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(54) **VACCINE COMPOSITION**

(76) Inventors: **Ralph Biemans**, Rixensart (BR); **Philippe Denoel**, Rixensart (BE); **Christiane Feron**, Rixensart (BE); **Carine Goraj**, Rixensart (BE); **Michael Paul Jennings**, Queensland (AU); **Jan Poolman**, Rixednsart (BE); **Vincent Weynants**, Rixensart (BE)

Correspondence Address:
SMITHKLINE BEECHAM CORPORATION
CORPORATE INTELLECTUAL
PROPERTY-US, UW2220
P. O. BOX 1539
KING OF PRUSSIA, PA 19406-0939 (US)

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(57) **ABSTRACT**

The present invention relates to the field of neisserial vaccine compositions, their manufacture, and the use of such compositions in medicine. More particularly it relates to processes of making novel engineered meningococcal strains which are less phase variable in terms of their LOS immunotype, and from which novel LOS subunit or meningococcal outer-membrane vesicle (or bleb) vaccines can be derived.

Figure 1

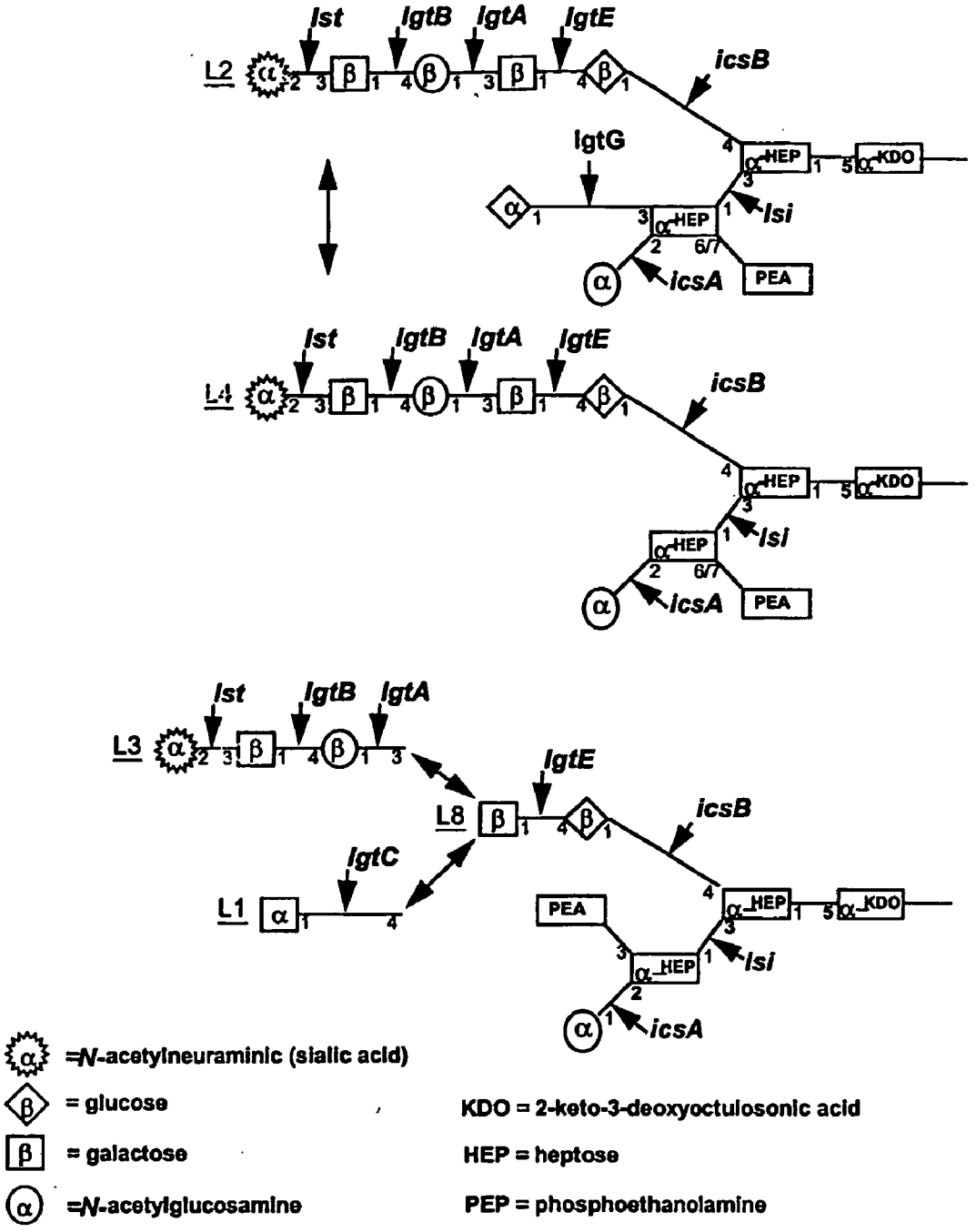

lst
lgtB
lgtA
lgtE
icsB
L2
lgtG
HEP
KDO
lsi
icsA
PEA
L4
L3
L8
lgtC
L1
=N-acetylneuraminic (sialic acid)
= glucose
= galactose
=N-acetylglucosamine
KDO = 2-keto-3-deoxyoctulosonic acid
HEP = heptose
PEP = phosphoethanolamine

Figure 2: L3 and L2 immunotypes (H44/76 , MC58 strains)

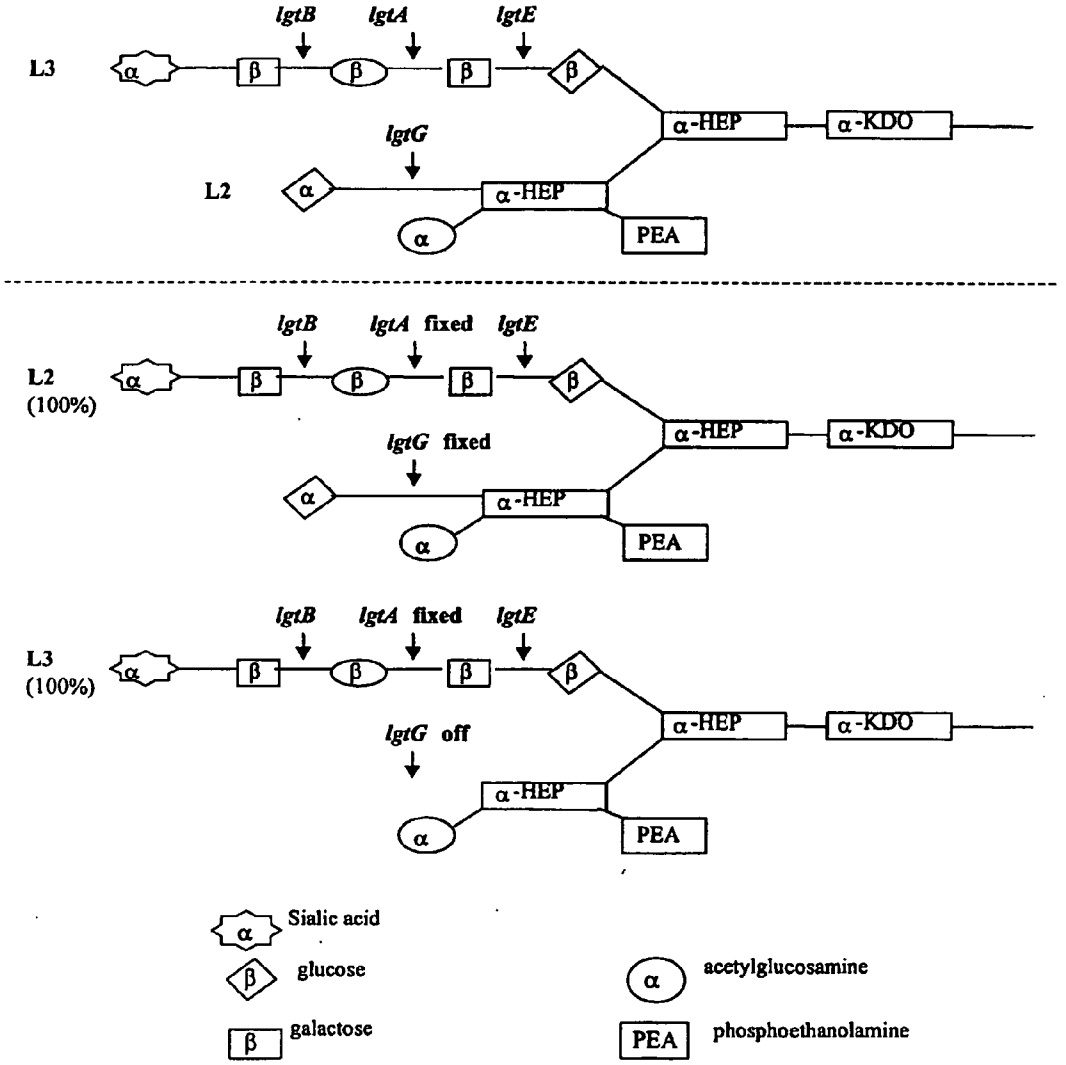
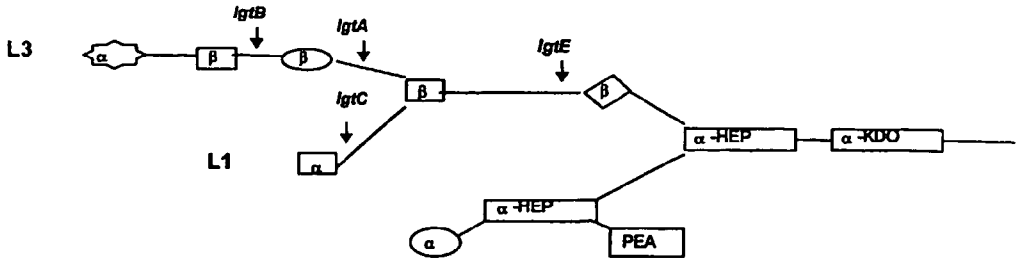
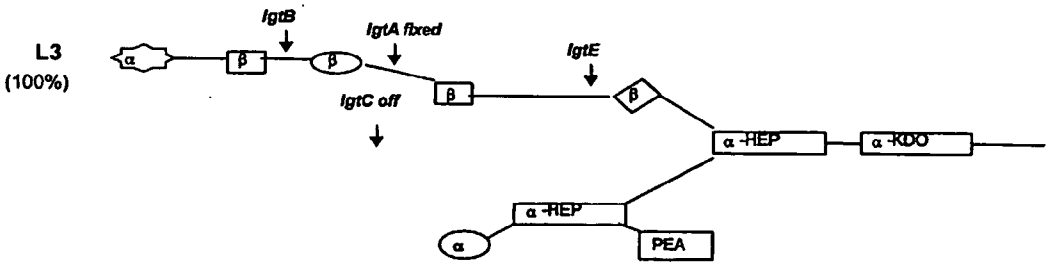


Figure 3 L3 and L1 Immunotypes (e.g. 126E strain)





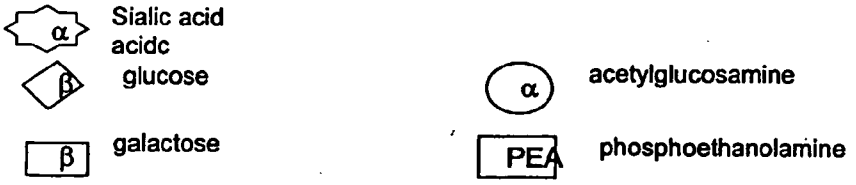


FIGURE 4

35E ATGAAGCTCAAAATAGACATTGCAACCAGCAACTTCAAACACGGCGGCGGCACGGAACGCTACACATTGGA
lgtGF ATGAAGCTCAAAATAGACATTGCAACCAACAACTTCAAACACGGCGGCGGCACGGAACGCTACACATTGGA

35E TTTGGTAAAGGGTCTGAACAGACAAAACATCACACCGGCCGTTTATGCGACGAAATTTGATCACGGCATTC
lgtGF TTTGGTAAAGGGTCTGAACAGACAAAACATCACACCGGCCGTTTATGCGACGAAATTTGATCACGGCATTC

35E CTGAATACGCCATGATCGAACCCCATCTTGTCGATCAACACCGGACGCTGAAAAAACTACGCCCATTCCTC
lgtGF CTGAATACGCCATGATCGAACCCCATCTTGTCGATCAACACCGGACGCTGAAAAAACTACGCCCATTCCTC

35E TTTTCAAGCCGGCTCACTCAAACCAGAAAAAACAGTGCCGCCAAACTGATTGCCTGCCACCACGCCGATTA
lgtGF TTTTCAAGCCGGCTCACTCAAACCAGAAAAAACAGTGCCGCCAAACTGATTGCCTGCCACCACGCCGATTA

35E CGCCGACCTCCTCATCTGCGGCGGCACACACTTGGGCTACCTGCACCATATGGCGCAAAAACCGAACCTGC
lgtGF CGCCGACCTCCTCATCTGCGGCGGCACACACTTGGGCTACCTGCACCATATGGCGCAAAAACCGAACCTGC

35E TCGACCGCCTCGCCATACGCCGTAACCGCAGCAACTACGCCACCG**CCAAACTGATTATGG**CGCATTCCCAT
lgtGF TCGACCGCCTCGCCATACGCCGTAACCGCAGCAACTACGCCACCG**CCAAACTGATTATGG**CGCATTCCCAT

35E ATGATGCGGTGCGAACTGGTCGGACTGTACGGCGTTCCCCCTGAAAGAATCCAAGTCGCCCCCCCCCCCGC
lgtGF ATGATGCGGTGCGAACTGGTCGGACTGTACGGCGTTCCCCCTGAAAGAATCCAAGTCG**CGCCGCCGCCC**GC

35E AGATACGGAACGCTTCTTCCCGCAACCCGGAGAAGCTGCCGACCTGCGCGCCAAATACGGCTTTGCCGACT
lgtGF AGATACGGAACGCTTCTTCCCGCAACCCGGAGAAGCTGCCGACCTGCGCGCCAAATACGGCTTTGCCGACT

35E ATGAAACCGTTTTCCTGTTCCCATCGACCGGCCACACGCGCAAAGGTCTGGAACTGCTTGCCGACTTTTTC
lgtGF ATGAAACCGTTTTCCTGTTCCCATCGACCGGCCACACGCGCAAAGGTCTGGAACTGCTTGCCGACTTTTTC

35E GAACATACCGGCCTGCCCGTCAAGCTCGCCGTTGTCGGCTC (SEQ ID NO: 13)
lgtGF GAACATACCGGCCTGCCCGTCAAGCTCGCCGTTGTCGGCTC (SEQ ID NO: 14)

VACCINE COMPOSITION

FIELD OF THE INVENTION

[0001] The present invention relates to the field of neisserial vaccine compositions, their manufacture, and the use of such compositions in medicine. More particularly it relates to processes of making novel engineered meningococcal strains which are less phase variable in terms of their LOS immunotype, and from which novel LOS subunit or meningococcal outer-membrane vesicle (or bleb) vaccines can be derived.

BACKGROUND OF THE INVENTION

[0002] *Neisseria meningitidis* (meningococcus) is a Gram negative bacterium frequently isolated from the human upper respiratory tract. It is a cause of serious invasive bacterial diseases such as bacteremia and meningitis. The incidence of meningococcal disease shows geographical, seasonal and annual differences (Schwartz, B., Moore, P. S., Broome, C. V.; Clin. Microbiol. Rev. 2 (Supplement), S18-S24, 1989). The bacterium is commonly classified according to the serogroup if its capsular polysaccharide.

[0003] Most disease in temperate countries is due to strains of serogroup B and varies in incidence from 1-10/100,000/year total population—sometimes reaching higher values (Kaczmarski, E. B. (1997), Commun. Dis. Rep. Rev. 7: R55-9, 1995; Scholten, R. J. P. M., Bijlmer, H. A., Poolman, J. T. et al. Clin. Infect. Dis. 16: 237-246, 1993; Cruz, C., Pavez, G., Aguilar, E., et al. Epidemiol. Infect. 105: 119-126, 1990).

[0004] Epidemics dominated by serogroup A meningococci, mostly in central Africa, sometimes reach incidence levels of up to 1000/100,000/year (Schwartz, B., Moore, P. S., Broome, C. V. Clin. Microbiol. Rev. 2 (Supplement), S18-S24, 1989). Nearly all cases as a whole of meningococcal disease are caused by serogroup A, B, C, W-135 and Y meningococci, and a tetravalent A, C, W-135, Y capsular polysaccharide vaccine is available (Armand, J., Arminjon, F., Mynard, M. C., Lafaix, C., J. Biol. Stand. 10: 335-339, 1982).

[0005] The frequency of *Neisseria meningitidis* infections has risen in the past few decades in many European countries. This has been attributed to increased transmission due to an increase in social activities (for instance swimming pools, theatres, etc.). It is no longer uncommon to isolate *Neisseria meningitidis* strains that are less sensitive or resistant to some of the standard antibiotics. This phenomenon has created an unmet medical need and demand for new anti-microbial agents, vaccines, drug screening methods, and diagnostic tests for this organism.

[0006] The available polysaccharide vaccines are currently being improved by way of chemically conjugating them to carrier proteins (Lieberman, J. M., Chiu, S. S., Wong, V. K., et al. JAMA 275: 1499-1503, 1996).

[0007] A serogroup B vaccine, however, is not available. The serogroup B capsular polysaccharide has been found to be nonimmunogenic—most likely because it shares structural similarity with host components (Wyle, F. A., Artenstein, M. S., Brandt, M. L. et al. J. Infect. Dis. 126: 514-522, 1972; Finne, J. M., Leinonen, M., Mäkelä, P. M. Lancet ii.: 355-357, 1983). Effort has therefore been focused in trying to develop serogroup B vaccines from outer membrane vesicles (or blebs) or purified protein components therefrom.

[0008] Alternative meningococcal antigens for vaccine development are meningococcal lipooligosaccharides (LOS). These are outer membrane bound glycolipids which differ from the lipopolysaccharides (LPS) of the Enterobacteriaceae by lacking the O side chains, and thus resemble the rough form of LPS (Griffiss et al. Rev Infect Dis 1988; 10: S287-295). Heterogeneity within the oligosaccharide moiety of the LOS generates structural and antigenic diversity among different meningococcal strains (Griffiss et al. Inf. Immun. 1987; 55: 1792-1800). This has been used to subdivide the strains into 12 immunotypes (Scholtan et al. J Med Microbiol 1994, 41:236-243). Immunotypes L3, L7, & L9 are immunologically identical and are structurally similar (or even the same) and have therefore been designated L3, 7, 9 (or, for the purposes of this specification, generically as "L3"). Meningococcal LOS L3, 7, 9 (L3), L2 and L5 can be modified by sialylation, or by the addition of cytidine 5'-monophosphate-N-acetylneuraminic acid. Although L2, L4 and L6 LOS are distinguishable immunologically, they are structurally similar and where L2 is mentioned herein, either L4 or L6 may be optionally substituted within the scope of the invention. Antibodies to LOS have been shown to protect in experimental rats against infection and to contribute to the bactericidal activity in children infected with *N. meningitidis* (Griffiss et al J Infect Dis 1984; 150: 71-79).

[0009] A problem associated with the use of LOS in a meningococcal vaccine, however, is its toxicity (due to its Lipid A moiety).

[0010] LOS is also present on the surface of meningococcal blebs. For many years efforts have been focused on developing meningococcal outer membrane vesicle (or bleb) based vaccines (de Moraes, J. C., Perkins, B., Camargo, M. C. et al. Lancet 340: 1074-1078, 1992; Bjune, G., Hoiby, E. A. Gronnesby, J. K. et al. 338: 1093-1096, 1991). Such vaccines have the advantage of including several integral outer-membrane proteins in a properly folded conformation which can elicit a protective immunological response when administered to a host. In addition, Neisserial strains (including *N. meningitidis* serogroup B—menB) excrete outer membrane blebs in sufficient quantities to allow their manufacture on an industrial scale. More often, however, blebs are prepared by methods comprising a 0.5% detergent (e.g. deoxycholate) extraction of the bacterial cells (e.g. EP 11243). Although this is desired due to the toxicity of LOS (also called endotoxin) as described above, it also has the effect removing most of the LOS antigen from the vaccine.

[0011] A further problem exists with the use of LOS (also known as LPS or lipopolysaccharide) as antigens in human vaccines, namely that they carry saccharide structures that are similar to human saccharide structures (for instance on human red blood cells), thus posing a safety issue with their use. Yet changing the LOS structure is problematic due to the structural sensitivity of the bactericidal effectiveness of the LOS antigen.

[0012] A further problem with using LOS as a vaccine antigen is that 12 LPS immunotypes exist with a diverse range of carbohydrate-structures (M. P. Jennings et al, Microbiology 1999, 145, 3013-3021; Mol Microbiol 2002, 43:931-43). Antibodies raised against one immunotype fail

to recognise a different immunotype. Although effort has been focused on producing a generic "core" region of the oligosaccharide portions of the LOS immunotypes (e.g. WO 94/08021), the bactericidal activity of antibodies generated against the modified LOS is lost. Thus a vaccine may need to have many LOS components of different immunotype to be effective.

[0013] Even if a few immunotypes could be selected, a final problem exists. To make LOS (or blebs containing LOS) of a certain immunotype a meningococcal strain needs to be cultured. A feature of meningococcal LOS is the reversible, high frequency switching of expression (phase variation) of terminal LOS structures (M. P. Jennings et al, Microbiology 1999, 145, 3013-3021). The phase variation exhibited by the LOS is an obstacle to the development of a cross-protective OMV or subunit vaccine based on the use of LOS as a protective antigen. For MenB strain H44/76, for example, the rate of switching from L3 to L2 immunotype is estimated at 1 in 1000 to 5000. Antibodies raised against the L3 structure failed to recognize the L2 immunotype and vice versa. Therefore it is extremely hard to maintain a LOS or bleb production strain with a constant, homogenous LOS immunotype.

[0014] The present invention presents processes for ameliorating one or more of the above problems, and presents methods for making novel vaccines based on meningococcal LOS as a protective antigen, particularly when present on an outer membrane vesicle.

SUMMARY OF THE INVENTION

[0015] The present invention relates to processes of making vaccine compositions for the effective prevention or treatment of neisserial, preferably meningococcal, disease. The processes of the invention involve making a genetically engineered meningococcal strain which has a fixed or locked LOS immunotype. In particular, methods are disclosed which allow L2 and L3 LOS immunotypes to be fixed. A process for making LOS or blebs from such engineered strains is further covered, as is a method of making an immunogenic composition comprising the steps of making the above LOS or blebs and mixing with a pharmaceutically acceptable excipient.

DESCRIPTION OF THE INVENTION

[0016] The subject matter of and information disclosed within the publications and patents or patent applications mentioned in this specification are incorporated by reference herein.

[0017] Reference to "lipooligosaccharide" (or "LOS") may also be referred to as "lipopolysaccharide" or "LPS".

[0018] The terms "comprising", "comprise" and "comprises" herein is intended by the inventors to be optionally substitutable with the terms "consisting of", "consist of", and "consists of", respectively, in every instance.

[0019] A locus containing various lgt genes is required for the biosynthesis of the terminal LOS structure (the sequences of which are known in the art—see M. P. Jennings et al, Microbiology 1999, 145, 3013-3021 and references cited therein; J. Exp. Med. 180:2181-2190 [1994]; WO 96/10086). Meningococci can change the immunotype of the expressed LOS via a mechanism of phase variable expression of some of these genes. The phase variable expression of LOS in L3 type menB strains (e.g. MC58, H44/76) operates via high frequency mutations in a homopolymeric G tract region of lgtA. A major difference between L2 and L3 (and other) immunotypes is the presence or absence of a glucose residue on the second heptose (see **FIG. 1**[with grey arrows showing phase variation] and **FIG. 2**). The addition of this residue is catalyzed by the lgtG gene product, which also exhibits phase variable expression. Other strains (e.g. 126E) can switch its LOS saccharidic structure from an L3 to an L1 immunotype through the expression of a third phase variable lgtC gene that catalyzes the extension of an additional galactose (**FIG. 1** and **FIG. 3**) (M. P. Jennings et al, Microbiology 1999, 145, 3013-3021).

[0020] The present inventors have overcome this problem by developing methods of producing neisserial vaccine production strains which are fixed (i.e. not phase variable) in their LOS immunotype. Thus in a first aspect the present invention provides a process of making a genetically engineered neisserial (preferably meningococcal, most preferably serogroup B) strain comprising the step of genetically engineering a neisserial (preferably meningococcal) strain with phase-variable LOS synthesis, to render LOS expression less phase variable (and preferably non-phase variable or fixed). By "reduced phase variability" or "less phase variable" in terms of LOS immunotype it is meant that one or more (preferably all) phase variable genes involved in the synthesis of the LOS immunotype or related LOS immunotypes is made less phase-variable or fixed so that the rate of switching between immunotypes is reduced (preferably by more than 2, 3, 5, 10 or 50 fold). By "fixed" and "non-phase variable" in terms of LOS immunotype it is meant that one or more (preferably all) phase variable genes involved in the synthesis of the LOS immunotype or related LOS immunotypes is fixed or made non-phase variable. By "reduced phase variability" or "less phase variable" in terms of LOS biosynthesis gene expression, it is meant that the chance of switching functional gene expression between on and off is reduced (preferably by more than 2, 3, 5, 10 or 50 fold), and "fixed" and "non-phase variable" in terms of LOS biosynthesis gene expression means that a gene previously susceptible to phase variation is rendered not susceptible to phase variation beyond the background chance of non site-specific switching on or off of functional gene expression.

[0021] In a specific embodiment, the process results in a reduced (preferably non) phase variable LOS having, preferably exclusively, an L2 immunotype (most preferably constitutively synthesised). Although this may be done with a strain with any immunotype (by switching on and off all appropriate genes, it is preferred that an L2 strain is used to perform this process of the invention.

[0022] Preferably such a process has a genetic engineering step comprising the element of reducing phase-variability of (preferably fixing) expression of the lgtA and/or lgtG gene products (i.e. fixing such that expression of full-length, functional gene product may not be switched off by phase variation—either or both the genes are constitutively expressed).

[0023] Clearly if either of the lgtA or lgtG genes is naturally in a fixed state in a neisserial strain to be used, only the gene that is still phase variable need be engineered.

[0024] Although fixing could take place in the present invention by inserting extra copies of either or both of the constitutively-expressed genes into the strain (whilst preferably inactivating the wild-type copy), this method is more convoluted than simply engineering the wild-type copy of the gene(s).

[0025] Preferably, the expression of either or both of lgtA and lgtG gene products is reduced in phase variability (preferably fixed) by reducing the length of the homopolymeric nucleotide tract (see Jennings et al. Microbiology 1999 145:3013) within the open-reading frame of the respective gene whilst maintaining the open-reading frame of the gene in frame.

[0026] For the homopolymeric G tract in the lgtA open-reading frame it is preferred that the tract is reduced to 8, more preferably 2, or most preferably 5 consecutive G nucleotides. Surprisingly the gene with 5 consecutive G nucleotides was optimal in terms of reduction of tract length and maintenance of LgtA enzyme function. A preferred embodiment is therefore a reduction of the tract to 5 nucleotides in combination with altering the codon usage within the tract as described below.

[0027] For the homopolymeric C tract in the lgtG open-reading frame it is preferred that the tract is reduced to 8, 5 or 2 consecutive C nucleotides.

[0028] Such tract reductions can be simply performed in general using homologous recombination (see WO 01/09350) between a plasmid construct containing the reduced tract and the genomic DNA of the strain to be changed after transformation of the strain with the plasmid.

[0029] Alternatively (or in addition), the expression of lgtA gene product can be reduced in phase-variability (preferably fixed) by changing the sequence of the homopolymeric nucleotide tract within the open-reading frame of the lgtA gene such that: one or more GGG codons encoding Glycine is changed to any other codon encoding glycine (GGA, GGC or GGT), or a codon encoding a conservative mutation, and/or the TCG codon encoding Serine (the final G being part of the tract) is changed to any other codon encoding Serine, or a codon encoding a conservative mutation, whilst maintaining the open-reading frame of the gene in frame. For instance, a 5G homopolymeric tract can advantageously have one GGG Glycine codon mutated to a nucleotide sequence GGG(A/C/T)G.

[0030] Furthermore, the expression of lgtG gene product can be alternatively or additively reduced in phase-variation (preferably fixed) by changing the sequence of the homopolymeric nucleotide tract within the open-reading frame of the lgtG gene such that: one or more CCC codons encoding Proline is changed to any other codon encoding Proline (CCA, CCG or CCT), or a codon encoding a conservative mutation, and/or the GCC codon encoding Alanine (the final CC pair being part of the tract) is changed to any other codon encoding Alanine, or a codon encoding a conservative mutation, whilst maintaining the open-reading frame of the gene in frame.

[0031] It is preferred that in the above scenarios codons are replaced with codons encoding the same amino acids, however where conservative mutations are used it is preferred that: 1) codons are selected containing 2 or (preferably) fewer nucleotides of the type making up the tract, & 2) the new encoded amino acid is a conservative mutation. Conservation mutations are understood by skilled persons in this field. However preferred substitutions are detailed in the table below.

Original residue	Exemplary substitutions	Preferred substitution
A	V, L, I	V
G	P, A	A
P	A, G	A
S	T, A	T

[0032] In such a scenario it is preferred that 2, 3 or, more preferably, 4 codons in the homopolymeric tract are changed, most preferably to encode the identical amino acid.

[0033] A combination of the above methods of the invention (reducing the tract length and altering the tract's codon usage) could be used to fix the lgtA and/or lgtG genes. For instance by both reducing the lgtA tract to 5 G residues, and replacing one of the GGG codons encoding Glycine to one of the other 3 codons encoding Glycine [yielding a final tract nucleotide sequence of GGG(A/C/T)G].

[0034] In an advantageous embodiment the expression of the lgtA gene product is fixed by reducing the length of the homopolymeric G nucleotide tract within the open-reading frame of the respective gene to 2 or 5 consecutive G nucleotides whilst maintaining the open-reading frame of the gene in frame, and the expression of lgtG gene product is fixed by changing the sequence of the homopolymeric nucleotide tract within the open-reading frame of the lgtG gene such that: 1, 2 or preferably 3 CCC codons encoding Proline is changed to any other codon encoding Proline (CCA, CCG, or CCT), or a codon encoding a conservative mutation, and/or the GCC codon encoding Alanine is changed to any other codon encoding Alanine, or a codon encoding a conservative mutation, whilst maintaining the open-reading frame of the gene in frame.

[0035] In a further specific embodiment, the process of the invention results in a reduced (preferably non) phase variable LOS having (preferably exclusively) an L3 immunotype (most preferably constitutively synthesised). Although this may be done with a strain with any immunotype (by switching on and off all appropriate genes, it is preferred that an L3 strain is used to perform this process of the invention.

[0036] As stated above, in this specification all reference to "L3" immunotype will be a reference to "L3, 7, 9", "L3", "L7", and "L9" immunotypes which have identical (or immunologically indistinguishable) carbohydrate structures.

[0037] In this process the genetic engineering step preferably comprises the elements of reducing phase-variability of (preferably fixing) the expression of the lgtA gene product [preferably such that expression of full-length, functional product may not be switched off by phase variation (i.e. is constitutively expressed as described above)], and/or permanently downregulating the expression of functional gene product from the lgtG gene.

[0038] By "downregulating the expression of functional gene product" it is meant that additions, deletions or substitutions are made to the promoter or open reading frame of

the gene such that the biosynthetic activity of the total gene product reduces (by 60, 70, 80, 90, 95 or most preferably 100%). Clearly frameshift mutations may be introduced, or weaker promoters substituted, however most preferably most or all of the open reading frame and/or promoter is deleted to ensure a permanent downregulation of the gene product. See WO 01/09350 for further methods of gene downregulation.

[0039] Clearly if lgtA expression is naturally fixed or lgtG expression is naturally down-regulated in a wild-type meningococcal strain to be altered, only the gene that is in need of change to fix the immunotype should be engineered.

[0040] Although fixing could take place in the process of the invention by inserting extra copies of the constitutively expressed lgtA gene into the organism (whilst preferably inactivating the wild-type copy), this method is more convoluted than simply engineering the wild-type copy of the gene(s).

[0041] The expression of lgtA gene product can be made less phase variable (preferably fixed) by reducing the length of the homopolymeric nucleotide tract within the open-reading frame of the gene whilst maintaining the open-reading frame of the gene in frame (preferably the homopolymeric G tract in the lgtA open-reading frame is reduced to 8, more preferably 2 or, most preferably, 5 consecutive G nucleotides) and/or by changing the sequence of the homopolymeric nucleotide tract within the open-reading frame of the lgtA gene such that: one or more GGG codons encoding Glycine is changed to any other codon encoding glycine, or a codon encoding a conservative mutation, and/or the TCG codon encoding Serine is changed to any other codon encoding Serine, or a codon encoding a conservative mutation, whilst maintaining the open-reading frame of the gene in frame (as described above). For instance, a 5G homopolymeric tract can advantageously have one GGG Glycine codon be mutated to a nucleotide sequence GGG(A/C/T)G.

[0042] A combination of the above methods of the invention (reducing the tract length and altering the tract's codon usage) could be used to fix the lgtA gene. For instance by both reducing the lgtA tract to 5 G residues, and replacing one of the GGG codons encoding Glycine to one of the other 3 codons encoding Glycine [yielding a final tract nucleotide sequence of GGG(A/C/T)G].

[0043] Preferably the expression of functional gene product from the IgtG gene is switched off (i.e. there is no or negligible IgtG gene product biosynthetic activity post mutation).

[0044] In an advantageous embodiment the expression of the lgtA gene product is fixed by reducing the length of the homopolymeric G nucleotide tract within the open-reading frame of the respective gene to 5 or 2 consecutive G nucleotides whilst maintaining the open-reading frame of the gene in frame, and the expression of functional gene product from the lgtG gene is switched off by deleting all or part of the promoter and/or open-reading frame of the gene.

[0045] Where the meningococcal strain to be altered has an lgtC gene (e.g. strain 126E), it is preferred that the processes of the invention have a genetic engineering step which comprises an element of permanently downregulating the expression of functional gene product from the lgtC gene, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene.

[0046] For potential safety implications, the above processes may be advantageously extended. The safety of antibodies raised to L3 or L2 LOS has been questioned, due to the presence of a structure similar to the lacto-N-neotetraose oligosaccharide group (Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-; **FIGS. 1-3**) present in human glycosphingolipids. Although a large number of people have been safely vaccinated with deoxycholate extracted vesicle vaccines containing residual amount of L3 LOS (G. Bjune et al, Lancet (1991), 338, 1093-1096; GVG. Sierra et al, NIPH ann (1991), 14, 195-210), if LOS is to be retained as an antigen as discussed herein, the deletion of a terminal part of the LOS saccharide structure has been found by the current inventors to be advantageous in preventing cross-reaction of the anti-LOS immune response with structures present at the surface of human tissues. In a preferred embodiment, inactivation of the lgtB gene results in an intermediate LOS structure in which the terminal galactose residue and the sialic acid are absent (see **FIG. 1-3**, the mutation leaves a 4GlcNAcβ1-3Galβ1-4Glcβ1-structure in L2 and L3 LOS). Such intermediates could be obtained in an fixed L3 (lgtA fixed on and/or lgtG fixed off) and a fixed L2 (lgtA and/or lgtG fixed on) LOS strain. An alternative and less preferred (short) version of the LOS can be obtained by turning off the lgtE gene.

[0047] Therefore, the above processes may also have a genetic engineering step comprising the element of downregulating (preferably permanently) the expression of functional gene product from the lgtB or lgtE gene, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene. A process involving rendering a strain $lgtB^{-}$is most preferred as the inventors have found that this is the optimal truncation for resolving the safety issue whilst still retaining an L2 or L3 LOS protective oligosaccharide epitope that can still induce a bactericidal (and even cross-bactericidal) antibody response.

[0048] Preferably the strains used in the processes of the invention are unable to synthesise capsular polysaccharide, where this is not the case it is advantageous to add a further process step of downregulating the expression of functional gene product critical for the production of capsular polysaccharide.

[0049] Where the process involves a wild-type meningococcus B strain, it is preferred that the genetic engineering step of the process comprises the element of permanently downregulating the expression of functional gene product from the siaD gene, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene. Such an inactivation is also described in WO 01/09350. The siaD (also known as synD) mutation is the most advantageous of many mutations that can result in removing the human-similar epitope from the capsular polysaccharide. This is because it is one of the only mutations that has no effect on the biosynthesis of the protective eptitopes of LOS, and thus it is advantageous in a process which aims at ultimately using LOS as a protective antigen, and has a minimal effect on the growth of the bacteria. Most preferably the processes of the invention

utilise a meningococcus B mutant strain with the lgtB and siaD genes downregulated or inactivated (preferably a lgtB$^-$ siaD$^-$strain).

[0050] Although siaD$^-$mutation is preferable for the above reasons, other mutations which switch off meningococcus B (or meningococcus in general) capsular polysaccharide synthesis may be used in the process of the invention. Thus the bleb production strain can be genetically engineered to permanently downregulate the expression of functional gene product from one or more of the following genes: ctrA, ctrB, ctrC, ctrD, synA (equivalent to synX and siaA), synB (equivalent to siaB) or synC (equivalent to siaC) genes, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene. The lgtE$^-$mutation may be combined with one or more of these mutations. Preferably the lgtB$^-$mutation is combined with one or more of these mutations.

[0051] A Neisserial locus containing various lgt genes, including lgtB and lgtE, and its sequence is known in the art (see M. P. Jennings et al, Microbiology 1999, 145, 3013-3021 and references cited therein; J. Exp. Med. 180:2181-2190 [1994]; WO 96/10086).

[0052] The processes of the invention may also include steps which render the LOS less toxic. Although this is not necessary for intranasal immunization with native OMV (J. J. Drabick et al, Vaccine (2000), 18, 160-172), for parenteral vaccination detoxification would present an advantage. LOS can be detoxified genetically by mutation/modification/inactivation of genes involved in Lipid A biosynthesis for example by downregulating the expression of functional gene product from the msbB and/or htrB genes, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene. Alternatively (or in addition) one or more of the following genes may be upregulated (by introducing a stronger promoter or integrating an extra copy of the gene): pmrA, pmrB, pmrE and pmrF.

[0053] See WO 01/09350 for more detail on the above detoxification methods, and for relevant promoter/gene sequences and upregulation and downregulation methods. The msbB and htrB genes of *Neisseria* are also called lpxL1 and lpxL2, respectively, (see WO 00/26384) and deletion mutations of these genes are characterised phenotypically by the msbB$^-$mutant LOS losing one secondary acyl chain compared to wild-type (and retaining 4 primary and 1 secondary acyl chain), and the htrB$^-$mutant LOS losing both secondary acyl chains. Such mutations are preferably combined with mutations to ensure that the neisserial production strain is capsular polysaccharide deficient (see above) to ensure the optimal presentation of detoxified LOS on the bleb, or to aid the purification of the detoxified subunit LOS.

[0054] A further aspect of the invention is a process of isolating L2 LOS comprising the steps of producing a genetically engineered neisserial strain with a reduced phase variable (preferably fixed) L2 immunotype by the process of the invention as described above, and isolating L2 LOS from the resulting strain. An additional advantageous step may be added to this process, namely conjugating the L2 LOS to a carrier comprising a source of T-cell epitopes (rendering the LOS an even better immunogen) and/or the step of presenting the L2 LOS in liposome formulations known in the art (see for instance WO 96/40063 and references cited therein).

[0055] The process of isolation of LOS from bacteria is well known in the art (see for instance the hot water-phenol procedure of Wesphal & Jann [Meth. Carbo. Chem. 1965, 5:83-91]). See also Galanos et al. 1969, Eur J Biochem 9:245-249, and Wu et al. 1987, Anal Bio Chem 160:281-289. Techniques for conjugating isolated LOS are also known (see for instance EP 941738 incorporated by reference herein).

[0056] For the purposes of this invention "a carrier comprising a source of T-cell epitopes" is usually a peptide or, preferably, a polypeptide or protein. Conjugation techniques are well known in the art. Typical carriers include protein D from non typeable *H. influenzae*, tetanus toxoid, diphtheria toxoid, CRM197, or outer membrane proteins present in bleb (particularly neisserial or meningococcal) preparations. Preferably the oligosaccharide portion of the LOS is conjugated.

[0057] Similarly a still further aspect of the invention is a process of isolating L3 LOS comprising the steps of producing a genetically engineered meningococcal strain with a reduced phase variable (preferably fixed) L3 immunotype by the process of the invention as described above, and isolating L3 LOS from the resulting strain. An additional advantageous step may be added to this process, namely conjugating the L3 LOS to a carrier comprising a source of T-cell epitopes and/or the step of presenting the L3 LOS in a liposome formulation.

[0058] For processes of the invention involving the isolation of LOS from strains with reduced phase variability, preferably the LOS is detoxified as part of the process. This may be done by known techniques of hydrazine or alkaline hydrolysis chemical treatments which remove acyl chains from the molecule (but which may reduce the protective efficacy of the molecule), but is preferably done by isolating the LOS from an htrB$^-$and/or msbB$^-$meningococcal mutant (as described above; particularly in capsule polysaccharide minus strains), or by adding a non-toxic peptide functional equivalent of polymyxin B [a molecule with high affinity to Lipid A] to the isolated LOS, in particular SAEP 2. See WO 93/14115, WO 95/03327, Velucchi et al (1997) J Endotoxin Res 4: 1-12, and EP 976402 for further details of non-toxic peptide functional equivalents of polymyxin B that may be used in the processes of this invention—particularly the use of the peptide SAEP 2 (of sequence KTKCKFLKKC where the 2 cysteines form a disulphide bridge).

[0059] Where the process of isolating fixed LOS of the invention introduces LOS into a liposome, outer membrane proteins may optionally also be added, and the LOS may be conjugated intra-liposome to such outer membrane proteins to render the oligosaccharide a T-dependent antigen. This may be done with a similar chemistry as described for intra-bleb LOS cross-linking as described below.

[0060] A further aspect of the invention is a process of isolating meningococcal blebs having an L2 or L3 LOS immunotype, comprising the steps of producing a genetically engineered meningococcal strain with a reduced phase variable (preferably fixed) L2 or L3 immunotype, respectively, by the processes of the invention as described above; and isolating blebs from the resulting strain.

[0061] Outer Membrane Vesicles (OMVs or blebs) can be isolated by many known techniques (Fredriksen et al, NIPH

Annals (1991), 14, 67-79; Zollinger et al, J. Clin Invest (1979), 63, 836-848; Saunders et al, Infect Immun (1999), 67, 113-119; J. J. Drabick et al, Vaccine (1999), 18, 160-172). These divide into 2 main groups—techniques which use deoxycholate (about 0.5%) to extract blebs from meningococcus, and techniques that use low levels of deoxycholate (DOC) or no deoxycholate at all. DOC free process blebs have the interesting feature of maintaining high level of LOS in the OMV—which is advantageous in a vaccine where LOS is a protective antigen. Compared to DOC extracted blebs, the concentration of L3 Ags in OMV obtained by a DOC free process is approximately ten times higher, also taking into account the fixing of lgtA. A detergent-free (preferably DOC-free) process of preparing blebs is preferred for the purposes of the processes of this invention for this reason, although extraction with a buffer containing low levels of detergent (preferably DOC) may also be advantageous in that the step would leave most of the tightly interacting LOS in the bleb whilst removing any more toxic loosely retained LOS. Typically 0-0.5% and preferably 0.02-0.4%, 0.04-3% or 0.06-2% detergent (preferably DOC) is used for bleb extraction, more preferably 0.08-0.15%, and most preferably around or exactly 0.1% is used to obtain an optimal amount of LOS to be stably present in the blebs. DOC free (or low DOC—0.3% or under (preferably 0.05-0.2%) DOC) extraction processes are particularly preferred where the LOS has been detoxified by one or more of the methods detailed above.

[**0062**] It is preferred that the LOS content of the blebs isolated by the process of the invention is 3-30, 5-25, 10-25, 15-22, and most preferably around or exactly 20% LOS content as measured by silver staining after SDS-PAGE electrophoresis using purified LOS as a standard (see method of Tsai, J. Biol. Standardization (1986) 14:25-33). Using Nmen L3 LOS as a standard in this method, in general LOS content in Nmen L3 immunotype blebs extracted with 0.1% DOC is about 20% LOS, with 0.2% DOC is about 15% LOS, with 0.3% DOC is about 10% LOS, and with 0.5% DOC is about 5% LOS.

[**0063**] The above bleb isolation processes of the invention may comprise an additional advantageous step of conjugating the L2 or L3 LOS in situ to an outer membrane protein (e.g. PorA or PorB) also present in the bleb preparation. Thus a further aspect of the invention is a process of the invention where the isolated bleb preparation is conjugated (through an integral outer-membrane protein) to LOS. Although LOS may be added to a bleb preparation for conjugation, it is preferred that the LOS is naturally present on the surface of the bleb preparation.

[**0064**] This process can advantageously enhance the stability and/or immunogenicity (providing T-cell help) and/or antigenicity of the LOS antigen within the bleb formulation—thus giving T-cell help for the T-independent oligosaccharide immunogen in its most protective conformation—as LOS in its natural environment on the surface of the outer membrane. In addition, conjugation of the LOS within the bleb can result in a detoxification of the LOS (without wishing to be bound by theory, the Lipid A portion may be more stably buried in the outer membrane if conjugated thus being less available to cause toxicity). Thus the detoxification methods mentioned above of isolating blebs from htrB$^-$or msbB$^-$mutants, or by adding non toxic peptide functional equivalent of polymyxin B to the composition may not be required (but which may be added in combination for additional security).

[**0065**] The processes of the invention may thus yield conjugated bleb preparations which are typically such that the toxicity of the LOS in the bleb is reduced compared to the same blebs with the same amount of totally unconjugated LOS. LOS toxicity may be readily determined by a skilled person, for example using the LOS rabbit pyrogenicity assay in the European Pharmacopoeia.

[**0066**] In particular, the inventors have found that a process of the invention yielding a composition comprising blebs wherein LOS present in the blebs has been conjugated in an intra-bleb fashion to outer membrane proteins also present in the bleb is advantageous in being part of a process to make a vaccine for the treatment or prevention of neisserial (preferably meningococcal) disease, wherein the process allows the vaccine to be of reduced toxicity and/or capable of inducing a T-dependent bactericidal response against LOS in its native environment.

[**0067**] This invention therefore further provides a method to make such an intra-bleb LOS conjugated bleb preparation from a strain of reduced phase variability (preferably fixed) LOS immunotype. By "intra bleb" it is meant that LOS naturally present in the bleb is conjugated to outer membrane protein present on the same bleb.

[**0068**] Such bleb preparations may be made by isolated blebs and then subjected them to known conjugation chemistries to link groups (e.g. NH_2 or COOH) on the oligosaccharide portion of LOS to groups (e.g. NH_2 or COOH) on bleb outer membrane proteins. Cross-linking techniques using glutaraldehyde, formaldehyde, or glutaraldehyde/formaldehyde mixes may be used, but it is preferred that more selective chemistries are used such as EDAC or EDAC/NHS (J. V. Staros, R. W. Wright and D. M. Swingle. Enhancement by N-hydroxysuccinimide of water-soluble carbodiimide-mediated coupling reactions. Analytical chemistry 156: 220-222 (1986); and Bioconjugates Techniques. Greg T. Hermanson (1996) pp173-176). Other conjugation chemistries or treatments capable of creating covalent links between LOS and protein molecules that could be used in this invention are described in EP 941738.

[**0069**] Preferably the bleb preparations are conjugated in the absence of capsular polysaccharide. The blebs may be isolated from a strain which does not produce capsular polysaccharide (naturally or via mutation), or may be purified from most (more than 60, 70, 80, 90, or 99% removed) and preferably all contaminating capsular polysaccharide. In this way, the intra-bleb LOS conjugation reaction is much more efficient.

[**0070**] Preferably more than 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 95% of the LOS present in the blebs is cross-linked/conjugated.

[**0071**] Preferably the blebs of the invention have been prepared such that the LOS content of the blebs is 3-30, 5-25, 10-25, 15-22, and most preferably around or exactly 20% LOS content as measured by silver staining after SDS-PAGE electrophoresis using purified LOS as a standard (see method of Tsai, J. Biol. Standardization (1986) 14:25-33). 20% LOS in meningococcal blebs can be achieved with

a 0.1% low DOC extraction, which may remove losely held LOS molecules, but conserve the majority of the antigen.

[0072] Where the intra-bleb conjugated blebs made by the process of the invention are derived from meningococcus, it is preferred that the strain from which they are derived is a mutant strain that cannot produce capsular polysaccharide (e.g. one of the mutant strains described above, in particular siaD$^-$).

[0073] A typical L3 meningococcal strain that can be used for the present invention is the H44/76 menB strain. A typical L2 strain is the B16B6 menB strain or the 39E meningococcus type C strain or strain 760676.

[0074] As stated above, the process of the invention allows the detoxification of blebs to some degree by the act of conjugation, and need not be detoxified any further, however further detoxification methods may be used for additional security, for instance by using blebs derived from a meningococcal strain that is htrB$^-$ or msbB$^-$ or adding a non-toxic peptide functional equivalent of polymyxin B [a molecule with high affinity to Lipid A] (preferably SEAP 2) to the bleb composition (as described above).

[0075] In the above way meningococcal blebs and immunogenic compositions comprising blebs can be made by the processes of the invention which have as an important antigen LOS of a certain immunotype (preferably L2 or L3) which is reproducibly made without phase variation, is reduced in toxicity (and preferably substantially non-toxic), devoid of autoimmunity problems, has a T-dependent character, and is present in its natural environment.

[0076] One or more of Men A, C, Y or W capsular polysaccharides or oligosaccharides (preferably at least MenC, or MenA and MenC, or Men C and MenY) may also be conjugated onto an outermembrane protein of the bleb in a process of the invention as well. Although this could be done in the same reaction as LOS cross-linking, it is preferred that this is done in a separate (preferably later) reaction.

[0077] Intrableb conjugation should preferably incorporate 1, 2 or all 3 of the following process steps: conjugation pH should be greater than pH 7.0, preferably greater than or equal to pH 7.5 (most preferably under pH 9); conditions of 1-5% preferably 2-4% most preferably around 3% sucrose should be maintained during the reaction; NaCl should be minimised in the conjugation reaction, preferably under 0.1M, 0.05M, 0.01M, 0.005M, 0.001M, and most preferably not present at all. All these process features make sure that the blebs remain stable and in solution throughout the conjugation process.

[0078] The EDAC/NHS conjugation process is a preferred process for intra-bleb conjugation. EDAC/NHS is preferred to formalydehyde which can cross-link to too high an extent thus adversely affecting filterability. EDAC reacts with carboxylic acids (such as KDO in LOS) to create an active-ester intermediate. In the presence of an amine nucleophile (such as lysines in outer membrane proteins such as PorB), an amide bond is formed with release of an isourea by-product. However, the efficiency of an EDAC-mediated reaction may be increased through the formation of a Sulfo-NHS ester intermediate. The Sulfo-NHS ester survives in aqueous solution longer than the active ester formed from the reaction of EDAC alone with a carboxylate. Thus, higher yields of amide bond formation may be realized using this two-stage process. EDAC/NHS conjugation is discussed in J. V. Staros, R. W. Wright and D. M. Swingle. Enhancement by N-hydroxysuccinimide of water-soluble carbodiimide-mediated coupling reactions. Analytical chemistry 156: 220-222 (1986); and Bioconjugates Techniques. Greg T. Hermanson (1996) pp173-176. Preferably 0.01-5 mg EDAC/mg bleb (by protein measured by Lowry) is used in the reaction, more preferably 0.05-1 mg EDAC/mg bleb. The amount of EDAC used depends on the amount of LOS present in the sample which in turn depends on the deoxycholate (DOC) % used to extract the blebs. At low % DOC (e.g. 0.1%), high amounts of EDAC are used (1 mg/mg and beyond), however at higher % DOC (e.g. 0.5%), lower amounts of EDAC are used (0.025-0.1 mg/mg) to avoid too much inter-bleb crosslinking.

[0079] A preferred process of the invention is therefore a process for producing intra-bleb conjugated LOS (preferably meningococcal) comprising the steps of producing reduced phase variable LOS, isolating blebs, conjugating blebs in the presence of EDAC/NHS at a pH between pH 7.0 and pH 9.0 (preferably around pH 7.5), in 1-5% (preferably around 3%) sucrose, and optionally in conditions substantially devoid of NaCl (as described above), and isolating the conjugated blebs from the reaction mix.

[0080] The reaction may be followed on Western separation gels of the reaction mixture using anti-LOS (e.g. anti-L2 or anti-L3) mAbs to show the increase of LOS molecular weight for a greater proportion of the LOS in the blebs as reaction time goes on.

[0081] Yields of 99% blebs can be recovered using such techniques.

[0082] EDAC was found to be an excellent intra-bleb cross-linking agent in that it cross-linked LOS to OMP sufficiently for improved LOS T-dependent immunogenicity, but did not cross link it to such a high degree that problems such as poor filterability, aggregation and inter-bleb cross-linking occurred. The morphology of the blebs generated is similar to that of unconjugated blebs (by electron microscope). In addition, the above protocol avoided an overly high cross-linking to take place (which can decrease the immunogenicity of protective OMPs naturally present on the surface of the bleb e.g. TbpA or Hsf).

[0083] A process for making immunogenic compositions or vaccines are also provided comprising the steps of producing isolated L2 LOS by the process of the invention as described above and/or producing isolated meningococcal blebs having an L2 LOS immunotype by the processes of the invention as described above, and formulating the L2 LOS and/or blebs with a pharmaceutically acceptable excipient.

[0084] Likewise a process for making immunogenic compositions or vaccines are also provided comprising the steps of producing isolated L3 LOS by the process of the invention as described above and/or producing isolated meningococcal blebs having an L3 LOS immunotype by the processes of the invention as described above, and formulating the L3 LOS and/or blebs with a pharmaceutically acceptable excipient.

[0085] An advantageous process of the invention is a process of making a multivalent immunogenic composition

or vaccine comprising the steps of producing one or both of isolated L2 LOS or isolated meningococcal blebs having an L2 LOS immunotype by the processes of the invention as described above, and producing one or both of isolated L3 LOS or isolated meningococcal blebs having an L3 LOS immunotype by the processes of the invention as described above, and mixing said L2 and L3 vaccine components together along with a pharmaceutically acceptable excipient. Preferably the process mixes isolated L2 and L3 LOS together which are made as described above (most preferably conjugated and in a liposome formulation). More preferably the process mixes L2 and L3 blebs together which are made as described above. Such compositions are advantageous as approximately 70% of meningococcus B immunotypes observed in disease isolates have an L3 structure, and 30% are L2. The invention therefore describes a process which can yield a universal meningococcus B vaccine.

[0086] The process of making immunogenic compositions or vaccines as described above may have an additional step of adding one or more (2, 3 or 4) meningococcal polysaccharides or oligosaccharides (either plain or conjugated to a carrier comprising T-cell epitopes) from serogroups A, C, Y or W to the composition. Preferably at least C is added (most preferably conjugated), and more preferably A and C or Y and C (preferably all conjugated) and most preferably A, C, Y and W (preferably all conjugated).

[0087] A further step that may be added to the above processes for making immunogenic compositions or vaccines as described above is the addition of a suitable adjuvant. Suitable adjuvants include an aluminium salt such as aluminum hydroxide gel (alum) or aluminium phosphate (preferably aluminum 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 derivatised polysaccharides, or polyphosphazenes.

[0088] Suitable Th1 adjuvant systems that may be added include, Monophosphoryl lipid A, particularly 3-de-O-acylated monophosphoryl lipid A (or other non-toxic derivatives of LPS), and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) [or non toxic LPS derivatives] together with an aluminium salt, preferably aluminium phosphate. An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 [or other saponin] and 3D-MPL [or non toxic LPS derivative] as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 [or saponin] is quenched with cholesterol as disclosed in WO96/33739. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil in water emulsion is described in WO95/17210 and is a preferred formulation that may be added. Other adjuvants that may be added comprise a saponin, more preferably QS21 and/or an oil in water emulsion and tocopherol. Unmethylated CpG containing oligo nucleotides (WO 96/02555) may also be added

[0089] Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M. F. & Newman M. J.) (1995) Plenum Press New York).

[0090] An immunoprotective dose of vaccines can be administered via the systemic or mucosal route. These administrations may include injection via the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or via mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Typically bleb quantity in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-100 μg of each bleb, preferably 5-50 μg, and most typically in the range 5-25 μg.

Ghost or Killed Whole cell Vaccines

[0091] The inventors envisage that the above processes concerning blebs can be easily extended to processes concerning ghost or killed whole cell preparations and vaccines (with identical advantages). Methods of making ghost preparations (empty cells with intact envelopes) from Gram-negative strains are well known in the art (see for example WO 92/01791). Methods of killing whole cells to make inactivated cell preparations for use in vaccines are also well known. The processes concerning blebs described throughout this document are therefore applicable to the processes concerning ghosts and killed whole cells for the purposes of this invention.

Growth of Neisserial Cells to High Cell Density in a Fermentor

[0092] The present inventors have also found that LOS phase variation is particularly problematic when trying to grow high cell densities of cells in fermentors. When nutrients become depleted it becomes more common for meningococcal LOS immunotype to change (in particular to shorter oligosaccharide chain LOS immunotypes). This can happen for L2 and L3 strains, and in particular truncated (e.g. lgtB) strains. The inventors have found that the processes of the invention in fixing LOS immunotype can alleviate this problem, and may allow high cell densities without change of immunotype. In particular, lgtA should be reduced in phase-variability, preferably fixed.

[0093] Thus, in a further aspect of the invention there is provided a process of growing a high cell density of a neisserial strain comprising the steps of:

[0094] a) genetically-engineering a neisserial strain to reduced the phase variability (and preferably fix) the LOS immunotype of said strain according to the processes of the invention above; and

[0095] b) growing the strain to high cell density in a fermentor.

[0096] Preferably an L2 or L3 meningococcal strain is grown (where preferably lgtA should be reduced in phase-variability, preferably fixed).

[0097] By "high cell density" it is meant a cell density of OD_{450} 10-19, preferably 12-16, in iron non-limiting conditions, or 6-12, preferably 8-10, in iron limited conditions.

[0098] The process may be extended by adding steps of isolating the LOS from the culture at high cell density. The LOS may then be conjugated to a carrier and/or introduced into a liposome as discussed above.

[0099] A bleb isolation step may alternately be added to obtain blebs from the culture of high cell density. This

should be done ideally with a low detergent, preferably DOC, % process, typically 0-0.3%, preferably 0.05-70.2%, most preferably around or exactly 0.1% deoxycholate.

[0100] The bleb LOS may advantageously be intra-bleb cojugated to an outer membrane protein also present in the blebs as described above.

[0101] The process may be extended to producing an immunogenic composition by formulating the LOS produced above with a pharmaceutically acceptable excipient.

[0102] Advantageously a process of making a multivalent immunogenic composition is provided comprising the steps of producing isolated LOS or isolated blebs of a certain immunotype (preferably L2) by the above process, and producing isolated LOS or isolated blebs of a different immunotype (preferably L3) by the above process, and mixing these LOS components components together along with a pharmaceutically acceptable excipient.

EXAMPLES

[0103] The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples are illustrative, but do not limit the invention.

Example 1

Making a Fixed L3 Strain (Fixed LgtA)

[0104] Genes encoding glycosyltransferases in *Neisseria meningitidis* often contain simple tandem repeats (for example, homopolymeric tracts) which mediate phase variation (high frequency reversible on/off switching of gene expression (Jennings et al 1995, Mol Micro 18 724; Jennings et al 1999, Microbiology 145 3013). The repeated sequences in these genes are present in the open reading frame and are transcribed and translated into protein. Phase variation may be eliminated by reducing (in frame) the homopolymeric tract. An alternative approach to deletion of the repeat sequences is to alter the nucleotide sequence in the repeat region so that it encodes the same amino acid sequence but does not constitute a repeat (see lgtG "fixed" mutant, Example 3). In this work we sought to "fix" the expression of certain glycosyltransferase genes in *Neisseria meningitidis* so that their expression was constituitively "on" or "off". In this way the LPS antigen expressed could be fixed to a defined structure, no longer subject to phase variation.

Mutation of the LgtA ene of Give Constitutive Expression of LgtA—the LgtA2G Mutant

[0105] In order to fix the expression of the lgtA gene so that it was fixed "on" we altered the homopolymeric tract of the lgtA gene so that only 2 G residues remained in the homopolymeric tract region (the wild type strain, MC58, has 14 G; Jennings et al 1995, supra). Using primers Lic31ext: 5'-CCT TTA GTC AGC GTA TTG ATT TGC G-3' and lgtAG2 5'-ATC GGT GCG CGC AAT ATA TTC CGA CTT TGC CAA TTC ATC-3' in PCR with *Neisseria meningitidis* strain MC58 chromosomal DNA as template we amplified the region to be altered. The latter primer incorporated the change in the lgtA sequence from 14G to 2G. The resulting PCR product was cloned into pT7Blue (Novogen), to create plasmid pT7lgtAG2. To reconstitute to complete lgtA gene so that the plasmid could be used to transform the new allele into *Neisseria meningitidis*, a BssHII fragment from plasmid p1B11 (Jennings et al 1995, supra) was cloned into the BssHII site of pT7lgtAG2 in the correct orientation. Nucleotide sequence analysis confirmed the correct orientation of the gene and that the sequence segment was identical to the corresponding section of the wild-type lgtA gene (Genbank accession NMU25839) apart from the alteration of the homopolymeric tract from 14 to 2 G residues. Using a similar process, variants of the lgtAG2 primer mutations were made so that a series of similar plasmids were created that contained lgtA alleles with 3, 4, 5, 7 and 10 G residues in the homopolymeric tract region.

[0106] Using a similar process the phase variation of the homopolymeric tract can also be fixed by altering the poly G regions so that the GGG codons are replaced with alternative glycine codons arranged so that the same amino acid sequence is encoded, but the nucleotide sequence does not have a repetitive nature and is unlikely to phase vary (see lgtG example below). In addition, a combination of the 2 methods could also be used—for instance the homopolymeric tract could be cut to 5 G residues & a GGG codon replaced with an alternative glycine codon.

Transformation of Strain MC58¢3 with pT7lgtAG2 to Transfer the LgtAG2to the Chromosome of *Neisseria Meningitidis* Strain MC58¢3

[0107] In order to transfer the lgtAG2 mutation to the chromosome of *Neisseria meningitidis* to make a mutant strain, the plasmid pT7lgtAG2 was linearized and used to transform *Neisseria meningitidis* strain MC58¢3 containing an lgtA::kan mutation (Jennings et al 1995, supra). Positive colonies were detected by mAb 4A8B2 in colony-immunoblot (Jennings et al 1999, supra). Confirmation that the LgtA positive phenotype (L3 immunotype structure) of the transformants was the result of the transfer of the lgtAG2 allele to the chromosome was confirmed by PCR of the relevant section of the lgtA gene using primers Lic31 ext and Lic16ext: 5'-CGA TGA TGC TGC GGT CTT TTT CCA T-3', followed by nucleotide sequencing with the same set of primers. The resulting strain 2G2 had the genotype: MC58 parent strain; siaD::ery lgtAG2). Strain 2G2 was subsequently transformed with the a plasmid containing an lgtB-::kan mutation (Jennings et al 1995, supra) to create strain 2G2ecoNI, this strain had the genotype: MC58 parent strain; siaD::ery lgtAG2 lgtB::kan

Example 2

Experiments with Fixed L3 and Intermediate (LgtB$^-$) DOC Free Blebs (Non-Detoxified LOS) Induced Cross-Bactericidal Antibodies

[0108] The MC58 derivative strain used is B:P1.7.16, opc-, siaD-. This strain was genetically modified to express either L3 (strain 2G2 [modified to reduce the homopolymeric tract to only 2 G nucleotides], lgtA fixed on) or an intermediate epitope (strain 2G EcoN1b-1, lgtA fixed on as with 2G2 but lgtB additionally turned off) or an LPS in short version (strain C6, lgtE off). OMV were produced according either a DOC process or DOC free process.

[0109] Mice (10 per group) were immunized three times by the intramuscular route on Day 0, 20 and 28. They received 1 or 10 μg (protein content) of blebs formulated on Al(OH)3. Blood samples were taken on day 28 (post II) and day 42 (post III).

[0110] Bactericidal assays were done on pooled sera and using homologous strains (MC58 and H44/76) and two heterologous strains (M97250687 and M9725078) with baby rabbit serum as source of exogenous complement.

[0111] The following table summarizes the results (bactericidal titers for 50% killing):

		Strain and serotype			
Antigen	Blood samples	MC58 P1.7.16	H44/76TT P1.7.16	M97250687 P1.19.15	M97252078 P1.4
c6 no doc 10 μg IM	Post II	>2560	>2560	>2560	98
c6 no doc 10 μg IM	Post III	1 353	>2560	>2560	90
c6 no doc 1 μg IM	Post II	247	620	247	<20
c6 no doc 1 μg IM	Post III	411	878	748	<20
2g2 no doc 10 μg IM	Post II	>320	>2560	>2560	>2560
2g2 no doc 10 μg IM	Post III	>2560	>2560	>2560	1407
2g2 no doc 1 μg IM	Post II	>2560	>2560	>2560	119
2g2 no doc 1 μg IM	Post III	>2560	>2560	>2560	348
2gecoN1b-1 no doc 10 μg IM	Post II	>2560	>2560	>2560	1162
2gecoN1b-1 no doc 10 μg IM	Post III	>2560	>2560	>2560	1213
2gecoN1b-1 no doc 1 μg IM	Post II	1 151	>2560	1 696	22
2gecoN1b-1 no doc 1 μg IM	Post III	2 220	>2560	1 947	135
c6 doc 10 μg IM	Post II	308	248	341	<20
c6 doc 10 μg IM	Post III	189	104	400	<20
c6 doc 1 μg IM	Post II	33	43	63	<20
c6 doc 1 μg IM	Post III	NC (>20)	24	156	<20
2g2 doc 10 μg IM	Post II	NC (>20)	25	360	<20
2g2 doc 10 μg IM	Post III	201	<20	647	<20
2g2 doc 1 μg IM	Post II	275	<20	299/644	<20
2g2 doc 1 μg IM	Post III	237	<20	728	<20
2gecoN1b-1 doc 10 μg IM	Post II	573	31	685	<20
2gecoN1b-1 doc 10 μg IM	Post III	NC (>40)	21	1 140	<20
2gecoN1b-1 doc 1 μg IM	Post II	261	NC	118	<20
2gecoN1b-1 doc 1 μg IM	Post III	348	NC	692	<20

[0112] Clearly, the presence of L3 (2g2) or intermediate (2gecon1b-1) epitope induces cross-bactericidal antibodies, while blebs from truncated LPS strain (C6) induce lower level of cross-reacting antibodies. This was particularly illustrated when 1 μg of OMV was injected.

[0113] Moreover, as shown with OMV purified with DOC, reducing the LPS content of blebs reduces the induction of cross-bactericidal antibodies. Aside from increased LPS, it is possible that DOC free blebs may also advantageously retain some proteins losely interacting with the OMVs such as lipoproteins.

Example 3

Mutation of the LgtG gene to Give Constitutive Expression of LgtG—the LgtG "Fixed" Mutant

[0114] Using strain *Neisseria meningitidis* strain 35E (L2 immunotype typing strain) as a template primer pair Lg1: 5'-ATG AAG CTC AAA ATA GAC ATT G-3' and Lg21: 5'-ATC TGC GGG CGG CGG CGC GAC TTG GAT-3', and primer pair LGdell8: 5'-GAA TTC GGA TCC AAC TGA TTG TGG CGC ATT CC-3' and Lg2UP: 5'-TGC CGT CTG AAG ACT TCA GAC GGC TTA TAC GGA TGC CAG CAT GTC-3' (underlined sequence denotes a *Neisseria meningitidis* uptake sequence) were used to produce two PCR products. These products were purified and then used in splice overlap PCR with primers Lg 1 and Lg2UP to produce a final product that was cloned into the pGEM-T Easy vector (Promega). The resulting plasmid, pL2+, was sequenced to confirm that the wild type sequence of 11C in the wild type polyC tract of lgtG had been replaced with 5'-CGCCGCCGCCC-3'. The sequence of the lgtG coding sequence in the region of the mutation is shown in **FIG. 4**[which shows the alignment of nucleotide sequence of the wild-type sequence of the lgtG gene of *Neisseria meningitidis* strain 35E and the lgtG "fixed" mutation (underlined, bold) contained on plasmid pL2+. Also shown is an XcmI restriction endonuclease cleavage site used to construct an IgtG::kan mutant].

Transformation of Strain MC58¢3lgtAG2 with pL2+ to Transfer the LgtG "Fixed" Mutation to the Chromosome.

[0115] In order to transform the lgtG "fixed" mutation and detect the LPS phenotype with immunocolony-blot screening it was necessary to create a strain that was fixed "off " expression for LgtG. A kanamycin cassette from pUK4kan was clones into the XcmI site of pL2+. The resulting plasmid, plgtG::kan, was used to transform 2G2 (see above) to kanamycion resistance and the correction position of the lgtG::kan allele was confirmed by PCR using primers Lg1 and Lg4 5'-AACCGTTTTCCTATTCCCAT-3', followed by nucleotide sequencing with the same primers. The resulting strain, ¢3lgtA2GlgtG::kan-3, had the genotype: MC58 parent strain; siaD::ery lgtAG2 lgtG::kan. This strain was then transformed with plasmid pL2+ and screened for colonies with an L2 phenotype and screen by colony-immuno blots (Mn 42F12.32). Positive colonies were picked and tested for by both kanamycin sensitivity and PCR using primers Lg1 and Lg8 5'-CAC CGA TAT GCC CGA ACT CTA-3' followed by sequencing with primer Lg5 5'-CAC CGC CAA ACT GAT TGT-3' to confirm the lgtG "fixed" mutation had replaced the lgtG::kan allele. The resulting strain ¢3lgtA2GlgtGL2+has the genotype: MC58 parent strain; siaD::ery lgtAG2 lgtG "fixed".

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aagctcgccg ttgtcggctc                                                680
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1. A process of making a genetically engineered neisserial strain with an L2 or L3 LOS immunotype of reduced phase variability for manufacture of an immunogenic composition comprising the steps of:

a) selecting a neisserial strain with phase-variable LOS synthesis,

b) genetically engineering the strain such that the homopolymeric nucleotide tract of a phase-variable lgtA and/or lgtG LOS oligosaccharide synthesis gene is modified to render the expression of the gene less phase variable,

c) isolating L2 or L3 LOS from the neisserial strain, and

d) formulating the isolated L2 or L3 LOS with a pharmaceutically acceptable excipient.

2. The process of claim 1, wherein the LOS oligosaccharide synthesis gene is modified to render the expression of the gene non-phase variable.

3. The process of claim 1, wherein the genetically engineered neisserial strain made has an LOS immunotype that is non-phase variable.

4. The process of claim 1, wherein the neisserial strain is selected from the group of a meningococcal strain and a meningococcus B strain.

5. The process of claim 1, wherein the genetically engineered neisserial strain made has L2 LOS immunotype.

6. The process of claim 5, wherein step a) a neisserial strain with phase-variable L2 LOS synthesis is selected.

7. The process of claim 5, wherein step b) comprises the step of fixing expression of an IgtA gene product.

8. The process of claim 7, wherein the expression of an IgtA gene product is fixed by reducing the length of the homopolymeric nucleotide tract within the open-reading frame of the gene and maintaining the open-reading frame in frame.

9. The process of claim 8, wherein the homopolymeric G tract in the IgtA open-reading frame is reduced to 8, 5 or 2 consecutive G nucleotides.

10. The process of claim 7, wherein the expression of an IgtA gene product is fixed by changing the sequence of the homopolymeric G nucleotide tract within the open-reading frame of the IgtA gene such that: one or more GGG codons encoding Glycine is changed to any other codon encoding Glycine, or a codon encoding a conservative mutation, and/or the TCG codon encoding Serine is changed to any other codon encoding Serine, or a codon encoding a conservative mutation, and maintaining the open-reading frame of the gene in frame.

11. The process of claim 10, wherein 2, 3 or 4 codons in the homopolymeric tract are changed and encode the identical amino acid or a different amino acid.

12. The process of claim 5, wherein step b) comprises the step of fixing the expression of an IgtG gene product.

13. The process of claim 12, wherein the expression of an IgtG gene product is fixed by reducing the length of the homopolymeric nucleotide tract within the open-reading frame of the gene and maintaining the open-reading frame in frame.

14. The process of claim 13, wherein the homopolymeric C tract in the IgtG open-reading frame is reduced to 8, 5 or 2 consecutive C nucleotides.

15. The process of claim 12, wherein the expression of an IgtG gene product is fixed by changing the sequence of the homopolymeric C nucleotide tract within the open-reading frame of the IgtG gene such that: one or more CCC codons encoding Proline is changed to any other codon encoding Proline, or a codon encoding a conservative mutation, or the GCC codon encoding Alanine is changed to any other codon encoding Alanine, or a codon encoding a conservative mutation, and maintaining the open-reading frame frame.

16. The process of claim 15, wherein 2, 3 or 4 codons in the homopolymeric tract are changed and encode the identical amino acid or a different amino acid.

17. The process of claim 5, wherein step b) comprises the steps of (1) fixing the expression of an IgtA gene product by reducing the length of the homopolymeric G nucleotide tract within the open-reading frame of the gene to 5 or 2 consecutive G nucleotides and maintaining the open-reading frame in frame or optionally changing the sequence of the homopolymeric G nucleotide tract such that one or more GGG codons encoding Glycine is changed to any other codon encoding glycine, or a codon encoding a conservative mutation, or the TCG codon encoding Serine is changed to any other codon encoding Serine, or a codon encoding a conservative mutation, and maintaining the open-reading frame of the gene in frame, and (2) fixing the expression of an IgtG gene product by changing the sequence of the homopolymeric C nucleotide tract within the open-reading frame of the IgtG gene such that 1, 2 or 3 CCC codons encoding Proline is changed to any other codon encoding Proline, or a codon encoding a conservative mutation, or the GCC codon encoding Alanine is changed to any other codon encoding Alanine, or a codon encoding a conservative mutation, whilst maintaining the open-reading frame in frame.

18. The process of claim 2, wherein the genetically engineered neisserial strain made has an L3 LOS immunotype.

19. The process of claim 18, wherein step a) a neisserial strain with phase-variable L3 LOS synthesis is selected.

20. The process of claim 18 or 19, wherein step b) comprises the step of fixing the expression of an IgtA gene product.

21. The process of claim 20, wherein the expression of the IgtA gene product is fixed by reducing the length of the homopolymeric nucleotide tract within the open-reading frame of the gene and maintaining the open-reading frame in frame.

22. The process of claim 21, wherein the homopolymeric G tract in the IgtA open-reading frame is reduced to 8, 5 or 2 consecutive G nucleotides.

23. The process of claim 20, wherein the expression of an IgtA gene product is fixed by changing the sequence of the homopolymeric G nucleotide tract within the open-reading frame of the IgtA gene such that one or more GGG codons encoding Glycine is changed to any other codon encoding glycine, or a codon encoding a conservative mutation, or the TCG codon encoding Serine is changed to any other codon encoding Serine, or a codon encoding a conservative mutation and maintaining the open-reading frame of the gene in frame.

24. The process of claim 23, wherein 2, 3 or 4 codons in the homopolymeric tract are changed and encode the identical amino acid or a different amino acid.

25. The process of claim 18, wherein step b) comprises the step of permanently downregulating the expression of a gene product from the IgtG gene.

26. The process of claim 25, wherein the expression of the gene product from the IgtG gene is switched off, optionally by deleting all or part of the promoter or open-reading frame of the gene.

27. The process of claim 18, wherein step b) comprises the steps of fixing the expression of the lgtA gene product by reducing the length of the homopolymeric G nucleotide tract within the open-reading frame of the gene to 2 consecutive G nucleotides and maintaining the open-reading frame in frame, and switching off the expression of gene product from the IgtG gene by deleting all or part of the promoter or open-reading frame of the gene.

28. The process of claim 5, wherein step b) comprises the step of permanently downregulating the expression of gene product from the IgtC gene, optionally by switching the gene off or by deleting all or part of the promoter or open-reading frame of the gene.

29. The process of claim 5, wherein step a) comprises the step of selecting a neisserial strain that is IgtB, or step b) comprises the step of genetically engineering the strain such that the expression of gene product from the IgtB or IgtE gene is permanently downregulated, optionally by switching the gene off or by deleting all or part of the promoter or open-reading frame.

30. The process of claim 5, wherein step a) comprises the step of selecting a neisserial strain that is unable to synthesise capsular polysaccharide, or step b) comprises the step of genetically engineering the strain such that it is unable to synthesize capsular polysaccharide by permanently downregulating the expression of gene product from one of the following genes: saiD, ctrA, ctrB, ctrC, ctrD, synA, synB or synC, optionally by switching the gene off or by deleting all or part of the promoter or open-reading frame.

31. The process of claim 5, wherein step a) comprises the step of selecting a neisserial strain that is msbB- or htrB-, or

step b) comprises the step of genetically engineering the strain such that the expression of functional gene product from the msbB or htrB gene(s) is permanently downregulated, optionally by switching the gene(s) off or by deleting all or part of the promoter or open-reading frame.

32. A process of isolating L2 LOS comprising the steps of producing a genetically engineered neisserial strain with a fixed L2 immunotype by the process of claim 5 or 28; and isolating L2 LOS from the resulting strain.

33. The process of claim 32, comprising the step of conjugating the L2 LOS to a carrier comprising a source of T-cell epitopes or the step of presenting the L2 LOS in a liposome formulation.

34. A process of isolating neisserial blebs having an L2 LOS immunotype, comprising the steps of producing a genetically engineered neisserial strain with a fixed L2 immunotype by the process of claim 5 or 28; and isolating blebs from the resulting strain.

35. The process of claim 34, where the step of isolating blebs involves extraction with about 0.03%, about 0.05-0.2% or about or exactly 0.1% deoxycholate.

36. The process of claim 34, comprising the step of intra-bleb conjugating the L2 LOS to an outer membrane protein present in the blebs.

37. A process of isolating L3 LOS comprising the steps of producing a genetically engineered neisserial strain with a fixed L3 immunotype by the process of claim 18; and isolating L3 LOS from the strain.

38. The process of claim 37, comprising the step of conjugating the L3 LOS to a carrier comprising a source of T-cell epitopes or the step of presenting the L3 LOS in a liposome formulation.

39. A process of isolating neisserial blebs having an L3 LOS immunotype, comprising the steps of producing a genetically engineered neisserial strain with a fixed L3 immunotype by the process of claim 18; and isolating blebs from the strain.

40. The process of claim 39, where the step of isolating blebs involves extraction with about 0.03%, about 0.05-0.2%, or about or exactly 0.1% deoxycholate.

41. The process of claim 39, comprising the step of intra-bleb conjugating the L3 LOS to an outer membrane protein present in the blebs.

42. A process of making an immunogenic composition comprising the steps of producing isolated L2 LOS by the process of claim 32 and formulating the L2 LOS with a pharmaceutically-acceptable excipient.

43. A process of making an immunogenic composition comprising the steps of producing isolated L3 LOS by the process of claim 37 and formulating the L3 LOS with a pharmaceutically acceptable excipient.

44. A process of making a multivalent immunogenic composition comprising the steps of producing isolated L2 LOS by the process of claim 32 producing isolated neisserial blebs having an L2 LOS immunotype, producing isolated L3 LOS or producing isolated neisserial blebs having an L3 LOS immunotype and mixing the L2 and L3 LOS components together along with a pharmaceutically acceptable excipient.

45. A process of growing a high cell density of an L2 or L3 neisserial strain comprising the steps of:

a) genetically-engineering a neisserial strain according to claim 5;

b) growing the strain to high cell density in a fermentor.

46. The process of claim 45, wherein the strain is grown to a cell density in iron non-limiting conditions of OD_{450} 10-19, or OD_{450} 12-16 or is grown to a cell density in iron limiting conditions of OD_{450} 6-12 or OD_{450} 8-10.

47. A process of isolating neisserial L2 or L3 LOS comprising the steps of growing an L2 or L3 neisserial strain to high cell density according to the process of claim 45, and isolating L2 or L3 LOS from the resulting strain.

48. The process of claim 47, comprising the step of conjugating the L2 or L3 LOS to a carrier comprising a source of T-cell epitopes or the step of presenting the L2 or L3 LOS in a liposome formulation.

49. A process of isolating neisserial blebs having an L2 or L3 LOS immunotype, comprising the steps of growing an L2 or L3 neisserial strain to high cell density according to the process of claim 45; and isolating blebs from the resulting strain.

50. The process of claim 49, where the step of isolating blebs involves extraction with about 0-0.3%, about 0.05-0.2%, or about or exactly 0.1% deoxycholate.

51. The process of claim 49, comprising the step of intra-bleb conjugating the L2 or L3 LOS to an outer membrane protein also present in the blebs.

52. A process of making an immunogenic composition comprising the steps of producing isolated L2 or L3 LOS by the process of claim 47 or producing isolated neisserial blebs having an L2 or L3 LOS immunotype and formulating the L2 or L3 LOS or blebs with a pharmaceutically acceptable excipient.

53. A process of making an immunogenic composition comprising the steps of producing isolated L2 LOS by the process of claim 47 or producing isolated neisserial blebs having an L2 LOS immunotype, producing isolated L3 LOS and mixing the L2 and L3 LOS components together along with a pharmaceutically acceptable excipient.

54. A process of making an immunogenic composition comprising the steps of producing isolated neisserial blebs having an L2 LOS immunotype by the process of claim 35, and formulating the L2 LOS or blebs with a pharmaceutically-acceptable excipient.

55. A process of making an immunogenic composition comprising the steps of producing isolated L3 LOS by the process of claims **39**, and formulating the L3 LOS or blebs with a pharmaceutically acceptable excipient.

56. A process of making an immunogenic composition comprising the steps of producing isolated neisserial blebs having an L2 LOS immunotype by the process of claims **34**, and mixing the L2 LOS or blebs with a pharmaceutically acceptable excipient.

57. A process of making an immunogenic composition comprising the steps of producing isolated neisserial blebs having an L3 LOS immunotype by the process of claim 39, and mixing the L3 LOS or blebs with a pharmaceutically acceptable excipient.

58. A process of making an immunogenic composition comprising the steps of producing isolated neisserial blebs having an L2 or L3 LOS immunotype by the process of claim 49, and formulating the L2 or L3 LOS or blebs with a pharmaceutically acceptable excipient.

59. A process of making an immunogenic composition comprising the steps of producing isolated neisserial blebs

having an L2 LOS immunotype by the process of claim 49, and mixing the L2 LOS or blebs and a pharmaceutically acceptable excipient.

60. A process of making an immunogenic composition comprising the steps of producing isolated neisserial blebs having an L3 LOS immunotype by the process of claim 49, and mixing the L3 LOS or blebs with a pharmaceutically acceptable excipient.

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