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(54) **Title**: TRANSFORMATION OF COMMENSAL NEISSERIA

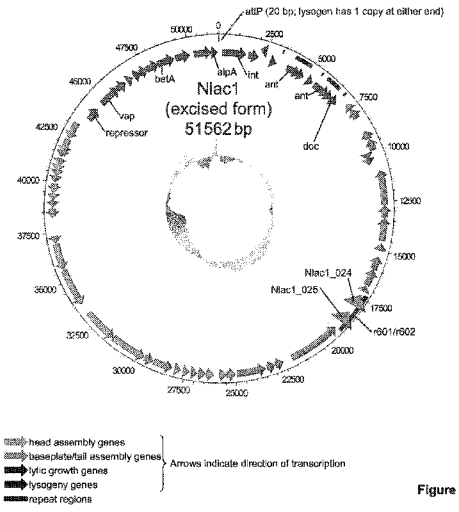

Figure 1

(57) **Abstract**: The present invention relates to methods and reagents, especially nucleic acid delivery vehicles, for transforming commensal Neisseria such as Neisseria lactamica. This is based on the r601 (SEQ ID NO: 1) and r602 (SEQ ID NO: 2) tandem repeat regions of the Neisseria NlLacl chromosomally integrated prophage and on two identical genes Nlacl_024 and Nlacl_025 genes (SEQ ID NO: 3) present in said r601 and r602 regions. There is provided a nucleic acid delivery vehicle, comprising an isolated polynucleotide sequence that is at least 300 nucleotides in length, wherein said polynucleotide sequence has at least 80% sequence identity to a nucleic acid sequence comprising at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2; and wherein said polynucleotide sequence includes an insertion site into which a first nucleotide sequence may be inserted.

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TRANSFORMATION OF COMMENSAL NEISSERIA

The present invention relates to methods and reagents for transforming commensal *Neisseria* such as *Neisseria lactamica*.

Infection by pathogenic organisms is one of the major causes of chronic and acute disease. In particular, infection resulting from microbial sources such as bacteria, viruses and protozoa, continue to claim millions of lives worldwide. With microbial species increasingly becoming resistant to conventional antibiotics, it would be desirable to provide alternative and preferably prophylactic means of protecting against and fighting microbial infection.

Neisseria meningitidis (*N. meningitidis*) is the causative agent of meningococcal meningitis and meningococcal septicaemia, and *Neisseria gonorrhoeae* (*N. gonorrhoeae*) is the causative agent of gonorrhoea.

Neisseria lactamica (*N. lactamica*) closely resembles *N. meningitidis*, and both species are common inhabitants of the human nasopharynx. However, unlike *N. meningitidis*, *N. lactamica* lacks the key genes required for invasive disease and is limited to a commensal existence in the nasopharynx. Natural colonisation by *N. lactamica* is thought reduce the risk of invasive meningococcal disease, probably by inducing a cross-protective immune response.

N. lactamica is important as the basis for experimental live vaccines and outer-membrane vesicle (OMV) vaccines against meningococcal disease caused by *N. meningitidis*. *N. lactamica* OMVs have been demonstrated to protect against lethal challenge in a mouse model of meningococcal disease (WO 00/50074).

OMVs from *N. meningitidis* have also been used in vaccines against meningococcal disease, and OMVs from *N. meningitidis* and *N. lactamica* have been used in a vaccine blend (WO 03/051379).

To exploit the full potential of *N. lactamica*, it would be useful to be able to modify it genetically.

Bacteria vary in their ability to take up and maintain foreign, heterologous DNA.

For instance, wild-type *E. coli* is resistant to transformation with DNA from non-*E. coli* sources, due to a restriction/ methylation system in which restriction endonucleases degrade foreign DNA while the cognate methylases protect "self" DNA by methylating any recognition sites for the restriction endonucleases promptly after DNA synthesis. Routine laboratory work therefore depends upon mutant strains of *E. coli* that are deficient in restriction endonuclease activity, for propagating and manipulating human or synthetic nucleic acids.

In contrast, many natural isolates of *N. meningitidis* are amenable to genetic engineering using plasmids or linear DNA. Laboratory transformation of *N. meningitidis* may therefore be exploiting loopholes that have evolved in order to allow natural transfer of DNA between related bacteria.

Unlike *N. meningitidis*, natural isolates of *N. lactamica* resist stable uptake of foreign, heterologous DNA.

In this regard, only two successful genetic modifications of *N. lactamica* are known: an NspA knockout and an NMB0033 (lytic murein transglycosylase A) knockout. These modified strains were obtained — at very low efficiencies — by disruptive insertion of antibiotic resistance genes. Such knockouts demonstrate that their targets are non-essential; however, attempts to insert larger DNA fragments into the *N. lactamica* chromosome have failed. Similarly, the rmpM gene of *N. meningitidis* can be knocked out and replaced with an extra copy of porA [Peeters *et al*. 1996, Vaccine (14); pages 1009-1015], but attempts to date to emulate this with *N. lactamica* have failed.

Like wild-type *E. coli*, the transformation barrier in *N. lactamica* is believed to arise from a restriction/ methylation system in which restriction endonucleases degrade foreign, heterologous DNA while the cognate methylases protect "self" DNA by methylating any recognition sites for the restriction endonucleases promptly after DNA synthesis.

At least five restriction endonucleases have been detected in *N. lactamica*, but only two of their cognate methylases are known, and no restriction-deficient mutants are available. One of the *N. lactamica* restriction endonucleases, NlaI, is an isoschizomer of the *Haemophilus ægyptus* enzyme HaeIII. NlaI may share with HaeIII an ability to cut single-stranded targets; if so, this may explain why conjugative transfer of single-stranded DNA has to date been unable to circumvent the *N. lactamica* transformation barrier. In this regard, conjugative transfer overcomes the transformation barrier in several organisms, but has failed with 53 isolates of *N. lactamica* [O'Dwyer CA *et al*. (2004), Infect. Immun., 72: pages 6511-6518].

The restriction systems of *Xanthomonas campestris* and *Clostridium botulinum* can be overcome by transformation with previously methylated DNA. However, this technique is not suitable for transformation of *N. lactamica* because the full set of methylase activities required to protect foreign DNA in *N. lactamica* is as yet unknown. Moreover, the probable NlaII methylation pattern is toxic to *E. coli* [Seeber S. *et al*., 1990, Gene 28;94(1):37-43].

There is therefore a need in the art to reliably and stably transform commensal *Neisseria* such as *N. lactamica* (eg. with a nucleotide sequence from a foreign, heterologous source).

There is also a need for an improved vaccine that provides protective immunity to infection by pathogenic organisms, such as pathogenic *Neisseria* (eg. *N.*

meningitidis or *N. gonorrhoea*), or that is immunostimulatory for the treatment of non-infectious disease, for example allergy and cancer.

In a first aspect, the present invention provides a nucleic acid delivery vehicle, comprising an isolated polynucleotide sequence that is at least 300 nucleotides in length, wherein said polynucleotide sequence has at least 80% sequence identity to a nucleic acid sequence comprising at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2; and wherein said polynucleotide sequence includes an insertion site into which a first nucleotide sequence may be inserted.

The present invention is based on homologous recombination of foreign DNA into the commensal Neisserial (eg. *N. lactamica*) chromosome. Once recombined into the genome of the commensal *Neisseria* (eg. *N. lactamica*), the foreign DNA sequence acquires the commensal host's methylation pattern; the foreign sequence is then protected from the host's restriction system, due to the requirement for the host to avoid self-restriction.

Integration of foreign DNA into a region of the commensal *Neisseria* genome (eg. *N. lactamica* genome) that the host is unable to discard further increases stability of transformation of the *N. lactamica* with the foreign sequence.

The present inventors have identified a prophage, termed 'Nlac1', residing in the genome of *N. lactamica* strain Y92-1009. Nlac1 is inserted into a region of the *N. lactamica* genome involved in respiration and the stress response. In *N. lactamica* strain Y92-1009, Nlac1 interrupts a serinyl-tRNA gene.

Multiplex PCR screening and dot blotting of 384 single colonies of *N. lactamica* strain Y92-1009 for 3 phage proteins indicated that the phage is consistently associated with the *N. lactamica* strain Y92-1009 host.

Furthermore, colony hybridisation and PCR analyses indicate that at least 10 other

N. lactamica isolates contain the same (or closely related) prophage. This indicates that the prophage Nlac1 is a typical component of commensal *Neisseria* such as *N. lactamica*.

Accordingly, the presence of the prophage Nlac1 is of relevance not only to the commercially relevant *N. lactamica* strain Y92-1009, but also to many other (potentially all) *N. lactamica* strains and to other commensal Neisserial species such as *Neisseria cinerea*, *Neisseria elongata*, *Neisseria flavescens*, *Neisseria polysaccharea, Neisseria sicca* and *Neisseria subflava.*

In contrast, screening a panel of seven disease-causing meningococcal strains identified no match to the prophage Nlac1.

The prophage Nlac1 has a 51562bp genome containing 65 genes. Sequence analysis by the inventors has identified that, in common with other bacteriophages, the Nlac1 sequence is divided into structural and regulatory regions, with genes for related functions clustered into contiguous blocks: the lysogeny block, the lytic growth block, the head-assembly block and the baseplate/ tail-assembly block (see Figure 1). The structural regions are passive during lysogeny.

Nlac1_024 and Nlac1_025 are two adjacent genes located within the tail-assembly block of the Nlac1 prophage. The nucleic acid sequence of Nlac1_024 and Nlac1_025 is 100% identical, and is represented by **SEQ ID NO: 3**.

The Nlac1_024 and Nlac1_025 genes lie within a broader tandem-repeated region made up of two 1057 bp elements, named r601 and r602, which overlap each other by 39 base-pairs and are identical except for three nucleotide substitutions at the 3' end of r602. Within the Nlac1 prophage, r601 is found at nucleotide positions 18335–17279, and r602 is found at nucleotide positions 19353–18297 (numbering according to the coordinate system of Figure 1, in which Nlac1_024 and Nlac1_025 are transcribed along the anticlockwise strand).

The nucleic acid sequence of r601 is represented by **SEQ ID NO: 1** and the nucleic acid sequence of r602 represented by **SEQ ID NO: 2**.

The Nlac_024 coding sequence lies within r601 (see residues 359-1018 of SEQ ID NO: 1) and the Nlac_025 coding sequence lies within r602 (see residues 359-1018 of SEQ ID NO: 2).

Due to the sequence identity of Nlac1_024 and Nlac1_025, these prophage genes are particularly attractive as sites for integration of foreign genetic material. In this regard, disruption of either one of the Nlac1_024 and Nlac1_025 genes is likely to leave the other gene intact to fulfil any unexpected functions that this pair of genes might have in maintaining lysogeny.

Furthermore, due to the substantial identity of r601 and r602, a homologous recombination cassette constructed based on any of SEQ ID NOs: 1, 2 or 3 (or a fragment thereof) effectively has a double target into which it can recombine.

A “commensal” organism coexists in an environment with another organism, such coexistence being beneficial to at least one of the organisms and generally not detrimental to either.

A number of different commensal *Neisseria* are known in the art and include *Neisseria lactamica, Neisseria cinerea*, *Neisseria elongata*, *Neisseria flavescens*, *Neisseria polysaccharea, Neisseria sicca* and *Neisseria subflava.* Different commensal *Neisseria* species are known to colonise the buccal or nasal areas.

Thus, in one embodiment, the commensal *Neisseria* is selected from *Neisseria lactamica*, *Neisseria cinerea*, *Neisseria elongata*, *Neisseria flavescens*, *Neisseria polysaccharea, Neisseria sicca* and *Neisseria subflava.*

In one embodiment, the commensal *Neisseria* is *Neisseria lactamica*. In one embodiment, the commensal *Neisseria* is *Neisseria lactamica* strain Y92-1009.

In the context of the present invention, a nucleic acid delivery vehicle is a nucleic acid construct comprising a "backbone" or "framework" polynucleotide sequence into which can be inserted a desired nucleotide sequence of interest.

The delivery vehicle of the invention comprises an isolated polynucleotide sequence that is at least 300 nucleotides in length, wherein said isolated polynucleotide sequence has at least 80% sequence identity to a nucleic acid sequence comprising at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2; wherein said polynucleotide sequence comprises an insertion site into which may be inserted a first nucleotide sequence.

In one embodiment, said isolated polynucleotide is at least 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000, 1025 or 1050 nucleotides in length.

In one embodiment, said isolated polynucleotide is at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 98% or 99% of the length of the nucleic acid sequence of SEQ ID NO: 1 or 2.

The polynucleotide sequence of SEQ ID NO: 1 or 2 may comprise one or more nucleic acid sequences that may function, under appropriate conditions, as an insertion site. Thus, in one embodiment, the isolated polynucleotide sequence comprises an insertion site that is naturally occurring in (ie. "endogenous" to) SEQ ID NO: 1 or 2.

In one embodiment, the isolated polynucleotide sequence comprises an insertion site that does not occur naturally at that position in SEQ ID NO: 1 or 2.

In accordance with this embodiment, the sequence of the isolated polynucleotide differs from the nucleic acid sequence of SEQ ID NO: 1 or 2 (or said at least 300 consecutive nucleotide region thereof). For example, the sequence of the polynucleotide is altered as compared with the nucleic acid sequence of SEQ ID NO: 1 or 2 (or said at least 300 consecutive nucleotide region thereof) so as to comprise said insertion site.

In one embodiment, in addition to any "endogenous" insertion site that occurs naturally at that position within SEQ ID NO: 1 or 2, the isolated polynucleotide sequence also comprises an insertion site that does not occur naturally at that position in the nucleic acid sequence of SEQ ID NO: NO: 1 or 2.

In accordance with this embodiment, the isolated polynucleotide sequence has less than 100% sequence identity to a nucleic acid sequence comprising at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2.

Thus, in one embodiment, the nucleic acid delivery vehicle comprises an isolated polynucleotide sequence that is at least 300 nucleotides in length, wherein said polynucleotide sequence has at least 80% sequence identity and less than 100% sequence identity to a nucleic acid sequence comprising at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2; wherein said polynucleotide sequence comprises an insertion site into which may be inserted a first nucleotide sequence.

In one embodiment, the delivery vehicle of the invention consists of an isolated polynucleotide sequence that is at least 300 nucleotides in length, wherein said polynucleotide sequence has at least 80% sequence identity to a nucleic acid sequence comprising (or consisting of) at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2; and wherein said polynucleotide sequence includes an insertion site into which a first nucleotide sequence may be inserted.

In one embodiment, the polynucleotide sequence has at least 85, 90, 92, 94, 95,

96, 97, 98 or 99% sequence identity to a nucleic acid sequence comprising (or consisting of) at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2. For example, the polynucleotide may have at least about 99.5% or 9.8% sequence identity to a nucleic acid sequence comprising (or consisting of) at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2.

In one embodiment, the nucleic acid sequence of the isolated polynucleotide differs from the at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2 at only about 100 nucleotide positions or fewer, such as at about 75, 50, 25, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 nucleotide positions. In one embodiment, the nucleic acid sequence of the isolated polynucleotide differs from the at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2 at about 4, 3, 2 or 1 nucleotide positions.

In one embodiment, any difference between the sequence of the isolated polynucleotide (as compared with the corresponding sequence of SEQ ID NO: 1 or 2) is selected from one or more nucleotide insertions, deletions and/ or substitutions. For example, the isolated polynucleotide sequence may have one or more nucleotide substitutions, as compared with the at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2.

In one embodiment, the nucleic acid sequence identity exists over the entire length of the nucleic acid sequence of SEQ ID NO: 1 or 2, or over the entire length of said at least 300 consecutive nucleotides thereof. Suitable conventional techniques for determining nucleic acid sequence identity are discussed below.

In one embodiment, said isolated polynucleotide has at least 80% identity to a nucleic acid sequence comprising at least 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000 or 1025 consecutive nucleotides of SEQ ID NO: 1 or 2.

The at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2 may be located

anywhere within the sequence of SEQ ID NO: 1 or 2. In this regard, the consecutive stretch of at least 300 nucleotides may start from any nucleotide position from nucleotide 1 to nucleotide 757 of SEQ ID NO: 1 or 2. In this regard, the consecutive stretch of at least 300 nucleotides may end at any nucleotide position from nucleotide 300 to nucleotide 1057 of SEQ ID NO: 1 or 2.

In one embodiment, said stretch of at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2 starts at nucleotide residue 1, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725 or 750 of SEQ ID NO: 1 or 2. In one embodiment, said stretch of at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2 ends at nucleotide residue 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000, 1025 or 1050 of SEQ ID NO: 1 or 2.

In one embodiment, said isolated polynucleotide sequence has at least 80% sequence identity to a nucleic acid sequence comprising nucleotide residues 1-300, 50-350, 100-400, 150-450, 200-500, 250-550, 300-600, 350-650, 400-700, 450-800, 500-850, 600-900, 650-950, 700-1000 or 750-1050 of SEQ ID NO: 1 or 2.

For example, in one embodiment, said isolated polynucleotide sequence has at least 80% sequence identity to a nucleic acid sequence comprising (or consisting of) at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2, wherein said at least 300 nucleotide nucleotides are from nucleotide residue 359 of SEQ ID NO: 1 or 2; or wherein said at least 300 nucleotide nucleotides end at nucleotide 1018 of SEQ ID NO: 1 or 2.

In one embodiment, said isolated polynucleotide sequence has at least 80% sequence identity to a nucleic acid sequence comprising at least 300 consecutive nucleotides of SEQ ID NO: 3 (ie. residues 359-1018 of SEQ ID NOs: 1 and 2).

In one embodiment, said isolated polynucleotide is at least 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 600, 625 or 650 nucleotides in length. In one embodiment, said isolated polynucleotide has a sequence length that is at least 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 98% or 99% of the length of the nucleic acid sequence of SEQ ID NO: 3.

The polynucleotide sequence of SEQ ID NO: 3 may comprise one or more nucleic acid sequences that may function, under appropriate conditions, as an insertion site. Thus, in one embodiment, the isolated polynucleotide sequence comprises an insertion site that is naturally occurring in (ie. "endogenous" to) SEQ ID NO: 3.

In one embodiment, the isolated polynucleotide sequence comprises an insertion site that does not occur naturally at that position in SEQ ID NO: 3.

In accordance with this embodiment, the sequence of the isolated polynucleotide differs from the nucleic acid sequence of SEQ ID NO: 3 (or said at least 300 consecutive nucleotide region thereof). For example, the sequence of the polynucleotide is altered as compared with the nucleic acid sequence of SEQ ID NO: 3 (or said at least 300 consecutive nucleotide region thereof) so as to comprise said insertion site.

In one embodiment, in addition to any "endogenous" insertion site that occurs naturally at that position within SEQ ID NO: 3, the isolated polynucleotide sequence also comprises an insertion site that does not occur naturally at that position in the nucleic acid sequence of SEQ ID NO: NO: 3.

In accordance with this embodiment, the isolated polynucleotide sequence has less than 100% sequence identity to a nucleic acid sequence comprising at least 300 consecutive nucleotides of SEQ ID NO: 3.

Thus, in one embodiment, the nucleic acid delivery vehicle comprises an isolated polynucleotide sequence that is at least 300 nucleotides in length, wherein said polynucleotide sequence has at least 80% sequence identity and less than 100% sequence identity to a nucleic acid sequence comprising at least 300 consecutive nucleotides of SEQ ID NO: 3; wherein said polynucleotide sequence comprises an insertion site into which may be inserted a first nucleotide sequence.

In one embodiment, the delivery vehicle of the invention consists of an isolated polynucleotide sequence that is at least 300 nucleotides in length, wherein said polynucleotide sequence has at least 80% sequence identity to a nucleic acid sequence comprising (or consisting of) at least 300 consecutive nucleotides of SEQ ID NO: 3; and wherein said polynucleotide sequence includes an insertion site into which a first nucleotide sequence may be inserted.

In one embodiment, the polynucleotide sequence has at least 85, 90, 92, 94, 95, 96, 97, 98 or 99% sequence identity to a nucleic acid sequence comprising (or consisting of) at least 300 consecutive nucleotides of SEQ ID NO: 3. For example, the polynucleotide may have at least about 99.5% or 9.8% sequence identity to a nucleic acid sequence comprising (or consisting of) at least 300 consecutive nucleotides of SEQ ID NO: 3.

In one embodiment, the nucleic acid sequence of the isolated polynucleotide differs from the at least 300 consecutive nucleotides of SEQ ID NO: 3 at only about 100 nucleotide positions or fewer, such as at about 75, 50, 25, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 nucleotide positions. In one embodiment, the nucleic acid sequence of the isolated polynucleotide differs from the at least 300 consecutive nucleotides of SEQ ID NO: 3 at about 4, 3, 2 or 1 nucleotide positions.

In one embodiment, any difference between the sequence of the isolated polynucleotide (as compared with the corresponding sequence of SEQ ID NO: 3) is selected from one or more nucleotide insertions, deletions and/ or substitutions.

For example, the isolated polynucleotide sequence may have one or more nucleotide substitutions, as compared with the at least 300 consecutive nucleotides of SEQ ID NO: 3.

In one embodiment, the nucleic acid sequence identity exists over the entire length of the nucleic acid sequence of SEQ ID NO: 3, or over the entire length of said at least 300 consecutive nucleotides thereof. Suitable conventional techniques for determining nucleic acid sequence identity are discussed below.

In one embodiment, said isolated polynucleotide has at least 80% identity to a nucleic acid sequence comprising at least 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640 or 650 consecutive nucleotides of SEQ ID NO: 3.

The isolated polynucleotide sequence of the invention comprises a site for insertion of a nucleotide sequence of interest.

In one embodiment, as discussed above, the polynucleotide sequence comprises an insertion site that is naturally present in (or "endogenous to") the nucleic acid sequence of SEQ ID NO: 1, 2 or 3.

In this regard, the present inventors have identified that the nucleic acid sequences of SEQ ID NOs: 1-2 and 3 comprise a number of naturally-occurring insertion sites, such as restriction sites.

For example, a *BclI* restriction site is located 62–57 residues upstream of the start of SEQ ID NO: 3 (= residues 297-302 of SEQ ID NOs: 1 and 2), a *BtrI* restriction site is located at residues 186-191 of SEQ ID NO: 3 (= residues 544-549 of SEQ ID NOs: 1 and 2), a *BsePI* restriction site is located at residues 59-64 of SEQ ID NO: 3 (= residues 417-422 of SEQ ID NOs: 1 and 2), and a *BsgI* restriction site is

located at residues 268-273 of SEQ ID NO: 3 (= residues 626-631 of SEQ ID NOs: 1 and 2).

Thus, in one embodiment, the isolated polynucleotide sequence comprises residues 297-302 of SEQ ID NO: 1 or 2, residues 544-549 of SEQ ID NO: 1 or 2, residues 417-422 of SEQ ID NO: 1 or 2, and/ or residues 626-631 of SEQ ID NO: 1 or 2. In one embodiment, the isolated polynucleotide sequence comprises residues 59-64 of SEQ ID NO: 3, residues 186-191 of SEQ ID NO: 3, and/ or residues 268-273 of SEQ ID NO: 3.

In one embodiment, as discussed above, an insertion site comprises a sequence of nucleic acid residues that is not naturally present at that location in the sequence of SEQ ID NO: 1, 2 or 3. Such an insertion site may conveniently be termed a “non-endogenous” insertion site, a “non-naturally occurring” insertion site, or an “artificial” insertion site.

Thus, in one embodiment, the isolated polynucleotide sequence of the invention includes an insertion site that is not naturally present in (not “endogenous to”) the corresponding nucleic acid sequence of SEQ ID NO: 1, 2 or 3.

In accordance with this embodiment of the invention, the isolated polynucleotide sequence has less than 100% identity to a nucleic acid sequence comprising at least 300 consecutive nucleotides of SEQ ID NO: 1, 2 or 3.

For example, a non-naturally occurring insertion site may be introduced into a polynucleotide sequence by mutation or sequence modification (eg. genetic modification/ engineering), as compared with the nucleic acid sequence of SEQ ID NO: 1, 2 or 3 (or said at least 300 consecutive nucleotides thereof).

An insertion site may be introduced by insertion of nucleotides into a polynucleotide sequence (as compared with the corresponding sequence of SEQ

ID NO: 1, 2 or 3). Alternatively, an insertion site may be created in a polynucleotide sequence by deletion or substitution of one or more nucleotides of the corresponding sequence of SEQ ID NO: 1, 2 or 3.

Conventional techniques are known in the art for genetically modifying a polynucleotide sequence by insertion, deletion and/ or substitution of one or more nucleotide residues.

The insertion site may be located anywhere in the polynucleotide sequence.

A skilled person is able to select a suitable location for the insertion site as a matter of routine. For example, selection of a suitable location to create an insertion site might be based on identifying a region of the polynucleotide sequence that requires little sequence modification (eg. only one nucleotide insertion, deletion or substitution) in order to create a nucleic acid sequence that comprises an insertion site.

As discussed in more detail below, insertion of the first nucleotide sequence into the delivery vehicle may depend on recognition of the nucleic acid sequence of the insertion site by an enzyme, such as a restriction enzyme or a recombinase enzyme. Alternatively, the insertion site may comprise a nucleic acid sequence having single-stranded DNA ends, and insertion of the nucleotide sequence of interest may comprise annealing between the single stranded ends of the insertion site and single-stranded ends of the nucleotide sequence of interest (eg. by ligation independent cloning, LIC).

It is important to note that the selection of an insertion site and the means by which the nucleotide sequence of interest is inserted into the delivery vehicle is in no way critical to the invention. A skilled person is aware of multiple conventional techniques for making recombinant DNA constructs and would be able to select a suitable technique and reagents as a matter of routine.

In one embodiment, the isolated polynucleotide sequence comprises an insertion site that comprises or consists of a nucleic acid sequence that is cleaved by a restriction enzyme.

Restriction enzymes are endonuclease enzymes that cleave the phosphodiester backbone of DNA. As such, restriction enzymes are also known as restriction endonucleases. A nucleic acid sequence that is cleaved by a restriction enzyme is known as a 'restriction site'.

By far the most common class of restriction enzymes used for cloning purposes are the Type II family. Most Type II restriction enzymes recognise and cleave a palindromic restriction site. However, Type II(S) restriction enzymes recognise a specific non-palindromic asymmetric sequence (recognition sequence) and cleave a restriction site located at a specific distance from the recognition sequence.

Thus, in one embodiment, the insertion site comprises or consists of a restriction site. In one embodiment, the restriction site is for a Type II restriction enzyme.

DNA restriction enzyme cleavage sequences have been well documented in the art, and corresponding DNA restriction enzymes that cleave said sequences are commercially available. It is therefore conventional for a skilled person to identify and select a suitable restriction site to ensure that a specific nucleotide sequence of interest may accurately be inserted into the delivery vehicle in the correct orientation. A skilled person is also familiar with conventional techniques for engineering a restriction site into a polynucleotide sequence.

For example, the insertion site may comprise or consist of a restriction site sequence that is cleaved by any of *Ava*I, *Avr*II, *BamH*I, *Bcl*I, *Bgl*II, *BsePI*, *Bsg*I, *Btr*I, *EcoR*I, *Hind*III, *Nde*I, *Nhe*I, *Pst*I, *Sal*I, *Spe*I, *Xba*I or *Xma*I. The nucleic acid sequences that are recognised and cleaved by these restriction enzymes are

provided below.

Restriction enzyme	Restriction site*
*Ava*I	C↓(C/T)CG(G/A)G
*Avr*II	C↓CTAGG
*BamH*I	G↓GATCC
*Bcl*I,	T↓GATCA
*Bgl*II	A↓GATCT
BsePI,	G↓CGCGC
*Bsg*I,	GTGCAGNNNNNNNNNNNNNNN↓**
*Btr*I,	C↓ACGTC
*Eco*RI	G↓AATTC
*Hind*III	A↓AGCTT
*Nde*I	C↓ATATG
*Nhe*I	G↓CTAGC
*Pst*I	CTGCA↓G
*Sal*I	G↓TCGAC
*Spe*I	A↓CTAGT
*Xba*I	T↓CTAGA
*Xma*I	C↓CCGGG

* where ↓ is the site of phosphodiester backbone cleavage;

** where N is any nucleic acid residue.

In one embodiment, the insertion site comprises or consists of a *BamHI* restriction site. For example, a *BamHI* restriction site (GGATCC) can be generated in SEQ ID NO: 3 by a single base substitution from T to G at nucleotide position 197 of SEQ ID NO: 3 (corresponds to nucleotide position 555 of SEQ ID NOs: 1 and 2).

In one embodiment, the insertion site comprises or consists of a *HindIII* restriction site. For example, a *HindIII* restriction site (AAGCTT) can be generated in SEQ ID NO: 3 by a single base substitution from G to C at nucleotide position 224 of SEQ ID NO: 3 (corresponds to nucleotide position 602 of SEQ ID NOs: 1 and 2).

Another conventional technique for inserting a nucleotide sequence of interest into a genetic construct such as a delivery construct is via site-specific recombination. Thus, in one embodiment, the insertion site comprises or consists of a nucleic acid sequence that is recognised and cleaved by a recombinase enzyme.

Conventional site-specific recombineering techniques include Invitrogen's Gateway® system. These techniques avoid the use of restriction enzymes, which might be desirable if the nucleotide sequence of interest comprises multiple restriction sites. A skilled person is aware of numerous conventional recombinase enzymes and their recognition sites – for example, the Cre recombinase of bacteriophage P1 (which catalyses recombination between two 34 base-pair loxP sites).

Another conventional technique for inserting a nucleotide sequence into a genetic construct such as a delivery vehicle, which also avoids the use of restriction enzymes, is ligation-independent cloning (LIC). Thus, in one embodiment, the insertion site comprises or consists of single-stranded nucleic acid sequences. The nucleotide sequence of interest is prepared with single-stranded nucleic acid overhangs (long 'sticky ends') that are complementary to the single-stranded nucleic acid sequences of the insertion site, and insertion of the nucleotide sequence of interest into the delivery vehicle is facilitated by complementary base pairing between the insertion site and the 'sticky ends' of the nucleotide sequence of interest.

In one embodiment, the isolated polynucleotide sequence comprises more than one insertion site. For example, the polynucleotide sequence may comprise 2, 3,

4 or 5 insertion sites.

The presence of multiple, defined insertion sites in the polynucleotide sequence permits accurate insertion of multiple (eg. 2, 3, 4 or 5) nucleotide sequences of interest into the delivery vehicle of the invention.

Each of the insertion sites may be selected from any of the types of insertion sites discussed above, and may be the same or may be different.

In one embodiment, the at least two insertion sites in the isolated polynucleotide sequence are spaced apart by a stretch of at least 10, 20, 30 or 40 nucleotides, such as by about 45 nucleotides.

Said multiple insertion sites may be a mixture of endogenous insertion sites (ie. naturally occurring in the sequence of SEQ ID NOs: 1, 2 or 3) and "non-endogenous"/ "engineered" insertion sites that do not occur naturally at that position in the sequence of SEQ ID NOs: 1, 2 or 3.

In one embodiment, multiple (eg. 2, 3, 4 or 5) insertion sites have been introduced (eg. engineered) into the sequence of said polynucleotide, as compared with the sequence of SEQ ID NO: 1, 2 or 3, or said at least 300 consecutive nucleotides thereof. Thus, in one embodiment, said polynucleotide sequence comprises multiple non-naturally occurring/ artificial insertion sites, as compared with the corresponding nucleic acid sequence of SEQ ID NO: 1, 2 or 3.

In one embodiment, the multiple insertion sites comprise multiple restriction sites.

Each of the multiple restriction sites may comprise or consist of the same or different nucleic acid sequences, and may be cleaved by the same restriction enzyme or by different restriction enzymes. In one embodiment, the isolated polynucleotide sequence comprises more than one (eg. 2 or 3) engineered

restriction sites that are cleaved by different restriction enzymes.

In one embodiment, the polynucleotide sequence comprises a restriction site for a *BamHI* restriction endonuclease and a restriction site for another restriction endonuclease or recombinase. In one embodiment, the *BamHI* restriction site is not naturally occurring in the sequence of SEQ ID NOs: 1, 2 or 3, and results from a single-nucleotide substitution (eg. as discussed above). The other restriction site may be endogenous or non-endogenous to SEQ ID NOs: 1, 2 or 3.

In one embodiment, the polynucleotide sequence comprises a restriction site for a *HindIII* restriction endonuclease and a restriction site for another restriction endonuclease or recombinase. In one embodiment, the *HindIII* restriction site is not naturally occurring in the sequence of SEQ ID NOs: 1, 2 or 3, and results from a single-nucleotide substitution (eg. as discussed above). The other restriction site may be endogenous or non-endogenous to SEQ ID NOs: 1, 2 or 3.

In one embodiment, the polynucleotide comprises a *BamHI* restriction site and a *HindIII* restriction site. In one embodiment, the *BamHI* and *HindIII* restriction sites do not naturally occur at that position in the sequence of SEQ ID NOs: 1, 2 or 3.

In one embodiment, the isolated polynucleotide sequence has at least 80% sequence identity (eg. 85, 90, 92, 94, 95, 96, 97, 98, 99 or 100% sequence identity) to the nucleic acid sequence of SEQ ID NO: 1 or 2, and comprises a single base substitution from T to G at nucleotide position 555 as compared with the nucleic acid sequence of SEQ ID NO: 1 and 2, and a single base substitution from G to C at nucleotide position 602 as compared with the nucleic acid sequence of SEQ ID NO: 1 and 2.

In one embodiment, the isolated polynucleotide sequence has at least 80% sequence identity (eg. 85, 90, 92, 94, 95, 96, 97, 98, 99 or 100% sequence identity) to the nucleic acid sequence of SEQ ID NO: 3, and comprises a single

base substitution from T to G at nucleotide position 197 as compared with the nucleic acid sequence of SEQ ID NO: 3 and a single base substitution from G to C at nucleotide position 224 as compared with the nucleic acid sequence of SEQ ID NO: 3.

SEQ ID NO: 4 is an example of an isolated polynucleotide sequence of the invention. In this regard, SEQ ID NO: 4 represents the nucleic acid sequence of the 1057 bp element r601 (shown in SEQ ID NO: 1) but with a single-nucleotide substitution from T to G at nucleotide position 555 as compared with the nucleic acid sequence of SEQ ID NO: 1, and a single-nucleotide substitution from G to C at nucleotide position 602 as compared with the nucleic acid sequence of SEQ ID NO: 1.

Thus, in one embodiment, the isolated polynucleotide sequence has at least 80% sequence identity (eg. 85, 90, 92, 94, 95, 96, 97, 98, 99 or 100% sequence identity) to a nucleic acid sequence comprising or consisting of at least 300 consecutive nucleotides of **SEQ ID NO: 4** (eg. at least 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000 or 1025 consecutive nucleotides thereof), wherein said polynucleotide sequence includes an insertion site into which may be inserted a first nucleotide sequence.

In one embodiment, the delivery vehicle of the invention comprises or consists of a polynucleotide sequence having the nucleic acid sequence of SEQ ID NO: 4.

The invention also provides a plasmid comprising a nucleic acid delivery vehicle of the invention, as described above.

Examples of plasmids include vectors such as DNA vectors and RNA vectors. In one embodiment, the plasmid is a transcription vector for replication of the delivery vehicle in a host cell such as *E. coli*. Plasmids typically contain control sequences

such as an origin of replication and multi-cloning site, which are known to those skilled in the art and may be selected depending upon the host cells. Plasmids may also include a selectable marker.

In one embodiment, the invention provides a host cell (such as *E. coli*) comprising a delivery vehicle or plasmid of the invention, as described above. The host cell replicates the delivery vehicle or plasmid. Thus, in one embodiment, the invention comprises propagating the delivery vehicle or plasmid of the invention in a host cell such as *E. coli.*

As used herein, "recombinant host cells", "host cells", "cells", "cell lines", "cell cultures", and other such terms refer to cells which can be, or have been, used as recipients for recombinant vector or other transfer DNA, and include the progeny of the original cell which has been transformed. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental or deliberate mutation.

Thus, in one aspect, the invention provides a method of producing a recombination cassette for integration of a first nucleotide sequence into the genome of a commensal *Neisseria* by homologous recombination, comprising inserting said nucleotide sequence of interest into a delivery vehicle of the invention as defined above.

Alternatively, a recombination cassette may be produced by inserting said first nucleotide sequence into a plasmid as defined above (which comprises said delivery vehicle of the invention).

A recombination cassette is prepared by inserting a first nucleotide sequence into the delivery vehicle or plasmid of the invention, via the insertion site.

Thus, a recombination cassette of the invention comprises a first nucleotide sequence, surrounded at both ends by flanking nucleic acid sequences from the delivery vehicle.

In the recombination cassette of the invention, the polynucleotide sequence of the delivery vehicle is arranged as 2 flanking regions on either side of the inserted first nucleotide sequence. The flanking region to the 5' of the first nucleotide sequence is known as the 5' flanking region, and the flanking region to the 3' of the first nucleotide sequence is known as the 3' flanking region.

The length and sequence of the flanking nucleic acid sequences on either side of the first nucleotide sequence will vary, depending on the location of the insertion site within the polynucleotide sequence of the delivery vehicle.

In the recombination cassette of the invention, there may be at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 500, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, 1000, 1010, 1020, 1030, 1040 or 1050 nucleotides of the polynucleotide sequence of the delivery vehicle, as defined above, arranged to the 5' of the first polynucleotide sequence.

In the recombination cassette of the invention, there may be at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 500, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850,

860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, 1000, 1010, 1020, 1030, 1040 or 1050 nucleotides of the polynucleotide sequence of the delivery vehicle, as defined above, arranged to the 3' of the first polynucleotide sequence.

In one embodiment, there are at least 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510 or 520 nucleotides of the polynucleotide sequence of the delivery vehicle arranged on both sides of the insertion site (and hence on both sides of the inserted first nucleotide sequence).

In one embodiment, there are at least 500 nucleotides of the polynucleotide sequence of the delivery vehicle arranged on both sides of the insertion site (and hence on both sides of the inserted first nucleotide sequence). For example, there may be about 550 (eg. 554 or 555) nucleotides of said polynucleotide sequence in the 5' flanking region, and about 500 (eg. 501, 502 or 503) nucleotides of said polynucleotide sequence in the 3' flanking region – or vice versa. In a specific example, the polynucleotide sequence may have at least 80% identity (but less than 100% identity) to SEQ ID NO: 1 or 2, with an insertion site located at position 555 as compared with the sequence of SEQ ID NOs: 1 or 2 into which the first nucleotide sequence is inserted. As such, the recombination cassette may comprise a 5' flanking region having about 555 nucleic acid residues of said polynucleotide sequence, and a 3' flanking region having about 502 nucleic acid residues of said polynucleotide sequence.

In one embodiment, there are at least 450 nucleotides of the polynucleotide sequence of the delivery vehicle arranged on both sides of the insertion site (and hence on both sides of the inserted first nucleotide sequence). For example, there may be about 600 (eg. 601 or 602) nucleotides of said polynucleotide sequence in the 5' flanking region, and about 455 (eg. 454, 455 or 456) nucleotides of said

polynucleotide sequence in the 3' flanking region – or vice versa. In a specific example, the polynucleotide sequence may have at least 80% identity (but less than 100% identity) to SEQ ID NO: 1 or 2, with an insertion site located at position 602 as compared with the sequence of SEQ ID NOs: 1 or 2 into which the first nucleotide sequence is inserted. As such, the recombination cassette may comprise a 5' flanking region having about 602 nucleic acid residues of said polynucleotide sequence, and a 3' flanking region having about 455 nucleic acid residues of said polynucleotide sequence.

In one embodiment, there are at least 75 nucleotides of the polynucleotide sequence of the delivery vehicle arranged on both sides of the insertion site (and hence on both sides of the inserted first nucleotide sequence in the recombination cassette). For example, there may be about 223 or 224 nucleotides of said polynucleotide sequence in the 5' flanking region, and about 75 (eg. 75, 76 or 77) nucleotides of said polynucleotide sequence in the 3' flanking region – or vice versa. In a specific example, the polynucleotide sequence may have at least 80% identity (but less than 100% identity) to SEQ ID NO: 3, with an insertion site located at position 224 as compared with the sequence of SEQ ID NOs: 3 into which the first nucleotide sequence is inserted. As such, the recombination cassette may comprise a 5' flanking region having about 224 nucleic acid residues of said polynucleotide sequence, and a 3' flanking region having about 76 nucleic acid residues of said polynucleotide sequence.

In one embodiment, there are at least 100 nucleotides of the polynucleotide sequence of the delivery vehicle arranged on both sides of the insertion site (and hence on both sides of the inserted first nucleotide sequence in the recombination cassette). For example, there may be about 196 or 197 nucleotides of said polynucleotide sequence in the 5' flanking region, and about 103 (eg. 102, 103 or 104) nucleotides of said polynucleotide sequence in the 3' flanking region – or vice versa. In a specific example, the polynucleotide sequence may have at least 80% identity (but less than 100% identity) to SEQ ID NO: 3, with an insertion site

located at position 197 as compared with the sequence of SEQ ID NOs: 3 into which the first nucleotide sequence is inserted. As such, the recombination cassette may comprise a 5' flanking region having about 197 nucleic acid residues of said polynucleotide sequence, and a 3' flanking region having about 103 nucleic acid residues of said polynucleotide sequence.

If the insertion site comprises a restriction site, the first nucleotide sequence may be cloned into the delivery vehicle or plasmid using a restriction enzyme that cleaves the restriction site. A skilled person is aware of conventional techniques and reagents for cloning a nucleotide sequence of interest into a restriction site.

Alternatively, if the insertion site comprises a recombinase recognition site, the first nucleotide sequence may be cloned into the delivery vehicle or plasmid by site-specific recombination using a recombinase restriction enzyme that cleaves the recognition site. A skilled person is aware of conventional techniques and reagents for cloning a polynucleotide sequence of interest into a polynucleotide sequence via site-specific recombination.

In one embodiment, the first nucleotide sequence is cloned into the insertion site of a plasmid-borne delivery vehicle, and the resulting recombination cassette is obtained from the plasmid. For example, the resulting recombination cassette is amplified from the plasmid-borne delivery vehicle (such as by PCR).

In one embodiment, the method of producing a recombination cassette comprises inserting multiple (eg. 2 or more) nucleotide sequences of interest into said delivery vehicle or plasmid. The resultant recombination cassette will therefore comprise multiple nucleotide sequences of interest (ie. "first", "second", "third" etc. nucleotide sequences).

The multiple nucleotide sequences may be inserted into the same insertion site, or may be inserted into different insertion sites. The multiple nucleotide sequences

of interest may be inserted into the delivery vehicle or plasmid substantially simultaneously, or they may be inserted sequentially (ie. one after the other).

The multiple nucleotide sequences inserted into the insertion site or sites may comprise the same nucleic acid sequence, or may comprise different nucleic acid sequences. For example, the multiple nucleic acid sequences may encode different polypeptides. Suitable nucleotide sequences of interest are discussed in more detail below.

The invention therefore provides a recombination cassette comprising a nucleic acid delivery vehicle of the invention, as described above, and further comprising a first nucleotide sequence inserted into said delivery vehicle via said insertion site.

In one embodiment, said recombination cassette is obtained (or obtainable) by a method described above.

The recombination cassette may be circular, or may be non-circular. In one embodiment, the recombination cassette is a linear nucleic acid molecule (ie. having free 5' and 3' ends). A linear nucleic acid sequence can be prepared from a closed circular molecule, such as a plasmid, by enzymatic digestion or physical disruption.

In one embodiment, said recombination cassette comprises more than one nucleotide sequence of interest. For example, the recombination cassette may comprise 2 or more nucleotide sequences of interest, such as 3, 4 or 5 or more nucleotide sequences of interest. Thus, in one embodiment, in addition to the first nucleotide sequence, the recombination cassette may comprise a second nucleotide sequence and optionally a third, fourth or fifth (or further) nucleotide sequence inserted into one or more insertion sites.

The recombination cassette of the invention is capable of homologous

recombination with genomic DNA of commensal *Neisseria* cells so as to introduce the nucleotide sequence(s) of interest into the commensal *Neisseria* cells' genomic DNA. In this regard, the recombination cassette of the invention is capable of "transforming" the commensal *Neisseria* cells.

A nucleotide sequence of interest sequence may comprise coding sequence (eg. one or more gene sequences), non-coding sequence (eg. regulatory sequences such as a promoter sequence or terminator sequence) or both coding and non-coding sequences.

In one embodiment, the nucleotide sequence of interest (eg. the first nucleotide sequence, as discussed above) comprises coding sequence. In this regard, the first nucleotide sequence may comprise one or more gene sequences, which encode one or more gene products.

In one embodiment, the first nucleotide sequence comprises a gene sequence that encodes a peptide, polypeptide or protein, or a fragment thereof.

In one embodiment, said first nucleotide sequence is a polycistronic nucleic acid sequence, comprising multiple (ie. at least two or more) polynucleotide sequences operably linked in the same reading frame. In one embodiment, the polycistronic nucleic acid sequence encodes a fusion protein. Alternatively, the polycistronic nucleic acid sequence encodes multiple (ie. at least two or more) individual, separate polypeptide sequences.

In one embodiment, the first nucleotide sequence comprises a nucleic acid sequence that is heterologous to said commensal *Neisseria* (ie. heterologous to the commensal *Neisseria* into which the nucleotide sequence of interest is to be integrated).

A polynucleotide sequence that is 'heterologous' to a commensal *Neisseria* is a

polynucleotide sequence that is 'not native to' or 'not normally present in' or 'not naturally occurring in' the commensal *Neisseria*. For example, said heterologous polynucleotide sequence does not naturally occur in the genome of the commensal *Neisseria*.

In one embodiment, the first nucleotide sequence comprises a coding sequence (eg. one or more gene sequences) that is heterologous to said commensal *Neisseria.*

The heterologous coding sequence may optionally be linked to non-coding sequence. As discussed below, the non-coding sequence may be heterologous to said commensal *Neisseria* or may alternatively be native to (naturally occurring in) said commensal *Neisseria.*

Heterologous genes encode heterologous gene products.

Thus, in one embodiment, the first nucleotide sequence comprises or consists of a gene that encodes a (at least one) gene product that is heterologous to said commensal *Neisseria*. A gene product that is heterologous to a commensal *Neisseria* is a gene product that is 'not native to' the commensal *Neisseria*, or is 'not normally present in' or 'not naturally occurring in' the commensal *Neisseria*. For example, the gene product is not naturally encoded by the genome of the commensal *Neisseria*.

Examples of gene products that are heterologous to said commensal *Neisseria* include polypeptides, and fragments thereof, such as antigens or epitopes.

Thus, in one embodiment, the first nucleotide sequence comprises a nucleic acid sequence that encodes a polypeptide, wherein said polypeptide is heterologous to said commensal *Neisseria*.

In one embodiment, said polypeptide comprises an antigenic polypeptide, or an antigenic fragment thereof (eg. an epitope) that is heterologous to said commensal *Neisseria*.

The terms 'antigen' and 'antigenic polypeptide' are synonymous and mean any polypeptide that can be recognized by the immune system and/ or that induces an immune response in a host organism (eg. a mammal, such as a human) exposed to the antigenic polypeptide.

For example, an antigenic polypeptide may stimulate a T-cell mediated immune response in the host organism and/ or may stimulate the generation of antibodies by the host organism. As such, an antigenic polypeptide is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or a T cell receptor.

For example, an antigenic polypeptide may provide a cell-mediated response involving T cells (eg. CD4+ and/ or CD8+ T cells). An antigenic polypeptide may have the ability to induce the secretion of Th1-type cytokines such as IFN-γ (eg. from predominantly CD4+ T cells).

An antigenic polypeptide comprises at least one antigenic determinant. The terms "antigenic determinant" and "epitope" are synonymous, and mean a part of an antigenic polypeptide that is recognised and bound by an antibody (or B cell or T cell) and elicits an immune response. In one embodiment, an antigenic polypeptide induces a neutralizing antibody response.

In one embodiment, an antigenic polypeptide provides protection (such as long term protection) against subsequent challenge.

In one embodiment, the first nucleotide sequence comprises a bacterial, viral, eukaryotic, fungal (eg. yeast) or protozoan nucleic acid sequence that is

heterologous to said commensal *Neisseria*. In one embodiment, said heterologous bacterial, viral, eukaryotic, fungal or protozoan nucleic acid sequence encodes a bacterial, viral, eukaryotic, fungal (eg. yeast) or protozoan polypeptide, such as an antigenic polypeptide, that is heterologous to said commensal *Neisseria*.

In one embodiment, said first nucleotide sequence comprises a nucleic acid sequence of a non-pathogenic (eg. commensal) organism, wherein said nucleic acid sequence is heterologous to said commensal *Neisseria*. In one embodiment, the first nucleotide sequence encodes a polypeptide of a non-pathogenic (eg. commensal) organism, wherein said polypeptide is heterologous to said commensal *Neisseria*.

In one embodiment, said first polynucleotide sequence may comprise a nucleic acid sequence of a commensal *Neisseria*, wherein said nucleic acid sequence encodes a polypeptide that is heterologous to the commensal *Neisseria* into which the nucleotide sequence of interest is to be transformed. For example, the nucleic acid sequence may be from a different commensal *Neisseria* species or strain from the commensal *Neisseria* species or strain into which the nucleotide sequence of interest is to be transformed.

By way of example, the heterologous gene may be a gene of *Neisseria lactamica, Neisseria cinerea*, *Neisseria elongata*, *Neisseria flavescens*, *Neisseria polysaccharea, Neisseria sicca* or *Neisseria subflava*.

In one embodiment, said first nucleotide sequence comprises or consists of a nucleic acid sequence of a pathogenic (disease causing) organism. In one embodiment, said first nucleotide sequence encodes a polypeptide that comprises or consists of a polypeptide of a pathogenic (disease causing) organism.

Examples of pathogenic organisms include pathogenic bacteria, viruses, fungi (eg.

yeast) and protozoa.

In one embodiment, said first nucleotide sequence comprises or consists of a nucleic acid sequence encoding a polypeptide of a pathogenic bacterium (eg. of a gram positive bacterium or gram negative bacterium). Thus, the first nucleotide sequence may encode a polypeptide comprising or consisting of a pathogenic bacterial polypeptide, such as a polypeptide of *Acinetobacter, Actinobacillus, Actinomycetes, Aeromonas, Bacillus, Bordetella, Borrelia, Branhamella, Brucella, Calymmatobacterium, Campylobacter, Chlamydia, Clostridia, Corynebacterium, Coxiella, Enterobacter, Erwinia, Erysipelothrix, Escherichia, Francisella, Haemophilus, Klebsiella, Legionella, Leptospira, Listeria, Moraxella, Mycobacterium, Mycoplasma, Neisseria, Nocardia, Pasturella, Pseudomonas, Proteus, Rickettsia, Salmonella, Shigella, Spirillum, Staphylococcus, Streptobacillus, Streptococcus, Streptomycetes, Treponema, Vibrio* or *Yersinia*.

In one embodiment, said first nucleotide sequence comprises or consists of a nucleic acid sequence encoding a polypeptide of a pathogenic virus (eg. a viral surface peptide or glycoprotein). Thus, the first nucleotide sequence may encode a polypeptide comprising or consisting of a pathogenic viral polypeptide, such as a polypeptide of polypeptide of rabies virus (eg. glycoprotein G), herpes simplex virus (eg. glycoprotein D), Epstein-Barr virus, vesicular stomatitis virus (eg. nucleoprotein), vaccinia virus, Human immunodeficiency virus (HIV), Hepatitis A virus (HAV), Hepatitis B (eg. hepatitis B virus surface antigen), Hepatitis C (HCV), human papillomavirus (HPV), Kaposi's Sarcoma-Associated Herpesvirus (KSHV), Respiratory Syncytial Virus, Ebola virus, Marburg virus, West Nile virus (WNV), St Louis Encephalitis virus (SLEV), Rift Valley Fever virus (RVFV), coronaviruses, rhinovirus, adenovirus, SIV, rotavirus, arbovirus, measles virus, polio virus, rubella virus, mumps virus, papova virus, varicella-zoster virus, varicella virus, hantavirus, arenavirus, bunyavirus, flavivirus, filovirus, cytomegalovirus, Tickborne hemorrhagic fever viruses, Tickborne encephalitis viruses and Influenza viruses.

In one embodiment, said first nucleotide sequence comprises or consists of a nucleic acid sequence encoding a polypeptide of a pathogenic fungus (eg. a pathogenic yeast). Thus, the first nucleotide sequence may encode a polypeptide comprising or consisting of a pathogenic fungal polypeptide, such as a polypeptide of *Acremonium, Alternaria, Amylomyces, Arthoderma, Aspergillus, Aureobasidium, Blastochizomyces, Botrytis, Candida, Cladosporium, Crytococcus, Dictyostelium, Emmonsia, Fusarium, Geomyces, Geotrichum, Microsporum, Neurospora, Paecilomyces, Penicillium, Pilaira, Pityrosporum, Rhizopus, Rhodotorula, Saccharomyces, Stachybotrys, Trichophyton, Trichoporon* or *Yarrowia*.

In one embodiment, said first nucleotide sequence comprises or consists of a nucleic acid sequence encoding a polypeptide of a pathogenic protozoan. Thus, the first nucleotide sequence may encode a polypeptide comprising or consisting of a pathogenic protozoan polypeptide, such as a polypeptide of *Plasmodium falciparum*; a *trypanosome*; or *Cryptosporidium*.

Said first nucleotide sequence may be up to about 10,000 nucleotides in length. In one embodiment, said first nucleotide sequence is up to about 7500, 5000, 4000, 3000, 2750, 2500, 2250, 2000, 1750, 1500, 1250 or 1000 nucleotides long. For example, said first nucleotide sequence may be in the region of about 100-3000 nucleotides long (eg. at least about 200, 300, 400, 500, 600, 700, 800, 900 or 1000 nucleotides in length).

In one embodiment, said first nucleotide sequence comprises a nucleic acid sequence (eg. a gene) of a pathogenic *Neisseria*, such as a *N. meningitidis* or *N. gonorrhoeae* nucleic acid sequence. In one embodiment, the pathogenic *Neisseria* nucleic acid sequence encodes a pathogenic *Neisseria* polypeptide (eg. *N. meningitidis* or *N. gonorrhoeae* polypeptide).

Examples of suitable pathogenic *Neisseria* polypeptides of interest (eg. *N. meningitidis* or *N. gonorrhoeae* polypeptides) include transferrin binding proteins,

factor H binding proteins (fHbp), NadA, superoxide dismutase (such as Cu,Zn-SOD), Neisserial Surface Protein A (NspA), a Porin (eg. PorA or PorB), Opa, Opc, NhhA, or any other outer membrane protein of pathogenic *Neisseria*.

Gene sequences for the majority of these antigens are known in the literature.

By way of example, a nucleic acid sequence encoding a transferrin binding protein is provided by **SEQ ID NO: 5**; a nucleic acid sequence encoding a factor H binding proteins (fHbp) is provided by **SEQ ID NO: 6**; a nucleic acid sequence encoding a NadA is provided by **SEQ ID NO: 7**; a nucleic acid sequence encoding a Cu,Zn-SOD is provided by **SEQ ID NO: 8**; a nucleic acid sequence encoding a Neisserial Surface Protein A (NspA) is provided by **SEQ ID NO: 9**; a nucleic acid sequence encoding a PorA is provided by **SEQ ID NO: 10**; and a nucleic acid sequence encoding a PorB is provided by **SEQ ID NO: 11**.

Variants and fragments of these nucleic acid sequences may be employed in the present invention – for example, the polynucleotide sequence of interest may comprise or consist of a nucleic acid sequence having at least 80% (eg. 82, 84, 86, 88, 90, 92, 94, 95, 96, 97, 98, 99 or 100%) sequence identity to any of **SEQ ID NOs: 5-11**, or a fragment thereof comprising or consisting of at least about 21 consecutive nucleotides thereof (eg. at least 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 or 1000 or more consecutive nucleotides thereof).

In one embodiment, said first nucleotide sequence comprises a heterologous gene that encodes an immunostimulatory gene product, such as an immunostimulatory polypeptide.

An immunostimulatory gene product may be immunostimulatory for treatment of non-infectious disease, for example allergy or cancer. For example, an immunostimulatory polypeptide may comprise a nut antigen polypeptide (eg.

peanut antigen), or may comprise a tumour-specific antigenic polypeptide (eg. melanoma-associated antigen "MAGE" or prostate specific antigen "PSA").

The embodiments of the invention as described above (and below) with respect to the first nucleotide sequence apply also to any other nucleotide sequence of interest that may be inserted into the delivery vehicle of the invention or that is inserted into the recombination cassette of the invention. For example, if the recombination cassette of the invention comprises multiple inserted nucleotide sequences of interest (ie. "second", "third", "fourth" or "fifth" nucleotide sequences, etc.) the embodiments discussed above (and below) apply to these "second", "third", "fourth" and "fifth" nucleotide sequences.

In one embodiment, the recombination cassette of the invention further comprises one or more Neisserial Uptake Sequences. A Neisserial Uptake Sequence is a short nucleic acid sequence (about 10 nucleotides in length) that promotes natural uptake of polynucleotide sequences by *Neisseria* sp.

An exemplary Neisserial Uptake Sequence comprises the nucleic acid sequence gccgtctgaa (shown in **SEQ ID NO: 12**), or the complement thereof. Thus, in one embodiment, the recombination cassette comprises a nucleic acid sequence having at least 80% sequence identity (eg. at least 85, 90, 95 or 100% sequence identity) to **SEQ ID NO: 12**, or a fragment thereof having at least 8 or 9 nucleotides.

The Neisserial Uptake Sequence may be naturally associated with the first nucleotide sequence. Thus, in one embodiment, the Neisserial Uptake Sequence is a part of the first nucleotide sequence. For example, the nucleic acid sequence encoding *N. meningitidis* Factor H binding protein (NMB1870) (**SEQ ID NO: 6**) comprises a Neisserial Uptake Sequence at residues 3-12 of SEQ ID NO: 6.

Alternatively, if the first nucleotide sequence does not inherently comprise a

Neisserial Uptake Sequence (eg. if the first nucleotide sequence does not comprise a nucleic acid sequence of a *Neisseria* sp.), it is an option to modify the recombination cassette to include one or more Neisserial Uptake Sequences.

For example, a Neisserial Uptake Sequence may be inserted into an insertion site of the recombination cassette. The Neisserial Uptake Sequence may be inserted into the same insertion site as the first nucleotide sequence (eg. so it is located at the 5' or 3'end of the nucleotide sequence of interest). Alternatively, the Neisserial Uptake Sequence may be inserted into a different insertion site from the site into which the first nucleotide sequence is inserted. In one embodiment, a Neisserial Uptake Sequence is inserted into the backbone of a plasmid vector that is used to propagate the recombination cassette prior to transformation into the intended recipient, or may be added to the ends of a linear recombination cassette by PCR amplification with a 10 bp tail on either one or both primers. Alternatively, the first nucleotide sequence may be modified to include a Neisserial Uptake Sequence (eg. at the 5' or 3' end of the first nucleotide sequence) prior to inserting the first nucleotide sequence into the insertion site.

The recombination cassette of the invention may further comprise a nucleic acid 'signal sequence' encoding a 'signal peptide'.

A signal peptide is a short peptide that, as a component of a larger polypeptide (eg. a polypeptide encoded by the nucleotide sequence of interest), directs the polypeptide to a desired intracellular or extracellular location (such as the plasma membrane of a commensal *Neisseria* cell).

Thus, in one embodiment, the first nucleotide sequence may comprise a gene sequence encoding a polypeptide operably linked to a nucleic acid 'signal sequence' encoding a signal peptide. A signal sequence encoding a signal peptide is commonly positioned 5' to the first nucleotide sequence, although certain signal sequences may be positioned elsewhere in the first nucleotide

sequence.

In one embodiment, the signal peptide is naturally associated with the encoded polypeptide. By way of example, *Neisseria* PorA polypeptide is naturally produced with a signal peptide that translocates the nascent PorA polypeptide through the inner membrane.

The signal peptide may be native to the commensal *Neisseria* into which the recombination cassette is to be transformed (eg. an *N. lactamica* signal peptide).

If the first nucleotide sequence does not encode a polypeptide comprising a signal peptide, a nucleic acid sequence encoding a signal peptide may be joined to the first nucleotide sequence in the correct reading frame. Thus, in one embodiment, the first nucleotide sequence is modified such that the encoded polypeptide is linked to a signal sequence from a different polypeptide, such as a commensal *Neisseria* polypeptide (eg. an *N. lactamica* polypeptide).

In one embodiment, the recombination cassette comprises one or more non-coding sequences that drive or regulate the expression and processing of the first nucleotide sequence. The non-coding sequence may be operably linked to the first nucleotide sequence (or may be comprised within the first nucleotide sequence, operably linked to a gene encoding a gene product of interest).

Examples of typical non-coding sequences include transcriptional regulatory elements such as leader sequences, promoter sequences, enhancer sequences, terminator sequences and polyadenylation signals; and translational regulatory elements, such as ribosomal binding sites and translation initiation and termination sequences.

For example, the first nucleotide sequence may comprise a nucleic acid sequence encoding a product of interest (eg. a polypeptide, as described above) operably

linked (at the 5' or 3' end) to a leader sequence. A leader sequence may affect processing of a primary DNA transcript to mRNA, and/ or may affect mRNA stability or translation efficiency.

The transcriptional and translational regulatory elements are functional in the commensal *Neisseria* into which the recombination cassette is transformed. In one embodiment, the transcriptional and translational regulatory elements are native to the commensal *Neisseria*. In one embodiment, the transcriptional and translational regulatory elements are naturally associated with the first nucleotide sequence.

The selection of suitable signal sequences, leader sequences, promoters, terminators and other regulatory elements is a matter of routine design within the level of ordinary skill in the art.

Generally, "operably linked" means that the nucleic acid sequences being linked are contiguous and arranged so that they function in concert for their intended purposes – for example, transcription initiates in the promoter and proceeds through the coding polynucleotide segment to the terminator. Where necessary to join two protein coding regions, the polynucleotide coding sequences should be contiguous and in reading frame.

In one embodiment, the recombination cassette comprises a selectable marker gene. A selectable marker gene encodes an identifiable gene product, which enables identification of a cell that has been transformed with the recombination cassette.

In one embodiment, the selectable marker gene encodes a protein necessary for the survival or growth of the transformed commensal *Neisseria*. This gene ensures the growth of only those commensal *Neisseria* that have been transformed. Conventional selection genes encode proteins that (a) confer

resistance to positive selection agents such as antibiotics or other toxic substances, eg. chloramphenicol, ampicillin, neomycin, methotrexate, etc.; (b) complement auxotrophic deficiencies; or (c) supply critical nutrients.

Thus, in one embodiment, the first nucleotide sequence comprises or consists of a selectable marker gene.

In one embodiment, recombination cassette comprises a first nucleotide sequence and at least one more nucleotide sequence, wherein one or more of said nucleotide sequences comprises or consists of a selectable marker gene. The nucleotide sequence encoding the selectable marker gene may be inserted into the same insertion site as the first or other nucleotide sequence of interest. Alternatively, the nucleotide sequence of interest encoding the selectable marker gene and the first (and/ or other) nucleotide sequence of interest may be inserted into the different insertion sites.

Thus, in accordance with one embodiment, the invention provides a method of producing a recombination cassette for integration of multiple nucleotide sequences into the genome of a commensal *Neisseria* by homologous recombination, wherein at least one of said nucleotide sequences comprises or consists of a selectable marker gene, said method comprising inserting said nucleotide sequences of interest into a delivery vehicle of the invention or into a plasmid of the invention.

In one embodiment, a selectable marker gene and a nucleotide sequence encoding an antigenic polypeptide are inserted into the delivery vehicle, to form a recombination cassette comprising both said selectable marker gene and said nucleotide sequence encoding an antigenic polypeptide. The antigenic polypeptide may be, for example, a polypeptide of a pathogenic organism, such as a pathogenic *Neisseria* sp.

In one specific embodiment illustrated in the Examples, the first nucleotide sequence encodes *N. meningitidis* PorA polypeptide, and the selectable marker gene is a chloramphenicol resistance gene (Cm^R gene).

In one aspect, the invention provides a method of transforming commensal *Neisseria* with a first nucleotide sequence, comprising introducing a recombination cassette of the invention, as described above, into said commensal *Neisseria*.

'Transformation' of commensal *Neisseria* refers to the heritable genetic alteration of commensal *Neisseria* cells resulting from the uptake, genomic incorporation, and expression of heterologous genetic material.

In one embodiment, the commensal *Neisseria* is *N. lactamica*.

A skilled person is familiar with conventional techniques for introducing a recombination cassette into bacteria such as commensal *Neisseria*. By way of example, suitable techniques may be pilus-dependent (eg. natural uptake) or pilus-independent uptake (eg. via chemical transformation or electroporation).

By virtue of the high level of sequence identity between the polynucleotide sequence in the recombination cassette and the target sequence in the commensal *Neisseria* genome (ie. the r601 or r602 sequence, or a fragment thereof such as the Nlac_024 or Nlac_025 coding sequences), the recombination cassette is capable of integration into the commensal *Neisseria* genome via homologous recombination.

In more detail, the recombination cassette recombines with a region of the commensal *Neisseria* genome located within the Nlac1 prophage. Specifically, the recombination cassette targets the identical genes Nlac1_024 and Nlac1_025 (each represented by **SEQ ID NO: 3**), which are located within a tandem-repeated region of the prophage made up of two 1057 bp elements, named r601 (**SEQ ID**

NO: 1, comprising Nlac1_024) and r602 (**SEQ ID NO: 2**, comprising Nlac1_025). Thus, once taken up by the commensal *Neisseria*, the recombination cassette of the invention integrates into the genome of the commensal *Neisseria* (eg. *N. lactamica*) by homologous, site-specific recombination, resulting in a transformed, recombinant commensal *Neisseria*.

The transformed, recombinant commensal *Neisseria* cells are known as "integrative transformants", because the heterologous nucleic acid sequence has become integrated into the genomic DNA of the commensal *Neisseria* cells.

In one embodiment, the method further comprises selecting for transformed, recombinant commensal *Neisseria* that have stably integrated the first nucleotide sequence (and additional nucleotide sequence(s) of interest, if present in the recombination cassette) into their genome.

Conventional selection techniques are known in the art, and include detecting expression of the nucleotide sequence(s) of interest.

By way of example, *Neisseria* colonies arising from the transformation may be screened directly for the presence of the first nucleotide sequence by PCR or by nucleic acid hybridisation. Such direct screening, though labour-intensive, has been found to be feasible in *Neisseria* spp. (Gunn JS & Stein DC 1996, Mol. Gen. Genet. 251:509–17).

Expression of a polypeptide encoded by the first nucleotide sequence may be verified using standard techniques such as Western blotting and flow-cytometry.

If the first nucleotide sequence encodes a detectable polypeptide such as a reporter peptide or selectable marker (eg. as discussed above), expression of the first nucleotide sequence may be verified by assaying for the reporter or selectable marker activity.

For example, the selectable marker may protect the recombinant, transformed commensal *Neisseria* from the effects of a positive selection agent. Cells may be cultured in the presence of this positive selection agent. Transformed cells that have undergone homologous recombination will be protected against exposure to said positive selection agent via expression of said selectable marker.

For example, in one embodiment, the commensal *Neisseria* to be transformed is sensitive to an antibiotic or other toxic substance, and the selectable marker confers resistance to said antibiotic or toxic substance. By way of example, the commensal *Neisseria* is sensitive to chloramphenicol (ie. Cm^S) and the selectable marker gene provides resistance to chloramphenicol. In accordance with this embodiment, selecting for transformed, recombinant commensal *Neisseria* comprises selecting for commensal *Neisseria* that have developed resistance to said antibiotic or toxic substance – eg. commensal *Neisseria* that are Cm^R.

Said selection method may comprise culturing the commensal *Neisseria* in the presence of said antibiotic or toxic substance (eg. on a medium comprising said antibiotic or toxic substance); growth of stably transformed, recombinant commensal *Neisseria* on this medium will be better than growth of commensal *Neisseria* that do not express the selectable marker.

Alternatively (or in addition), the commensal *Neisseria* to be transformed has an auxotrophic deficiency, which is complemented by a product encoded by a nucleotide sequence of interest provided by the recombination cassette. Integrative transformation of the commensal *Neisseria* can be verified by detecting the substantial absence or reduction of the auxotrophic deficiency.

In one aspect, the invention provides a recombinant commensal *Neisseria,* such as *Neisseria lactamica*, that has a first nucleotide sequence of interest (eg. a heterologous gene sequence) stably integrated within its genome.

In one embodiment, the first nucleotide sequence is stably integrated into the genome of said recombinant commensal *Neisseria* within the commensal *Neisseria* Nlac1 prophage.

In one embodiment, the first nucleotide sequence is stably integrated within the r601 or r602 tandem repeated region of the Nlac1 prophage, defined by SEQ ID NOs: 1 or 2. In one embodiment, the first nucleotide sequence is stably integrated within the Nlac_024 or Nlac_025 genes, defined by SEQ ID NO: 3 – ie. a Δ*Nlac1_024*::*foreign gene* (or Δ*Nlac1_025*::*foreign gene*) genotype.

In one embodiment, the recombinant commensal *Neisseria* is obtainable (or has been obtained) by a transformation method as described above.

The integrated nucleotide sequence acquires the commensal *Neisseria* methylation pattern, and is therefore protected from the commensal *Neisseria* restriction system.

The recombinant commensal *Neisseria* expresses the first nucleotide sequence of interest. For example, in one embodiment, said recombinant commensal *Neisseria* expresses a polypeptide (eg. as defined herein) encoded by the integrated first nucleotide sequence. By way of example, the polypeptide may comprise or consist of a heterologous polypeptide, such as an antigenic polypeptide, for example, a polypeptide of a pathogenic organism.

In one embodiment, the invention provides a recombinant commensal *Neisseria,* such as *N. lactamica*, that expresses a polypeptide of a pathogenic Neisseria (eg. *N. meningitidis* or *N. gonorroheae*), wherein said polypeptide is encoded by a first nucleotide sequence that is stably integrated into the genome of said commensal *Neisseria*.

In one embodiment, said recombinant commensal *Neisseria,* such as *N. lactamica*, expresses an *N. meningitidis* PorA gene and a selectable marker such as an antibiotic resistance gene from one or more nucleotide sequences that are stably integrated into the genome of said commensal *Neisseria*. In one embodiment, said PorA coding sequence and said selectable marker gene are stably integrated within the Nlac1 prophage of said commensal *Neisseria* genome (eg. within the r601 or r602 sequence, such as within the Nlac_024 or Nlac_025 gene of said prophage – ie. a Δ*Nlac1_024*::*foreign gene* genotype or Δ*Nlac1_025*::*foreign gene* genotype).

In one embodiment, the invention provides a method of transforming a commensal *Neisseria,* such as *N. lactamica*, with a first nucleotide sequence, comprising introducing into said commensal *Neisseria* chromosomal DNA obtained from a transformed, recombinant commensal *Neisseria* of the invention, as described above.

Chromosomal DNA from the genome of the transformed, recombinant commensal *Neisseria* (including the integrated first nucleotide sequence of interest) has the correct commensal *Neisseria* methylation pattern and is therefore protected from the commensal *Neisseria* restriction enzymes. This enables high efficiency 'secondary transformation' of other, wild-type, commensal *Neisseria*.

Chromosomal DNA may be obtained from the transformed, recombinant commensal *Neisseria* using conventional techniques with which a skilled person is familiar. Likewise, suitable transformation techniques (pilus-dependent or pilus-independent) are also well known in the art – eg. as discussed above.

Successful secondary transformation of the commensal *Neisseria* may be determined using any of the conventional selection techniques described above.

The invention also provides a 'secondarily' transformed, recombinant commensal

Neisseria, obtainable by a method as described above.

Recombinant commensal *Neisseria* obtained by this secondary transformation method will not necessarily have Δ*Nlac1_024*::*foreign gene* (or Δ*Nlac1_025*::*foreign gene*) genotypes. Instead, the properly methylated DNA (including the nucleotide sequence of interest) may integrate into another locus in the commensal *Neisseria* genome.

In one embodiment, the product of the first nucleotide sequence (eg. an antigenic polypeptide) is at least partially exposed at the surface of the commensal *Neisseria* cell.

The transformed, recombinant commensal *Neisseria* may be live, or may be killed. For example, commensal *Neisseria* may be killed using heat or by suspension in a mixture of bactericidal agents such as thiomersal and formaldehyde.

Live transformed commensal *Neisseria* may be attenuated. However, it is usually not required to attenuate commensal *Neisseria* because these organisms are avirulent.

An immunogenic component or extract may be obtained from the recombinant, commensal *Neisseria* of the invention, as described herein. Said immunogenic component or extract may comprise a gene product (typically a polypeptide, such as an antigenic polypeptide) encoded by the first nucleotide sequence.

An immunogenic component or extract of the transformed, recombinant commensal *Neisseria* may comprise an outer membrane preparation, such as an outer membrane vesicle (OMV) preparation, or may comprise a protein fraction.

The outer membrane preparation (eg. OMVs) or protein fraction may comprise a gene product (typically a polypeptide, such as an antigenic polypeptide as

described herein) encoded by the first nucleotide sequence.

Outer membrane vesicles (OMVs), also referred to as 'blebs', are discrete vesicles formed or derived from fragments of the outer membrane of a Gram negative bacterium such as *Neisseria* (eg. commensal *Neisseria* such as *N. lactamica*). OMVs typically comprise outer membrane proteins (OMPs), lipids, phospholipids, periplasmic material and lipopolysaccharide (LPS). OMVs have a mean diameter of around 120nm and typically within the range of 80-200nm.

A number of conventional techniques are known in the art for extraction of outer membrane components such as OMVs, protein fractions, lipooligosaccharides and lipopolysaccharides from cell preparations (see WO 00/50074), and are suitable to obtain the immunogenic components or extracts of the invention.

Outer membrane preparations and protein fractions can be obtained from commensal *Neisseria* cultured in the presence or absence of iron. For example, a protein fraction of commensal *Neisseria* is conveniently obtained by suspending commensal *Neisseria* cells or membranes in the presence of detergent and incubating the suspension so as to extract proteins therefrom.

OMVs can also be obtained from commensal *Neisseria* according to a number of methods known in the art, for example by deoxycholate extraction, Tris/HCl/EDTA extraction, and lithium acetate extraction. Protocols for performing such extractions are described in detail in the literature. However, it will be appreciated by the skilled person that virtually any chemical and/ or physical technique that enables disruption of the commensal *Neisseria* outer membrane in order to release sufficient OMVs for purification and isolation, is suitable for preparation of the compositions of the invention.

Thus, in one aspect, the invention further provides a method of preparing an immunogenic component or extract of the recombinant, commensal *Neisseria* of

the invention, as described herein.

In one embodiment, the method comprises (i) suspending said recombinant commensal *Neisseria* in the presence of detergent; and (ii) incubating the suspension so as to extract an immunogenic component or extract from the recombinant commensal *Neisseria*. Step (ii) of said method may further comprise the steps of (iii) centrifuging the suspension to separate the suspension into a supernatant and a pellet; and (iv) fractionating the an immunogenic component or extract from the supernatant.

This specific method can be modified according to the extraction protocol selected by the user.

For example, instead of or as well as using detergent in the initial step (i), alternative conventional techniques may be used, such as high salt concentration, chaotropic agents, high or low pH, enzymic digestion and/ or mechanical disruption.

The invention also provides an immunogenic composition, comprising a transformed, recombinant commensal *Neisseria* as described above, and optionally a pharmaceutically acceptable carrier.

The invention also provides an immunogenic composition comprising an immunogenic component or extract of a transformed, recombinant commensal *Neisseria* of the invention, as described herein; and optionally a pharmaceutically acceptable carrier.

In one embodiment, said immunogenic component or extract comprises a gene product (typically a polypeptide such as an antigenic polypeptide) encoded by said first nucleotide sequence. In one embodiment, said immunogenic component or extract comprises outer membrane vesicles.

The invention also provides a method of preparing an immunogenic composition, comprising combining a transformed, recombinant commensal *Neisseria*, as described above, with a pharmaceutically acceptable carrier.

The invention also provides a method of preparing an immunogenic composition, comprising: obtaining an immunogenic component or extract from a recombinant commensal *Neisseria* of the invention (eg. via a method as described above); and combining said immunogenic component or extract with a pharmaceutically acceptable carrier.

In one embodiment, the immunogenic component or extract obtained from the recombinant commensal *Neisseria* comprises a polypeptide encoded by said first nucleotide sequence. In one embodiment, said immunogenic component or extract comprises outer membrane vesicles.

Non-limiting examples of pharmaceutically acceptable carriers include water, saline, and phosphate-buffered saline. In some embodiments, however, the composition is in lyophilized form, in which case it may include a stabilizer, such as BSA. In some embodiments, it may be desirable to formulate the composition with a preservative, such as thiomersal or sodium azide, to facilitate long term storage.

Optionally, said methods of preparing an immunogenic composition comprise the step of combining the components described above with one or more of a excipient, diluent, adjuvant, buffering agent, immunoregulatory agent and/ or antimicrobial compound that is pharmaceutically acceptable and compatible with the active ingredient.

Thus, immunogenic compositions of the invention comprise active immunogenic ingredients (ie. transformed, recombinant commensal *Neisseria*, or immunogenic

component or extract thereof) and a pharmaceutically acceptable carrier, and may optionally comprise one or more of a excipient, diluent, adjuvant, buffering agent, immunoregulatory agent and/ or antimicrobial compound, as described below.

Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/ or adjuvants, which enhance the effectiveness of the vaccine.

Examples of adjuvants that may be effective include but are not limited to: complete Freunds adjuvant (CFA), Incomplete Freunds adjuvant (IVA), Saponin, a purified extract fraction of Saporin such as Quil A, a derivative of Saporin such as QS-21, lipid particles based on Saponin such as ISCOM/ISCOMATIX, E. coli heat labile toxin (LT) mutants such as LTK63 and/ or LTK72, aluminium hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine ("thr-MDP"), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, "nor-MDP"), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryl oxy)-ethylamine (CGP 19835A, "MTP-PE"), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2 % squalene/ Tween 80 emulsion.

Alternatively, the vaccine of the invention may be "substantially free of adjuvant". In this context, "substantially free of adjuvant" means that there is less than 0.05% adjuvant, such as less than 0.025% adjuvant, or less than 0.001% adjuvant. In one embodiment, the vaccine may be completely free of adjuvant.

Examples of buffering agents include, but are not limited to, sodium succinate (pH 6.5), and phosphate buffered saline (PBS; pH 6.5 and 7.5).

In one embodiment, the immunogenic composition (eg. vaccine) of the invention

may further comprise one or more immunoregulatory agents selected from, for example, immunoglobulins, antibiotics, interleukins (eg. IL-2, IL-12), and/ or cytokines (eg. IFNγ).

In one embodiment, the immunogenic composition is a therapeutic or prophylactic formulation, or medicament, such as a vaccine.

As used, herein, a "vaccine" is a formulation that, when administered to a subject stimulates a protective immune response against infection or stimulates or desensitises the immune system in the treatment of a non-infectious medical condition such as an allergy or cancer. The immune response may be a humoral and/ or cell-mediated immune response. As described in more detail below, a vaccine of the invention can be used, for example, to protect a subject from the effects of infection by a pathogenic organism, such as *Neisserial* invention (eg. *N. meningitidis* or *N. gonorrhoeae* infection).

Administration of immunogenic compositions (eg. vaccines) of the invention to a subject is generally by conventional routes e.g. intravenous, subcutaneous, intraperitoneal, or mucosal routes. The administration may be by parenteral injection, for example, a subcutaneous or intramuscular injection.

Accordingly, the therapeutic formulations, medicaments and prophylactic formulations (eg. vaccines) of the invention are typically prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid prior to injection may alternatively be prepared. The preparation may also be emulsified, or the peptide encapsulated in liposomes or microcapsules.

An immunogenic composition comprising transformed commensal *Neisseria* of the invention that are buccal colonizers may be administered in a mouthwash.

Oral formulations may include conventional excipients such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders.

An immunogenic composition comprising transformed commensal *Neisseria* of the invention that are nasal colonizers may be administered in a nasal spray.

Formulations for intranasal administration may in the form of nasal droplets or a nasal spray. An intranasal formulation may comprise droplets having approximate diameters in the range of 100-5000 µm, such as 500-4000 µm, 1000-3000 µm or 100-1000 µm. Alternatively, in terms of volume, the droplets may be in the range of about 0.001-100 µl, such as 0.1-50 µl or 1.0-25 µl, or such as 0.001-1 µl.

Alternatively, the immunogenic composition (eg. vaccine) may be an aerosol formulation. The aerosol formulation may take the form of a powder, suspension or solution. Aerosol particles may be delivered using a nebulizer (eg. via the mouth) or nasal spray. An aerosol formulation may optionally contain a propellant and/ or surfactant. The size of aerosol particles is relevant to the delivery capability of an aerosol. Smaller particles may travel further down the respiratory airway towards the alveoli than would larger particles. In one embodiment, the aerosol particles have a diameter distribution to facilitate delivery along the entire length of the bronchi, bronchioles, and alveoli. Alternatively, the particle size distribution may be selected to target a particular section of the respiratory airway, for example the alveoli. In the case of aerosol delivery of the medicament, the particles may have diameters in the approximate range of 0.1-50 µm, such as 1-25 µm, or 1-5µm.

Preferred dose ranges for administration of whole-cell transformed, recombinant commensal *Neisseria* are 0.2µg to 100µg. Preferred dose ranges for

administration of immunogenic components or extracts of said transformed, recombinant commensal *Neisseria* are from 0.2µg to 100µg.

The immunogenic composition (eg. vaccine) of the invention may contain 5% to 95% of active ingredient, such as at least 10% or 25% of active ingredient, or at least 40% of active ingredient or at least 50, 55, 60, 70 or 75% active ingredient.

The immunogenic composition (eg. vaccine) may be administered in a single dose schedule, or in a multiple dose schedule. In a single dose schedule, the full dose is given at substantially one time. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or re-enforce the immune response, for example (for human subjects), at 1-4 months for a second dose, and if needed, a subsequent dose(s) after a further 1-4 months. The dosage regimen will, at least in part, be determined by the need of the individual and be dependent upon the judgment of the practitioner.

In one embodiment, the vaccine of the present invention may be administered as part of a 'prime-boost' vaccination regime.

Prime-boost vaccination regimes involve: Priming – ie. exposing a subject to one or more antigens or a vaccine; and subsequently: Boosting – ie. exposing the subject to one or more antigens or a vaccine. The 'boost' antigen/ vaccine is typically different from the 'primer' antigen/ vaccine (known as "heterologous" prime-boost). In this regard, heterologous prime-boost immunization strategies have been shown to induce higher levels of effector T cell responses in subjects as compared with homologous boosting with the same vaccine.

Thus, in one embodiment of the invention, the subject's immune system is 'primed' by administration of a conventional vaccine and then 'boosted' by administration of the vaccine of the present invention. Alternatively, a subject's immune system

may be 'primed' by administration of the vaccine of the present invention, and then 'boosted' by administration of a conventional vaccine.

The 'priming' step may be carried out on the subject at any age – in the case of mammalian subjects (eg. human subjects), priming is conventionally carried out neonatally, or during infancy, adolescence or adulthood. The 'boosting' step may be carried out at any time after the 'priming' step. In the case of mammalian subjects (eg. human subjects), a boosting step may be carried out at least about 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 weeks after the priming step, or at least about 3, 6, 8 or 12 months after the priming step, or at least about 2, 5, 10, 15, 20, 25, 30, 35, or 40 or more years after the boosting step. In one embodiment, for a human subject, the priming step is carried out during infancy and the boosting step is carried out during adolescence.

In one embodiment, the immunogenic composition (eg. a vaccine) of the invention can be administered to a subject simultaneously or sequentially with one or more immunoregulatory agents selected from, for example, immunoglobulins, antibiotics, interleukins (eg. IL-2, IL-12), and/ or cytokines (eg. IFNγ) and/ or one or more antimicrobial compounds, such as conventional anti-tuberculosis drugs (eg. rifampicin, isoniazid, ethambutol or pyrizinamide).

The therapeutic formulation, medicament or prophylactic formulation (eg. a vaccine) is administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/ or therapeutically effective.

In this regard, as used herein, an "effective amount" is a dosage or amount that is sufficient to achieve a desired biological outcome. As used herein, a "therapeutically effective amount" is an amount which is effective, upon single or multiple dose administration to a subject for treating, preventing, curing, delaying, reducing the severity of, ameliorating at least one symptom of a disorder or recurring disorder, or prolonging the survival of the subject beyond that expected

in the absence of such treatment.

Accordingly, the quantity of active ingredient to be administered, which is generally in the range of 5 micrograms to 250 micrograms of antigen per dose, depends on the subject to be treated, capacity of the subject's immune system to generate a protective immune response, and the degree of protection desired.

Precise amounts of active ingredient required to be administered may depend on the judgment of the practitioner and may be particular to each subject.

The transformed, recombinant commensal *Neisseria*, or immunogenic component or extract thereof, or immunogenic composition, stimulates an immune response in a subject.

Thus, in one embodiment, the invention provides a transformed, recombinant commensal *Neisseria*, or immunogenic component or extract thereof, or immunogenic composition, as described above, for use in stimulating an immune response in a subject.

The invention also provides the use of a transformed, recombinant commensal *Neisseria*, or immunogenic component or extract thereof, or immunogenic composition, as described above, for the manufacture of a medicament for stimulating an immune response in a subject.

The invention further provides a method for stimulating an immune response in a subject, comprising administering to the subject a transformed, recombinant commensal *Neisseria*, or immunogenic component or extract thereof, or immunogenic composition, as described above.

In one embodiment, immune stimulation is measured by a protective effect in an *in vivo* survival assay. In one embodiment, immune stimulation is measured by an

increased frequency in T lymphocytes specific for an antigen in the vaccine (ie. a T cell immune response). In one embodiment, the immune stimulation is a memory T cell immune response, such as a central memory T cell response (eg. a CCR7+ response).

In one embodiment, immune stimulation is measured by an increase in antibody titer that is specific for an antigen in the vaccine.

In one embodiment, the immune response is against a pathogenic organism, such as pathogenic *Neisseria* (eg. *N. meningitidis* or *N. gonorrhoeae*).

Thus, in one embodiment, the invention provides a transformed, recombinant commensal *Neisseria*, or immunogenic component or extract thereof, or immunogenic composition, as described above, for use in treating or preventing an infection in a subject.

The invention also provides the use of a transformed, recombinant commensal *Neisseria*, or immunogenic component or extract thereof, or immunogenic composition, as described above, for the manufacture of a medicament for treating or preventing an infection in a subject.

The invention further provides a method for treating or preventing an infection in a subject, comprising administering to the subject a transformed, recombinant commensal *Neisseria*, or immunogenic component or extract thereof, or immunogenic composition, as described above.

The term “infection” includes the proliferation of a pathogenic organism within and/ or on the tissues of a host organism. The term "preventing an infection" includes preventing the initiation of an infection and/ or reducing the severity or intensity of an infection. The term "treating an infection" embraces therapeutic or preventative/ prophylactic measures, and includes post-infection therapy and

amelioration of an infection.

In one embodiment, the infection is a Neisserial infection – such as infection by *N. meningitidis* or *N. gonorrhoeae*.

In one embodiment, administration of the recombinant commensal *Neisseria*, or immunogenic component or extract thereof, or immunogenic composition, as described above, is useful for treating or preventing meningococcal disease. In one embodiment, administration of the recombinant commensal *Neisseria*, or immunogenic component or extract thereof, or immunogenic composition, as described above, is useful for treating or preventing meningitis or gonorrhoea.

In one embodiment, the immune response is against an antigen, such as an allergen, or a disease (eg. tumour)-specific antigen.

Thus, in one embodiment, the invention provides a transformed, recombinant commensal *Neisseria*, or immunogenic component or extract thereof, or immunogenic composition, as described above, for use in stimulating or desensitizing the immune system in a subject.

The invention also provides the use of a transformed, recombinant commensal *Neisseria*, or immunogenic component or extract thereof, or immunogenic composition, as described above, for the manufacture of a medicament for stimulating or desensitizing the immune system in a subject.

The invention also provides a method for stimulating or desensitizing the immune system in a subject, comprising administering to the subject a transformed, recombinant commensal *Neisseria*, or immunogenic component or extract thereof, or immunogenic composition, as described above.

Methods and uses for stimulating or desensitizing the immune system in a subject

may be useful for treating or preventing allergies (eg. nut allergies) or cancers.

As used herein, the "efficacy" of a vaccine describes the ability of the vaccine to protect a subject (typically a mammalian subject eg. a human, bovine, porcine or equine subject) from challenge with a pathogen, or from a non-infectious medical condition. By way of example, "vaccine efficacy" may refer to the efficacy of a vaccine in preventing the initiation of an infection or non-infectious medical condition and/ or reducing the severity/ intensity of an infection or non-infectious medical condition.

An immunogenic composition of the invention (eg. vaccine) may be administered to a subject already having an infection, or a condition or symptoms associated with an infection, to treat or prevent said infection. In one embodiment, the subject is suspected of having come in contact with an infectious organism, such as pathogenic *Neisseria*, or has had known contact with an infectious organism, but is not yet showing symptoms of exposure.

An immunogenic composition of the invention (eg. vaccine) may be administered to a subject already having a non-infectious medical condition, or a condition or symptoms associated with a non-infectious medical condition, to treat or prevent said non-infectious medical condition. In one embodiment, the subject is suspected of having come in contact with an allergen, or has had known contact with an allergen, but is not yet showing symptoms of exposure.

When administered to a subject that already has an infection or a non-infectious medical condition, or is showing symptoms associated with an infection or non-infectious medical condition, the therapeutic composition/ medicament (eg. vaccine) may cure, delay, reduce the severity of, or ameliorate one or more symptoms, and/ or prolong the survival of a subject beyond that expected in the absence of such treatment.

Alternatively, a therapeutic/ prophylactic composition or medicament (eg. vaccine) may be administered to a subject who ultimately may acquire an infection or a non-infectious medical condition, in order to prevent, cure, delay, reduce the severity of, or ameliorate one or more symptoms of said infection or non-infectious medical condition, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment.

In one embodiment, the subject has previously been exposed to an allergen or pathogen (eg. pathogenic *Neisseria*). For example, the subject may have had an infection in the past (but is optionally not currently infected). The subject may be latently infected with a pathogen. Alternatively, or in addition, the subject may have been vaccinated against the allergen or pathogen in the past. In one embodiment, the subject has been pre-exposed to a conventional vaccine against the allergen or pathogen in the past (eg. the subject's immune system has been 'primed').

In the context of the therapeutic uses and methods of the invention, a 'subject' is any animal subject that would benefit from stimulation of an immune response. Typical animal subjects are mammals, for example, human, bovine, porcine, ovine, caprine, equine, corvine, canine or feline subjects. In one embodiment, the subject is human, bovine, porcine or equine. Some pathogenic organisms such as pathogenic *Neisseria* are obligate human pathogens. Thus, in the context of these pathogens, the 'subject' is a human.

The treatments and preventative therapies of the present invention are applicable to a variety of different subjects of different ages. In the context of humans, the therapies are applicable to children (eg. infants, children under 5 years old, older children or teenagers) and adults. In the context of other animal subjects, the therapies are applicable to immature subjects and mature/ adult subjects. The treatments and preventative therapies of the present invention are applicable to subjects who are immunocompromised or immunosuppressed (eg. human

patients who have HIV or AIDS, or other animal patients with comparable immunodeficiency diseases), subjects who have undergone an organ transplant, bone marrow transplant, or who have genetic immunodeficiencies.

A neutralisation test examines the capability of antisera raised to a specific antigen to neutralise (ie. inhibit or prevent) a particular biological process associated with the functionality of the antigen.

In general terms, antisera are raised to the antigen in an appropriate animal model (for example, mice, guinea pigs, rabbits, goats, sheep, horse) using the immunisation protocol that is appropriate to the host. For example, a standard immunisation protocol for guinea pigs might be immunisation with 0.5 nmoles antigen on day 1, followed by two further 0.5 nmole inoculums over an eight-week period. Two weeks after the final dose, the sera would be obtained from the animals and used for neutralisation test.

A neutralisation test is typically performed by mixing the sera with the active antigen, or a protein containing the antigen, *in vitro* prior to assessment of the functionality of the antigen in an appropriate test.

A challenge test examines the capability of the antigen to raise a sufficient host response in order to neutralise (ie. inhibit or prevent) the pathogenesis of the agent from which the antigen is derived. In general terms, susceptible animals are inoculated with appropriate doses of the test antigen over an appropriate time period. For example, a standard immunisation protocol in mice might be initial immunisation with 5µg antigen on day 1, followed by 5µg antigen on day 14 and 5µg antigen on day 28.

On completion of the immunisation schedule, the animals are challenged with a test dose of the test agent and observed for susceptibility to the agent. An antigen

that demonstrated potential as a vaccine candidate would protect the animals from succumbing to the effects of the agent.

The challenge test therefore differs conceptually from the neutralisation test. The neutralisation test assesses the ability of anti-antigen sera to inactivate an agent *in vitro*. The challenge test assesses the ability of an antigen to raise a host response to the test agent – ie. the challenge test assesses *in vivo* efficacy.

As used herein, the terms "nucleic acid sequence", "nucleotide sequence", "polynucleotide sequence" and "polynucleotide" are used interchangeably and do not imply any length restriction. The terms "nucleic acid" and "nucleotide" are also used interchangeably. The terms "peptide", "polypeptide" and "protein" are used interchangeably and do not imply any length restriction.

Polynucleotide sequences include nucleic acid sequences that have been removed from their naturally occurring environment, recombinant or cloned DNA isolates, and chemically synthesized analogues or analogues biologically synthesized by heterologous systems.

When applied to a nucleic acid sequence, the term "isolated" denotes that the polynucleotide sequence has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences (but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators), and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment. Methods for isolating nucleic acid sequences are known in the art.

The term "recombinant polynucleotide" means a polynucleotide of genomic, cDNA, semi-synthetic, or synthetic origin which, by virtue of its origin or manipulation: (1)

is not associated with all or a portion of a polynucleotide with which it is associated in nature; or (2) is linked to a polynucleotide other than that to which it is linked in nature; and (3) does not occur in nature. This artificial combination is often accomplished by via conventional chemical synthesis techniques, or by the artificial manipulation of isolated segments of nucleic acids – eg. by conventional genetic engineering techniques.

Polynucleotides may be prepared by any means known in the art. For example, large amounts of polynucleotides may be produced by replication in a suitable host cell such as *E. coli*. Natural or synthetic DNA fragments can be incorporated into recombinant nucleic acid constructs, typically DNA constructs, capable of introduction into and replication in a prokaryotic or eukaryotic cell.

Polynucleotides may also be produced by chemical synthesis, eg. by the phosphoramidite method or the triester method, and may be performed on commercial automated oligonucleotide synthesizers. A double-stranded fragment may be obtained from the single stranded product of chemical synthesis either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

A nucleic acid sequence can be also obtained by conventional cloning procedures, such as PCR, or can be synthesized using nucleic acid synthesis machines. An alternative way to prepare a full-length polynucleotide is to synthesize a specified set of overlapping oligonucleotides (eg. 40 to 100 nucleotides). Other sequences may be added that contain signals for proper initiation and termination of transcription and translation.

In view of the degeneracy of the genetic code, considerable sequence variation is possible among the nucleotide sequences of the present invention. Degenerate codons encompassing all possible codons for a given amino acid are set forth

below:

Amino Acid	Codons	Degenerate Codon
Cys	TGC TGT	TGY
Ser	AGC AGT TCA TCC TCG TCT	WSN
Thr	ACA ACC ACG ACT	ACN
Pro	CCA CCC CCG CCT	CCN
Ala	GCA GCC GCG GCT	GCN
Gly	GGA GGC GGG GGT	GGN
Asn	AAC AAT	AAY
Asp	GAC GAT	GAY
Glu	GAA GAG	GAR
Gln	CAA CAG	CAR
His	CAC CAT	CAY
Arg	AGA AGG CGA CGC CGG CGT	MGN
Lys	AAA AAG	AAR
Met	ATG	ATG
Ile	ATA ATC ATT	ATH
Leu	CTA CTC CTG CTT TTA TTG	YTN
Val	GTA GTC GTG GTT	GTN
Phe	TTC TTT	TTY
Tyr	TAC TAT	TAY
Trp	TGG	TGG
Ter	TAA TAG TGA	TRR
Asn/ Asp		RAY
Glu/ Gln		SAR
Any		NNN

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding each amino acid. For example, some polynucleotides encompassed by the degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequences of the present invention.

A “variant” nucleic acid sequence has substantial sequence homology or substantial sequence identity to a reference nucleic acid sequence (or a fragment thereof). A nucleic acid sequence or fragment thereof is “substantially homologous” (or “substantially identical”) to a reference sequence if, when optimally aligned (with appropriate nucleotide insertions or deletions) with the other nucleic acid (or its complementary strand), there is nucleotide sequence identity in at least about 80, 82, 84, 86, 88, 90, 92, 94, 96, 98 or 99% of the nucleotide bases.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences may be compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequent coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percentage sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Alternatively, a nucleic acid sequence is substantially homologous (or substantially similar) to a corresponding naturally-occurring sequence when the two molecules are capable of hybridizing under selective hybridization conditions. Selectivity of hybridization exists when hybridization occurs which is substantially more selective than total lack of specificity. Typically, selective hybridization will occur when there is at least 80% homology over a stretch of at least about 300 nucleotides, such as at least about 80, 82, 84, 86, 88, 90, 92, 94, 96, 98 or 99% homology. The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will often be over a stretch of at least about 400, 500 or 600 nucleotides or more, for example over at least about 700, 800, 900 or 1000 or more nucleotides.

Nucleic acid hybridization will be affected by such conditions as salt concentration

(eg. NaCl), temperature, or organic solvents, in addition to the base composition, length of the complementary strands, and the number of nucleotide base mismatches between the hybridizing nucleic acids, as will be readily appreciated by those skilled in the art. Stringent hybridisation conditions are preferably employed, and generally include temperatures in excess of 30°C, typically in excess of 37°C and preferably in excess of 45°C. Stringent salt conditions will ordinarily be less than 1000 mM, typically less than 500 mM, and preferably less than 200 mM. The pH is typically between 7.0 and 8.3. The combination of parameters is much more important than the measure of any single parameter.

A "fragment" of a polynucleotide comprises a series of consecutive amino acid residues from the sequence of said full-length polynucleotide. By way of example, a "fragment" of a polynucleotide may comprise (or consist of) at least 300 consecutive nucleic acid residues from the sequence of said polynucleotide (eg. at least 350, 400, 450, 500, 550, 600, 650, 700, 750, 800 850, 900, 950 or 1000 consecutive nucleic acid residues of said polynucleotide). A fragment may include at least one antigenic determinant and/ or may encode at least one antigenic epitope of the corresponding polypeptide of interest.

Aspects and embodiments of the invention are illustrated by the Examples provided below, and with reference to the following Figures, in which:

Figure 1 shows a map of bacteriophage Nlac1. The two copies of the duplicated structural gene (Nlac1_024 and Nlac1_025) are shown as double-width arrowheads. Nlac1 is represented in excised form for ease of illustration: the chromosomally integrated prophage is linearised by homologous recombination between attP and an identical 20 bp element in the *N. lactamica* chromosome.

Figure 2 shows a map of bacteriophage Nlac1, with porA and Cm^R integrated into Nlac1_024.

Figure 3 shows a map of Nlac1_024, showing the positions of two engineered restriction sites, BamHI (G^GATC_C) and HindIII (A^AGCT_T) – the altered nucleotides are shown in capitals. The nucleotide numbering is from 5' end of attP (the numbering in parentheses is for the identical gene Nlac1_025). Nlac1_024 and Nlac1_025 are on the negative strand. Note that Figure 3 is reverse-complemented relative to Figure 1.

Figure 4 shows an alignment of the sequences of repeats r601 and r602 of the Nlac1 prophage. The first and last codons of Nlac1_024 (within r601) and Nlac1_025 (within r602) are marked by their amino acid in bold. The three nucleotide sequence differences between repeat r601 and repeat r602 are shown as white text in black boxes. Owing to the overlap between r601 and r602, the 39 nucleotides at the 5′ end of r601 are also the 39 nucleotides at the 3′ end of r602. Figure 4 is reverse-complemented relative to Figure 1.

Figure 5 shows flow-cytometric measurement of PorA surface-expression levels in four secondary recombinant clones of *N. lactamica* (each obtained by transformation with chromosomal DNA from a primary Nlac1_024-targeted recombinant clone). Data from *N. meningitidis* MC58 and from a ΔporA::KmR derivative of *N. meningitidis* H44/76 are included as positive and negative controls respectively.

Figure 6 shows SDS-PAGE (left) and western blot (right) of OMVs from *N. meningitidis* MC58, porA+ engineered *N. lactamica* Y92-1009 and wild-type *N. lactamica*. The gel was stained with colloidal Coomassie Blue. The blot was probed with monoclonal anti-PorA P1.7, and visualised using an alkaline phosphatase-conjugated second antibody and AP New Magenta.

KEY TO SEQ ID NOs:

SEQ ID NO: 1	r601 polynucleotide sequence
SEQ ID NO: 2	r602 polynucleotide sequence
SEQ ID NO: 3	Nlac1_024 polynucleotide sequence (= 100% identical to Nlac1_025 polynucleotide sequence)
SEQ ID NO: 4	modified r601 with a BamHI and a HindIII site arising from single-nucleotide substitutions at positions 555 and 602
SEQ ID NO: 5	coding sequence of transferrin binding protein A from *Neisseria meningitidis* K454
SEQ ID NO: 6	coding sequence of Factor H binding protein from *Neisseria meningitidis* MC58
SEQ ID NO: 7	coding sequence of NadA (NMB1994) from *Neisseria meningitidis* MC58
SEQ ID NO: 8	coding sequence of CU-Zn-superoxide dismutase (NMB1398) from *Neisseria meningitidis* MC58
SEQ ID NO: 9	coding sequence of Neisserial surface protein A (NspA) (NMB0663) from *Neisseria meningitidis* MC58
SEQ ID NO: 10	coding sequence of PorA (NMB1429) from *Neisseria meningitidis* MC58

SEQ ID NO: 11 coding sequence of PorB (NMB2039) from *Neisseria meningitidis* MC58

SEQ ID NO: 12 Neisserial Uptake Sequence

EXAMPLES

Example 1: Modification of the r601 element for use in recombination cassettes

The 1057 bp r601 element (SEQ ID NO: 1) was amplified by PCR and modified to contain two unique restriction sites, BamHI and HindIII, 45 base pairs apart, by means of the single-nucleotide substitutions listed in Table 1 and illustrated in Figure 3.

Table 1:

Enzyme	Recognition site (substitution in capitals)	Nucleotide position affected by substitution		
		In SEQ ID NO: 1 (r601)	Equivalent position in SEQ ID NO: 2 (r602)	In SEQ ID NO: 3 (Nlac_024 and Nlac_025)
BamHI	gGatcc	T**555**→G	T**555**→G	T**197**→G
HindIII	aagCtt	G**602**→C	G**602**→C	G**224**→C

The modified r601 element was propagated as an insert in an *E. coli* plasmid.

Example 2: Incorporation of porA and Cm^R genes into *N. lactamica*

The Cm^R (chloramphenicol resistance) and porA (42 kDa porin) genes, from

pACYC184 and *N. meningitidis* MC58 respectively, were cloned into the BamHI and HindIII sites in the plasmid-borne modified r601 element.

The resulting recombination cassette was amplified from the plasmid using the same primer-pair that was previously used to amplify r601 from *N. lactamica*.

The linear cassette was transformed into *N. lactamica* (Cm^S $porA^-$) and recombinant colonies were obtained by selection on TSA-yeast agar containing 0·5 μg ml^{-1} chloramphenicol.

Expression of PorA by the recombinant *N. lactamica* was verified by Western blotting and flow-cytometry.

Example 3: secondary transfer of *por*A and CmR genes from the primary recombinant *N. lactamica* into wild-type *N. lactamica*

Chromosomal DNA extracted from the initial Cm^R *por*A^+ recombinant *N. lactamica* was used to re-transform wild-type cells of the same species using techniques originally developed for the "naturally transformable" Neisseriae (*N. meningitidis* and *N. gonorrhoeae*).

The higher efficiency transformation afforded by DNA with the correct methylation pattern resulted in multiple clones differing in their PorA expression levels (Figure 3). SDS-PAGE and Western-blotting data for one of these clone is shown in Figure 4. Flow cytometry shows that PorA is correctly translocated to the surface of recombinant *N. lactamica*.

Recombinant *N. lactamica* strains obtained this way (secondary transformation following an initial targeting of Nlac1_024/Nlac1_025) do not necessarily have

Δ*Nlac1_024*::*foreign gene* (or Δ*Nlac1_025*::*foreign gene*) genotypes. Properly methylated DNA may integrate into other loci.

Example 4: Preparation of vaccine containing killed whole cells

Transformed *N. lactamica* strain Y92-1009 (prepared according to Example 2 or 3 above) is grown in Mueller Hinton broth (MHB) containing 5μgml^{-1} ethylenediamine-N, N'bis(2-hydroxyphenylacetic acid) (EDDHA), and incubated at 37°C with shaking (140rpm) for approximately 6h.

Bacteria are then harvested by centrifugation and resuspended in phosphate buffered saline (PBS) containing 1% (v/v) formaldehyde and 0.1% (w/v) thiomersal, and left to stand overnight at 2-8°C. Killed cells are then resuspended in PBS to an OD_{650} of 1.0 (equivalent to 2 x 10^9 CFUml^{-1}) and alhydrogel is added to 25% (V/V), yielding a product suitable for subcutaneous administration.

This method is suitable also for *N. cinerea, N. elongata, N. flavescens, N. polysaccharea, N. sicca* and *N. subflava*.

Example 5: Preparation of vaccine containing *N. lactamica* outer membrane (OM) preparations

Transformed *N. lactamica* strain Y92-1009 (prepared as per Example 2 or 3) is grown in MHB with and without the addition of 5μgml^{-1} EDDHA overnight at 37°C with shaking. Iron limited (with EDDHA) and iron replete cells were then treated separately. Bacteria from 1.5 litres are harvested by centrifugation and resuspended in 20ml 200mM Lithium acetate, 5mM EDTA, pH 6.0 and incubated for 3h at 37°C with shaking. Bacteria are then passed 7 times through a 21 gauge needle and pelleted at 8000g for 10min.

The supernatant is recovered and membranes pelleted by centrifugation at 100,000g for 1h at 4°C. The membranes are then resuspended in 10mM HEPES, pH 7.4, containing 0.1% (v/v) 10mM PMSF, yielding OM-containing vaccinating preparations. The protein content of the OM vaccine preparations is determined using the bicinchoninic acid assay (Sigma, UK). OMs are diluted in sterile deionized water to give a protein concentration of 100μgml^{-1}. This is then mixed with an equal volume of Alhydrogel, to give a final protein concentration of 50μgml^{-1}, and emulsified thoroughly.

Alhydrogel (Superfoss, Denmark) is used for the primary dose, and for subsequent boosts.

CLAIMS:

1. A nucleic acid delivery vehicle, comprising an isolated polynucleotide sequence that is at least 300 nucleotides in length, wherein said polynucleotide sequence has at least 80% sequence identity to a nucleic acid sequence comprising at least 300 consecutive nucleotides of SEQ ID NO: 1 or 2; and wherein said polynucleotide sequence includes an insertion site into which a first nucleotide sequence may be inserted.

2. A nucleic acid delivery vehicle according to Claim 1, wherein said polynucleotide sequence has at least 80% sequence identity to a nucleic acid sequence comprising at least 300 consecutive nucleotides of SEQ ID NO: 3.

3. A nucleic acid delivery vehicle according to Claim 1, wherein said polynucleotide sequence has at least 80% sequence identity to a nucleic acid sequence comprising at least 300 consecutive nucleotides of SEQ ID NO: 4.

4. A nucleic acid delivery vehicle according to any of Claims 1-3, wherein the insertion site comprises a nucleic acid sequence that can be cleaved by a restriction endonuclease or a recombinase enzyme.

5. A plasmid comprising a nucleic acid delivery vehicle according to any previous claim.

6. A method of producing a recombination cassette for integration of a first nucleotide sequence into the genome of a commensal *Neisseria* by homologous recombination, comprising inserting said first nucleotide sequence into a nucleic acid delivery vehicle according to any of Claims 1-4, or into a plasmid according to Claim 5.

7. A recombination cassette comprising a nucleic acid delivery vehicle

according to any of Claims 1-4, and further comprising a first nucleotide sequence inserted into said delivery vehicle via said insertion site.

8. A recombination cassette according to Claim 7, wherein said recombination cassette is a linear nucleic acid molecule.

9. A recombination cassette according to Claim 7 or 8, wherein said first nucleotide sequence comprises a nucleic acid sequence that is heterologous to said commensal *Neisseria*.

10. A recombination cassette according to Claim 9, wherein said heterologous nucleic acid sequence encodes an antigenic polypeptide that is heterologous to said commensal *Neisseria*, such as a polypeptide of a pathogenic organism.

11. A recombination cassette according to Claim 9, wherein said pathogenic organism is a pathogenic *Neisseria*, such as *N. meningitidis* or *N. gonorrhoeae*.

12. A recombination cassette according to Claim 10, wherein said pathogenic *Neisseria* polypeptide is selected from the group consisting of a transferrin binding protein, a factor H binding protein (fHbp), NadA, a superoxide dismutase (for example a Cu,Zn-SOD), Neisserial surface protein A (NspA), a porin (eg. PorA or PorB), Opa, Opc or NhhA.

13. A recombination cassette according to any of Claims 7-12, further comprising a Neisserial Uptake Sequence and/ or a signal sequence.

14. A recombination cassette according to any of Claims 7-13, further comprising a reporter gene, such as an antibiotic resistance gene.

15. A method of transforming commensal *Neisseria* with a first nucleotide sequence, comprising introducing a recombination cassette according to any of

Claims 7-14 into said commensal *Neisseria*.

16. A method according to Claim 15, further comprising selecting for recombinant commensal *Neisseria* that express the first nucleotide sequence.

17. A recombinant commensal *Neisseria,* such as *Neisseria lactamica*, that has a first nucleotide sequence recombinantly integrated into the genome of said recombinant commensal *Neisseria*.

18. A recombinant commensal *Neisseria* according to Claim 17, wherein said first nucleotide sequence is stably integrated into the Nlac1 prophage in the genome of said recombinant commensal *Neisseria*.

19. A recombinant commensal *Neisseria* according to Claim 17 or 18, obtainable by a transformation method according to Claim 15 or 16.

20. A recombinant commensal *Neisseria* according to any of Claims 17-19, wherein said recombinant commensal *Neisseria* expresses a heterologous antigenic polypeptide encoded by said first nucleotide sequence.

21. A method of transforming a commensal *Neisseria* with a first nucleotide sequence, comprising introducing chromosomal DNA obtained from a recombinant commensal *Neisseria* according to any of Claims 17-20 into said commensal *Neisseria*.

22. A method of obtaining an immunogenic component or extract from a recombinant commensal *Neisseria* according to any of Claims 17-20, comprising:

(i) suspending said recombinant commensal *Neisseria* in the presence of detergent; and

(ii) incubating the suspension so as to extract an immunogenic component or extract from the recombinant commensal *Neisseria*.

23. A method of preparing an immunogenic composition, comprising combining a recombinant commensal *Neisseria* according to any of Claims 17-20 with a pharmaceutically acceptable carrier.

24. A method of preparing an immunogenic composition, comprising:

(i) obtaining an immunogenic component or extract from a recombinant commensal *Neisseria* according to any of Claims 17-20; and

(ii) combining said immunogenic component or extract with a pharmaceutically acceptable carrier.

25. An immunogenic composition, such as a vaccine, comprising a recombinant commensal *Neisseria* according to any of Claims 17-20, or an immunogenic component or extract thereof.

26. A method according to Claim 22 or 24 or an immunogenic composition according to Claim 25, wherein said immunogenic component or extract comprises outer membrane vesicles.

27. An immunogenic composition for use in stimulating an immune response in a subject, wherein said immunogenic composition is an immunogenic composition according to Claim 25.

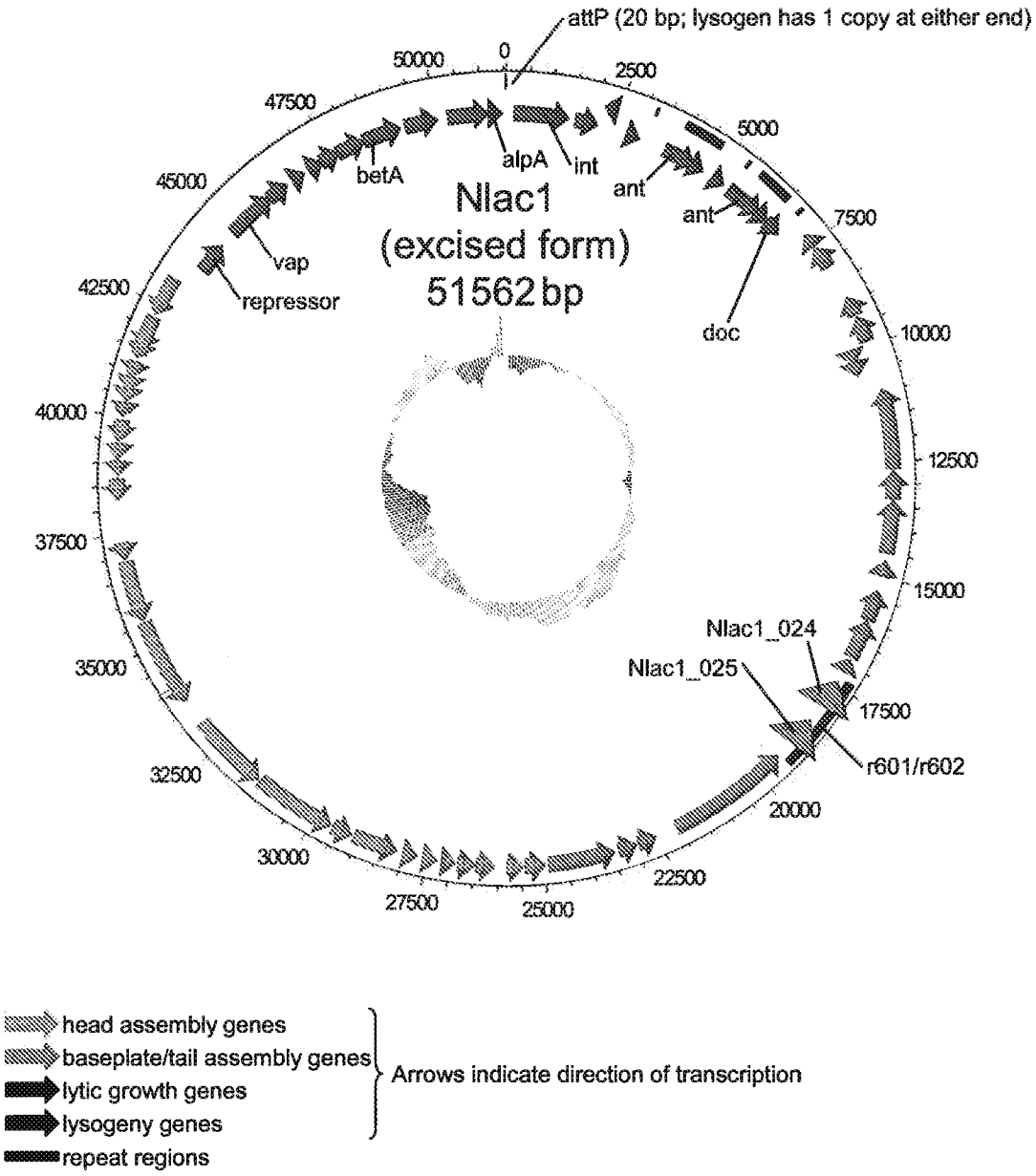
28. Use of an immunogenic composition according to Claim 25, for the manufacture of a medicament for stimulating an immune response in a subject.

29. A method of stimulating an immune response in a subject, comprising administering to the subject an immunogenic composition according to Claim 25.

30. An immunogenic composition for use according to Claim 27, a use according to Claim 28, or a method according to Claim 29, for treating or

preventing an infection in a subject, such as a Neisserial infection.

31. A nucleic acid delivery vehicle, plasmid, recombination cassette, recombinant commensal *Neisseria*, immunogenic composition, method or use as hereinbefore described and/ or as illustrated in any of the Examples and/ or Figures.


attP (20 bp; lysogen has 1 copy at either end)
0
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5000
7500
10000
12500
15000
17500
20000
22500
25000
27500
30000
32500
35000
37500
40000
42500
45000
47500
50000
Nlac1
(excised form)
51562 bp
alpA
int
ant
ant
doc
betA
vap
repressor
Nlac1_024
Nlac1_025
r601/r602
head assembly genes
baseplate/tail assembly genes
lytic growth genes
lysogeny genes
repeat regions
Arrows indicate direction of transcription

Figure 1

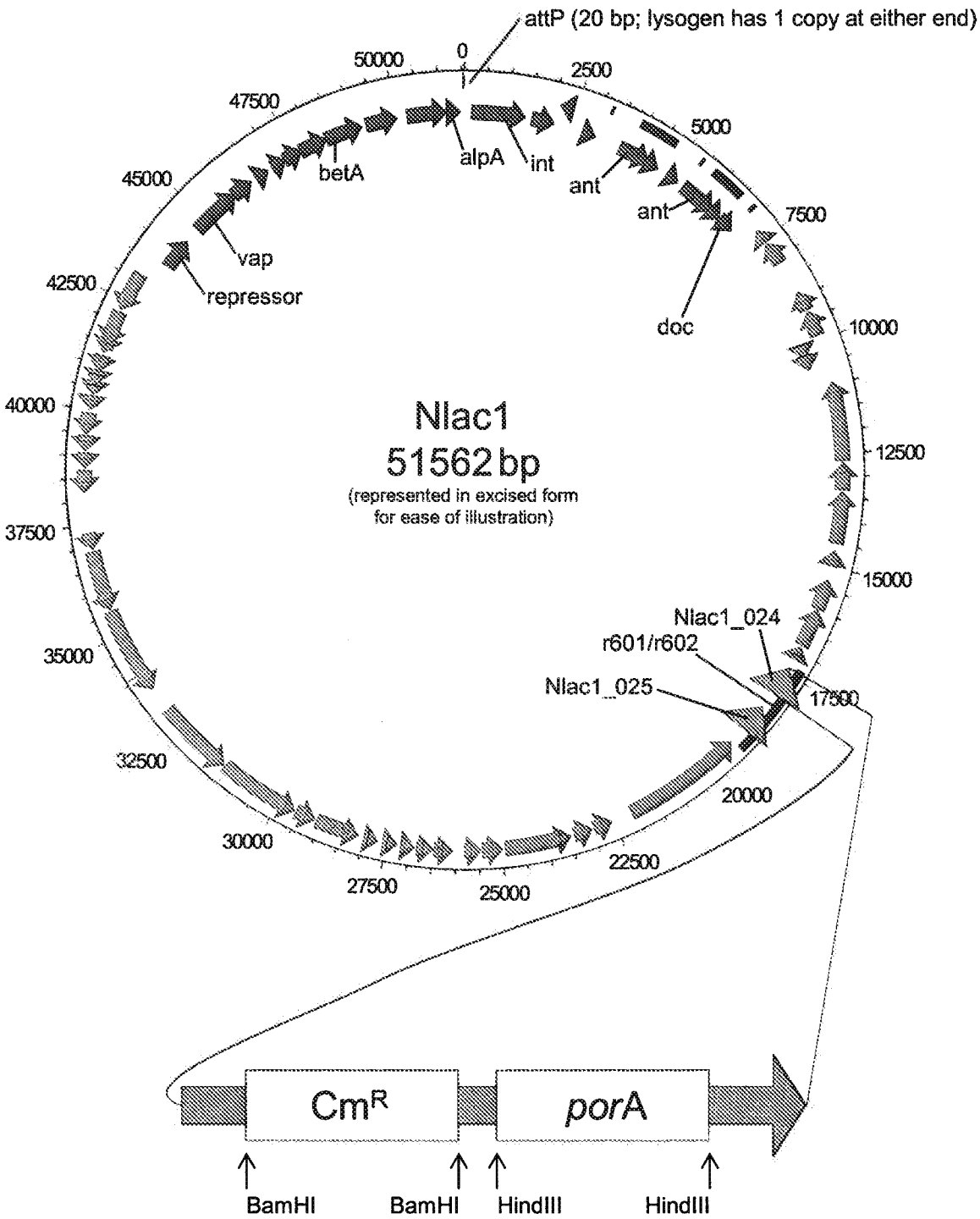
attP (20 bp; lysogen has 1 copy at either end)
0
2500
5000
7500
10000
12500
15000
17500
20000
22500
25000
27500
30000
32500
35000
37500
40000
42500
45000
47500
50000
alpA
int
ant
ant
doc
betA
vap
repressor
Nlac1
51562 bp
(represented in excised form
for ease of illustration)
Nlac1_024
r601/r602
Nlac1_025
Cm^R
porA
BamHI
BamHI
HindIII
HindIII

Figure 2

```
M  A  W  N  S  I  G  I  P  N  V  P  N  L  P  R  N  T  G  G      20
atggcttggaactcaatcggcattccgaatgtgccgaatctcccaagaaacaccggcggc 17918 (18936)

A  L  I  K  F  G  G  A  A  L  A  N  A  V  F  G  N  Y  W  G      40
gcgctgattaagttcggcggtgcggcactcgccaacgccgttttcggcaactattgggggc 17858 (18876)

I  F  G  Q  N  G  I  P  L  L  L  S  D  N  V  T  S  V  K  H      60
atcttcggacaaaacggcatcccgctgctgttgtcggacaacgtcacttcggtcaaacac 17798 (18816)

Q  N  T  S  K  V  S  N  A  P  V  E  R  G  S  F  A  S  Y  N      80
caaaacacgtccaaagtatccaatgcgcctgtcgaacgcggctcgttcgccagctacaac 17738 (18756)
               gGatcc
                    \
                    BamHI

K  V  G  D  P  F  T  V  T  V  Q  M  S  K  G  S  G  G  V  F     100
aaggttggcgacccgtttacggtaacggtgcagatgagcaagggcagcggcggcgtgttc 17678 (18696)
aagCtt
    \
   HindIII

E  R  G  A  F  L  G  L  L  D  T  L  A  N  S  T  D  L  F  L     120
gagcgcgggggcgtttctggcctgttggatacgctggccaacagcacggatctgttcttg 17618 (18636)

V  I  T  P  E  A  V  Y  P  N  M  A  I  T  G  Y  D  Y  A  R     140
gttatcacgccggaagcggtgtatccgaatatggcgattacgggctacgactacgcccgc 17558 (18576)

E  A  S  D  G  A  R  L  L  K  V  N  I  H  L  A  E  V  R  Q     160
gaggcttcagacggcgcacggctgttgaaggtcaatatccatttggccgaagtgcggcag 17498 (18516)

A  E  V  K  Y  T  K  T  K  S  E  G  A  Q  A  Q  A  D  G  G     180
gcggaagtgaaatataccaaaaccaaatccgaaggcgcacaggcacaggcagacggaggc 17438 (18456)

R  V  Q  P  K  P  I  Q  N  N  E  S  I  L  S  K  S  R  G  S     200
agggtacagcccaagccgatacaaaacaatgaatccatcttgtctaagtcgcgcggttcg 17378 (18396)

V  G  G  W  L  G  K  I  T  D  T  F  G  K  G  F  G  P  D       219
gtcggcggttggcttgggaaaataaccgatacattcggaaaggggttcgggccggat    17315 (18333)
```

Figure 3


r601 AAAA[redacted]CGAAACCCCGTGAAGCG[redacted]GCAACTTC[redacted]CGGGGTTCTGTATTTAATCCTTATGGGCAAGGAGAAAATC
r602 AAAA[redacted]TGAAACCCCGTGAAGCG[redacted]GCAACTTC[redacted]CGGGGTTCTGTATTTAATCCTTATGGGCAAGGAGAAAATC
1.....10.....20.....30.....40.....50.....60.....70.
r601 ATAAGTGGATTTTAACGTAAACACGGAGGCAAGGAAAGTGGCAGAACTGTTGGAAAAATCAAAAACACTCCG
r602 ATAAGTGGATTTTAACGTAAACACGGAGGCAAGGAAAGTGGCAGAACTGTTGGAAAAATCAAAAACACTCCG
.....80.....90.....100.....110.....120.....130.....140..
r601 CCGCCTTGCATGGGGTTTGATACTGATGACCCCGACAACGGCGCTGATATGGAAATTGGCAGATATTCTGAC
r602 CCGCCTTGCATGGGGTTTGATACTGATGACCCCGACAACGGCGCTGATATGGAAATTGGCAGATATTCTGAC
.....150.....160.....170.....180.....190.....200.....210....
r601 CGCGCTGAAATAGGCCGTCTGAAAAGCGAAACCCCGTGAGGCGCGGCGCAATTTTTGGCACTTCGTGGCAGG
r602 CGCGCTGAAATAGGCCGTCTGAAAAGCGAAACCCCGTGAGGCGCGGCGCAATTTTTGGCACTTCGTGGCAGG
...220.....230.....240.....250.....260.....270.....280.....
Met
r601 GATTTGTCTGATCATCACGGTATGGAAAATATTTACCGTGATCGAACTGCTTGTGAAATAAAGGTTTGTTAT
r602 GATTTGTCTGATCATCACGGTATGGAAAATATTTACCGTGATCGAACTGCTTGTGAAATAAAGGTTTGTTAT
.290.....300.....310.....320.....330.....340.....350.....
r601 GGCTTGGAACTCAATCGGCATTCCGAATGTGCCGAATCTCCCAAGAAACACCGGCGGCGCGCTGATTAAGTT
r602 GGCTTGGAACTCAATCGGCATTCCGAATGTGCCGAATCTCCCAAGAAACACCGGCGGCGCGCTGATTAAGTT
361.....370.....380.....390.....400.....410.....420.....430
r601 CGGCGGTGCGGCACTCGCCAACGCCGTTTTCGGCAACTATTGGGGCATCTTCGGACAAAACGGCATCCCGCT
r602 CGGCGGTGCGGCACTCGCCAACGCCGTTTTCGGCAACTATTGGGGCATCTTCGGACAAAACGGCATCCCGCT
.....440.....450.....460.....470.....480.....490.....500..
r601 GCTGTTGTCGGACAACGTCACTTCGGTCAAACACCAAAACACGTCCAAAGTATCCAATGCGCCTGTCGAACG
r602 GCTGTTGTCGGACAACGTCACTTCGGTCAAACACCAAAACACGTCCAAAGTATCCAATGCGCCTGTCGAACG
.....510.....520.....530.....540.....550.....560.....570....
r601 CGGCTCGTTCGCCAGCTACAACAAGGTTGGCGACCCGTTTACGGTAACGGTGCAGATGAGCAAGGGCAGCGG
r602 CGGCTCGTTCGCCAGCTACAACAAGGTTGGCGACCCGTTTACGGTAACGGTGCAGATGAGCAAGGGCAGCGG
...580.....590.....600.....610.....620.....630.....640.....
r601 CGGCGTGTTCGAGCGCGGGGCGTTTCTCGGCCTGTTGGATACGCTGGCCAACAGCACGGATCTGTTCTTGGT
r602 CGGCGTGTTCGAGCGCGGGGCGTTTCTCGGCCTGTTGGATACGCTGGCCAACAGCACGGATCTGTTCTTGGT
.650.....660.....670.....680.....690.....700.....710.....
r601 TATCACGCCGGAAGCGGTGTATCCGAATATGGCGATTACGGCTACGACTACGCCCGCGAGGCTTCAGACGG
r602 TATCACGCCGGAAGCGGTGTATCCGAATATGGCGATTACGGCTACGACTACGCCCGCGAGGCTTCAGACGG
721.....730.....740.....750.....760.....770.....780.....790
r601 CGCACGGCTGTTGAAGGTCAATATCCATTTGGCCGAAGTGCGGCAGGCGGAAGTGAAATATACCAAAACCAA
r602 CGCACGGCTGTTGAAGGTCAATATCCATTTGGCCGAAGTGCGGCAGGCGGAAGTGAAATATACCAAAACCAA
.....800.....810.....820.....830.....840.....850.....860..
r601 ATCCGAAGGCGCACAGGCACAGGCAGACGGAGGCAGGGTACAGCCCAAGCCGATACAAAACAATGAATCCAT
r602 ATCCGAAGGCGCACAGGCACAGGCAGACGGAGGCAGGGTACAGCCCAAGCCGATACAAAACAATGAATCCAT
.....870.....880.....890.....900.....910.....920.....930....
r601 CTTGTCTAAGTCGCGCGGTTCGGTCGGCGGTTGGCTTGGGAAAATAACCGATACATTCGGAAAGGGGTTCGG
r602 CTTGTCTAAGTCGCGCGGTTCGGTCGGCGGTTGGCTTGGGAAAATAACCGATACATTCGGAAAGGGGTTCGG
...940.....950.....960.....970.....980.....990.....1000.....
Glu***
r601 GCCGGATTAAAAAATCGAAACCCCGTGAAGCGGGCAACTTCACGGGGTT
r602 GCCGGATTAAAAAATCGAAACCCCGTGAAGCGGGCAACTTCACGGGGTT
.1010.....1020.....1030.....1040.....1050....

Figure 4

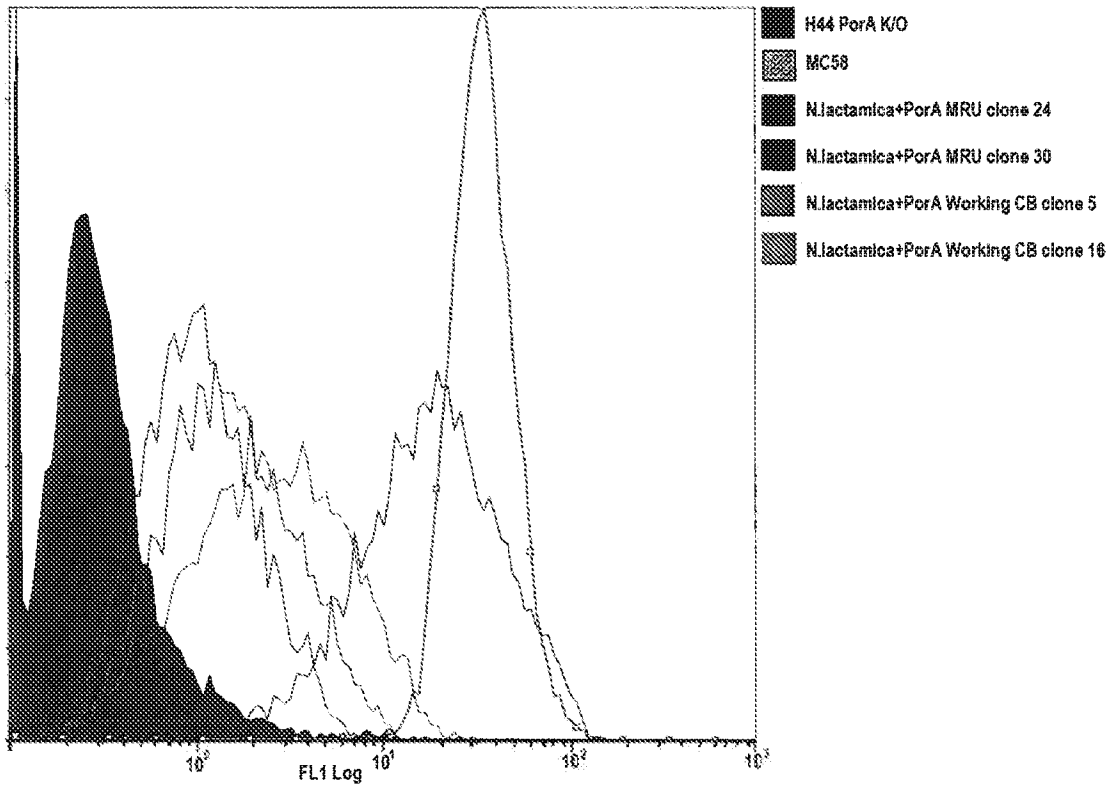
H44 PorA K/O
MC58
N.lactamica+PorA MRU clone 24
N.lactamica+PorA MRU clone 30
N.lactamica+PorA Working CB clone 5
N.lactamica+PorA Working CB clone 16
FL1 Log

Figure 5

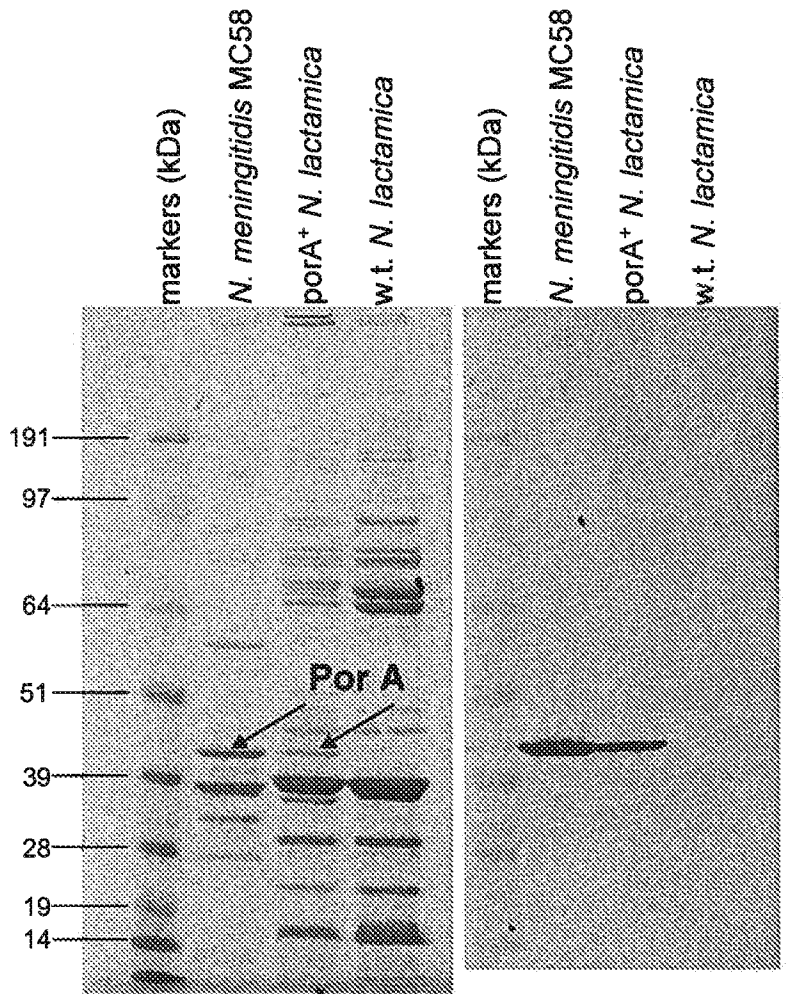
markers (kDa)
N. meningitidis MC58
porA+ N. lactamica
w.t. N. lactamica
markers (kDa)
N. meningitidis MC58
porA+ N. lactamica
w.t. N. lactamica
191
97
64
51
39
28
19
14
Por A

Figure 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2010/052179

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/74 C12R1/36 C07K16/12 A61K39/095
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12R C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HOKE C ET AL: "TAXONOMY OF THE NEISSERIAE DNA BASE COMPOSITION INTERSPECIFIC TRANSFORMATION AND DNA HYBRIDIZATION", INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY, vol. 32, no. 1, 1982, pages 57-66, XP002623611, ISSN: 0020-7713	17-26,31
Y	the whole document -----	21-31
Y	WO 00/50074 A2 (MICROBIOLOGICAL RES AUTHORITY [GB]; IMP COLLEGE SCHOOL OF SCIENCE [GB]) 31 August 2000 (2000-08-31) cited in the application the whole document -----	21-31
	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 February 2011	11/03/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Madruga, Jaime

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ROBERTS M C ET AL: "TRANSFER OF BETA LACTAMASE PLASMIDS FROM NEISSERIA-GONORRHOEAE TO NEISSERIA-MENINGITIDIS AND COMMENSAL NEISSERIA SPECIES BY THE 25.2-MEGADALTON CONJUGATIVE PLASMID", ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 32, no. 9, 1988, pages 1430-1432, XP002623612, ISSN: 0066-4804 the whole document	21-31
A	O'DWYER C A ET AL: "EXPRESSION OF HETEROLOGOUS ANTIGENS IN COMMENSAL NEISSERIA SPP.: PRESERVATION OF CONFORMATIONAL EPITOPES WITH VACCINE POTENTIAL", INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, US, vol. 72, no. 11, 1 November 2004 (2004-11-01), pages 6511-6518, XP008044838, ISSN: 0019-9567, DOI: DOI:10.1128/IAI.72.11.6511-6518.2004 cited in the application the whole document	1-31
A	PIEKAROWICZ ANDRZEJ ET AL: "Characterization of the dsDNA prophage sequences in the genome of Neisseria gonorrhoeae and visualization of productive bacteriophage", BMC MICROBIOLOGY, BIOMED CENTRAL, LONDON, GB, vol. 7, no. 1, 5 July 2007 (2007-07-05), page 66, XP021028186, ISSN: 1471-2180, DOI: DOI:10.1186/1471-2180-7-66 the whole document	1-31
A	QVARNSTROM YVONNE ET AL: "Variations in gene organization and DNA uptake signal sequence in the folP region between commensal and pathogenic Neisseria species", BMC MICROBIOLOGY, vol. 6, February 2006 (2006-02), XP002623613, ISSN: 1471-2180 the whole document	1-31

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2010/052179

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DUNNING HOTOPP J C ET AL: "Comparative genomics of Neisseria meningitidis: Core genome, islands of horizontal transfer and pathogen-specific genes", MICROBIOLOGY 200612 GB LNKD-DOI:10.1099/MIC.0.29261-0, vol. 152, no. 12, December 2006 (2006-12), pages 3733-3749, XP002623614, ISSN: 1350-0872 the whole document ----	1-31
A	WO 2008/102173 A1 (HEALTH PROT AGENCY [GB]; SERUM INST OF INDIA LTD [IN]; KAPRE SUBHASH V) 28 August 2008 (2008-08-28) the whole document ----	1-31

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2010/052179

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