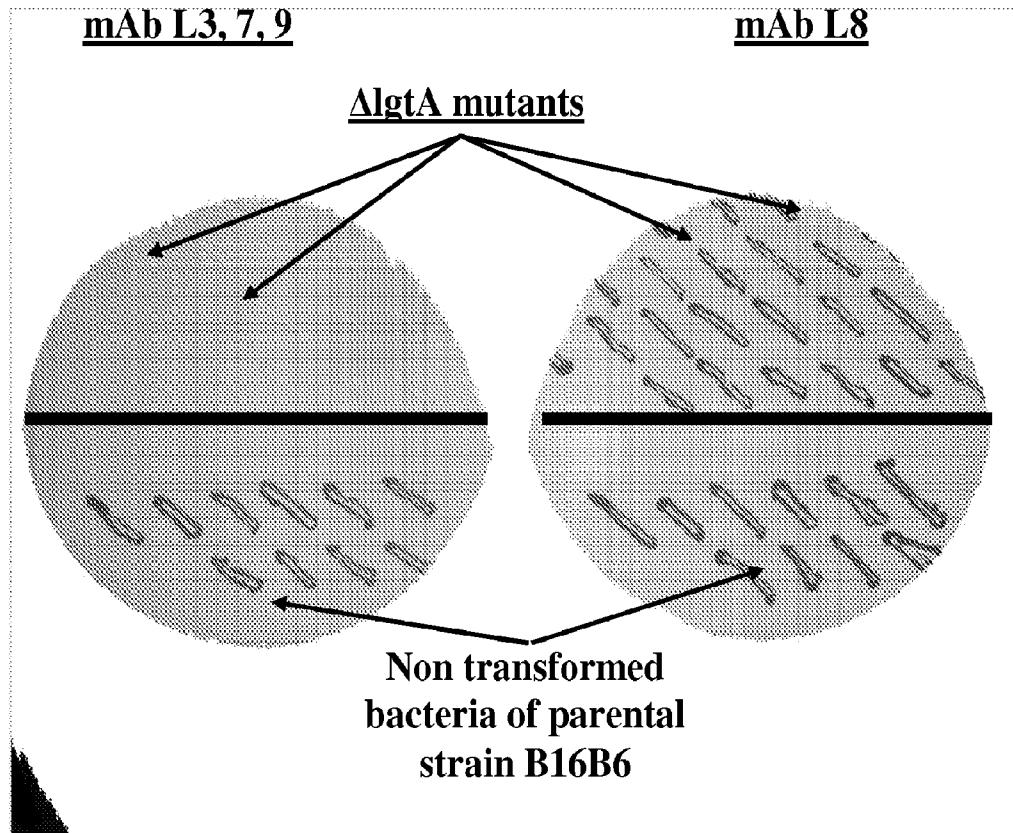
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Stable expression of L8-5  
LOS and Opc

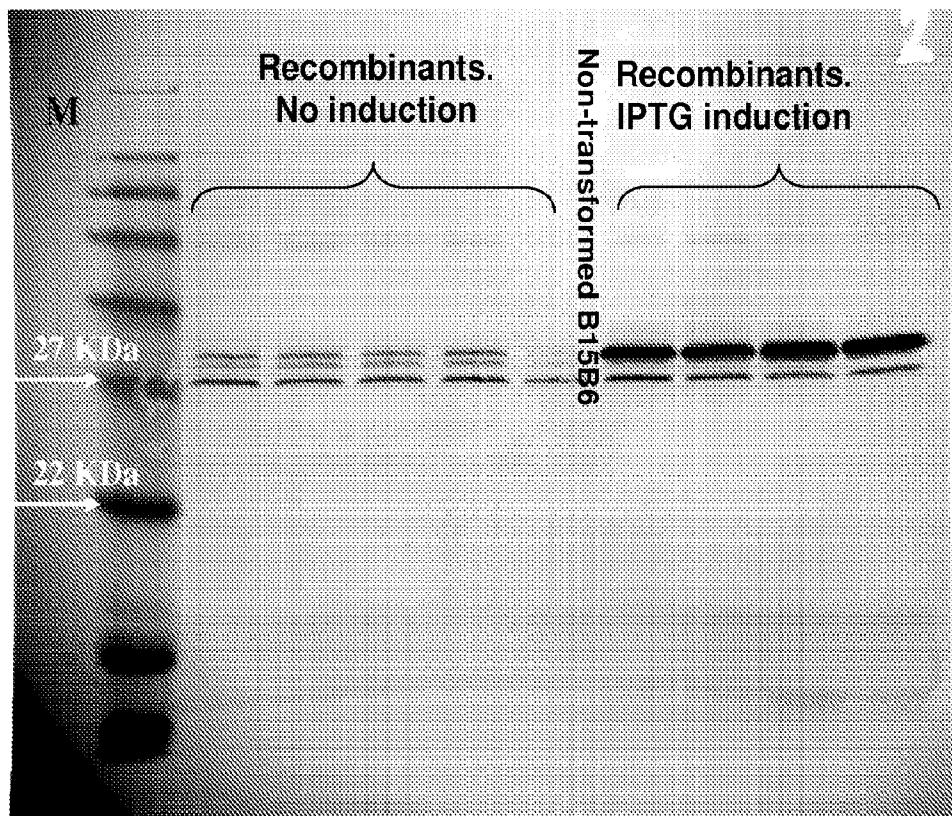
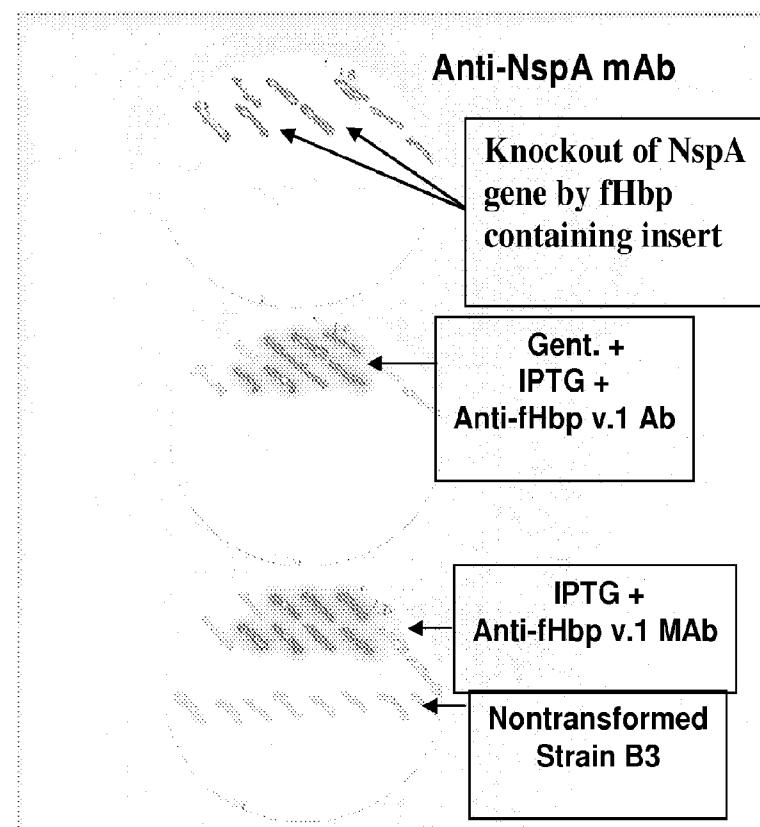
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Add 2<sup>nd</sup> PorA (P1.22-1,4)  
Over express fHbp(v.2)  
Stable expression of L8-2

16/18  
FIGURE 19

## FIGURE 20

**A.****B.**

18/18  
FIGURE 21

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SEQUENCE LISTING

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Donets, Mkhail  
Schmied, Deborah  
Loni n, Boris  
Marques, Ryan  
Moran, Elizabeth E.

<120> MENINGOCOCCAL MULTI VALENT NATIVE OUTER MEMBRANE VACCINE, METHODS OF MAKING AND USE THEREOF

<130> 20554WC01

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