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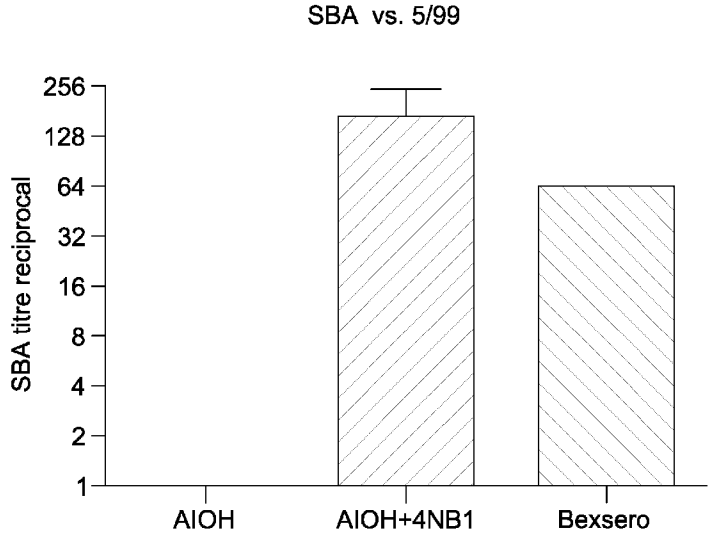

Figure 49

(57) Abstract: The invention relates to a modified *Neisseria lactamica,* wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein; a method of prophylactic treatment for pathogenic infection of a subject comprising nasopharyngeal inoculation of a modified *Neisseria lactamica*; a method of reducing or preventing meningococcal colonisation of a subject; a method of modifying the microbiome of a subject; a wild-type *Neisseria lactamica* for use for the prophylactic treatment of meningococcal infection of a subject or reducing colonisation of a subject, wherein the prophylactic treatment comprises nasopharyngeal inoculation of the wild- type *Neisseria lactamica*; associated nucleic acid for mutagenesis of *Neisseria lactamica;* methods of mutagenesis; outer membrane vesicle (OMV) vaccines; and associated compositions and methods thereof.

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Meningococcal infection and modified *Neisseria lactamica*

The present invention relates to a modified *Neisseria lactamica*, methods of prophylactic treatment with modified *Neisseria lactamica*, methods of mutagenesis of *Neisseria lactamica*, a series of cloning vectors for modifying *Neisseria lactamica*, and modified *Neisseria lactamica*-generated outer membrane vesicles (OMVs).

Asymptomatic oropharyngeal carriage of *Neisseria meningitidis* is common in many communities and in high income counties the highest rates are seen in young adults, particularly in semi closed communities with active social mixing such as educational facilities. In contrast, invasive meningococcal disease, in the form of septicaemia and/or meningitis, has an incidence of <1 per 100,000 persons per annum in most high income countries. In populations vaccinated with glycoconjugate vaccines, disease incidence of the vaccine serogroups is dramatically reduced due to herd protection conferred by reduced carriage and transmission. A large proportion of current meningococcal carriage and disease is due to bacteria expressing serogroup B capsule, for which no glycoconjugate vaccines exist. A new sub-capsular vaccine 4CMenB induces bactericidal antibodies against a range of strains, but the effect on carriage of serogroup B *N. meningitidis* is relatively modest. Reduction of carriage of *N. meningitidis* will be a key strategy in future control of meningococcal disease. A number of studies indicate that carriage of the related but non-invasive *Neisseria lactamica* may confer natural herd protection of infants. Age-specific rates of *N. meningitidis* carriage and disease were inversely proportional to carriage of *N. lactamica* and a modelling study has suggested a mean 4.7 year delay in meningococcal carriage following carriage of *N. lactamica.* The mechanism of this relationship is undetermined, but is unlikely to be due to cross-protective antibody production, as the early years of life that are associated with high rates of *N.lactamica* carriage pre-date considerably the development of natural bactericidal antibodies to *N. meningitidis*. Furthermore, in a controlled infection study, intranasal inoculation of young adults with live *N. lactamica* was followed by development of humoral immunity to *N. lactamica* in those who carried the inoculated strain, but this did not induce significant cross reactive antibodies against *N. meningitidis*.

Outer Membrane Vesicles (OMV) are generated during the growth of the Gram negative bacterium, *Neisseria lactamica* and have been demonstrated to induce immunity to protein antigens following administration to humans. The components of OMV-based vaccines are naturally packaged in a manner that promotes a strong immune response, and have been successfully trialled for use in vaccination against meningococcal disease. The problem with OMV-based vaccines is that the immunity they elicit is constrained by the genetic repertoire of the bacterium from which the OMV is derived, so cross-protective immunity generated in response to exposure to OMV-based vaccine is only against immunogenic antigens that are highly similar in both *Neisseria lactamica* and the closely-related bacterium *Neisseria meningitidis* (the meningococcus). Comparative genomic analyses have repeatedly demonstrated there a large number of important, immunogenic virulence determinants coded for in the meningococcal genome that are absent in the *N. lactamica* genome, meaning that vaccines based on wild type *N. lactamica* OMV would require supplementation with exogenously-derived meningococcal antigens in order to provoke cross-protective immunity to *Neisseria meningitidis* (e.g. a situation similar to 4CMenB vaccine – aka Bexsero™, in which two exogenously-derived meningococcal outer membrane protein conjugates are supplemented into a meningococcal-derived OMV preparation).

In order to modify the outer membrane components of *Neisseria lactamica,* and to have them presented in a biologically and immunologically relevant ('native') orientation in the membrane, it is preferential to introduce genetic material into the genome of *Neisseria lactamica.* This material would contain genes that encode the desired protein components, under the control of either constitutive or inducible gene promoters depending on the relevant application. The transcriptional and translational machinery of the bacterium would then produce the desired antigen and direct it to the membrane. However, despite being a naturally competent bacterial species, which constitutively takes up exogenous DNA from the environment, *Neisseria lactamica* has proven to be refractory to targeted mutagenesis or directed genetic change. As such, there are currently no molecular systems for the manipulation of the *Neisseria lactamica* genome.

An aim of the invention is to improve, or at least provide alternatives to, current methods of treatment or prevention of meningococcal infection and to provide modified *Neisseria lactamica* having enhanced properties for use in such methods.

Invention Summary

According to a first aspect of the invention, there is provided a modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

According to another aspect of the invention, there is provided a method of prophylactic treatment for meningococcal infection of a subject comprising nasopharyngeal inoculation of a modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

According to another aspect of the invention, there is provided a method of reducing or preventing meningococcal colonisation of a subject comprising nasopharyngeal inoculation of a modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

According to another aspect of the invention, there is provided a method of modifying the microbiome of a subject comprising nasopharyngeal inoculation of a modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

According to another aspect of the invention, there is provided a method of modifying the microbiome of a subject comprising nasopharyngeal inoculation of wild-type *Neisseria lactamica*.

According to another aspect of the invention, there is provided a method of preventing meningococcal colonisation of a subject comprising nasopharyngeal inoculation of a wild-type *Neisseria lactamica*.

According to another aspect of the invention, there is provided a method of prophylactic treatment for pathogenic infection of a subject comprising nasopharyngeal inoculation of a modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

According to another aspect of the invention, there is provided a modified *Neisseria lactamica* for use for the prophylactic treatment of meningococcal infection of a subject, wherein the prophylactic treatment comprises nasopharyngeal inoculation of the modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

According to another aspect of the invention, there is provided a wild-type *Neisseria lactamica* for use for the prophylactic treatment of meningococcal infection of a subject, wherein the prophylactic treatment comprises nasopharyngeal inoculation of the *Neisseria lactamica*.

According to another aspect of the invention, there is provided a modified *Neisseria lactamica* for use for reducing or preventing colonisation of *Neisseria meningitidis* in a subject, the use comprising nasopharyngeal inoculation of the modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

According to another aspect of the invention, there is provided a cloning vector for modification of *Neisseria lactamica* comprising one or more Heterologous Antigen Expression Cassettes (HAEC), wherein the HAEC comprises:

-a heterologous nucleic acid sequence insertion site;

-a first promoter, upstream of the heterologous nucleic acid sequence;

-a second promoter, downstream of the heterologous nucleic acid sequence; and

-a selection marker downstream of the second promoter;

wherein the HAEC is flanked by a sequence homologous to a region of *Neisseria lactamica* chromosome.

According to another aspect of the invention, there is provided a cloning vector for modification of *Neisseria lactamica* comprising one or more *Neisseria* Heterologous Antigen Expression Cassettes (HAEC), wherein the HAEC comprises:

-a heterologous nucleic acid sequence insertion site;

-a selection marker; and

-a promoter upstream of the heterologous nucleic acid sequence insertion site and selection marker;

wherein the HAEC is flanked by a sequence homologous to a region of *Neisseria lactamica* chromosome.

According to another aspect of the invention, there is provided a cloning vector for mutagenesis of *Neisseria lactamica* comprising one or more Heterologous Antigen Expression Cassettes (HAEC), wherein the HAEC comprises:

-a heterologous nucleic acid sequence;

-a first promoter upstream of the heterologous nucleic acid sequence;

-a second promoter downstream of the heterologous nucleic acid sequence; and

-a selection marker downstream of the second promoter;

wherein the HAEC is flanked by a sequence homologous to a region of *Neisseria lactamica* chromosome.

According to another aspect of the invention, there is provided a cloning vector for mutagenesis of *Neisseria lactamica* comprising one or more Heterologous Antigen Expression Cassettes (HAEC), wherein the HAEC comprises:

-a heterologous nucleic acid sequence;

-a selection marker; and

-a promoter upstream of the heterologous nucleic acid sequence and selection marker;

wherein the HAEC is flanked by a sequence homologous to a region of *Neisseria lactamica* chromosome.

According to another aspect of the invention, there is provided a nucleic acid for mutagenesis of *Neisseria lactamica* comprising one or more Heterologous Antigen Expression Cassettes (HAEC), wherein the HAEC comprises:

-a heterologous nucleic acid sequence;

-a first promoter upstream of the heterologous nucleic acid sequence;

-a second promoter downstream of the heterologous nucleic acid sequence; and

-a selection marker downstream of the second promoter;

wherein the HAEC is flanked by a sequence homologous to a region of *Neisseria lactamica* chromosome.

According to another aspect of the invention, there is provided a nucleic acid for mutagenesis of *Neisseria lactamica* comprising one or more Heterologous Antigen Expression Cassettes (HAEC), wherein the HAEC comprises:

-a heterologous nucleic acid sequence;

-a selection marker; and

-a promoter upstream of the heterologous nucleic acid sequence and selection marker;

wherein the HAEC is flanked by a sequence homologous to a region of *Neisseria lactamica* genome.

According to another aspect of the invention, there is provided a method of mutagenesis of *Neisseria lactamica* comprising transformation of *Neisseria lactamica* with the cloning vector according the invention herein.

According to another aspect of the invention, there is provided a method of mutagenesis of *Neisseria lactamica* comprising transformation of *Neisseria lactamica* with hypermethylated nucleic acid.

According to another aspect of the invention, there is provided an outer membrane vesicle (OMV) vaccine, wherein the OMV is an OMV of the modified *Neisseria lactamica* described herein.

According to another aspect of the invention, there is provided a composition comprising the OMV according to the invention herein; or the modified *Neisseria lactamica* according to the invention herein.

According to another aspect of the invention, there is provided an OMV; modified *Neisseria lactamica*; or composition according to the invention herein for use in a vaccine/vaccination.

According to another aspect of the invention, there is provided a method of vaccination of a subject for the prevention of infection or colonisation of a pathogen comprising the administration of the OMV or the modified *Neisseria lactamica* or the composition according to the invention herein.

This invention describes a process to circumvent the barriers to transformation of *Neisseria lactamica* and allow targeted genetic modification of this organism to be performed for the first time. The invention allows stable integration of DNA constructs into loci of the *Neisseria lactamica* chromosome, with utility for both deletion of existing genes (i.e. targeted mutagenesis) or insertion of genes from other biotic sources.

Detailed Description

It is understood that reference to protein or nucleic acid "variants", it is understood to mean a protein or nucleic acid sequence that has at least 70%, 80%, 90%, 95%, 98%,

99%, 99.9% identity with the sequence of the fore mentioned protein or nucleic acid. The percentage identity may be calculated under standard NCBI blast p/n alignment parameters. "Variants" may also include truncations of a protein or nucleic acid sequence. The skilled person will understand that various truncations of a protein can still provide a protein which retains its function and/or antigenic activity.

The term "colonisation" is defined as the synthesis of multiple cellular and subcellular processes leading to the retention of a given living organism in close association within, upon or beneath host cells or extracellular matrix. An organism, colonising a surface within a human or animal host, may or may not cause disease, and may be recoverable from that surface by various detection techniques, which would verify the fact that colonization has occurred. Colonisation can be induced by inoculating an organism into the human, so that it lives on a given surface within the body.

The term "heterologous protein" or "heterologous DNA" or " heterologous nucleic acid sequence" is understood to mean that the protein or sequence is derived from a different species or strain relative to the organism, e.g. it is not homologous. In the context of the invention, heterologous may be used interchangeably with "exogenous".

The term "exogenous" refers to any nucleic acid or protein that originates outside of the organism of concern. The term "endogenous" refers to nucleic acid or protein that is originating or produced within the same organism.

The term "homologous DNA" or "homologous nucleic acid sequence" is understood to mean that the sequence is derived from or is of the same structure as the DNA/nucleic acid sequence found in the species or strain of concern.

The term "recombinant DNA" refers to DNA molecules formed by laboratory methods of genetic recombination (such as molecular cloning) to bring together genetic material, perhaps from multiple sources, creating sequences that would not otherwise be found in the organism.

The term "infection", in particular "meningococcal infection" may refer both to the invasive disease state, wherein the meningococcus has penetrated into the blood stream from a colonized mucosal surface and is actively growing within the host, inducing sepsis; and the benign carriage of the meningococcus in the nasopharynx with no adverse effects.

The term "meningococcal colonisation" may refer to benign carriage of the meningococcus in the nasopharynx with no adverse effects.

The term "cassette" may refer to a genetic element that may contain a selection of genetic features such as a gene, a gene insert site, a recombination site, restriction sites, selection markers, promoters and enhancers.

The term "selection marker" is intended to cover a "screening marker" and the terms may be used interchangeably herein. The selection marker is a genetic element, such as an antibiotic resistance gene, that can be used to identify cells carry and expressing the selection marker.

In some of the descriptions herein, the term 'phase variation' will be used to refer to a variety of genetic mechanisms that cause a subset of bacterial cells from a given Neisserial population to silence expression of a narrow range of genes, whilst retaining the nucleotide sequence of the appropriate gene in the chromosome. Phase variation is a stochastic process, driven by a range of molecular mechanisms, and takes place at the level of individual bacteria, such that given populations of bacteria are phenotypically heterologous for a "phase variable" gene product.

In some of the descriptions herein, the term 'construct' may be used in reference to the plasmid/cloning vector under discussion. The term 'cassette' may be used in reference to the part of the construct intended for uptake and assimilation into the genome of *Neisseria lactamica*.

In some of the descriptions herein, the term '**DUS**' may be used to refer to the canonical Neisserial DNA Uptake Sequence (5' – GCCGTCTGAA – 3') (SEQ ID NO: 1) and '**AT-DUS**' may be used to refer to the canonical AT-flanked Neisserial DNA Uptake Sequence (5' – ATGCCGTCTGAA – 3') (SEQ ID NO: 2). Where the inverse (i.e. the reverse complement) of either sequence is present and noted as part of any construct (i.e. the canonical version of either element is present in the complementary DNA strand to that containing the coding sequences of the appropriate genes), the prefix '**inv-**' may precede the appropriate acronym. In other aspects of the invention, other forms of the Neisserial DNA Uptake Sequences could be substituted (though they would work at reduced efficiency).

In some of the descriptions herein, the term '**NHCIS(X)**' may be used to refer to "*Neisseria* Heterologous Construct Insertion Site X", where X represents a number, arbitrarily assigned to an appropriate chromosomal locus in the order of development. NHCIS are areas of relative gene paucity in the *N. lactamica* chromosome, to where exogenous genetic material can be targeted for integration into the genome with minimal disruption to other open reading frames (hereafter, ORF). It should be noted that although there are multiple potential NHCIS loci (based upon data on the size of *N. lactamica* intergenic gaps), the transformation procedure described herein, and detailed in **PROTOCOL A** allows for *any* locus to be targeted for chromosomal integration of Cassettes.

In some of the descriptions herein, the term '**HAEC(Y)**' may be used to refer to "Heterologous Antigen Expression Construct Y", where Y represents a number, arbitrarily assigned to the HAEC sequence in the order of development. HAEC are tandemly-linked promoter sequences that drive gene expression in *N. lactamica* (but are not necessarily of *N. lactamica* derivation), flanked and punctuated by restriction sites for ease of downstream cloning. The skilled person will understand that any promoter sequence that enables gene expression in bacteria could be incorporated into a HAEC. Constructs and cassettes containing HAEC are referred to as HAEC(Y):(Z)-(Z'), whereby Z and Z' represent the genes placed downstream and under the control of each

of the respective promoters contained in the HAEC, in the order of 5' to 3' on the coding strand.

In some of the descriptions herein, the term 'donor material' shall be used to refer to a nucleic acid molecule suitable for uptake by and for chromosomal integration into *N. lactamica*. These molecules can be from any source, including but not limited to: (hypermethylated) PCR products, extracted chromosomal or linearised, plasmid DNA.

Modified *Neisseria lactamica*

According to a first aspect of the invention, there is provided a modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

In one embodiment, the modified *Neisseria lactamica* is capable of expressing the heterologous protein encoded by the recombinant DNA. In another embodiment, the modified *Neisseria lactamica* expresses the heterologous protein encoded by the recombinant DNA. The expression of the heterologous protein encoded by the recombinant DNA may be inducible or constitutive.

The heterologous protein may comprise a eukaryote protein. The heterologous protein may comprise a viral protein, such as a capsid protein. The heterologous protein may comprise a protein of a non-*Neisseria* prokaryote. The heterologous protein may comprise a protein. The heterologous protein may comprise a protein of a different strain of *Neisseria*. The DNA encoding the heterologous protein may comprise eukaryote DNA. The DNA encoding the heterologous protein may comprise a processed coding sequence (for example, a gene sequence with introns removed). The DNA encoding the heterologous protein may comprise of sequence not found in *wild-type N. lactamica*. The modified *Neisseria lactamica* may be transformed with recombinant DNA encoding two or more heterologous proteins.

The heterologous protein may comprise fluorescent protein, such as green, red, cyan, yellow fluorescent protein, or the like, and their derivatives. The heterologous protein may be CLOVER. The heterologous protein may comprise a protein capable of enabling or enhancing *Neisseria lactamica* colonisation of a subject, for example nasopharyngeal colonisation of the subject. The heterologous protein may comprise meningococcal adhesin NadA, or a functional homologue, variant or derivative thereof. The heterologous protein may comprise an antigen, for example a disease-associated antigen. The heterologous protein may comprise a *Neisseria meningitidis* protein, or a variant or part thereof. The heterologous protein may comprise a *Neisseria meningitidis* antigen. The heterologous protein may comprise a *Neisseria meningitidis* outer membrane protein, or a variant or part thereof. The *Neisseria meningitidis* protein/antigen may comprise *Neisseria meningitidis* serotype A, B, C, Y, W135 and X protein/antigen. The *Neisseria meningitidis* protein/antigen may comprise protein/antigen of non-typeable isolates of *Neisseria meningitidis*. The *Neisseria meningitidis* protein/antigen may comprise PorA. The PorA may comprise (P1.7,16) or PorA (P1.7-2,4). The *Neisseria meningitidis* protein/antigen may comprise any protein/antigen selected from the group comprising heparin sulphate proteoglycan binding protein, Opc (encoded by the *opcA* gene); NadA; factor H binding protein, fHbp; Heparin-Binding Antigen, NHBA, or Gna2123 (encoded by *gna2132* or *nhba* gene) (see Serruto *et al.*, Proc Natl Acad Sci U S A. 2010 Feb 23;107(8):3770-5); hia/hsf homologue, NhhA (coded for by *gna0992*) (see Scarselli *et al.*, Mol Microbiol. 2006 Aug;61(3):631-44. Epub 2006 Jun 27.); tbpA (transferrin binding protein A); lbpAB (lactoferrin binding protein); *Morexella* IgD binding protein (MID); and variants, derivatives, hybrids or partial fragments thereof; or combinations thereof. In one embodiment, the *Neisseria meningitidis* protein/antigen may comprise Opc. In another embodiment, both Opc and NadA may be provided.

In one embodiment, two or more, such as a plurality of, heterologous proteins may be encoded and/or expressed by the modified *Neisseria lactamica*. In another embodiment, three or more heterologous proteins are encoded and/or expressed by the modified *Neisseria lactamica*.

The recombinant DNA may be stably transformed to provide the modified *Neisseria lactamica*. The recombinant DNA may be chromosomally integrated. The recombinant DNA may be inserted into an intergenic chromosomal sequence. The intergenic chromosomal sequence may be between genes NLY_27080 and NLY_27100 of *Neisseria lactamica* strain Y92-1009, or equivalent loci thereof in other *Neisseria lactamica* strains. The intergenic chromosomal sequence may be between genes NLY_36160 and NLY_36180 of *Neisseria lactamica* strain Y92-1009, or equivalent loci thereof in other *Neisseria lactamica* strains. In one embodiment, the recombinant DNA is inserted into the NHCIS loci of the modified *Neisseria lactamica*. The recombinant DNA may be inserted into NHCIS1 or NHCIS2. The recombinant DNA may be inserted into NHCIS1 or NHCIS2, or an equivalent thereof in other *Neisseria lactamica* strains. NHCIS1 and NHCIS2 may allow expression of protein/antigen at different levels relative to each other. Therefore, if a high level of protein expression is desired, then the gene coding for the heterologous protein can be inserted into NHCIS2; conversely, genes coding for either potentially toxic products, or proteins that have a detrimental, concentration-dependent effect on the host organism can be inserted into NHCIS1 for a lower level of expression relative to NHCIS2 inserts. Alternatively if a high level of protein expression is desired, then the gene coding for the heterologous protein can be inserted into NHCIS1; conversely, genes coding for either potentially toxic products, or proteins that have a detrimental, concentration-dependent effect on the host organism can be inserted into NHCIS2 for a lower level of expression relative to NHCIS1 inserts.

In another embodiment, the recombinant DNA may not be inserted into an intergenic chromosomal sequence. The recombinant DNA may not be inserted into an essential gene (i.e. a gene essential for survival and/or growth of *N. lactamica*). The recombinant DNA may not be inserted into a wild-type *N. lactamica* gene. The recombinant DNA may not be inserted into a wild-type *N. lactamica* operon. The recombinant DNA may not be inserted into a wild-type *N. lactamica* regulatory sequence. The recombinant DNA may comprise part of a gene or genes flanking an intergenic sequence.

The DNA encoding the heterologous protein (i.e. the endogenous or heterologous gene

to be inserted) may be at least 600bp in length. In another embodiment, the DNA encoding the heterologous protein may be at least 800bp in length. In another embodiment, the DNA encoding the heterologous protein may be at least 1kbp in length. In another embodiment, the DNA encoding the heterologous protein may be at least 1.5kbp in length.

The *Neisseria lactamica* may comprise *Neisseria lactamica* Y92-1009. The skilled person will understand that other *Neisseria lactamica* isolates may be suitable for modification according to the invention herein.

In one embodiment the modified *Neisseria lactamica* is a β-galactosidase (*lacZ*) deficient mutant (Δ *lacZ*). The modified *Neisseria lactamica* may not comprise a functional or non-functional wild-type *lacZ* gene. The modified *Neisseria lactamica* may not comprise any substantial parts of the wild-type *lacZ* gene. Substantial parts of the wild-type *lacZ* gene sequence may comprise at least 10 nucleotides of the wild-type *lacZ* gene sequence.

Advantageously, providing a modified *Neisseria lactamica* with no wild-type *lacZ* gene or parts thereof, minimises the chance of undesirable homologous recombinations where *lacZ* may be used as a selection marker on a cloning vector for transforming the modified *Neisseria lactamica.*

Therefore, according to another aspect of the present invention, there is provided a modified *Neisseria lactamica,* wherein the modified *Neisseria lactamica* does not comprise wild type *lacZ* gene sequence, or substantial parts thereof.

The modified *Neisseria lactamica* may have been derived from a wild-type strain of *Neisseria lactamica* that normally has a functional *lacZ* gene. Such a wild-type strain may be modified to form the modified *Neisseria lactamica* by *lacZ* gene knockout or substantial removal of *lacZ* gene sequence.

Advantageously, the provision of a modified *Neisseria lactamica* with wild type *lacZ*

gene sequence, or substantial parts thereof removed from the chromosome can avoid non-specific recombination events with Nlac *lacZ*-containing Cassettes and concomitant mis-targeting of the Cassette to loci other than that intended. This strain provides a background for insertion of genes coding for heterologous proteins without need for antibiotic resistance markers, a preferred state for potential human challenge with recombinant *Neisseria lactamica*.

In one embodiment the modified *Neisseria lactamica* is a β-galactosidase (*lacZ*) deficient mutant (*Δ lacZ*), which is also deficient for the *Neisseria* Heparin Binding Antigen (NHBA or Gna2132). The modified *Neisseria lactamica* may not comprise a functional or non-functional wild-type *lacZ* gene. The modified *Neisseria lactamica* may not comprise any substantial parts of the wild-type *lacZ* gene. Substantial parts of the wild-type *lacZ* gene sequence may comprise at least 10 nucleotides of the wild-type *lacZ* gene sequence. The modified *Neisseria lactamica* may not comprise a functional or non-functional wild-type *nhba* gene. The modified *Neisseria lactamica* may not comprise any substantial parts of the wild-type *nhba* gene. Substantial parts of the wild-type *nhba* gene sequence may comprise at least 10 nucleotides of the wild-type *nhba* gene sequence.

The modified *Neisseria lactamica* may comprise a synthetic version (i.e. not wild-type) of the *lacZ* gene (synth.*lacZ*), in which the coding sequence has been modified to diversify the sequence from the endogenous Nlac version of the *lacZ* gene, whilst maintaining the fidelity of the encoded amino acid sequence of β-galactosidase. In one embodiment, the synthetic version of the *lacZ* gene may comprise the sequence of SEQ ID NO: 20 (Figure 37) or a variant thereof. A variant of the synthetic version of the *lacZ* gene may comprise may comprise a sequence of at least 80% identity with the sequence of SEQ ID NO: 20 (Figure 37), whilst maintaining β-galactosidase function. In another embodiment, a variant of the synthetic version of the *lacZ* gene may comprise a sequence of at least 85%, 90%, 95%, 98%, 99%, or 99.5% identity with the sequence of SEQ ID NO: 20 (Figure 37), whilst maintaining β-galactosidase function. The skilled person will understand that fewer or more amino acid substitutions which either diversify the sequence away from wild-type or conform the sequence back towards

wild-type may be envisaged, and still retain function and sufficient diversity to avoid inadvertent recombination with wild-type sequence. For example, up to 40% of the substitutions to form the synthetic *lacZ* gene may be reverted back to wild-type (although with an increased likelihood of inadvertent recombination). In another embodiment, up to 5%, 10%, 15%, 20%, 25% or 30% of the substitutions to form the synthetic *lacZ* gene may be reverted back to wild-type.

Providing a synthetic version of the *lacZ* gene advantageously introduces a functional copy of the *lacZ* gene back into the chromosome of a modified Nlac strain having a previously knocked out *lacZ* gene via an heterologous nucleic acid insertion, so that β-galactosidase activity is maintained. For example, double mutants encoding heterologous NadA and Opc can retain β-galactosidase activity.

The recombinant DNA may further comprise a selection marker. The selection marker may be expressed under the control of an exogenous promoter. The selection marker may be heterologous to wild-type *N. lactamica.* The selection marker may comprise a *Neisseria lactamica* β-galactosidase (*lacZ*) gene or a non-*Neisseria lactamica* β-galactosidase (*lacZ*) gene. The selection marker may comprise the synthetic *lacZ* gene described herein. In one embodiment, the selection marker may not comprise an antibiotic resistance marker/gene. In one embodiment, the selection marker may comprise any one of kanamycin, spectinomycin, erythromycin, tetracycline, or gentamycin resistance genes.

The recombinant DNA may encode a promoter. In one embodiment, the recombinant DNA encodes at least two promoters. The promoter may promote expression of the gene encoding the heterologous protein and/or the selection marker. In embodiments where at least two promoters are encoded, a first promotor may promote expression of the heterologous protein and a second promoter may promote expression of the selection marker, or vice versa. The first and second promoters may be constitutive promoters. Alternatively, the first and second promoters may be inducible promoters. Alternatively, first promoter may be constitutive and the second promoter may be inducible, or vice versa.

The first and/or second promoter may comprise a *Neisseria lactamica* promoter. i.e. a promoter that is recognised by the transcriptional apparatus of *Neisseria lactamica* and capable of promoting expression in *Neisseria lactamica*. The first and second promoters may be different, for example, a different promoter sequence. The first and second promotors may be promotors originating from different genes.

The first promoter may comprise the promoter from the α-2,3 sialyltransferase (*lst)* gene. Alternatively, the second promoter may comprise the promoter from the α-2,3 sialyltransferase (*lst*) gene.

The second promotor may comprise the promotor for the gene coding for Porin B (*porB*). Alternatively, the second promotor may comprise the promotor for the gene coding for Porin B (*porB*).

In one embodiment, the first promoter comprises the promoter from the α-2,3 sialyltransferase (*lst)* gene and the second promotor comprises the promotor for the gene coding for Porin B (*porB*). In an alternative embodiment, the first promoter comprises the promoter for the gene coding for Porin B (*porB*) and the second promotor comprises the promotor from the α-2,3 sialyltransferase (*lst)* gene.

The first promotor may be a constitutive or inducible gene promoter. The second promoter may be a constitutive or inducible gene promoter. The first promotor may be a constitutive promoter and the second promoter may be an inducible promoter, or vice versa. The inducible promoter may be a phase variable promoter.

Advantageously, using a first promoter, such as a constitutive promoter or an inducible promoter, would express a large amount of an antigen, for example in quantities similar to wild type *N. meningitidis*. In instances where the host develops an immune response against that antigen, then it could potentially lead to immune clearance of the modified *N. lactamica* and a termination of colonisation. In an alternative embodiment using a phase variable promoter, a subset of the modified *N. lactamica* population – those with

the antigen in the phase OFF position – may escape immunological clearance, persist in the nasopharynx and be present later on to re-express the gene (in the subset of the survivor population that revert to the phase ON phenotype). This can effectively constitute a re-challenge with the antigen of choice and potentially serve to mature an immune response against the target protein.

The first promoter may comprise the hybrid *porA/porB* promoter described herein. Alternatively, the second promoter may comprise hybrid *porA/porB* promoter described herein. The *porA* sequence may be a modified form having the 5' polyadenosine tract removed.

An enhancer sequence may also be provided with the promoter. The enhancer may comprise a 200bp sequence upstream of the -35 box of the RNA Polymerase binding site. The enhancer may comprise a 250bp sequence upstream of the -35 box of the RNA Polymerase binding site. In one embodiment, the enhancer is a native enhancer for the promoter. In one embodiment, the enhancer is a *porA* enhancer (see Figure 25 and Figure 26).

A hybrid promoter may be provided having a hybrid *porA/porB* promoter coupled with a *porA* enhancer (for example see Figure 25 and Figure 26). The hybrid *porA/porB* promoter may comprise a *porA* sequence wherein the homopolymeric tract of 'G' nucleotides (that renders the wild type *porA* gene phase variable) has been replaced with sequence derived from the wild type, non-phase variable *porB* promoter of *N.lactamica*. A hybrid promoter may be provided having the promoter from the sialyltransferase gene coupled with a *porA* enhancer (see Figure 21).

The promoter with enhancer sequence may comprise or consist of the sequence of any one of *lst*:*lacZ*; *lst*(50):*lacZ*; *lst*(100):*lacZ*; *lst*(150):*lacZ*; *lst*(200):*lacZ*; *lst*(250):*lacZ*; or *lst*(400):*lacZ* as depicted in Figure 21. Variants of such promoter and enhancer sequences may be provided, for example *lst*(1-400):*lacZ* with 1-400 denoting the length of the sequence extending 5' from the promoter in accordance to Figure 21. The variant may comprise *lst*(50-400):*lacZ* or *lst*(100-300):*lacZ* or *lst*(150-250):*lacZ*.

The hybrid *porA/porB* promoter may be preceded by 200 bp or 250 bp of transcriptional enhancer sequence, derived from the wild type *porA* gene of *N. meningitidis*. The *porA/porB* hybrid promoter may be followed (3') by a second hybrid promoter, wherein the 17 bp that separate the -10 and -35 boxes of the RNA Polymerase binding site of the wild type *porB* gene have been replaced with 17 bp of sequence derived from the *lst* promoter.

The hybrid, synthetic promoters advantageously combine elements of the various wild type promoters, along with the enhancer sequence of *porA* to ensure they remain phase on, and express the genes they control to high levels.

In one embodiment, the promoter sequence comprises a homopolymeric 'G' tract, which separates the -10 and -35 boxes of the promoter, such as the wild type *porA* promoter. The homopolymeric 'G' tract may comprise about 9 to 17 contiguous guanosine nucleotide residues. The homopolymeric 'G' tract may comprise about 9 to 15 contiguous guanosine nucleotide residues. The homopolymeric 'G' tract may comprise about 10 to 15 contiguous guanosine nucleotide residues. The homopolymeric 'G' tract may comprise about 10 to 12 contiguous guanosine nucleotide residues. In one embodiment, the homopolymeric 'G' tract comprises or consists of 11 contiguous guanosine nucleotide residues. In another embodiment, the homopolymeric 'G' tract comprises or consists of 10 contiguous guanosine nucleotide residues.

In another embodiment, the recombinant DNA may be promoterless, but is inserted into a chromosomal site under the influence of an endogenous promoter.

In one embodiment, the modified *N. lactamica* is modified by chromosomal integration of an endogenous gene to enhance expression of the endogenous gene. In another embodiment, the modified *N. lactamica* is modified by chromosomal integration of an endogenous gene that has been modified to enhance a property and/or expression of the endogenous gene. In another embodiment, the modified *N. lactamica* is modified by chromosomal integration of a regulatory element, such as a promoter and/or enhancer,

which can enhance expression of an endogenous gene.

Reducing meningococcal colonisation and prophylactic inoculation of modified *Neisseria lactamica*

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According to another aspect of the invention, there is provided a method of prophylactic treatment for pathogenic infection of a subject comprising nasopharyngeal inoculation of a modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

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The pathogenic infection may comprise meningococcal infection.

According to another aspect of the invention, there is provided a method of reducing or preventing meningococcal colonisation of a subject comprising nasopharyngeal
15 inoculation of a modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

The meningococcal colonisation may comprise colonisation of *Neisseria meningitidis*.

20 According to another aspect of the invention, there is provided a method of modifying the microbiome of a subject comprising nasopharyngeal inoculation of a modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

25 According to another aspect of the invention, there is provided a method of modifying the microbiome of a subject comprising nasopharyngeal inoculation of wild-type *Neisseria lactamica*.

According to another aspect of the invention, there is provided a method of preventing
30 meningococcal colonisation of a subject comprising nasopharyngeal inoculation of a wild-type *Neisseria lactamica*.

According to another aspect of the invention, there is provided a method of prophylactic

treatment for pathogenic infection of a subject comprising nasopharyngeal inoculation of a modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

According to another aspect of the invention, there is provided a modified *Neisseria lactamica* for use for the prophylactic treatment of meningococcal infection of a subject, wherein the prophylactic treatment comprises nasopharyngeal inoculation of the modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

According to another aspect of the invention, there is provided a wild-type *Neisseria lactamica* for use for the prophylactic treatment of meningococcal infection of a subject, wherein the prophylactic treatment comprises nasopharyngeal inoculation of the *Neisseria lactamica*.

According to another aspect of the invention, there is provided a modified *Neisseria lactamica* for use for reducing or preventing colonisation of *Neisseria meningitidis* in a subject, the use comprising nasopharyngeal inoculation of the modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

Advantageously, the invention provides a prophylactic means of displacing resident *N. meningitidis* and preventing the (re-)acquisition of new *N. meningitidis* into the nasopharynx. The invention promotes herd immunity in a given population of humans, on the basis that the presence of *Neisseria lactamica* in the nasopharynx prevents co-colonisation with the more pathogenic *N. meningitidis*. If the *N. meningitidis* is not present in an individual's nasopharynx then it cannot be transmitted to other individuals; meaning it cannot possibly cause disease in these other individuals. During epidemic spread of a meningococcal outbreak, close contacts of patients with meningococcal disease can be prophylactically administered *Neisseria lactamica*, as a cheap alternative to antibiotic prophlyaxis.

The modified *Neisseria lactamica* for use for the prophylactic treatment of meningococcal infection may be according the invention herein described. The modified *Neisseria lactamica* for use for reducing or preventing colonisation of *Neisseria meningitidis* in a subject may be according the invention herein described.

The meningococcal infection may comprise *Neisseria meningitidis* serotype A, B, C, Y, W-135, and/or X infection. The meningococcal infection may comprise non-typeable *Neisseria meningitidis*. The *Neisseria meningitidis* colonisation may comprise *Neisseria meningitidis* serotype A, B, C, Y, W135 and/or X colonisation. The *Neisseria meningitidis* colonisation may comprise non-typeable *Neisseria meningitidis*. In one embodiment, the *Neisseria meningitidis* comprises *Neisseria meningitidis* serotype B.

The modified or wild-type *Neisseria lactamica* may be provided in a suspension. The suspension may comprise between about 2×10^2 per ml and about 2×10^8 per ml of the modified or wild-type *Neisseria lactamica*. The suspension may comprise between about 2×10^2 per ml and about 2×10^7 per ml of the modified or wild-type *Neisseria lactamica*. The suspension may comprise between about 2×10^2 per ml and about 2×10^6 per ml of the modified or wild-type *Neisseria lactamica*. Alternatively, the suspension may comprise between about 2×10^3 per ml and about 2×10^5 per ml of the modified or wild-type *Neisseria lactamica*. Alternatively, the suspension may comprise about 2×10^4 per ml of the modified or wild-type *Neisseria lactamica*. The suspension may comprise PBS buffer. The suspension of modified or wild-type *Neisseria lactamica* may comprise a pharmaceutically acceptable carrier. In one embodiment, the pharmaceutically acceptable carrier comprises glycerol. The pharmaceutically acceptable carrier may comprise Frantz medium. Alternatively, the pharmaceutically acceptable carrier may comprise Frantz medium with glycerol. The glycerol may be provided in an amount of between 20% and 40% v/v. The glycerol may be provided in an amount of about 30% v/v.

In one embodiment, the nasopharyngeal inoculation of the modified *Neisseria lactamica* comprises exposing nasopharyngeal tissue of the subject to the modified *Neisseria lactamica*. In one embodiment, the nasopharyngeal inoculation of the modified

Neisseria lactamica comprises applying a suspension of the modified *Neisseria lactamica* onto nasopharyngeal tissue of the subject. The exposure/application may be by spraying, or by droplet of a modified *Neisseria lactamica* suspension. The exposure/application may be by drinking and swilling a suspension of the modified *Neisseria lactamica* around the mouth.

The modified *Neisseria lactamica* may comprise an inducible promoter, which only promotes significant expression of a gene in the presence of an inducer agent. Additionally or alternatively, the modified *Neisseria lactamica* may comprise a transient gene, which is only expressed in one, two or three generations of the modified *Neisseria lactamica.*

In an embodiment, wherein the modified *Neisseria lactamica* comprises an inducible promoter the subject may be administered with an agent capable of inducing the promoter. The administration may be concurrent with inoculation of the modified *Neisseria lactamica*, pre-inoculation of the modified *Neisseria lactamica*, or post-inoculation of the modified *Neisseria lactamica.*

***Neisseria lactamica* Cloning Vector**

According to another aspect of the invention, there is provided a cloning vector for modification of *Neisseria lactamica* comprising one or more Heterologous Antigen Expression Cassettes (HAEC), wherein the HAEC comprises:

-a heterologous nucleic acid sequence insertion site;

-a first promoter, upstream of the heterologous nucleic acid sequence;

-a second promoter, downstream of the heterologous nucleic acid sequence; and

-a selection marker downstream of the second promoter;

wherein the HAEC is flanked by a sequence homologous to a region of *Neisseria lactamica* chromosome.

Each flanking sequence homologous to a region of *Neisseria lactamica* genome may be between about 50 bp and about 1500bp. In another embodiment, each flanking sequence homologous to a region of *Neisseria lactamica* genome may be between about 75 bp and about 1200bp. In another embodiment, each flanking sequence homologous to a region of *Neisseria lactamica* genome may be between about 100 bp and about 1200bp. In another embodiment, each flanking sequence homologous to a region of *Neisseria lactamica* genome may be between about 200 bp and about 1200bp. In another embodiment, each flanking sequence homologous to a region of *Neisseria lactamica* genome may be between about 100 bp and about 1000bp.

According to another aspect of the invention, there is provided a cloning vector for modification of *Neisseria lactamica* comprising one or more *Neisseria* Heterologous Antigen Expression Cassettes (HAEC), wherein the HAEC comprises:

-a heterologous nucleic acid sequence insertion site;

-a selection marker; and

-a promoter upstream of the heterologous nucleic acid sequence insertion site and selection marker;

wherein the HAEC is flanked by a sequence homologous to a region of *Neisseria lactamica* chromosome.

In one embodiment, the modification of *Neisseria lactamica* may be mutagenesis of *Neisseria lactamica*. The mutagenesis may comprise a double crossover event, leading to recombination of the heterologous expression construct into the chromosome of *Neisseria lactamica*.

The heterologous nucleic acid sequence insertion site may comprise a restriction enzyme recognition sequence. The heterologous nucleic acid sequence insertion site may comprise a multiple cloning site, for example, a region of DNA comprising a plurality of different restriction enzyme recognition sequences. The restriction enzyme recognition sequence may be for recognition of a restriction enzyme which leaves blunt ends, or nucleotide overhangs. The restriction enzyme recognition sequence may be for recognition of a restriction enzyme selected from *BamHI, XbaI, SalI, XhoI, NotI, NdeI,*

and *HindIII,* or combinations thereof.

Advantageously, providing nucleotide overhangs provides the ability to select or design heterologous nucleic acid sequences that will orientate in a preferred orientation (directional cloning).

In one embodiment, a heterologous nucleic acid sequence may be provided in the heterologous nucleic acid sequence insertion site.

Therefore, according to another aspect of the invention, there is provided a cloning vector for mutagenesis of *Neisseria lactamica* comprising one or more Heterologous Antigen Expression Cassettes (HAEC), wherein the HAEC comprises:

-a heterologous nucleic acid sequence;

-a first promoter upstream of the heterologous nucleic acid sequence;

-a second promoter downstream of the heterologous nucleic acid sequence; and

-a selection marker downstream of the second promoter;

wherein the HAEC is flanked by a sequence homologous to a region of *Neisseria lactamica* chromosome.

According to another aspect of the invention, there is provided a cloning vector for mutagenesis of *Neisseria lactamica* comprising one or more Heterologous Antigen Expression Cassettes (HAEC), wherein the HAEC comprises:

-a heterologous nucleic acid sequence;

-a selection marker; and

-a promoter upstream of the heterologous nucleic acid sequence and selection marker;

wherein the HAEC is flanked by a sequence homologous to a region of *Neisseria lactamica* chromosome.

According to another aspect of the invention, there is provided a nucleic acid for

mutagenesis of *Neisseria lactamica* comprising one or more Heterologous Antigen Expression Cassettes (HAEC), wherein the HAEC comprises:

-a heterologous nucleic acid sequence;

-a first promoter upstream of the heterologous nucleic acid sequence;

-a second promoter downstream of the heterologous nucleic acid sequence; and

-a selection marker downstream of the second promoter;

wherein the HAEC is flanked by a sequence homologous to a region of *Neisseria lactamica* chromosome.

According to another aspect of the invention, there is provided a nucleic acid for mutagenesis of *Neisseria lactamica* comprising one or more Heterologous Antigen Expression Cassettes (HAEC), wherein the HAEC comprises:

-a heterologous nucleic acid sequence;

-a selection marker; and

-a promoter upstream of the heterologous nucleic acid sequence and selection marker;

wherein the HAEC is flanked by a sequence homologous to a region of *Neisseria lactamica* genome.

The nucleic acid may be a cloning vector. The nucleic acid may be a PCR product. The nucleic acid may be a hypermethlyated PCR product. Additionally or alternatively, the nucleic acid may be devoid of one or more restriction enzyme recognition sites selected from NlaI, NlaII, Drg, NlaIII, NlaIV and NgoMIV recognition sites, or combinations thereof.

The heterologous expression construct may comprise a Heterologous Antigen Expression Cassette (HAEC). The heterologous expression construct may comprise the sequence of HAEC1 (see Figure 12), HAEC2 (see Figure 13), HAEC3 (see Figure 25), or HAEC4 (see Figure 26).

The HAEC may encode an antigen, for example which is heterologous for *Neisseria lactamica*. Alternatively, the HAEC may encode an antigen, for example which is homologous for *Neisseria lactamica*.

The promoter, such as the first and/or second promoter may comprise a *Neisseria lactamica* promoter. i.e. a promoter that is recognised and capable of promoting expression in *Neisseria lactamica*. The first and second promoters may be different, for example, a different promoter sequence. The first and second promotors may be promotors originating from different genes.

The promoter, such as the first promoter, may comprise the promoter from the sialyltransferase gene. Alternatively, the second promoter may comprise the promoter from the sialyltransferase gene.

The promoter, such as the first promoter, may comprise the promoter for the gene coding for Porin B (*porB*). Alternatively, the second promoter may comprise the promoter for the gene coding for Porin B (*porB*). The first promoter may comprise the hybrid *porA/porB* promoter described herein. Alternatively, the second promoter may comprise hybrid *porA/porB* promoter described herein.

An enhancer sequence may also be provided with the promoter. The enhancer may comprise a 200bp sequence upstream of the -35 box of the RNA Polymerase binding site. The enhancer may comprise a 250bp sequence upstream of the -35 box of the RNA Polymerase binding site. In one embodiment, the enhancer is a native enhancer for the promoter. In one embodiment, the enhancer is a *porA* enhancer (see Figure 25 and Figure 26).

A hybrid promoter may be provided having a hybrid *porA/porB* promoter coupled with a *porA* enhancer (for example see Figure 25 and Figure 26). The hybrid *porA/porB* promoter may comprise a *porA* sequence wherein the homopolymeric tract of 'G' nucleotides (that renders the wild type *porA* gene phase variable) has been replaced with sequence derived from the wild type, non-phase variable *porB* promoter of *N.lactamica*.

A hybrid promoter may be provided having the promoter from the sialyltransferase gene coupled with a *porA* enhancer (see Figure 21).

The promoter with enhancer sequence may comprise or consist of the sequence of any one of *lst*:*lacZ*; *lst*(50):*lacZ*; *lst*(100):*lacZ*; *lst*(150):*lacZ*; *lst*(200):*lacZ*; *lst*(250):*lacZ*; or *lst*(400):*lacZ* as depicted in Figure 21. Variants of such promoter and enhancer sequences may be provided, for example *lst*(1-400):*lacZ* with 1-400 denoting the length of the sequence extending 5' from the promoter in accordance to Figure 21. The variant may comprise *lst*(50-400):*lacZ* or *lst*(100-300):*lacZ* or *lst*(150-250):*lacZ*.

The hybrid *porA*/*porB* promoter may be preceded by 200 bp or 250 bp of transcriptional enhancer sequence, derived from the wild type *porA* gene of *N. meningitidis*. The *porA*/*porB* hybrid promoter may be followed (3') by a second hybrid promoter, wherein the 17 bp that separate the -10 and -35 boxes of the RNA Polymerase binding site of the wild type *porB* gene have been replaced with 17 bp of sequence derived from the *lst* promoter.

The hybrid, synthetic promoters advantageously combine elements of the various wild type promoters, along with the enhancer sequence of *porA* to ensure they remain phase on, and express the genes they control to high levels.

In one embodiment, the promoter sequence comprises a homopolymeric 'G' tract, which separates the -10 and -35 boxes of the promoter, such as the wild type *porA* promoter. The homopolymeric 'G' tract may comprise about 9 to 17 contiguous guanosine nucleotide residues. The homopolymeric 'G' tract may comprise about 9 to 15 contiguous guanosine nucleotide residues. The homopolymeric 'G' tract may comprise about 10 to 15 contiguous guanosine nucleotide residues. The homopolymeric 'G' tract may comprise about 10 to 12 contiguous guanosine nucleotide residues. In one embodiment, the homopolymeric 'G' tract comprises or consists of 11 contiguous guanosine nucleotide residues. In another embodiment, the homopolymeric 'G' tract comprises or consists of 10 contiguous guanosine nucleotide residues.

In one embodiment, the first promoter comprises the promoter from the sialyltransferase (*lst*) gene and the second promotor comprises the promotor for the gene coding for Porin B (*porB*). In an alternative embodiment, the first promoter comprises the promoter for the gene coding for Porin B (*porB*) and the second promotor comprises the promotor from the sialyltransferase (*lst*) gene.

The first promotor may be a constitutive or inducible gene promoter. The second promoter may be a constitutive or inducible gene promoter. The first promotor may be a constitutive promoter and the second promoter may be an inducible promoter, or vice versa.

The selection marker may comprise a β-galactosidase (*lacZ*) gene. In one embodiment, the selection marker comprises a *Neisseria lactamica* β-galactosidase (*lacZ*) gene. In an embodiment wherein two or more HAEC are provided, each HAEC may comprise a different β-galactosidase (*lacZ*) gene, for example from different natural or synthetic sources. In one embodiment, the selection marker may not comprise an antibiotic resistance marker. In one embodiment, the selection marker may comprise any one of kanamycin, spectinomycin, erythromycin, tetracycline, or gentamycin resistance genes.

The promoter, such as the first and/or second promoter sequences may be flanked by a plurality of unique restriction sites. The term "unique" is understood to mean that a restriction site is provided only once in the cloning vector sequence.

The nucleic acid, such as the cloning vector, PCR product or heterologous expression construct, may comprise a canonical Neisserial DNA Uptake Sequence (DUS), for example 5' – GCCGTCTGAA – 3' (SEQ ID NO: 1), or a reverse compliment thereof. The nucleic acid may comprise a canonical AT-flanked Neisserial DNA Uptake Sequence (AT-DUS), for example 5' – ATGCCGTCTGAA – 3' (SEQ ID NO: 2), or a reverse compliment thereof.

Advantageously, the provision of a Neisserial DNA Uptake Sequence (DUS) or AT-flanked Neisserial DNA Uptake Sequence (AT-DUS) in the nucleic acid can enhance

uptake of the nucleic acid during transformation (see Figure 9).

The nucleic acid may be suitable for uptake by, and for chromosomal integration into, *N. lactamica*. The nucleic acid may be from any source, for example selected from PCR product, hypermethylated PCR product, extracted chromosomal DNA, or plasmid DNA. The nucleic acid may be provided in linearised form.

The heterologous nucleic acid may be hypermethylated, whereby all the deoxycytosine residues of the heterologous nucleic acid have been replaced with methyl-deoxycytosine. The heterologous expression construct and flanking homologous sequences may be hypermethylated, whereby all the deoxycytosine residues of the heterologous nucleic acid have been replaced with methyl-deoxycytosine. In one embodiment, the cloning vector may be hypermethylated, whereby all the deoxycytosine residues of the cloning vector have been replaced with methyl-deoxycytosine.

In one embodiment, the heterologous expression cassette and flanking homologous sequences may not comprise one or more NlaIII restriction recognition sites. The heterologous expression construct and flanking homologous sequences may not comprise the sequence 5' – CATG – 3'. In one embodiment, the heterologous expression construct and flanking homologous sequences may not comprise any one of NlaI, NlaII, Drg, NlaIII, NlaIV and NgoMIV recognition sites, or combinations thereof. In one embodiment, the heterologous nucleic acid may not comprise a NlaIII restriction recognition site. The heterologous nucleic acid may not comprise the sequence 5' – CATG – 3'. In one embodiment, heterologous nucleic acid may not comprise any one of NlaI, NlaII, Drg, NlaIII, NlaIV and NgoMIV recognition sites, or combinations thereof. In one embodiment, the cloning vector may not comprise a NlaIII restriction recognition site. The cloning vector may not comprise the sequence 5' – CATG – 3'. In one embodiment, the cloning vector may not comprise any one of NlaI, NlaII, Drg, NlaIII, NlaIV and NgoMIV recognition sites, or combinations thereof.

The transformation efficiency of *N. lactamica* is highest when the cloning vector is

hypermethylated PCR product, but other sources of nucleic acid can successfully transform *N. lactamica,* albeit at lower efficiency. With properly-designed constructs that are free of 'CATG' sequences (NlaIII restriction enzyme cut sites), it is possible to successfully transform *N. lactamica* with normal PCR products (at very low efficiency). In particular, despite being a naturally competent bacterial species, which constitutively takes up exogenous DNA from the environment, *Neisseria lactamica* has proven to be refractory to targeted mutagenesis or directed genetic change. The most likely cause of this is the battery of restriction enzymes encoded in the genome of *Neisseria lactamica*, most notably the 4-cutter restriction enzyme, NlaIII, which cuts dsDNA at the short palindromic sequence 'CATG'. In the absence of selective pressure against the accumulation of these sequences, this motif can occur with relatively high frequency in a given stretch of nucleotides, meaning that uptake of DNA containing one or more of these sequences results in the intracellular degradation of the material before homologous recombination can take place and integrate the exogenous DNA into the *Neisseria lactamica* chromosome. As such, there are currently no molecular systems for the manipulation of the *Neisseria lactamica* genome. Advantageously, this invention circumvents the barriers to transformation of *Neisseria lactamica* and allows targeted genetic modification of this organism for the first time. In particular, methylation of restriction sites can inhibit the DNA cleavage action of restriction endonucleases (see Figure 7), which prevents degradation of the product following uptake and therefore provides sufficient time for homologous recombination to take place and the construct to become integrated into the chromosomal locus of choice. This allows stable integration of DNA constructs into loci of the *Neisseria lactamica* chromosome, with utility for both deletion of existing genes (i.e. targeted mutagenesis) or insertion of genes from other sources, such as genes from other Gram negative bacteria and eukaryotic proteins.

The cloning vector may be modified from the standard cloning vector pUC19 (Norrander et al, Gene 1983 Dec;26(1):101-6.). For example, the cloning vector may comprise pUC19 sequence, or substantial parts thereof. The cloning vector may comprise pUC19 sequence, or substantial parts thereof, with the *lacZ* promotor encoding sequence removed. The cloning vector may comprise the

pUC19NHCIS(X)::HAEC(Y):(Z)-*lacZ* vector system described herein. In one embodiment, the cloning vector may comprise pUC19NHCIS1::HAEC1:(Z)-*lacZ*. In another embodiment, the cloning vector may comprise pUC19NHCIS2::HAEC1:(Z)-*lacZ*. In another embodiment, the cloning vector may comprise pUC19NHCIS1::HAEC2:*porA*plusprom-*lacZ*. In another embodiment, the cloning vector may comprise pUC19NHCIS1::HAEC4:*nadA-lacZ*. In another embodiment, the cloning vector may comprise pUC19NHCIS1::HAEC4:*nadA-lacZ*. In another embodiment, the cloning vector may comprise pSC101NHCIS1::HAEC4:*opcA-lacZ*. In another embodiment, the cloning vector may comprise pUC19Δnhba::HAEC3:(Z)-lacZ. In another embodiment, the cloning vector may comprise pNHCIS(X)::HAEC(Y):(Z)-*lacZ*. In another embodiment, the cloning vector may comprise pNHCIS2::HAEC1:(Z)-*lacZ*. In another embodiment, the cloning vector may comprise pUC19*Δnhba*::HAEC1:(Z)-*lacZ*. In another embodiment, the cloning vector may comprise pUC19*Δnhba*.

The cloning vector may comprise pUC19 sequence, or substantial parts thereof, comprising the pUC origin of replication (pMB1). Alternatively, the cloning vector may comprise pUC19 sequence, or substantial parts thereof, with the pUC origin of replication (pMB1) has been substituted for the *repA/ori*, minimally-required replicatory region of plasmid pSC101. (see Chang and Cohen J. 1978. Construction and characterisation of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. Bact; and Vocke and Bastia 1983. Primary Structure of the essential replicon of the plasmid pSC101. PNAS). In one embodiment, the cloning vector comprises pSC101NHCIS1::HAEC4:*opcA-lacZ*.

The cloning vector may comprise an *N. lactamica* codon-optimized version of the *opcA* gene (eg. NMB1053), optionally under the control of the optimally enhanced, modified *porA/porB* hybrid promoter, and further optionally flanked on either side by sequences derived from the 5' and 3' ends of the Nlac *lacZ* gene. In one embodiment, the cloning vector comprises pSC101::*ΔlacZ:opcA*.

The cloning vector may comprise a synthetic version (i.e. not wild-type) of the *lacZ*

gene (synth.*lacZ*), in which the coding sequence has been modified to diversify the sequence from the endogenous Nlac version of the *lacZ* gene, whilst maintaining the fidelity of the encoded amino acid sequence of β-galactosidase. In one embodiment, the synthetic version of the *lacZ* gene may comprise the sequence of SEQ ID NO: 20 (Figure 37) or a variant thereof. A variant of the synthetic version of the *lacZ* gene may comprise may comprise a sequence of at least 80% identity with the sequence of SEQ ID NO: 20 (Figure 37), whilst maintaining β-galactosidase function. In another embodiment, a variant of the synthetic version of the *lacZ* gene may comprise a sequence of at least 85%, 90%, 95%, 98%, 99%, or 99.5% identity with the sequence of SEQ ID NO: 20 (Figure 37), whilst maintaining β-galactosidase function. The skilled person will understand that fewer or more amino acid substitutions which either diversify the sequence away from wild-type or conform the sequence back towards wild-type may be envisaged, and still retain function and sufficient diversity to avoid inadvertent recombination with wild-type sequence. For example, up to 40% of the substitutions to form the synthetic *lacZ* gene may be reverted back to wild-type (although with an increased likelihood of inadvertent recombination). In another embodiment, up to 5%, 10%, 15%, 20%, 25% or 30% of the substitutions to form the synthetic *lacZ* gene may be reverted back to wild-type.

In one embodiment, the cloning vector comprises pSC101::*ΔlacZ*-synth.*lacZ*-3'ENDNHCIS1.

Providing a synthetic version of the *lacZ* gene, for example in the *ΔlacZ*-synth.*lacZ*-3'ENDNHCIS1 construct, advantageously introduces a functional copy of the *lacZ* gene back into the chromosome of an Nlac strain having a previously knocked out *lacZ* gene via an heterologous nucleic acid insertion, for example Nlac strain *ΔlacZ* NHCIS1::HAEC4:*nadA-ΔlacZ:opcA*, so that β-galactosidase activity is maintained. For example, double mutants encoding heterologous NadA and Opc can retain β-galactosidase activity.

In one embodiment, the cloning vector comprises pSC101NHCIS1::HAEC4:*porA*(P1.7,16)-*lacZ*. In another embodiment, the cloning

vector comprises pSC101NHCIS1::PV*porA*(P1.7,16)-*lacZ*.

Advantageously, the copy number of pSC101-derived plasmids is tightly controlled by the presence of the self-encoded RepA protein, and the plasmids exist at a much lower copy number per bacterial cell (=5) than the pUC plasmids (=50-300), which can facilitate successful transformation of constructs encoding gene products that are potentially toxic to the host cell.

The sequence homologous to a region of *Neisseria lactamica* genome may comprise a sequence of NHCIS1 or NHCIS2. In one embodiment, the sequence homologous to a region of *Neisseria lactamica* genome comprise a sequence of NHCIS1.

Method of mutagenesis

According to another aspect of the invention, there is provided a method of mutagenesis of *Neisseria lactamica* comprising transformation of *Neisseria lactamica* with the cloning vector according to the invention herein.

According to another aspect of the invention, there is provided a method of mutagenesis of *Neisseria lactamica* comprising transformation of *Neisseria lactamica* with hypermethylated nucleic acid.

The hypermethylated nucleic acid may comprise or consist of a hypermethylated PCR product. The hypermethylated nucleic acid may comprise or consist of a hypermethylated cloning vector.

The method of mutagenesis may further comprise screening for successful transformants. Successful transformants may be screened by the use of the selection marker. For example, where the selection marker comprises a β-galactosidase (*lacZ*) gene, the screening may comprise the testing for β-galactosidase activity. Where the selection marker comprises an antibiotic resistance gene, the screening may comprise the testing for ability to grow or survive in the presence of the antibiotic. Other selection

methods may be used, for example restoration of function for auxotrophic mutants. In one embodiment, successful transformants may be selected for by screening in a *ΔlacZ* mutant of *Neisseria lactamica,* for example a *ΔlacZ* mutant of Y92-1009. In one embodiment, successful transformants may be selected for by screening in a *ΔlacZ Δnhba* mutant of *Neisseria lactamica,* for example a *ΔlacZ Δnhba* mutant of Y92-1009.

Advantageously, the use of a non-antibiotic related selection marker, such as β-galactosidase (*lacZ*) provides a more clinically acceptable strain of a modified *Neisseria lactamica* resulting from this method. The resulting modified *Neisseria lactamica* strain may be used as a platform for the generation of outer membrane vesicles (OMV), for example for vaccines. An antibiotic selection marker may be used for strains that may ultimately be used as a platform for the generation of outer membrane vesicles (OMV).

Outer Membrane Vesicle (OMV) vaccine

According to another aspect of the invention, there is provided an outer membrane vesicle (OMV) vaccine, wherein the OMV is an OMV of the modified *Neisseria lactamica* described herein.

The OMV may comprise a protein, or a variant or part thereof, which is heterologous to the modified *Neisseria lactamica*. The heterologous protein may comprise PorA, such as PorA of N. *meningitidis.*

According to another aspect of the invention, there is provided a composition comprising the OMV according to the invention herein; or the modified *Neisseria lactamica* according to the invention herein.

The composition may be a pharmaceutically acceptable composition.

According to another aspect of the invention, there is provided an OMV; modified *Neisseria lactamica*; or composition according to the invention herein for use in a vaccine/vaccination.

According to another aspect of the invention, there is provided a method of vaccination of a subject for the prevention of infection or colonisation of a pathogen comprising the administration of the OMV or the modified *Neisseria lactamica* or the composition according to the invention herein.

The vaccination may be for prevention of infection or colonisation of *Neisseria meningitidis* in the subject.

The skilled person will understand that optional features of one embodiment or aspect of the invention may be applicable, where appropriate, to other embodiments or aspects of the invention.

Embodiments of the invention will now be described in more detail, by way of example only, with reference to the accompanying drawings.

Figure 1: Plasmid map of pUC19*ΔnlaIII*::CLOVER-*aphA3*.

Figure 2: Nucleotide sequence (SEQ ID NO: 3) of tandemly-expressed, *N. lactamica*-codon-optimised *CLOVER* and *aphA3* genes. The sequence of the CLOVER gene is shown as white text against a black background and the *aphA3* sequence is shown as boxed black text. The NotI and NdeI restriction sites are shown as black text against a grey background. The DUS and RBS are shown in lower case letters, with the DUS sequence underlined.

Figure 3: Confocal microscopy of wild type and CLOVER-expressing strains of *Neisseria lactamica* Y91-1009.

Figure 4: Plasmid map of pUC19*ΔnlaIII*::*aphA3*.

Figure 5: Transformation efficiency of wild type *Neisseria lactamica* using (hypermethylated) PCR products amplified from pUC19*ΔnlaIII*::*aphA3*.

Figure 6: Transformation efficiency of wild type *Neisseria lactamica* using (hypermethylated) PCR products amplified from pUC19*ΔnlaIII*::CLOVER-*aphA3* and derivatives thereof, wherein site-directed mutagenesis has been used to remove 'CATG' sequences from the CLOVER coding sequence.

Figure 7: Hypermethylation of PCR product blocks restriction activity of NlaIII.

Figure 8: Effect of the length of the flanking region and amount of DNA used to transform wild type *Neisseria lactamica* using PROTOCOL A.

Figure 9: Effect of the neisserial DNA Uptake Sequence (DUS) on the transformation efficiency of *Neisseria lactamica* using PROTOCOL A.

Figure 10: Chromosomal schematic and nucleotide sequence of NHCIS1 (*N.lactamica* Y92-1009) (SEQ ID NOs: 4 and 5).

Figure 11: Chromosomal schematic and nucleotide sequence of NHCIS2 (*N.lactamica* Y92-1009) (SEQ ID NOs: 6 and 7).

Figure 12: Annotated nucleotide sequence of HAEC1 (SEQ ID NO: 8).

Figure 13: Annotated nucleotide sequence of HAEC2 (SEQ ID NO: 9).

Figure 14: Plasmid map of pUC19NHCIS1::HAEC1:(Z)-*lacZ*.

Figure 15: Plasmid map of pUC19NHCIS1::HAEC2:(Z)-*lacZ*.

Figure 16: Plasmid map of pUC19NHCIS2::HAEC1:(Z)-*lacZ*.

Figure 17: Bar graph showing Specific Activity of *lacZ*, expressed from different NHCIS loci.

Figure 18: Plasmid map of pUC19NHCIS1::HAEC2:*porA*plusprom-*lacZ.*

Figure 19: Expression of PorA in recombinant *N.lactamica* has no appreciable effect on growth rate in TSB.

Figure 20: PorA is surface-expressed in recombinant *N.lactamica* strain 2Pp7.A.

Figure 21: Promoter constructs for investigating transcriptional enhancement by sequence associated with the *N.meningitidis porA* gene (SEQ ID NOs: 10-16).

Figure 22: Transcriptional enhancement of the *lst* promoter by sequence cloned from upstream of the *porA* gene from *Neisseria meningitidis* strain MC58.

Figure 23: Promoter construct designed to investigate the mechanism of *porA* transcriptional enhancement (SEQ ID NO: 17).

Figure 24: DNA bending is partially responsible for the transcriptional enhancement activity of the *porA* enhancer sequence.

Figure 25: Annotated nucleotide sequence of HAEC3 (SEQ ID NO: 18).

Figure 26: Annotated nucleotide sequence of HAEC4 (SEQ ID NO: 19).

Figure 27: Plasmid map of pUC19NHCIS1::HAEC4:*nadA-lacZ*

Figure 28: Plasmid map of pUC19NHCIS1::HAEC4:(Z)-*lacZ*

Figure 29: NadA-expressing *N.lactamica* have increased adherence to HEP-2 cells.

Figure 30: Plasmid map of pSC101NHCIS1::HAEC4:*opc-lacZ*

Figure 31: Plasmid map of pUC19*Δnhba*::HAEC3:(Z)-*lacZ*

Figure 32: Plasmid map of pUC19*Δnhba*

Figure 33: Repeated transformation of *N. lactamica* does NOT select for a more transformable phenotype.

Figure 34: Effect of the amount of donor material used to transform wild type *Neisseria lactamica*: supplemental.

Figure 35: Plasmid map of pSC101::*ΔlacZ:opcA*

Figure 36: Plasmid map of pSC101::*ΔlacZ*-synth.*lacZ*-3'ENDNHCIS1

Figure 37 (SEQ ID NO: 20): Coding sequence of synth.*lacZ*

Figure 38: Expression of NadA and Opc outer membrane proteins on the surface of recombinant strains of *N. lactamica*

Figure 39: Expression of NadA, Opc or a combination of both antigens in recombinant *N. lactamica* has no appreciable effect on growth rates in TSB

Figure 40: NadA-expressing strains of *N. lactamica* have increased adherence to HEP-2 cells, whilst Opc-expressing strains of *N. lactamica* are internalized by HEP-2 cells in significantly larger numbers

Figure 41: The NadA-expressing strain of *N. lactamica*, 4NB1, is recovered in significantly higher numbers from within human nasopharyngeal tissue explants than is the wild type

Figure 42: Recombinant strains of *N. lactamica* expressing meningococcal adhesin proteins have similar sensitivity profiles to front-line antibiotics as the wild type

Figure 43: Recombinant strains of *N. lactamica* expressing meningococcal adhesin proteins are competent for uptake of exogenous DNA and can be transformed with efficiencies similar to the wild type

Figure 44: Wild type and recombinant strains of *N. lactamica* are completely refractory to transformation by chromosomal DNA derived from *N. meningitidis*

Figure 45: Plasmid map of pSC101NHCIS1::HAEC4:*porA*(P1.7,16)-*lacZ*

Figure 46: Plasmid map of pSC101NHCIS1::PV*porA*(P1.7,16)-*lacZ*

Figure 47: Recombinant Nlac strains express Porin A (P1.7, 16) at the cell surface and generate PorA-containing OMV

Figure 48: Immunisation with OMV from recombinant, PorA-expressing Nlac generates anti-meningococcal SBA

Figure 49: Immunisation with OMV from recombinant, NadA-expressing Nlac generates anti-meningococcal SBA

Trials of experimental human nasopharyngeal inoculation with wild type *Neisseria lactamica* strain Y92-1009 have shown that successful colonisation of volunteers by the bacterium can lead to persistent carriage of the organism for up to six months, and that inoculation with the live bacterium also broadens the range of humoral immune responses directed against a narrow panel of *Neisseria meningitidis* isolates. Colonisation of the nasopharynx by *Neisseria lactamica* is completely benign. Therefore, the ability to modify the outer membrane components of *Neisseria lactamica* to include proteins not present in the wild type organism would be extremely beneficial, as it would allow the use of these modified strains as either a means of generating novel

OMV that contain antigens from biotic sources other than *Neisseria lactamica*, or as a safe means of delivering antigen to the nasopharyngeal mucosa for prolonged periods as an alternative means of vaccination.

The most likely cause of the lack of methods to manipulate the genetics of *Neisseria lactamica* is the battery of restriction enzymes encoded in the genome of *Neisseria lactamica*, most notably the 4-cutter restriction enzyme, NlaIII, which cuts dsDNA at the short palindromic sequence 'CATG'. By random chance and in the absence of negative selection pressure, this sequence motif occurs with high frequency in a given stretch of nucleotides, meaning that uptake of DNA containing one or more of these sequences results in the intracellular degradation of the material before homologous recombination can take place and integrate the exogenous DNA into the *Neisseria lactamica* chromosome. This invention describes a process to circumvent the barriers to transformation of *Neisseria lactamica* and allow targeted genetic modification of this organism to be performed for the first time. The invention allows stable integration of DNA constructs into loci of the *Neisseria lactamica* chromosome, with utility for both deletion of existing genes (i.e. targeted mutagenesis) or insertion of genes from other biotic sources. This procedure differs from normal methods used to transform *Neisseria* in its use of hypermethylated PCR products as the donor material, whereby all the deoxycytosine residues of the nucleotide product have been replaced with methyl-deoxycytosine. Methylation of restriction sites can inhibit the DNA cleavage action of restriction endonucleases, which prevents degradation of the product following uptake and therefore provides sufficient time for homologous recombination to take place and the construct to become integrated into the chromosomal locus of choice. The restriction enzyme NlaIII is inhibited in this way (see Figure 7), and the use of hypermethylated PCR products to transform *Neisseria lactamica* is demonstrably more efficient than using non-methylated, 'normal' PCR products (see Figure 6).

A number of cloning vectors have been developed in accordance with the invention. These cloning vectors are based upon the standard cloning vector pUC19, but come in two varieties dependent upon the application. For most routine manipulations and

cloning strategies, plasmids containing the pMB1 origin of replication (*ori*), which maintains a relatively high number of plasmids per cell, can be used. pMB1 is the original *ori* for pUC19, and all plasmids described in accordance with this invention that contain pMB1 are described as pUC19 derivatives (e.g. pUC19*ΔnlaIII*::CLOVER-*aphA3*). In instances where a gene produces a product toxic to *E. coli* (the organism used for the maintenance and amplification of the plasmids), or where high-level expression of a gene is deleterious, a variant plasmid backbone has been developed in which the pMB1 *ori* has been replaced with the minimally required replication region of pSC101 (*repA/ori*). Whilst the rest of the plasmid backbone, including the selection marker, remains unchanged from pUC19, these plasmids are maintained at a much lower copy number per cell (i.e. five). The reduction in copy number may therefore prove permissive for cloning of deleterious sequences with high fidelity. All plasmids that contain the *repA/ori* region of pSC101 are described as pSC101 derivatives (i.e. pSC101NHCIS1::HAEC4:*opcA-lacZ*).

The majority of cloning vectors developed in accordance with this invention contain large regions of homology to the *Neisseria lactamica* chromosome, with added restriction sites for ease of cloning. The regions of homology are designated the *Neisseria* Heterologous Construct Insertion Sites (NHCIS) and are located in areas of relative gene paucity in the chromosome of the vaccine strain of *Neisseria lactamica*, Y92-1009. The purpose of these plasmids is to streamline the process of inserting DNA coding for heterologous antigen(s) into the Y92-1009 chromosome, specifically into loci where there will be minimal disruption of other genes that are perhaps essential or beneficial to the biological fitness of the bacterium. Using these vectors we have also developed derivatives that contain a novel means of screening for successful transformation of *Neisseria lactamica*, whereby the regions of homology flank Heterologous Antigen Expression Constructs (HAEC), each of which consists of two *Neisseria lactamica* promoter sequences surrounded by a number of unique restriction sites for streamlined manipulation and directional cloning of desired sequences for downstream introduction into the Y92-1009 genome. At the current time there are 4 such constructs, designated HAEC1 through HAEC4, named in the order in which they were created. HAEC1 contains the *lst* promoter followed (3') by the *porB* promoter,

and this is reversed in HAEC2. HAEC3 contains a hybrid *porA/porB* promoter, wherein the homopolymeric tract of 'G' nucleotides that renders the wild type *porA* gene phase variable has been replaced with sequence derived from the wild type, non-phase variable *porB* promoter of *N.lactamica*. This promoter is preceded by 250 bp of transcriptional enhancer sequence, derived from the wild type *porA* gene of *N.meningitidis*. The *porA/porB* hybrid promoter is followed (3') by a second hybrid promoter, wherein the 17 bp that separate the -10 and -35 boxes of the RNA Polymerase binding site of the wild type *porB* gene have been replaced with 17 bp of sequence derived from the *lst* promoter. The rest of the promoter is identical to that of the wild type, *N.lactamica porB* gene (Figure 25). HAEC4 contains an identical *porA/porB* hybrid promoter to HAEC3, but which is preceded by only 200 bp of *porA*-derived enhancer sequence. This length of enhancer sequence has been demonstrated to be more potent than the 250 bp version (see Figure 22). In HAEC4, the *lst* promoter follows (3') the highly transcriptionally active hybrid promoter (Figure 26). In each plasmid designed to transform *N. lactamica* in a way suitable for use in human challenge, the second promoter sequence of each HAEC drives a copy of the *Neisseria lactamica* β-galactosidase (*lacZ*) gene, which we have used as a means of screening for successful transformation of a *ΔlacZ* mutant derivative of Y92-1009. To construct the *ΔlacZ* mutant, the procedure described in Protocol A was utilised to completely remove the coding sequence of the *lacZ* gene – thus minimising the potential for off-target recombination of NHCIS-targeted constructs. Taken together, the *ΔlacZ* Y92-1009 derivative and the NHCIS(X)::HAEC(Y):(Z)-*lacZ* vector system constitute a useful tool for generating the recombinant strains of *Neisseria lactamica* described above in a way free from the use of antibiotic resistance cassettes, which would otherwise preclude their use in human challenge studies.

In some examples a *ΔlacZ* mutant derivative of *N. lactamica* Y92-1009 is further genetically manipulated. This strain was created using the transformation protocol detailed in Protocol A. The strain has had all of the coding sequence for the wild type *lacZ* gene removed from the chromosome to avoid non-specific recombination events with the Nlac *lacZ*-containing Cassettes and concomitant mis-targeting of the Cassette to loci other than that intended. This strain provides the background for insertion of

genes coding for heterologous antigens without need for antibiotic resistance markers, a preferred state for potential human challenge with recombinant *Neisseria lactamica*.

When referring to the use of restriction sites for restriction/ligation cloning of sequences into various constructs, the same restriction site must be absent from the oligonucleotide sequence being cloned.

PROTOCOL A: Transformation protocol for *Neisseria lactamica*:

1. Inoculate TSB with multiple colonies of the recipient strain of *Neisseria lactamica* (hereafter, the Culture). This could be a recognised laboratory strain such as Y92-1009, a mutant derivative thereof, or wild type or mutant strains of *N. lactamica* isolated from volunteers.
2. Incubate the Culture at 37 °C, 5 % CO_2 with shaking until an OD_{600nm} is reached of 0.3.
3. Dilute the Culture x100 in fresh TSB.
4. Spot 10 µl aliquots of the x100 dilution of the Culture onto Tryptone Soy Broth + 0.2 % yeast extract agar plates (hereafter, TSB agar) and allow to dry in a Microbiological Safety Cabinet.
5. Replace the lids and transfer the plate(s) (right side up) to a CO_2 incubator set to 30 °C, 5 % CO_2. Incubate for 6 h.
6. Onto the surface of the now-visible *N.lactamica* colonies, pipette 10 µl of Tris HCl buffer containing an appropriate amount of Donor material and allow to dry in a Microbiological Safety Cabinet.
7. Transfer the plate(s) to the incubator at 30 °C, 5 % CO_2 and incubate for 9-10 h.

PROTOCOL A.1: Alternative transformation protocol for *Neisseria lactamica*:

1. Grow the recipient strain of *N. lactamica* on suitable (i.e. growth sustaining) agar plates overnight at 37 °C, 5 % CO_2. This could be a recognised laboratory strain such as Y92-1009, a mutant derivative thereof, or wild type or mutant strains of *N. lactamica* isolated from volunteers.
2. Using a sterile microbiological loop, transfer a multitude of colonies to fresh, sterile TSB medium supplemented with 10 mM Mg^{2+} ions (solutions of either $MgSO_4$ or

$MgCl_2$ are suitable). Continue this process until the OD_{600nm} of the suspension is greater than or equal to 0.3.

3. Transfer aliquots of the concentrated bacterial suspension to fresh, sterile microcentrifuge tubes and adjust the OD_{600nm} to 0.3 using fresh, sterile TSB medium supplemented with 10 mM Mg^{2+}.
4. Prepare the Donor material in sterile PBS. The Donor material could be genomic DNA, a PCR or hypermethylated PCR product, or an artificially synthesized nucleic acid.
5. Add the Donor material to the microcentrifuge tubes, mix by vortexing or pipetting and incubate the tubes at 37 °C, 5 % CO_2 for 3 h.

Continue with Protocol B for constructs using β-galactosidase activity as the means of identifying transformed bacteria; alternatively, plate the suspensions on an appropriately selective agar medium prior to overnight incubation.

PROTOCOL B: Screening for transformed *Neisseria lactamica* containing β-galactosidase as part of the genetic construct:

8. Harvest each patch of putatively-transformed *N. lactamica* into 1 ml of fresh, sterile TSB.
9. Perform a 10x serial dilution of the bacterial suspension in TSB: i.e. x10, $x10^2$ and $x10^3$.
10. Further dilute the $x10^3$ suspension x5 (i.e. 100 µl of 10^3 bacterial suspension in to 400 µl TSB).
11. On 5 separate TSB agar plates supplemented with 20 ng/ml 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (hereafter X-GAL), spread 100 µl of the 5 x 10^{-3} bacterial suspension and allow to dry in a Microbiological Safey Cabinet.
12. Incubate overnight at 37 °C, 5 % CO_2.
13. Isolate each BLUE colony into 1 ml TSB and culture at 37 °C, 5 % CO_2 for production of stocks and isolation of DNA.

Plasmid map of pUC19*ΔnlaIII*::CLOVER-*aphA3*.

With reference to Figure 1, the pUC19*ΔnlaIII::CLOVER-aphA3* Construct is a pUC19-derived plasmid designed to introduce codon-optimised CLOVER and *aphA3*, under the control of the promoter from the α-2,3-Sialyltransterase gene (*lst*), into the ORF of the

restriction enzyme, NlaIII (*nlaIII*, NLY_28620). The Cassette, which has been cloned into the HincII restriction site of pUC19 via Gibson Assembly (NEB), consists of sequence amplified from the region of the *N.lactamica* chromosome containing the *nlaIII* gene, including most of the *nlaIII* coding sequence, into which has been cloned versions of the *CLOVER* and *aphA3* genes that are codon-optimised for expression in *Neisseria lactamica*. Expression of these genes is controlled by a modified *lst* promoter, into the 5' untranslated region (hereafter, 5' UTR) of which was introduced an XhoI restriction site to simplify downstream manipulation of the Cassette. The *CLOVER* and *aphA3* genes are tandemly expressed from this promoter, and are separated by (in 5' to 3' order), a NotI restriction site (to facilitate substitution of the *CLOVER* gene for any other nucleotide sequence), a DUS (to enhance uptake of the Cassette by *N. lactamica* as part of PROTOCOL A), and a modified Ribosome Binding Site (hereafter, RBS) (to ensure translation of mRNA coding *aphA3*) (see Figure 2). Immediately 3' of *aphA3*, the chromosomal sequence has been modified to introduce an XbaI restriction site. This site enables XbaI-digestion of the Construct to release the Cassette, for use as Donor material in PROTOCOL A. The sequence derived from the *N. lactamica nlaIII*-containing chromosomal region also contains an inv. AT-DUS, 5' of the start codon of the *nlaIII* gene. The plasmid map is presented showing all relevant features, including the insertionally-inactivated ORF for *nlaIII*, and detailing the location of unique restriction sites with recognition sequences 6 nucleotides or greater.

Nucleotide sequence of tandemly-expressed, *N. lactamica*-codon-optimised *CLOVER* and *aphA3* genes.

With reference to Figure 2, the sequences of these genes have been codon-optimised for expression in *N. lactamica*. The activity of NlaIII is a significant barrier to successful transformation of *N. lactamica* (see Figure 6). This piece of the Cassette could be controlled from any *N. lactamica*-compatible promoter sequence and be flanked by a contiguous nucleotide sequence from theoretically any locus in the *N. lactamica* chromosome.

Confocal microscopy of wild type and CLOVER-expressing strains of *Neisseria lactamica* Y91-1009.

With reference to Figure 3, wild type *N. lactamica* Y92-1009 was transformed with donor material derived by hypermethylated PCR from pUC19*ΔnlaIII*::CLOVER-*aphA3* (Figure 1), as described in PROTOCOL A. Transformants, which were selected for on the basis of resistance to kanamycin (50 μg/ml in TSB agar plates), were isolated and cultured. Chromosomal integration of the donor material into the *nlaIII* locus was determined by PCR (data not shown). Stationary phase (overnight) colonies of both wild type Y92-1009 (A) and a transformed, putatively CLOVER-expressing derivative thereof (B), were dispersed into sterile PBS, spread across the surface of a microscope slide and allowed to air dry in a Class II microbiological safety cabinet. Bacteria were visualised under the confocal microscope using light at a wavelength of 988 nm and the images digitally captured. It is important to note that whilst the overall level of fluorescence is low, there is green fluorescence of the CLOVER-transformed bacteria, which is not evident in the wild type, parental strain. This is the first demonstration of significant eukaryotic gene expression in *N. lactamica*. Furthermore, this is the first demonstration of heterologous protein expression in this species of bacterium.

Plasmid map of pUC19 *ΔnlaIII*::*aphA3*.

With reference to Figure 4, the pUC19*ΔnlaIII*::*aphA3* Construct is a derivative of pUC19*ΔnlaIII*::*CLOVER-aphA3*, wherein the *aphA3* gene has been placed under direct transcriptional control of the *lst* promoter, and that which remains of the *nlaIII* coding sequence has been further truncated. The sequence derived from the *N. lactamica* *nlaIII*-containing chromosomal region also contains an inv. AT-DUS, 5' of the start codon of the *nlaIII* gene. The plasmid map is presented showing all relevant features, including the insertionally-inactivated ORF for *nlaIII*, and detailing the location of unique restriction sites with recognition sequences 6 nucleotides or greater.

Transformation efficiency of wild type *Neisseria lactamica* using (hypermethylated) PCR products amplified from pUC19*ΔnlaIII*:*aphA3*.

With reference to Figure 5, XbaI-digested pUC19*ΔnlaIII*::*aphA3* was used as a template to amplify the *ΔnlaIII*::*aphA3* Cassette using both traditional (Non-methylated) and 'Hypermethylated' PCR. Traditional PCR uses an equimolar mixture of the four, unmodified deoxyribonucleotides; whereas 'hypermethylated' PCR uses a nucleotide

mixture that substitutes 5-methyl-dCTP instead of unmodified dCTP and results in a PCR product in which every C residue is methylated. The primers used in the reaction produced products of identical sequence with 1200 bp of 'flanking' DNA on either side of the *aphA3* gene. The transformation was carried out according to Protocol A, using a total of 1000 ng of each PCR product (=0.46 pmol). The number of transformants was considered to be equal to the number of kanamycin-resistant colonies that grew overnight on selective agar plates (TSB + 0.2 % yeast extract supplemented with 50 µg/ml kanamycin). Indeed, PCR analysis of the *nlaIII* chromosomal locus from 50 individual, kanamycin resistant colonies derived from transformation with either construct showed that the *aphA3* gene had been accurately targeted. The graph shows that transformation into the *nlaIII* locus is significantly higher through the use of hypermethylated PCR product as compared to an identical, unmodified PCR product. However, it is important to note that the *ΔnlaIII*::*aphA3* Cassette is free of 'CATG' sites, the recognition and cleavage site of the NlaIII restriction enzyme.

Transformation efficiency of wild type *Neisseria lactamica* using (hypermethylated) PCR products amplified from pUC19*ΔnlaIII*::CLOVER-*aphA3* and derivatives thereof, wherein site-directed mutagenesis has been used to remove 'CATG' sequences from the CLOVER coding sequence.

With reference to Figure 6, the coding sequence of the *Neisseria lactamica*-codon optimised CLOVER gene contains two 'CATG' sequences, which is the recognition and cleavage site for the NlaIII restriction enzyme. As a four-cutter restriction enzyme, the frequency of this sequence in exogenous genetic material is likely to be high, and we hypothesise that the restriction activity of NlaIII is one of the main components of the observed resistance of *Neisseria lactamica* to genetic manipulation. To investigate this, a series of plasmids were generated in which one or both of the two 'CATG' sequences had been removed from the CLOVER gene, using the Q5 Site-directed mutagenesis kit (NEB). The remainder of the construct contained no CATG sequences. These plasmids were used as templates for traditional (i.e. Non-methylated) or 'hypermethylated' PCR. Identical primers were used for both types of PCR, producing PCR products with 600 bp of sequence flanking either side of the CLOVER-*aphA3* cassette. Transformation of wild type *Neisseria lactamica* was carried out as described in Protocol A, using 634 ng

(=0.46 pmol) of each PCR or hmPCR product. The number of transformants was considered to be equal to the number of kanamycin-resistant colonies that grew overnight on selective agar plates (TSB + 0.2 % yeast extract supplemented with 50 μg/ml kanamycin), adjusted for dilution and plating volume. Where the transformation efficiency was below the limits of detection, the appropriate data points were calculated as if the transformation derived a single transformant. The graph shows that the transformation efficiency of wild type *Neisseria lactamica* is significantly effected by the presence of 'CATG' sequences, wherein the introduction of a single 'CATG' sequence into an otherwise CATG-free construct reduces the transformation efficiency by an average of 33 fold. The graph also shows that the use of hypermethylated PCR products enhances the transformation efficiency of wild type *Neisseria lactamica*, in keeping with the data presented in Figure 4. Where two 'CATG' sequences are present in the transformation construct, use of hypermethylated PCR product is approximately 4000 times more efficient at transforming wild type *Neisseria lactamica* than using an equivalent, Non-methylated PCR product. Importantly, there are no differences in the transformation efficiencies measured using any of the hypermethylated PCR products, which implies complete blocking of all restriction endonuclease recognition and cleavage sites. Hypermethylation is also likely to block the restriction function of other endonuclease enzymes in *Neisseria lactamica*, as transforming bacteria with a hypermethylated PCR product derived from the CATG-free template was on average 22 times more efficient than using a Non-methylated PCR product.

Hypermethylation of PCR product blocks restriction activity of NlaIII.

With reference to Figure 7, the series of pUC19*ΔnlaIII*::CLOVER-*aphA3* plasmids, each with a different number of 'CATG' motifs within the CLOVER coding sequence, were used as templates in both normal (i.e. Non-methylated) and hypermethylated PCR. Amplification of the *ΔnlaIII*::CLOVER-*aphA3* Cassettes was followed by *in vitro* digestion of 1 μg of each with recombinant NlaIII (NEB) in Cutsmart buffer. Two hundred nanograms of each digested product was then loaded onto a 0.7% agarose gel and electrophoresis was carried out to separate any products. An inverted gel image was captured from the UV transilluminator using a digital camera. Whilst each hypermethylated PCR product (Hm) ran at a slightly increased molecular weight cf. its

non-methylated equivalent (N), the former was completely protected from NlaIII enzymatic digest.

Effect of the length of the flanking region and amount of DNA used to transform wild type *Neisseria lactamica* using PROTOCOL A.

With reference to Figure 8, to investigate the effect of the size of the regions of homology to the *N. lactamica* chromosome on transformation efficiency (A), a series of hypermethylated PCR products of different lengths were derived from XbaI-digested pUC19*ΔnlaIII*::*aphA3* plasmid. In each product the *aphA3* gene and its promoter were identical, whilst the length of the *nlaIII* chromosomal regions surrounding this sequence was varied. The flanking lengths used in this experiment (i.e. the length of the DNA sequence on each side of the *aphA3* gene) were: 75 bp, 150 bp, 300 bp, 600bp and 1200 bp. As shown in Figures 6 and 7, the fact that the PCR products were hypermethylated ruled out the presence of 'CATG' sequences in shorter products as a confounding factor, as it is likely that all restriction endonuclease recognition and cleavage sites were blocked by the inclusion of mdCTP. Transformation of wild type *Neisseria lactamica* was carried out according to Protocol A, using 0.46 pmol of each hypermethylated PCR product. The number of transformants was considered to be equal to the number of kanamycin-resistant colonies that grew overnight on selective agar plates (TSB + 0.2 % yeast extract supplemented with 50 μg/ml kanamycin), adjusted for dilution and plating volume. Graph A shows that with increasing length of the Cassette flanking sequence, there is an increase in the transformation efficiency of *Neisseria lactamica* into the *nlaIII* locus. However, the presence of longer flanking sequences results in increased inter-experimental variation in transformation efficiency.

To investigate the effect of the amount of donor DNA on the transformation efficiency of *Neisseria lactamica* (B), increasing amounts of hypermethylated PCR product with 600 bp of flanking sequence were used as described in Protocol A. This PCR product was used because there is inherently less inter-experimental variability then when using a product that contains 1200 bp of flanking sequence, implying a better signal-to-noise ratio in the event the effect was a subtle one. Wild type *Neisseria lactamica* were transformed using 0.06, 0.26, 0.46, 0.66, 0.86 and 1.06 pmol of hypermethylated PCR

product. The number of transformants was considered to be equal to the number of kanamycin-resistant colonies that grew overnight on selective agar plates (TSB + 0.2 % yeast extract supplemented with 50 μg/ml kanamycin), adjusted for dilution and plating volume. Graph B shows that there is a potentially bi-phasic peak in transformation efficiency, although the mechanism for this remains even theoretically unclear. The only significant difference in transformation efficiency is between the transformations carried out using 0.26 and 0.46 pmol of DNA, where the lower amount of DNA yields the most transformants. However, this is most probably a statistical anomaly that we predict will disappear with repetition of the experiment.

Effect of the neisserial DNA Uptake Sequence (DUS) on the transformation efficiency of *Neisseria lactamica* using PROTOCOL A.

With reference to Figure 9, the non-palindromic repeat sequence 5' – GCCGTCTGAA – 3' (SEQ ID NO: 1), or close derivatives/relatives thereof, occurs with high frequency in the chromosomes of many *Neisseria* species. Previous studies have demonstrated that bacterial uptake of DNA molecules containing these sequences is enhanced, hypothetically through a charge interaction with a positively charged region of ComP, a pilus-associated protein. The effect of the standard DNA Uptake Sequence (hereafter, DUS) on transformation efficiency is further enhanced by the presence of 'AT' nucleotides at the 5' end. The AT-variant of the DUS (hereafter, AT-DUS) is the 'dialect' of DUS repeated at the highest frequency in the wild type *Neisseria lactamica* chromosome. The *nlaIII* chromosomal region included in pUC19*ΔnlaIII::aphA3* contains an inverted AT-DUS sequence in its 5' end. As a result, the primer pairs used to generate the PCR products containing 300 bp, 150 bp and 75 bp of flanking *nlaIII* chromosomal sequence (see Figure 6) each contain only one copy of the DUS (that which was deliberately included after the *aphA3* coding sequence), as compared to the PCR products containing 600 bp and 1200 bp of flanking *nlaIII* sequence, which contain two. To investigate the role of an additional AT-DUS on the transformation efficiency of wild type *Neisseria lactamica*, two versions of each PCR product were amplified from XbaI-digested pUC19*ΔnlaIII:aphA3*, using alternative 5' primers: one primer in each set contained a canonical, inverted AT-DUS at the 5' terminus (DUS, black circles), whilst the alternate version contained a scrambled DUS (S-DUS, red

squares). The S-DUS contains the same proportions of the same nucleotides as the DUS, but in a configuration designed to ensure no similarity to the various dialects of DUS. Transformation of wild type *Neisseria lactamica* was carried out as described in Protocol A, using 0.46 pmol of each PCR or hmPCR product. The number of transformants was considered to be equal to the number of kanamycin-resistant colonies that grew overnight on selective agar plates (TSB + 0.2 % yeast extract supplemented with 50 μg/ml kanamycin), adjusted for dilution and plating volume. The graph shows that inclusion of a second DUS in the PCR products used to transform *Neisseria lactamica* increases the transformation efficiency, and that this effect is more enhanced (to the point of becoming statistically significant) with increasing flanking sequence length.

Chromosomal schematic and nucleotide sequence of NHCIS1 (*N. lactamica* Y92-1009).

With reference to Figure 10, the schematic shows the chromosomal locus of NHCIS1 and its surrounding genes, which are shaded (greyscale) on the basis of GC nucleotide pair content (modified and reproduced from: http://www.xbase.ac.uk/genome/neisseria-lactamica-y92-1009/CACL01000001/NLY_27080;/viewer). The nucleotide sequences of the NHCIS1 regions are as detailed on the plasmid map for pUC19NHCIS1::HAEC1:(Z)-*lacZ* (Figure 14) (i.e. 5'ENDNHCIS1 and 3'ENDNHCIS1).

Chromosomal schematic and nucleotide sequence of NHCIS2 (*N. lactamica* Y92-1009).

With reference to Figure 11, the schematic shows the chromosomal locus of NHCIS2 and its surrounding genes, which are shaded (greyscale) on the basis of GC nucleotide pair content (modified and reproduced from: http://www.xbase.ac.uk/genome/neisseria-lactamica-y92-1009/CACL01000018/NLY_36160;/viewer). The sequences of the NHCIS2 regions as detailed on the plasmid map for pUC19NHCIS2::HAEC1:(Z)-*lacZ* (Figure 16) (i.e. 5'ENDNHCIS2 and 3'ENDNHCIS2). The *italicised* text represent nucleotides comprising part of the coding sequence for NLY_36160. The 5' NHCIS2 sequence contains an AT-DUS and an inv-AT-DUS, which are highlighted in bold text.

Annotated nucleotide sequence of HAEC1.

With reference to Figure 12, the sequence consists of the *N. lactamica lst* promoter, preceded by a DUS and a SalI restriction site (to facilitate cloning and replacement of the *lst* promoter sequence), separated from the *N. lactamica porB* promoter sequence by an XhoI restriction site, an interchangeable LINKER sequence and a NotI restriction site. It is important to note that either LINKER sequence can be any given nucleotide sequence (represented as poly-N). By including an XhoI restriction site immediately 5' of the START codon and a NotI restriction site immediately 3' of the STOP codon of any given coding sequence (e.g. through the use of extended primer sequences that incorporate these sites), that sequence can be directionally cloned, IN-FRAME into the Construct for expression from the *lst* promoter. The featured NdeI restriction site can be used in conjunction with the HindIII restriction site present immediately 5' of the *porB* terminator sequence to clone any given coding sequence into the Construct. By including an NdeI restriction site immediately 5' of the START codon and a HindIII restriction site immediately 3' of the STOP codon of any given coding sequence (e.g. through the use of extended primer sequences that incorporate these sites), that sequence can be directionally cloned, IN-FRAME into the Construct for expression from the *porB* promoter. Immediately after the HindIII restriction site, there is a transcriptional terminator sequence, derived from downstream of the *N. lactamica porB* gene. This is immediately followed by an AT-DUS. In the context of heterologous antigen expression in recombinant *Neisseria lactamica*, HAEC1 is flanked on either side by sequences homologous to the *N. lactamica* chromosome (e.g. NHCIS1).

Annotated nucleotide sequence of HAEC2.

With reference to Figure 13, the sequence consists of the *N. lactamica porB* promoter, preceded by a DUS and a SalI restriction site (to facilitate cloning and replacement of the *lst* promoter sequence), separated from the *N. lactamica lst* promoter sequence by an XhoI restriction site, an interchangeable LINKER sequence and a NotI restriction site. It is important to note that either LINKER sequence can be any given nucleotide sequence (represented as poly-N). By including an XhoI restriction site immediately 5' of the START codon and a NotI restriction site immediately 3' of the STOP codon of

any given coding sequence (e.g. through the use of extended primer sequences that incorporate these sites), that sequence can be directionally cloned, IN-FRAME into the Construct for expression from the *porB* promoter. The featured NdeI restriction site can be used in conjunction with the HindIII restriction site present immediately 5' of the *porB* terminator sequence to clone any given coding sequence into the Construct. By including an NdeI restriction site immediately 5' of the START codon and a HindIII restriction site immediately 3' of the STOP codon of any given coding sequence (e.g. through the use of extended primer sequences that incorporate these sites), that sequence can be directionally cloned, IN-FRAME into the Construct for expression from the *lst* promoter. Immediately after the HindIII restriction site, there is a transcriptional terminator sequence, derived from downstream of the *N. lactamica porB* gene. This is immediately followed by an AT-DUS. In the context of heterologous antigen expression in recombinant *Neisseria lactamica*, HAEC2 is flanked on either side by sequences homologous to the *N. lactamica* chromosome (e.g. NHCIS1).

Plasmid map of pUC19NHCIS1::HAEC1:(Z)-*lacZ*.

With reference to Figure 14, the pUC19NHCIS1::HAEC1:(Z)-*lacZ* plasmid consists of a Cassette, comprised of the intergenic chromosomal sequence between NLY_ 27080 and NLY_27100 of *N. lactamica* (i.e. NHCIS1, see Figure 10), disrupted by HAEC1:(Z)-*lacZ* (where Z represents any given coding sequence) (Figure 12); cloned into pUC19 between two XbaI restriction sites. In one aspect of the invention, the *N. lactamica lacZ* gene is included as a screening marker, to enable identification of successfully transformed *N. lactamica* on the basis of BLUE/WHITE colony formation on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). This is deemed essential to the production of strains of *N. lactamica* for potential inoculation into human volunteers. Immediately 3' of the *lacZ* gene the Construct includes the terminator sequence from the *N. lactamica porB* gene, to ensure there is no translation of downstream, cistronic transcripts. Immediately 5' to the *porB* terminator sequence there is a unique HindIII restriction site, to facilitate removal of the *lacZ* gene from the Construct and its substitution with potentially any other oligonucleotide sequence. In other aspects of the invention, the Construct can be manipulated to introduce either one or two of potentially any given oligonucleotide sequence into the chromosome of *N.*

lactamica at the NHCIS1 locus, although it is anticipated that one of the two sequences encodes a marker gene to enable screening or selecting for successfully transformed *N. lactamica*. The marker gene could plausibly encode proteins conferring antibiotic resistance, but it is preferable to avoid the use of such markers for producing strains intended for human challenge. The plasmid map is presented showing all relevant features and detailing the location of unique restriction sites with recognition sequences 6 nucleotides or greater.

Plasmid map of pUC19NHCIS1::HAEC2:(Z)-*lacZ*.

With reference to Figure 15, the pUC19NHCIS1::HAEC2:(Z)-*lacZ* Construct contains many elements identical to pUC19NHCIS1::HAEC1:(Z)-*lacZ*; except that HAEC1 (Figure 12) has been replaced by HAEC2 (Figure 13). The functional difference of this change is that the expression of any gene of interest cloned correctly into this plasmid will be driven by the *N. lactamica porB* promoter, whilst the expression of *lacZ* is driven by the *N. lactamica lst* promoter. The plasmid map is presented showing all relevant features and detailing the location of unique restriction sites with recognition sequences 6 nucleotides or greater.

Plasmid map of pNHCIS2::HAEC1:(Z)-*lacZ*.

With reference to Figure 16, the pNHCIS2::HAEC1:(Z)-*lacZ* plasmid consists of a Cassette, comprised of the intergenic chromosomal sequence between NLY_ 36160 and NLY_36180 of *N. lactamica* (i.e. NHCIS2, see Figure 11), disrupted by HAEC1:(Z)-*lacZ* (where Z represents any given coding sequence) (Figure 12); cloned into a truncated version of pUC19 between two XbaI restriction sites. The plasmid map is presented showing all relevant features and detailing the location of unique restriction sites with recognition sequences 6 nucleotides or greater.

Specific Activity of *lacZ*, expressed from different NHCIS loci.

With reference to Figure 17, a *ΔlacZ* mutant derivative of *N. lactamica* Y92-1009 was transformed with Cassettes derived from either pUC19NHCIS1::HAEC1:(Z)-*lacZ* or pNHCIS2::HAEC1:(Z)-*lacZ* according to the procedure detailed in PROTOCOL A. Chromosomal integration of HAEC1:(Z)-*lacZ* into either NHCIS1 (Strain NHCIS1) or

NHCIS2 (Strain NHCIS2) was confirmed by PCR of the respective locus (data not shown) and functionality of the *lacZ* gene was confirmed by growth of the strains as BLUE colonies on Tryptone Soy Broth + 0.2 % yeast extract agar plates (hereafter, TSB agar), supplemented with X-Gal (20 ng ml^{-1}). Wild type Y92-1009, the *ΔlacZ* mutant derivative thereof and Strains NHCIS1/2 were cultured in Tryptone Soy Broth, supplemented with 0.2 % yeast extract (hereafter, TSB) at 37 °C, 5 % CO_2 and 320 rpm, until reaching mid-log phase (OD_{600nm} = 0.3). Bacteria were pelleted by centrifugation, washed once in sterile PBS then resuspended into 200 µl of Bacterial Lysis Buffer (BLB) before being lysed through sonication. The supernatant was diluted 5x with BLB, and 5, 2 and 1 µl aliquots were assayed for B-galactosidase activity using the chromogenic substrate ONPG. The activities in each of these aliquots were averaged to provide a value for each sample in each experiment. The protein concentration of each supernatant was measured using the DC Protein Assay Reagent (Bio Rad) and these values were used to normalise the measured β-galactosidase activities (yielding Specific Activity). Values shown are the mean of three independent experiments. Error bars represent the standard error of the mean. Where no error bars are visible, they fall within the line at the top of the column.

These data show that gene expression, driven by identical promoter sequences, is different when the same gene construct is chromosomally integrated into different loci. Assuming that β-galactosidase specific activity is proportional to the amount of β-galactosidase enzyme present, which is itself proportional to the level of transcriptional activity from the *porB* promoter sequence; the graph shows a significantly higher β-galactosidase activity, indicative of increased levels of gene transcription, were measured in Strain NHCIS2 than in Strain NHCIS1. In both instances expression of the *lacZ* gene is being controlled by the *porB* promoter. In the wild type and *ΔlacZ* strains, β-galactosidase expression is being controlled by its native, inducible *lac* promoter, except that the coding sequence for β-galactosidase is missing in the *ΔlacZ* strain, eliminating almost all of β-galactosidase activity. This graph shows that the choice of locus for chromosomal integration of genes coding for heterologous antigen is important. Based on these data; if a high level of protein expression is desired, then the gene coding for the heterologous antigen could be targeted to NHCIS2; conversely,

genes coding for either potentially toxic products, or proteins that have a detrimental, concentration-dependent effect on the host organism should be targeted to NHCIS1, where the overall level of gene activity is lower.

Plasmid map of pUC19NHCIS1::HAEC2:*porA*plusprom-*lacZ*

With reference to Figure 18, the pUC19NHCIS1::HAEC2:*porA*plusprom-*lacZ* plasmid is a derivative of pUC19NHCIS1::HAEC2:(Z)-*lacZ* (Figure 15), whereby the *porA* gene sequence, derived from *Neisseria meningitidis* strain H44/76, has been cloned, together with the native *porA* promoter and 107 bp of upstream chromosomal sequence, in between the SalI and NotI restriction sites of pUC19NHCIS1::HAEC2:(Z)-*lacZ*. Note that the plasmid map is based upon the *ideal* sequence for this plasmid, wherein the homopolymeric 'G' tract, which separates the -10 and -35 boxes of the native *porA* promoter contains 11 contiguous guanosine nucleotide residues. The plasmid map is presented showing all relevant features and detailing the location of unique restriction sites with recognition sequences 6 nucleotides or greater.

Expression of PorA in recombinant *N. lactamica* has no appreciable effect on growth rate in TSB.

With reference to Figure 19, the *ΔlacZ* mutant derivative of *N. lactamica* Y92-1009 was transformed with donor material derived by hypermethylated PCR from pUC19NHCIS1::HAEC2:*porA*plusprom-*lacZ* (Clone ♯7), as described in PROTOCOL A. Individual transformants, screened for on the basis of BLUE/WHITE colony formation on X-gal-containing TSB agar plates, were isolated. Chromosomal integration of the HAEC2:*porA*plusprom-*lacZ* cassette into the NHCIS1 locus was demonstrated through PCR (data not shown). Following DNA sequencing of the *porA* coding sequence in this locus, Strain A (hereafter: 2Pp7.A) was determined to contain genetic material with 100 % identity to the *porA* gene.

Overnight cultures of wild type *N. meningitidis* strain, H44/76 (grown in Mueller Hinton Broth: MHB), the *ΔlacZ* mutant derivative of *N. lactamica* strain, Y92-1009 and the recombinant *N. lactamica* strain, 2Pp7.A (both grown in TSB + 0.2 % yeast extract), were washed once in PBS then resuspended into Bacterial Lysis Buffer (hereafter;

BLB) supplemented with 100 μg/ml lysozyme, protease inhibitor cocktail and 1 mM PMSF. Suspensions were incubated at 30 °C for 30 minutes before the bacteria were lysed via sonication on ice (3 x 15 second bursts). Sonicated lysates were supplemented with 2 μl of a 5 x dilution of rDNase and adjusted to a final [Mg^{2+}] of 2 mM before a further 30 minute incubation at 30 °C. Bacterial membrane fractions were collected by centrifugation at 17,000 *g* for 30 minutes at 4 °C, then resolubilised for 10 minutes at 95 °C into 1 x LDS reducing sample buffer (Life Technologies). Insoluble material was removed by centrifugation at 17,000 *g* for 10 minutes at 4 °C, following which the protein concentration of the solubilised membrane fraction was measured using the *RC DC* Protein Assay kit from Bio Rad. A total of 50 μg of protein was loaded into each well of a 4-12 % polyacrylamide gel and proteins were separated by electrophoresis. The proteins were then transferred to a methanol-activated PVDF membrane and the membrane was blocked using 5 % milk-TBS for 1 h. The membrane was interrogated with a 1:1000 dilution of SM300 (anti-PorA P 1,7.16 monoclonal antibody) in 5 % milk-TBS and an anti-mouse IgG-HRP conjugate (1:10,000 dilution in 5 % milk-TBS). Washed membranes were exposed to ECL reagents, and bands were visualised on photographic film.

The Western blot (*left*) of *Neisseria* membrane fractions shows that the recombinant strain of *N. lactamica*, 2Pp7.A produces a membrane-associated protein that is recognised by the SM300 MAb, similar to but in much less abundance than the meningococcal strain known to express the cognate PorA protein, H44/76. The absence of a similar band in the membrane fraction derived from the *ΔlacZ* mutant derivative of *N. lactamica* Y92-1009 suggests that this band is the PorA protein. This is, to the best of our knowledge, the first example of meningococcal gene expression in *N. lactamica*. The comparitive growth curve (*right*) of different *N. lactamica* strains in TSB medium supplemented with 0.2 % yeast extract shows that the expression of PorA by 2Pp7.A is not detrimental to the aerobic growth of the bacterium.

It is important to note that the relatively low levels of PorA expression in strain 2Pp7.A (as compared to wild type *N. menigitidis* strain H44/76), are attributable to the phase-variable nature of the endogenous *porA* promoter. Sequencing of the *porA* promoter

region from the chromosome of 2Pp7.A revealed that the homopolymeric 'G' tract that separates the -10 and -35 boxes (RNA Polymerase binding sites) in this promoter was only 10 nucleotides long. Previous work has shown that when replicating this promoter, slipped strand mispairing mutagenesis can lead to variation in the number of 'G' residues in this tract, resulting in a heterologous bacterial population. Whilst a tract length of 10 nucleotides is permissive for gene expression in *N. meningitidis*, the overall levels of gene expression are lower than from *porA* promoters that contain a tract length of 11 nucleotides (the optimum length). Future iterations of recombinant *N. lactamica* strains expressing PorA will need to either contain non phase variable promoters or have the optimum number of 'G' residues in the homopolymeric tract.

PorA is surface-expressed in recombinant *N. lactamica* strain 2Pp7.A

With reference to Figure 20, both wild type *N. lactamica* strain Y92-1009 and its recombinant derivative, the PorA (P1.7,16) expressing strain, 2Pp7.A, were cultured to mid-log phase (OD_{600nm} = 0.4) in TSB supplemented with 0.2 % yeast extract. Aliquots of each culture containing 1 x 10^7 cfu were transferred to fresh microcentrifuge tubes and washed twice in PBS containing 5 % foetal calf serum (hereafter, Wash Buffer). Washed bacteria were resuspended into 100 µl of Wash Buffer, supplemented with a 1:100 dilution of SM300 MAb and incubated at 4 °C for 30 minutes. The primary Mab was removed by washing twice with 1 ml of Wash Buffer, and the bacteria were resuspended into 100 µl of Wash Buffer containing a 1:100 dilution of anti-mouse IgG-AlexaFluor488. The secondary antibody was allowed to bind over the course of 30 minutes at 4 °C, before the bacteria were again washed twice in Wash Buffer. After labelling, the bacteria were resuspended into 100 µl formalin and were fixed for 10 minutes at room temperature. Once formalin had been removed and the labelled, fixed bacteria washed twice more in Wash Buffer, they were resuspended into 200 µl of Wash Buffer and transferred to FACS tubes for quantitative analysis of AlexaFluor488 fluorescence by Flow Cytometry.

The graph shows that the Mean fluorescence intensity of the sample composed of the recombinant strain of *N. lactamica* (2Pp7.A; BLUE plot), is greater than the Mean fluorescence intensity of the sample composed of wild type *N. lactamica* (BLACK

plot). This indicates surface expression of the target for SM300, which is most plausibly the (P1.7, 16) PorA protein. Together with the data from Figure 19, these data show that the gene for the meningococcal-derived antigen *porA* has been successfully introduced into the chromosome of *N. lactamica* strain Y92-1009, that the PorA protein is associated with the membrane of the bacterium, and that the PorA protein is expressed on the surface of recombinant strain 2Pp7.A.

Promoter constructs for investigating transcriptional enhancement by sequence associated with the *N. meningitidis porA* gene

With reference to Figure 21, in previous work conducted on the expression of the *porA* gene in *N. meningitidis*, it was remarked upon that the sequence immediately 5' of the -35 box of the *porA* promoter bore resemblance to *transcriptional enhancer elements*, originally characterised in *E. coli*. These sequences are characterised by multiple poly-'A' and/or poly-'T' nucleotide tracts, which confers on those molecules an ability to physically bend. It has been shown that such DNA bending brings distal DNA sequence elements into close proximity, such that proteins bound to these sequence elements are brought together to interact and potentially to form complexes. To investigate the role of a putative *porA* enhancer sequence on gene expression, a series of plasmids were developed in which the *N. lactamica lacZ* gene was placed under the transcriptional control of the *lst* promoter and targeted to NHCIS1 (Figure 10). Other plasmids in this series were otherwise identical, but also included incrementally larger sequences derived from immediately upstream of the -35 box from the *porA* promoter in *N. meningitidis* strain MC58. The length of the putative enhancer sequence preceding and abutted to the *lst* promoter ranged from 50 bp to 400 bp (GREY text). In the diagram, each promoter and enhancer sequence shows restriction sites as black text against a boxed, grey background, the -10 and -35 RNA Polymerase binding sites as white text against a black background and the *lst* 5' UTR as lower case letters, the 17 bp separating the -10 and -35 boxes of the *lst* promoter are underlined. The half site shown, CAT, is from the NdeI restriction site immediately preceding the *lacZ* coding sequence. The second half of this site is the ATG START codon of the *lacZ* gene.

With the 'naked' (i.e. unenhanced) *lst* promoter providing a basal level of *lacZ* gene

activity in the NHCIS1 locus, it was hypothesised that any enhancement to gene expression mediated through the upstream, putative enhancer sequences would lead to increased *lacZ* gene expression, higher amounts of β-galactosidase per bacterium and therefore increased levels of β-galactosidase Specific Activity.

Transcriptional enhancement of the *lst* promoter by sequence cloned from upstream of the *porA* gene from *Neisseria meningitidis* strain MC58.

With reference to Figure 22, the series of plasmids containing the *N. lactamica lacZ* gene under transcriptional control of the *lst* promoter and incrementally larger, putative enhancer sequences derived from chromosomal sequence immediately 5' to the -35 box of the *porA* gene of *N. meningitidis* strain MC58, were used as PCR templates for the generation of donor genetic material with which to transform the *ΔlacZ* mutant derivative of *N. lactamica*. Transformation was carried out using hypermethylated PCR product as described in PROTOCOL A. Individual transformants, screened for on the basis of BLUE/WHITE colony formation on X-gal-containing TSB agar plates, were isolated. Chromosomal integration of the *lst*(X)::*lacZ* cassettes into the NHCIS1 locus was demonstrated through PCR (data not shown). Following DNA sequencing of the *lacZ* promoter sequences in this locus, individual clones containing constructs with identical sequences were pooled.

Recombinant *N. lactamica* strains containing the *lst* promoter-driven *lacZ* gene, were cultured in TSB at 37 °C, 5 % CO_2 and 320 rpm, until reaching mid-log phase (OD_{600nm} = 0.3). Bacteria were pelleted by centrifugation, washed once in sterile PBS then resuspended into 200 µl of BLB supplemented with protease inhibitor cocktail, before being lysed through sonication (3 x 15 seconds pulses) on ice. Immediately after the last round of sonication, lysates were supplemented to a final concentration of 1 mM PMSF. Cell debris was removed by centrifugation at 17,000 *g* for 10 minutes ands the supernatant transferred to a fresh microcentrifuge tube. The supernatant was diluted 5x with BLB, and 5, 2 and 1 µl aliquots were assayed for β-galactosidase activity using the chromogenic substrate ONPG. The activities in each of these aliquots were averaged to provide a value for each sample in each experiment. The protein concentration of each supernatant was measured using the DC Protein Assay Reagent (Bio Rad) and these

values were used to normalise the measured β-galactosidase activities (yielding Specific Activity). Values shown are the mean of three independent experiments. Error bars represent the standard error of the mean. Where no error bars are visible, they fall within the line at the top of the column.

These data show that the sequence immediately upstream of the RNA Polymerase binding site of the meningococcal *porA* gene acts as a transcriptional *enhancer*. The native *lst* promoter provides a baseline level of β-galactosidase activity when *lacZ* is expressed from this promoter at the NHCIS1 locus, but the Specific Activity of β-galactosidase is significantly increased when the upstream sequence of nucleotides is at least 150 bp long and optimal at 200 bp. Further increases to the length of the enhancer sequence disrupt its function in the NHCIS1 context and leads to a reduced Specific Activity in these samples (i.e. at 250 and 400 bp).

Promoter construct designed to investigate the mechanism of *porA* transcriptional enhancement

With reference to Figure 23, to investigate whether DNA bending is responsible for the enhancement of gene expression observed in Figure 22, site-directed mutagenesis using the Q5 site-directed mutagenesis kit (NEB) was employed to introduce 5 additional nucleotides into the junction between the enhancer sequence (GREY text) and the -35 box of the *lst*(200):*lacZ* promoter. The additional five residues, along with the ultimate 'A' residue of the enhancer region (shown as white text), constitute a novel restriction site for NsiI in this construct (shown as black text in grey box: "ATGCAT"). The resulting construct was termed NHCIS1::*lst*(200+5):*lacZ*. The presence of an additional 5 nucleotides at the junction of the enhancer and promoter sequences has the effect of turning the enhancer region through half a helix relative to the promoter, given there are 10-11 nucleotide residues per turn of the DNA helix. This hypothetically reverses the directionality of the DNA bending, such that the DNA still bends, but in the opposite direction to the original construct. The consequence of this is that any distal sequence elements and their associated proteins, which would normally be brought into close association with RNA Polymerase and act to enhance open complex formation, would instead be brought into close association with the non-coding strand of the DNA, on the

opposite 'face' of the DNA to RNA Polymerase. It was hypothesised that, if the *porA* enhancer sequence functions through a DNA bending phenomenon, then there will be a significantly lower level of β-galatosidase Specific Activity in *N. lactamica* transformed with the *lst*(200+5):*lacZ* construct, as compared to *N. lactamica* transformed with *lst*(200):*lacZ*. In the diagram, restriction sites are shown as black text against a boxed, grey background, the -10 and -35 RNA Polymerase binding sites are shown as white text against a black background and the *lst* 5' UTR as lower case letters, with the 17 bp separating the -10 and -35 boxes of the *lst* promoter underlined. The half site shown, CAT, is from the NdeI restriction site immediately preceding the *lacZ* coding sequence. The second half of this site is the ATG START codon of the *lacZ* gene.

DNA bending is at least partially responsible for the transcriptional enhancement activity of the sequence upstream of the *N. meningitidis porA* gene.

With reference to Figure 24, the *ΔlacZ* mutant derivative of *N. lactamica* strain Y92-1009 and the recombinant *N. lactamica* strains *lst*:*lacZ*, *lst*(200):*lacZ* and *lst*(200+5):*lacZ*, were cultured in TSB at 37 ^{o}C, 5 % CO_2 and 320 rpm, until reaching mid-log phase (OD_{600nm} = 0.3). Bacteria were pelleted by centrifugation, washed once in sterile PBS then resuspended into 200 µl of BLB supplemented with protease inhibitor cocktail, before being lysed through sonication (3 x 15 seconds pulses) on ice. Immediately after the last round of sonication, lysates were supplemented to a final concentration of 1 mM PMSF. Cell debris was removed by centrifugation at 17,000 *g* for 10 minutes and the supernatant transferred to a fresh microcentrifuge tube. The supernatant was diluted 5x with BLB, and 5, 2 and 1 µl aliquots were assayed for β-galactosidase activity using the chromogenic substrate ONPG. The activities in each of these aliquots were averaged to provide a value for each sample in each experiment. The protein concentration of each supernatant was measured using the DC Protein Assay Reagent (Bio Rad) and these values were used to normalise the measured β-galactosidase activities (yielding Specific Activity). Values shown are the mean of three independent experiments. Error bars represent the standard error of the mean. Where no error bars are visible, they fall within the line at the top of the column.

These data show that the addition of 5 extra nucleotides at the junction of the 200

nucleotide-long *porA* transcriptional enhancer and the -35 box of the *lst* promoter reduces by approximately 50 % the Specific Activity of β-galactosidase measured in bacterial lysates. The graph shows that the addition of the enhancer sequence to the 5' end of the *lst* promoter results in a large increase in β-galactosidase Specific Activity, as compared to bacteria expressing *lacZ* from the 'naked' (i.e. unenhanced) *lst* promoter. Despite being otherwise identical to the *lst*(200):*lacZ* construct, the presence of 5 extra nucleotides in the *lst*(200+5):*lacZ* construct significantly reduces the Specific Activity of β-galactosidase, indicating less transcriptional activity from this promoter construct.

Annotated nucleotide sequence of HAEC3.

With reference to Figure 25, this Cassette has been designed to enable non-phase variable, high level gene expression of heterologous antigen. The sequence consists of a modified *N. meningitidis porA* promoter, wherein the phase variable, 17 bp tract that separates the -10 and -35 boxes in the wild type *porA* promoter has been replaced with 17 bp of sequence identical to that which separates the -10 and -35 boxes of the *N. lactamica porB* promoter. The -35 box of the modified *porA* promoter is preceded by 250 bp of the *porA*-derived transcriptional enhancer sequence (Figure 22), and most distally a SalI restriction site, to facilitate cloning and replacement of the promoter sequence (when used in conjunction with the XhoI restriction site). The modified, optimally-enhanced *porA* promoter is separated from the 3', modified *N. lactamica porB* promoter sequence by an XhoI restriction site, an interchangeable LINKER sequence and a NotI restriction site. It is important to note that either LINKER sequence can be any given nucleotide sequence (represented as poly-N). It is also important to note that the 17 bp sequence separating the -10 and -35 boxes of the RNA Polymerase binding site in this *porB* promoter have been replaced by 17 bp of sequence derived from the *lst* promoter. This is to avoid recombination between identical sequences within the Cassette that might otherwise lead to loss of the gene coding for heterologous antigen. By including an XhoI restriction site immediately 5' of the START codon and a NotI restriction site immediately 3' of the STOP codon of any given coding sequence (e.g. through the use of extended primer sequences that incorporate these sites), that sequence can be directionally cloned, IN-FRAME into the Construct for expression from the modified, optimally-enhanced *porA* promoter. The

featured NdeI restriction site can be used in conjunction with the HindIII restriction site present immediately 5' of the *porB* terminator sequence to clone any given coding sequence into the Construct. By including an NdeI restriction site immediately 5' of the START codon and a HindIII restriction site immediately 3' of the STOP codon of any given coding sequence (e.g. through the use of extended primer sequences that incorporate these sites), that sequence can be directionally cloned, IN-FRAME into the Construct for expression from the *lst* promoter. Immediately after the HindIII restriction site, there is a transcriptional terminator sequence, derived from downstream of the *N. lactamica porB* gene. This is immediately followed by an AT-DUS. In the context of heterologous antigen expression in recombinant *Neisseria lactamica*, HAEC3 is flanked on either side by sequences homologous to the *N. lactamica* chromosome (e.g. NHCIS1).

Annotated nucleotide sequence of HAEC4.

With reference to Figure 26, this Cassette has been designed to enable non-phase variable, high level gene expression of heterologous antigen. The sequence consists of a modified *N. meningitidis porA* promoter, wherein the phase variable, 17 bp tract that separates the -10 and -35 boxes in the wild type *porA* promoter has been replaced with 17 bp of sequence identical to that which separates the -10 and -35 boxes of the *N. lactamica porB* promoter. The -35 box of the modified *porA* promoter is preceded by 200 bp of the *porA*-derived transcriptional enhancer sequence (Figure 22), and most distally a SalI restriction site, to facilitate cloning and replacement of the promoter sequence (when used in conjunction with the XhoI restriction site). The modified, optimally-enhanced *porA* promoter is separated from the 3' *N. lactamica lst* promoter sequence by an XhoI restriction site, an interchangeable LINKER sequence and a NotI restriction site. It is important to note that either LINKER sequence can be any given nucleotide sequence (represented as poly-N). By including an XhoI restriction site immediately 5' of the START codon and a NotI restriction site immediately 3' of the STOP codon of any given coding sequence (e.g. through the use of extended primer sequences that incorporate these sites), that sequence can be directionally cloned, IN-FRAME into the Construct for expression from the modified, optimally-enhanced *porA* promoter. The featured NdeI restriction site can be used in conjunction with the HindIII

restriction site present immediately 5' of the *porB* terminator sequence to clone any given coding sequence into the Construct. By including an NdeI restriction site immediately 5' of the START codon and a HindIII restriction site immediately 3' of the STOP codon of any given coding sequence (e.g. through the use of extended primer sequences that incorporate these sites), that sequence can be directionally cloned, IN-FRAME into the Construct for expression from the *lst* promoter. Immediately after the HindIII restriction site, there is a transcriptional terminator sequence, derived from downstream of the *N. lactamica porB* gene. This is immediately followed by an AT-DUS. In the context of heterologous antigen expression in recombinant *Neisseria lactamica*, HAEC4 is flanked on either side by sequences homologous to the *N. lactamica* chromosome (e.g. NHCIS1).

Plasmid map of pUC19NHCIS1::HAEC4:*nadA-lacZ*

With reference to Figure 27, the pUC19NHCIS1::HAEC4:*nadA-lacZ* plasmid is a derivative of pUC19NHCIS1::HAEC2:*porA*plusprom-*lacZ* (Clone ♯7) (Figure 18), wherein the majority of the plasmid architecture is identical to pUC19NHCIS1::HAEC2:*porA*plusprom-*lacZ;* but where the wild type, non-optimally enhanced *porA* promoter and the *porA* coding sequence have been replaced by the optimally enhanced, modified *porA/porB* hybrid promoter and a *N. lactamica* codon-optimised version of the *nadA* gene. The *nadA* gene codes for the membrane associated adhesin, *Neisseria* Adhesin A, one of the four immunogenic components of the Bexsero anti-meningococcal serogroup B vaccine. The hybrid promoter and the *nadA* coding sequence were synthesised as a gBLOCK gene fragment (Integrated DNA Technologies) and cloned into the SalI-NotI double-digested plasmid vector using Gibson Assembly (NEB). The plasmid map is presented showing all relevant features and detailing the location of unique restriction sites with recognition sequences 6 nucleotides or greater.

Plasmid map of pUC19NHCIS1::HAEC4:(Z)-*lacZ*

With reference to Figure 28, the pUC19NHCIS1::HAEC4:(Z)-*lacZ* plasmid is a derivative of pUC19NHCIS1::HAEC4:*nadA-lacZ*, wherein the *nadA* coding sequence has been replaced by a 14 bp LINKER sequence. To create this plasmid, the *nadA*

coding sequence was excised from pUC19NHCIS1::HAEC4:*nadA-lacZ* using PCR and the plasmid re-circularised using the KLD enzyme mix from the Q5 site-directed mutagenesis kit (NEB). The plasmid map is presented showing all relevant features and detailing the location of unique restriction sites with recognition sequences 6 nucleotides or greater.

NadA-expressing *N. lactamica* have increased adherence to HEP-2 cells.

With reference to Figure 29, the pUC19NHCIS1::HAEC4:*nadA-lacZ* and pUC19NHCIS1::HAEC4:(Z)-*lacZ* plasmids were used as templates for hypermethylated PCR. Hypermethylated donor genetic material was used to transform the *ΔlacZ* mutant derivative of *N. lactamica* strain Y92-1009 as described in PROTOCOL A. Individual transformants, screened for on the basis of BLUE/WHITE colony formation on X-gal-containing TSB agar plates, were isolated. Chromosomal integration of either the HAEC4:(Z)-*lacZ* or HAEC4:*nadA-lacZ* cassettes into the NHCIS1 locus was demonstrated through PCR (data not shown). Following DNA sequencing of this locus, NadA-expressing *N. lactamica* strain B1 (hereafter: 4NB1) was determined to contain genetic material with 100 % identity to the *nadA* gene, the modified *porA/porB* hybrid promoter and *porA* enhancer sequence. NB: Transformation with the HAEC4:(Z)-*lacZ* cassette provides a procedural control and a transformed derivative (hereafter: 4YB2) that contains identical elements to 4NB1, but without the *nadA* coding sequence.

Two days prior to infection, HEP-2 cells were seeded into 24 well plates at a density of 2×10^5 cells/well. HEP-2 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % Foetal Calf Serum (FCS) at 37 ^{o}C, 5 % CO_2. On the morning of infection, duplicate wells of cells were treated with trypsin/EDTA and the average number of cells per well was measured by counting using a haemocytometer. Wild type *N. lactamica* strain Y92-1009, the NadA-expressing strain 4NB1 and the control strain 4YB2 were cultured in TSB at 37 ^{o}C, 5 % CO_2 and 320 rpm, until reaching mid-log phase (OD_{600nm} = 0.3). Aliquots of each culture were isolated, pelleted by centrifugation and then resuspended in fresh DMEM + FCS. The final concentration of bacteria was adjusted so that 500 µl of DMEM contained sufficient

bacteria to infect cells at MOI = 100. At t=ZERO, HEP-2 cells were washed twice in sterile PBS, then infected at MOI = 100 with the relevant bacterial strain. Plates were transferred to the incubator and cultured at 37 °C, 5 % CO_2 until the appropriate time point. At t = 2h, 4h and 6h the plates were removed from the incubator, supernatants were carefully aspirated and each well washed 5 x with excess, sterile PBS. The final two washes were accompanied by complete aspiration of liquid from the well. A 250 µl aliquot of a 2 % saponin solution in PBS was added to each well and the plates were again incubated for 15 minutes at 37 °C, 5 % CO_2. To fully disrupt the HEP-2 monolayers and disperse adherent/internalised bacteria, 750 µl of sterile PBS was added to each well and the cells were mechanically agitated through pipetting. The diluted lysate was serially diluted 10-fold in PBS, and the number of viable bacteria determined by plating onto Columbia Blood Agar (CBA) plates. The viability of each lysate was normalised to the estimated number of HEP-2 cells per well.

The graph shows the number of 'HEP-2 adherent' bacteria of each different strain recovered from infected wells at 2h, 4h and 6h. At all time points there is a trend for the transformed strain, that which putatively expresses the NadA adhesin (4NB1), to bind more readily to HEP-2 cells than either the wild type strain or the transformation control strain (4YB2). Over time, this difference becomes more pronounced, but is similarly more variable. Whether this simple difference in adherence will reflect a higher propensity of this strain to colonise the human nasopharynx has yet to be determined.

Plasmid map of pSC101NHCIS1::HAEC4:*opcA-lacZ*

With reference to Figure 30, each of the pUC-derived plasmids used in this series has a large copy number (50-300 copies per bacterial cell). Whilst this simplifies the harvest of these plasmids, the high copy number may also result in toxicity – especially if the plasmid codes for a genetic system designed to express high levels of a particular product. The metabolic burden to *E. coli* transformed with these plasmids perhaps renders them non-viable, meaning that the only plasmids recovered during transformation are those with mutations in either the coding sequence or the promoter region. One potential solution to the problem of gene dose-mediated toxicity is to change the plasmid's origin of replication. By substituting the pUC origin of replication

(modified pMB1) for the minimally-required region for propagation of plasmid pSC101 (*repA/ori*), the derivative plasmids will have a copy number of only 5 in daughter cells. It is hypothesised that the propensity for mutated plasmids to be selected for will be lower in the pSC101-based plasmids than the pUC19-based ones, increasing the frequency with which high fidelity plasmids containing the correct sequence for the gene of interest are recovered.

The pSC101NHCIS1::HAEC4:*opcA-lacZ* plasmid contains a *N. lactamica* codon-optimised version of the *opcA* gene under the control of the optimally-enhanced, modified *porA/porB* hybrid promoter. The plasmid is designed for use as a template in hypermethylated PCR, so as to generate donor genetic material suitable for the transformation of *N. lactamica* according to PROTOCOL A. The *opcA* and *lacZ* genes are targeted to NHCIS1. The plasmid map is presented showing all relevant features and detailing the location of unique restriction sites with recognition sequences 6 nucleotides or greater.

Plasmid map of pUC19*Δnhba*::HAEC1:(Z)-*lacZ*

With reference to Figure 31, the pUC19*Δnhba*::HAEC1:(Z)-*lacZ* Construct comprises many features identical to those of pUC19NHCIS1::HAEC1:(Z)-*lacZ* (Figure 14), but where the sequences homologous to the NHCIS1 locus are replaced by sequences homologous to the *N.lactamica nhba* gene (alternatively, *gna2132* OR NLY_32180). The genome of *N.lactamica* strain 020-06 contains the gene: NLA_20270, which codes for the putative lipoprotein GNA2132, otherwise known as the *Neisseria* Heparin Binding Antigen (NHBA). NHBA is one of the four immunogenic components of the 4CMenB (Bexsero) vaccine. The genome of *N.lactamica* strain Y92-1009 contains the NLY_32180 open reading frame, which has an 87.5 % similarity with NLA_20270 at the nucleotide sequence level, and codes for a protein with the characteristic, arginine-rich region of NHBA. Therefore NLY_32180 likely codes for the Y92-1009 homologue of NHBA.

The pUC19*Δnhba*::HAEC1:(Z)-*lacZ* Construct was designed as the first step in of a two-step strategy to truncate the coding sequence (and therefore effectively delete) the

NHBA homologue from the *ΔlacZ* derivative of Y92-1009, without the need for a screening/selection marker being present in the final strain. Initially, *ΔlacZ N.lactamica* were transformed with the *Δnhba*::HAEC1:(Z)-*lacZ* Cassette according to PROTOCOL A, which disrupted the *nhba* gene with a functional copy of the *N.lactamica lacZ* gene. Successful transformants were screened for on medium containing X-Gal in accordance with PROTOCOL B, and grew as blue colonies. Successful transformants were verified by PCR of the *nhba* locus and had the genotype: *ΔlacZ Δnhba*::HAEC1:(Z)-*lacZ*. One of these strains provided the background for the second transformation event, which used the *Δnhba* Cassette, amplified from pUC19*Δnhba* (Figure 32), to remove the *lacZ* coding sequence and replace the *nhba* gene with a truncated version of itself (*Δnhba*). The plasmid map is presented showing all relevant features and detailing the location of unique restriction sites with recognition sequences 6 nucleotides or greater.

Plasmid map of pUC19*Δnhba*

With reference to Figure 32, the pUC19*Δnhba* Construct comprises a truncated copy of the *N.lactamica nhba* gene cloned into a modified, truncated version of pUC19. The pUC19*Δnhba* Construct was designed as the second step in a two-step strategy to truncate the coding sequence (and therefore effectively delete) the NHBA homologue from the *ΔlacZ* derivative of Y92-1009, without the need for a screening/selection marker being present in the final strain. The *Δnhba* Cassette was amplified from this plasmid and transformed into *ΔlacZ Δnhba*::HAEC1:(Z)-*lacZ N.lactamica* according to PROTOCOL A. Successful transformants were screened for on medium containing X-Gal in accordance with PROTOCOL B, and grew as white colonies. Successful transformants were verified by PCR of the *nhba* locus and had the genotype: *ΔlacZ Δnhba*. These strains will provide the background for subsequent transformation events, wherein components of the 4CMenB (Bexsero) vaccine will be expressed in *N.lactamica* in a way suitable for future use of those strains in human challenge. The plasmid map is presented showing all relevant features and detailing the location of unique restriction sites with recognition sequences 6 nucleotides or greater.

Repeated transformation of *N. lactamica* does NOT select for a more transformable phenotype.

With reference to Figure 33, a justified cause for concern in using genetically modified *N. lactamica* in a human challenge model is whether recombinant derivatives are more readily transformable than their parent cells. A higher propensity to become transformed by genetic material from the nasopharyngeal milieu means an increased risk of recombinant *N. lactamica* taking up and integrating into its genome the means to synthesise extracellular capsule, which could transition the organism from harmless commensal to potential pathogen. If the process of transforming *N. lactamica* also unintentionally selects for an enhanced inherent transformability, then the serial transformation of strains is likely to result in increased transformation efficiency under experimental conditions.

In creating a *Δnhba* mutant in *N. lactamica*, bacteria were serially transformed a total of three times. The first transformation removed the coding sequence for β-galactosidase (*lacZ*) from the wild type organism (to produce *ΔlacZ*). The *ΔlacZ* strain provided the background for the knock-out of the *nhba* gene, which was achieved using a two-step approach. The first step was the introduction of the HAEC1:(Z)-*lacZ* cassette) into the *nhba* chromosomal locus (to produce *ΔlacZ Δnhba*::HAEC1:(Z)-*lacZ*). The HAEC1 cassette was subsequently removed from sequence-verified transformants, along with a large section of the *nhba* coding sequence (to produce *ΔlacZ Δnhba*). Each of the above steps was dependent upon BLUE/WHITE colony formation on X-gal-supplemented TSB agar plates.

Each of the above strains was experimentally transformed with 0.46 pmol of hypermethylated PCR product, amplified from pUC19*ΔnlaIII*:*aphA3* (Figure 4). The number of transformants was considered to be equal to the number of kanamycin-resistant colonies that grew overnight on selective agar plates (TSB + 0.2 % yeast extract supplemented with 50 μg/ml kanamycin), adjusted for dilution and plating volume. The graph shows that there are no significant INCREASES in the transformation efficiencies of any of the serially-transformed strains, which suggests that we are *not* inadvertently selecting for an inherently more transformable phenotype.

OMV production from *Neisseria meningitidis* and *Neisseria lactamica*

Note: *Neisseria meningitidis* is a BSL-2 organism that is spread via creation of aerosols and droplets. Infection rate among laboratory workers is very low, estimated to be 13/100,000, but is approximately 10-fold higher than the general population. Mortality among infected workers is approximately 50%. Historical cases of laboratory-acquired infection are traceable to manipulation of the organism on the open bench by clinical microbiologists. Therefore, all manipulations of the live organism, including sub-culturing of colonies from plates, must only be performed within a class II containment hood.

Effect of the amount of donor material used to transform wild type *Neisseria lactamica*: supplemental.

With reference to Figure 34, wild type *N. lactamica* strain Y92-1009 was transformed with a broad range of dilutions of a hypermethylated PCR construct amplified from pUC19*ΔnlaIII:aphA3*, (*ΔnlaIII:aphA3*(600)) according to Protocol A. The number of transformants was considered to be equal to the number of kanamycin resistant colonies evident after overnight growth on selective media (TSB agar supplemented with 50 μg/ml kanamycin), corrected for dilution factor and plating volume. The total number of viable bacteria was determined by tenfold serial dilution of each suspension of putatively transformed *N. lactamica*. This graph shows a strong relationship between the amount of donor material and the frequency of transformation of wild type *N. lactamica*. The relationship appears to be linear over the dilution range (0.05 pmol – 0.000005 pmol), but appears to approach saturation when transforming using 0.5 pmol of donor material. This is consistent with Figure 8 (B), wherein small changes in the amount of available donor material when provided in relatively large quantities (0.06 pmol – 1 pmol) do not appear to appreciably impact the frequency of Nlac transformation. Points represent the Mean ± SEM of six biological replicates.

Plasmid map of pSC101::*ΔlacZ:opcA*

With reference to Figure 35, the vector component of this plasmid, comprising the origin of replication (*repA/ori*) from pSC101 and the ampicillin resistance gene and M13 primer binding sites of pUC19, is identical to that of

pSC101NHCIS1::HAEC4:*opc-lacZ* (**Figure 30**). The pSC101::*ΔlacZ:opcA* plasmid contains an *N. lactamica* codon-optimized version of the *opcA* gene (eg. NMB1053) under the control of the optimally enhanced, modified *porA/porB* hybrid promoter and flanked on either side by sequences derived from the 5' and 3' ends of the Nlac *lacZ* gene. The purpose of the *ΔlacZ:opcA* construct is to introduce the *opcA* gene into the coding sequence of the *lacZ* gene, which was itself introduced into the NHCIS1 locus along with the *nadA* gene (using the NHCIS1::HAEC4:*nadA-lacZ* construct – **Figure 27**). Transformation of *N. lactamica* strain 4NB1 with this construct generated the strain *ΔlacZ* NHCIS1::HAEC4:*nadA-ΔlacZ:opcA*, which contained chromosomal sequences with 100 % identity to both the *porA*(P1.7,16) and *opcA* genes and grew as WHITE colonies on X-gal-containing TSB agar plates. The plasmid map is presented showing all relevant features and unique restriction sites.

Plasmid map of pSC101::*ΔlacZ*-synth.*lacZ*-3'ENDNHCIS1

With reference to Figure 36, the vector component of this plasmid, comprising the origin of replication (*repA/ori*) from pSC101 and the ampicillin resistance gene and M13 primer binding sites of pUC19, is identical to that of pSC101NHCIS1::HAEC4:*opc-lacZ* (**Figure 30**). The pSC101::*ΔlacZ*-synth.*lacZ*-3'ENDNHCIS1 plasmid contains a synthetic version of the *lacZ* gene (synth.*lacZ*), in which the coding sequence has been adjusted to maximize its diversity from the endogenous Nlac version of the *lacZ* gene at the nucleotide sequence level, whilst maintaining the fidelity of the amino acid sequence of β-galactosidase (see **Figure 37**). The synth.*lacZ* gene is flanked at its 5' end by nucleotide sequence identical to the 3' end of the endogenous Nlac version of the *lacZ* gene, and at its 3' end by nucleotide sequence identical to the 3' end of NHCIS1. The purpose of the *ΔlacZ*-synth.*lacZ*-3'ENDNHCIS1 construct is to introduce a functional copy of the *lacZ* gene back into the chromosome of Nlac strain *ΔlacZ* NHCIS1::HAEC4:*nadA-ΔlacZ:opcA*, so that all strains in the NadA/Opc mutant panel retain β-galactosidase activity. Transformation of *N. lactamica* strain *ΔlacZ* NHCIS1::HAEC4:*nadA-ΔlacZ:opcA* with this construct generated the strain, *ΔlacZ* NHCIS1::HAEC4:*nadA-ΔlacZ:opcA-lacZ* (hereafter, 4NOA1), which contained chromosomal sequences with 100 % identity to both the *porA*(P1.7,16) and *opcA* coding sequences, and grew as BLUE colonies on X-gal-

containing TSB agar plates. The plasmid map is presented showing all relevant features and unique restriction sites.

Coding sequence of synth.*lacZ*

With reference to Figure 37, this coding sequence has been designed to maximize diversity from the nucleotide sequence of the endogenous Nlac version of *lacZ*, whilst maintaining the fidelity of the amino acid sequence of β-galactosidase (CAI: 0.687). These adjustments were necessary in order to minimize the likelihood of the synthetic *lacZ* gene undergoing homologous recombination with the remaining *lacZ* fragments left in the NHCIS1 locus of Nlac strain *ΔlacZ* NHCIS1::HAEC4:*nadA-ΔlacZ:opcA*, which would have most probably resulted in the excision of the *opcA* gene from the chromosome. Where nucleotides have been manually substituted in order to increase nucleotide sequence diversity, they are in lower case.

Expression of NadA and Opc outer membrane proteins on the surface of recombinant strains of *N. lactamica*

With reference to Figure 38, wild type *N. lactamica* strain Y92-1009 and its recombinant derivatives, the putatively NadA-expressing strain, 4NB1 and the putatively Opc-expressing strain, 4OA2, were cultured to mid-log phase (OD_{600nm} = 0.4) in TSB supplemented with 0.2 % yeast extract. Aliquots of each culture containing 2 x 10^7 CFU were transferred to fresh microcentrifuge tubes and washed twice in Wash Buffer. Washed bacteria were resuspended into 100 µl of Wash Buffer, supplemented with either a 1:200 dilution of anti-NadA monoclonal antibody, 6E3 (WT and 4NB1) or a 1:50 dilution of anti-Opc monoclonal antibody, 279/5c (WT and 4OA2) and incubated at 4 °C for 30 minutes. The primary mAbs were removed by washing twice with 1 ml of Wash Buffer, and the bacteria were resuspended into 100 µl of Wash Buffer containing a 1:100 dilution of anti-mouse IgG-AlexaFluor488 (1.5 mg/ml). The secondary antibody was allowed to bind over the course of 30 minutes at 4 °C, before the bacteria were again washed twice in Wash Buffer. After labelling, the bacteria were resuspended into 100 µl formalin and were fixed for 10 minutes at room temperature. Once formalin had been removed and the labelled, fixed bacteria washed twice more in Wash Buffer, they were resuspended into 200 µl of Wash Buffer and transferred to

FACS tubes for quantitative analysis of AlexaFluor488 fluorescence by Flow Cytometry.

The graphs in this figure show that, in both instances, the Mean fluorescence intensity of the samples composed of the recombinant strains of *N. lactamica* (either 4NB1, GREEN plot or 4OA2, BLUE plot), are greater than the Mean fluorescence intensity of the samples composed of wild type *N. lactamica* (RED plots). This indicates surface expression of the targets for 6E3 and 279/5c mAbs, which are most plausibly the NadA and Opc outer membrane proteins, respectively. Combined with the binding and internalisation data collected from analysis of the interaction of these recombinant Nlac strains with HEP-2 cells (**Figure 40**) and (in the case of 4NB1) human nasopharyngeal tissue explants (**Figure 41**), these graphs suggest the NadA and Opc outer membrane proteins are functionally expressed on the surface of recombinant Nlac strains 4NB1 and 4OA2, respectively.

Expression of NadA, Opc or a combination of both antigens in recombinant *N. lactamica* has no appreciable effect on growth rates in TSB

With reference to Figure 39, it is plausible that expression of one or more outer membrane protein(s) in additional to the usual complement of Nlac proteins, might constitute a metabolic burden for recombinant strains of *N. lactamica*. A significant metabolic burden might manifest as impairment in the growth characteristics of the strains as compared to wild type. To investigate this, wild type *N. lactamica* strain Y92-1009 and the mutant derivatives thereof, 4NB1, 4OA2, 4NOA1 and 4YB2 were cultured in TSB and the OD_{600nm} and viability of each culture was measured hourly. Area Under Curve analyses of these data show that there are no significant differences between the growth rates or viability of these strains, suggesting that expression of additional outer membrane protein(s) do(es) not constitute a significant metabolic burden in rich medium. Points represent Mean $\pm$ SD of four biological replicates, where no bars are visible, they fall within the points.

NadA-expressing strains of *N. lactamica* have increased adherence to HEP-2 cells, whilst Opc-expressing strains of *N. lactamica* are internalized by HEP-2 cells in significantly larger numbers

With reference to Figure 40, wild type *N. lactamica* strain Y92-1009 and the recombinant, (meningococcal adhesin-expressing) derivatives thereof: 4NB1, 4OA2, 4NOA1 and 4YB2 were grown to mid-log phase, washed, resuspended into Dulbecco's modified Eagles medium (DMEM) supplemented with 10 % foetal bovine serum (FBS) and used to infect duplicate confluent monolayers of HEP-2 epithelial cells at a multiplicity of infection (MOI) of 100 (i.e. 100 bacteria per HEP-2 cell). Infected cells were incubated at 37 °C, 5 % CO2 and samples were processed every 2 h for a total of 6 h. Thirty minutes prior to each sampling, the infected supernatant was removed from one of each pair of duplicates and the HEP-2 cells were gently washed with sterile PBS. Pre-warmed (37 °C) DMEM + 10% FBS, supplemented with 100 μg/ml gentamicin and 10 μg/ml penicillin G was then added to each aspirated well and the plates returned to the incubator, with the intention of killing all bacteria adherent to the surface of the cells. At the appropriate time points, all media was aspirated from the HEP-2 cells, which were subsequently washed 5 times with an excess of sterile PBS. Following aspiration of the final wash, 250 μl of a 2 % saponin solution in PBS was added to each well and, after 15 minutes incubation at 37 °C and repeated pipetting to break up the HEP-2 monolayers, was supplemented with 750 μl of sterile PBS (yielding all HEP-2 associated bacteria, or all HEP-2 internalised bacteria from a given well in a total volume of 1 ml). These suspensions were serially diluted in sterile PBS and the number of viable CFU enumerated on CBA agar. Viable counts were normalized to the number of HEP-2 cells present in each well. These data show that: (A). By 4 h of infection (light gray bars), Nlac strains expressing the meningococcal adhesin NadA on their surface (4NB1 and 4NOA1) associate in significantly higher numbers with HEP-2 cells than do any of the other strains examined. The wild type parental strain (WT), the Opc-expressing strain (4OA2) and the transformation procedure-control strain (4YB2) all associate in similar numbers with HEP-2 cells at every time point studied, but in consistently lower numbers when compared to both 4NB1 and 4NOA1. For all strains, these data suggest that the binding capacity of the HEP-2 cells becomes saturated at or before 4 h of infection, but that a significantly larger population of the NadA-expressing

strains can associate with this cell line. This likely indicates the existence of an as-yet unidentified receptor for NadA binding on the HEP-2 cell surface, which is not bound by the surface armamentarium of wild type Nlac. $*p \leq 0.05$, RM 2-way ANOVA with Tukey's Multiple Comparisons test, n = 4. (B). By 6 h of infection (dark gray bars, also shown in isolation in blow-out below main graph), the Nlac strain expressing the meningococcal adhesin Opc on its surface (4OA2) becomes internalized by HEP-2 cells in significantly higher numbers than every other strain examined except for 4NOA1. Although 4NOA1 also expresses Opc along with NadA, the internalization of this strain by HEP-2 cells is not significantly different from that of any other strain examined. Whether the presence of NadA in addition to Opc somehow interferes with the internalization process is not yet clear, although the relative expression levels of each adhesin on the surface of these bacteria, as compared to strains expressing one or the other protein, have not yet been determined. Bars represent Mean ± SD; $*p \leq 0.05$, $***p \leq 0.001$ and $****p \leq 0.0001$, RM 2-way ANOVA with Tukey's Multiple Comparisons test, n = 4.

The NadA-expressing strain of *N. lactamica*, 4NB1, is recovered in significantly higher numbers from within human nasopharyngeal tissue explants than is the wild type

With reference to Figure 41, wild type *N. lactamica* strain Y92-1009 and the recombinant, NadA-expressing derivative thereof, 4NB1 were grown to mid-log phase, washed, resuspended in sterile PBS and used to infect the epithelium of agarose-mounted, transwell-suspended nasopharyngeal tissue explants derived from human turbinets from consenting donors. Each strain was used to infect four replicate explants. Explants were incubated at 37 ^{o}C, 5 % CO2 for 24 h, after which they were carefully removed from the agarose, weighed and processed. Duplicate explants were washed either three times in sterile PBS by vortexing (30 seconds per wash) or were vortexed for 30 seconds in a 0.2 % solution of sodium taurocholate (bile salts) in PBS, followed by an additional 2 washes in sterile PBS. The wash in bile salts effectively sterilizes the surface of the explant, isolating the population of viable bacteria that have penetrated the epithelium (i.e. 'sub-epithelial bacteria'). Each explant was subsequently disrupted into 1 ml of a sterile 2 % saponin solution in PBS, using a high-pressure, one-shot tissue

disruptor. The resultant cell suspension was incubated for 15 minutes at 37 °C, 5 % CO2 to release any intracellular bacteria. An aliquot of each saponised lysate was serially diluted and plated on CBA agar to enumerate viable bacteria (CFU/ml). Viable counts were normalized to the weight of each explant (g). Points on each graph represent the average of values derived from duplicate explants. Paired Explants (derived from the same donor but infected with different bacterial strains) are joined by dotted lines. Although there is no significant difference between the total number of wild type or 4NB1 bacteria associated with the explants (i.e. surface-bound and those within the body of the explant), a significantly larger number of CFU were recovered from inside explants infected with strain 4NB1 than with wild type Nlac ($*p = 0.0313$, Wilcoxon matched pairs signed rank test, n = 5). These data suggest that although surface expression of meningococcal NadA by *N. lactamica* does not significantly enhance the ability of the strain to bind to the epithelial surface of nasopharyngeal explants compared to wild type, a significantly larger number of 4NB1 bacteria are able to penetrate the epithelium. Whether these sub-epithelial bacteria are intracellular or not remains to be elucidated; however, given the existence of sub-epithelial *Neisseria* microcolonies in tonsillar crypts (Sim et al, 2000), we hypothesize that an increased propensity to cross the nasopharyngeal epithelium *ex vivo* may impact upon the colonisation dynamics of this recombinant strain in human challenge. Penetration of the mucosal epithelium could plausibly mean the bacteria are protected from elements of the innate immune system operating at the mucosal service, effectively sheltering the strain from killing and promoting its persistence in a given human host. However, we must be mindful that the sub-epithelial location of these bacteria might preclude their recovery by swabbing of the nasopharynx, leading to false negative results in terms of an individual's colonization status.

Recombinant strains of *N. lactamica* expressing meningococcal adhesin proteins have similar sensitivity profiles to front-line antibiotics as the wild type

With reference to Figure 42, and in the context of potentially using these strains in experimental human challenge, it is important to determine whether the process of creating recombinant strains of *N. lactamica* has decreased their sensitivity to clinically relevant antibiotics. Although the expectation is that these strains pose no threat to

human health, the ability to kill these bacteria using front-line antibiotics should it become necessary needs to be assessed. Wild type *N. lactamica* strain Y92-1009 and recombinant Nlac strains 4NB1, 4OA2, 4NOA1 and 4YB2 were cultured in TSB to mid log phase. Sterile cotton swabs were saturated with these suspensions, which were then used to inoculate the surface of TSB agar plates supplemented with 5 % horse blood in such a way as to generate a confluent lawn of bacteria after overnight growth. Onto the surface of each inoculated plate, an E-test strip was placed containing one of rifampicin, ciprofloxacin or ceftriaxone across a range of concentrations. The minimum inhibitory concentration (MIC) of each antibiotic was determined to be the concentration at which the bacterial lawn failed to develop following overnight growth, characterized by an area of clearance surrounding the E-test strip (Table 1). With reference to Table 2, which details the MIC and antibiotic 'sensitivity' levels of the pathogenic species of *Neisseria* to each of these antimicrobials, it is evident that each strain remains "sensitive" to these antibiotics. These data demonstrate that the process of generating our recombinant strains of *N. lactamica* has not significantly altered the susceptibility profile of these bacteria to the antimicrobial agents used to treat infections of the pathogenic *Neisseria* species.

Recombinant strains of *N. lactamica* expressing meningococcal adhesin proteins are competent for uptake of exogenous DNA and can be transformed with efficiencies similar to the wild type

In reference to Figure 43, wild type *N. lactamica* strain Y92-1009 and recombinant Nlac strains 4NB1, 4OA2, 4NOA1 and 4YB2 were transformed with 0.5 pmol of hypermethylated PCR construct: *ΔnlaIII:aphA3*(600), as described in Protocol A. The number of transformants was considered to be equal to the number of kanamycin-resistant colonies that grew overnight on selective agar plates (TSB + 0.2 % yeast extract supplemented with 50 μg/ml kanamycin), adjusted for dilution factor and plating volume. This experiment was performed to determine whether serial transformation of *N. lactamica* had inadvertently selected for a "more transformable" phenotype compared to the WT strain. This is important, as our argument that recombinant Nlac is safe for use in human challenge experiments is predicated on the fact that they are no more likely to assimilate genetic information from the environment than the parental

strain. An increased propensity to take up exogenous DNA could plausibly translate into an increased likelihood to assimilate capsule synthesis genes from the nasopharyngeal milieu, with the potential to transform commensal strains into facultative pathogens. Strains 4NB1, 4OA2 and 4YB2 have each undergone two transformations from the wild type background, whereas strain 4NOA1 has been transformed a total of four times. These data show no significant differences in the transformation efficiency between any of the strains investigated and is consistent with the data presented in **Figure 33**. This implies that isolation of serially-transformed bacteria does not also select for an inherently 'more transformable' phenotype.

Wild type and recombinant strains of *N. lactamica* are completely refractory to transformation by chromosomal DNA derived from *N. meningitidis*

In reference to Figure 44, the wild type meningococcal strain MC58, along with the wild type *N. lactamica* strain Y92-1009 and the recombinant derivatives thereof, 4NB1, 4OA2 and 4NOA1, were exposed to 1 x 10^{-4} pmol of chromosomal DNA isolated from four mutant strains of *Neisseria,* as described in Protocol A.1. Chromosomal DNA was isolated from the Nlac mutant derivative of Y92-1009 containing an insertionally-inactivated copy of the *nlaIII* gene (*ΔnlaIII*) and mutant derivatives of MC58 containing insertionally-inactivated copies of the *opc*, *nadA* and *siaD* genes, (*Δopc, ΔnadA and ΔsiaD*, respectively). All insertionally-inactivated meningococcal genes contained the full coding sequence of the appropriate gene, disrupted by identical copies of the kanamycin resistance gene, *aphA3*, transcriptionally controlled by the Nlac *lst* gene promoter. The same antibiotic resistance-conferring marker was used to disrupt the *nlaIII* coding sequence. The number of transformants was considered to be equal to the number of kanamycin-resistant colonies that grew overnight on selective agar plates (Columbia agar supplemented with horse blood and saturated then allowed to dry with 3 ml of a 2 μg/ml kanamycin solution), adjusted for dilution factor and plating volume. This experiment was designed to determine the propensity of (recombinant) Nlac strains to take up and become transformed with chromosomal DNA from a meningococcal donor, which is the most plausible source of capsule synthesis genes these strains might encounter in the nasopharyngeal milieu. As such, all the strains were exposed to chromosomal DNA derived from MC58 *ΔsiaD*. Because Nlac does not express capsule,

and instead contains the capsule null locus (*cnl*) in a position homologous to the capsule synthesis locus in Nmen, the incorporation of the insertionally-inactivated *siaD* gene into the Nlac chromosome would represent a *de novo*, untargeted recombination event and could theoretically occur at any locus. This is also true for all the recombinant Nlac strains. However, each of the recombinant strains contains at least one additional region of similarity to the meningococcal chromosome not present in the WT strain, i.e. the *opc* and *nadA* genes incorporated into NHCIS1. Both coding sequences for the Nlac versions of *opc* and *nadA* are approximately 80 % similar to the appropriate MC58 homologue, which could plausibly facilitate homologous recombination of meningococcal DNA into the Nlac genome. As such, strains 4NB1 and 4OA2 were exposed to DNA derived from MC58 *ΔnadA* and MC58 *Δopc,* respectively, whilst strain 4NOA1 was exposed to DNA derived from both of these sources. Because we hypothesize that exogenous, non-Nlac derived chromosomal DNA taken up into *N. lactamica* will be degraded by potent restriction endonuclease activities (specifically, NlaIII), it was important to demonstrate that each of the Nlac strains in this experiment were actually competent for DNA uptake and homologous recombination. As such, each Nlac strain was also exposed to chromosomal DNA isolated from Y92-1009 *ΔnlaIII*, which reproducibly transformed wild type Nlac with high efficiency in preliminary experiments. As a demonstration that the isolated, meningococcal chromosomal DNA was capable of transforming a competent and *compatible* recipient, wild type MC58 bacteria were exposed to chromosomal DNA from all mutant sources. These data demonstrate that each strain of Nlac is highly competent for DNA uptake and homologous recombination when transformed with chromosomal DNA from a compatible source (*ΔnlaIII*, black bars). Interestingly, *ΔnlaIII* chromosomal DNA was also capable of transforming wild type MC58 with low efficiency, in what is presumably a *de novo*, untargeted recombination event due to the absence of an *nlaIII* coding sequence in the MC58 genome. This observation is in keeping with data that demonstrates horizontal gene transfer from Nlac into the pathogenic *Neisseria in vivo*. Each pool of chromosomal DNA derived from the MC58 mutant strains was capable of transforming wild type MC58 with low efficiency, as compared to the analogous back-cross into Nlac. However, each strain of Nlac was completely refractory to transformation with meningococcal DNA isolated from any mutant source (*Δopc,*

checked bars; *ΔnadA*, striped bars or *ΔsiaD*, dotted bars), with absolutely no recovery of kanamycin-resistant colonies across biological replicates (n = 6). Bars denote Mean ± SD.

Plasmid map of pSC101NHCIS1::HAEC4:*porA*(P1.7,16)-*lacZ*

With reference to Figure 45, the pSC101NHCIS1::HAEC4:*porA*(P1.7,16)-*lacZ* plasmid is a derivative of pSC101NHCIS1::HAEC4:*opcA-lacZ* (**Figure 30**), wherein the majority of the plasmid architecture is identical to pSC101NHCIS1::HAEC4:*opcA-lacZ*; but where the *opcA* coding sequence has been replaced by an Nlac codon-optimized version of the gene coding for Porin A (P1.7,16) (*porA*(P1.7,16)). Important to note is the fact that the nucleotide sequence of *porA* (P1.7,16) has been adjusted to remove the homopolyadenosine tract present at the 5' end of the wild type *porA* gene, so as to reduce the likelihood of *porA*(P1.7,16) expression being downregulated through phase variation. Transformation of strain *ΔlacZ* with hypermethylated NHCIS1::HAEC4:*porA*(p1.7,16)-*lacZ* construct gave rise to strain *ΔlacZ* NHCIS1::HAEC4:*porA*(p1.7,16)-*lacZ* (hereafter, 4PA1), which contained chromosomal sequences with 100% identity to the *porA*(P1.7,16) gene and grew as BLUE colonies on TSB agar plates supplemented with X-gal. The plasmid map is presented showing all relevant features and detailing the location of unique restriction sites.

Plasmid map of pSC101NHCIS1::PV*porA*(P1.7,16)-*lacZ*

With reference to Figure 46, the pSC101NHCIS1::PV*porA*(P1.7,16)-*lacZ* plasmid is a derivative of pSC101NHCIS1::HAEC4:*opcA-lacZ* (**Figure 30**), wherein the majority of the plasmid architecture is identical to pSC101NHCIS1::HAEC4:*opcA-lacZ*; but where the *porA/porB* hybrid promoter and the *opcA* coding sequence have been replaced with the native *porA* promoter and *porA*(P1.7,16) coding sequence, amplified from the chromosome of wild type meningococcal strain H44/76. It is important to note that, unlike pUC19NHCIS1::HAEC2:*porA*plusprom-*lacZ* (**Figure 18**), in which there was significant truncation of the *porA* transcriptional enhancer sequence (**Figures 21 & 22**), the pSC101NHCIS1::PV*porA*(P1.7,16)-*lacZ* plasmid contains the optimum length of 200 bp of enhancer sequence upstream of the *porA* promoter. Note that the plasmid

map is based upon the *ideal* sequence for this plasmid, wherein the homopolymeric 'G' tract, which separates the -10 and -35 boxes of the native *porA* promoter contains 11 contiguous guanosine nucleotide residues. The plasmid map is presented showing all relevant features and detailing the location of unique restriction sites.

Recombinant Nlac strains express Porin A (P1.7, 16) at the cell surface and generate PorA-containing OMV

In reference to Figure 47, wild type *N. lactamica* strain Y92-1009 and the (putatively) PorA-expressing strains 2Pp7.A and 4PA1 were assessed for Porin A expression by western blot and flow cytometry (A and B, respectively). Outer membrane vesicles of the wild type and 4PA1 strains were analysed for PorA content by western blotting (C) prior to their use to immunize mice (see **Figure 48**).

(A) Wild type (WT) Nlac and strains 2Pp7.A and 4PA1 were grown to mid-log phase and lysed with sonication. Equal amounts (50 μg) of each crude membrane preparation were separated by SDS-PAGE and transferred to activated PVDF. Expression of PorA in the membrane was detected by interrogation with anti-P1.7, 16 mAb, SM300. PVDF was subsequently stripped and reprobed with antibody directed against the constitutively-expressed membrane protein LpdA.

(B) WT Nlac and strains 2Pp7.A and 4PA1 were grown to mid-log phase and 2 x 10^7 whole cells were labelled with anti-meningococcal serosubtype P1.7 mAb (NIBSC) and a goat-derived, anti-murine IgG-Alexafluor488 conjugate. After labelling, the bacteria were fixed in formalin prior to analysis on a FACSCalibur flow cytometer. Graph shows the Median Fluorescence Intensity of three independent bacterial cultures, lines denote Mean MFI. These data are consistent with that presented in **Figure 20**, which showed a low level of PorA expression on the cell surface of strain 2Pp7.A. The addition of approximately 100 bp to the 5' end of the *porA* enhancer sequence (**Figures 21 & 22**) and the substitution of the native homopolymeric G tract present in the wild type *porA* promoter for 17 bp derived from the non-phase variable *porB* promoter, have resulted in an increased level of PorA expression in strain 4PA1.

(C) WT Nlac and strain 4PA1 were grown overnight in modified Catlin medium (MC.7) to produce OMV, which was harvested with deoxycholate-extraction. Five

micrograms (5 μg) of each OMV preparation were analysed for PorA by western blotting with anti-P1.7,16 mAb, SM300.

Immunisation with OMV from recombinant Nlac generates anti-meningococcal SBA

In reference to Figure 48, mice were immunised i/p using a standard prime/boost strategy. Mice were injected with either Alum alone (AlOH) or Alum combined with deoxycholate-extracted OMV derived from one of: wild type Nlac (WT), the PorA-expressing recombinant Nlac strain 4PA1 (4PA1) or wild type serogroup B meningococcal strain H44/76 (H44/76). NB: H44/76 expresses the cognate PorA serosubtype, P1.7, 16.

Sera from 5 immunised mice per group were pooled and doubling dilutions were assessed for SBA against **(A)** H44/76 and **(B)** MC58 (which expresses PorA serosubtype P1.7, 16-2). The maximum dilution of sera tested was 1:1024. OMV derived from strain 4PA1 elicited strong SBA against H44/76. Serum from these mice was more potent against strain MC58 than mice immunised with OMV from H44/76, suggesting Nlac OMV could provide broader anti-meningococcal adjuvant properties than Nmen-derived OMV.

In reference to Figure 49, mice were immunised i/p using a standard prime/boost strategy. Mice were injected with either Alum alone (AlOH) or Alum combined with deoxycholate-extracted OMV derived from the NadA-expressing recombinant Nlac strain 4NB1 (4NB1+AlOH). Sera from 5 immunised mice per group were pooled and doubling dilutions were assessed for SBA against *Neisseria meningitidis* strain 5/99 (a reference strain that expresses NadA to high levels). OMV derived from strain 4NB1 elicited strong SBA against 5/99 (SBA titre reciprocal: 170.67 ± 74). Serum from these mice was more potent against strain 5/99 than antisera from humans immunised with the 4CMenB (Bexsero) anti-meningococcal vaccine (SBA titre reciprocal: 64 ± 0). Bars represent Mean SBA titre reciprocal $\pm$ SD. $n \geq 2$.

Materials

50ml bio-reaction tubes (50 ml tubes with 0.22µm filter cap) (GPE Scientific)

250ml shaker flask, baffled with vented cap (Fisherbrand, pack of 12, Cat no 11735253): These flasks are fully autoclavable, including cap.

Glass homogeniser and plunger (VWR: Vessel = 5ml capacity, cat no. 432-0201. Plunger = 5ml, cat no. 432-0207.

*Ultracentrifuge bottles for rotor 55.2 Ti fixed-angle rotor: Polycarbonate bottle x 6 with cap assembly, 26.3ml 25 x 89 mm. Beckman Coulter, Cat no. 355618.

Yeast extract powder (500g, Fisher Scientific, Cat no. 10108202)

Agar plates

Tryptone soya broth media powder (500g, Fisher Scientific, Cat no. 10198002)

Add 15g of powder to 500ml of water. Supplement with 0.2% (1g) yeast extract. Sterilise by autoclaving at 121°C for 15 minutes to make broth.

To make agar, add 7.5g of bacteriological agar to the above mix prior to autoclaving. Then swirl to mix agar and allow to cool until the bottle can comfortably be held in the palm of the bare hand.

Agar bacteriological (agar no. 1) (500g, Fisher Scientific, Cat no. 10351303)

Pour into petri dishes within a class II containment hood. Leave half uncovered to cool before replacing lids to avoid build up of condensation.

Media

Modified Catlin medium (MC.7)

Dissolve the following components in distilled water for a final volume of 1000ml and 500ml respectively. pH will be approximately 7.

	1000ml	500ml
NaCl	5.8g	2.9g
K_2HPO_4	4g	2g
NH_4Cl	1g	0.5g
K_2SO_4	1g	0.5g
D-(+)-glucose	10g	5g
$MgCl_2 \cdot 6H_2O$	0.4g	0.2g
$CaCl_2 \cdot 2H_2O$	0.03g	0.015g
Yeast extract	0.8g	0.4g
HEPES	5.96g	2.98g
EDDA	0.005g	0.0025g

Dissolve the following reagents in distilled water and filter sterilise using a 0.2µm syringe filter.

L-glutamic acid	2 x 1.95g	1.95g

Weigh 1.95g into 21ml 1M HCl. Warm to 60 °C and add 6 M HCl dropwise until it dissolves. Bring pH back to 7 using 6 M NaOH. Top up to 50ml with distilled water and filter sterilise. Make up 2 x 50 ml tube for 1000 ml of medium.

L-Cysteine•HCl	0.1g	0.05g

For 500 ml make up to 25 ml with water and filter sterilise. For 100 ml make up to 50 ml

Add the above to the autoclaved medium within a class II containment hood to keep medium sterile.

After autoclaving, the medium is yellow and may contain a large white precipitate that will not dissolve. In the event of precipitate formation, ensure thorough resuspension of the precipitate before use.

Buffers

Buffer 1

0.1M Tris-HCl pH 8.6
10mM EDTA
0.5% (W/V) Deoxycholic acid sodium salt

Buffer 2
50mM Tris-HCl pH 8.6
2mM EDTA
1.2% Deoxycholic acid sodium salt
20% sucrose (W/V)

After addition of EDTA to above buffers, heat to 60 ^{o}C in a water batch to encourage it to dissolve. If it does not fully dissolve, add 1 M NaOH whilst stirring until a solution is obtained. Then add remaining reagents.

Buffer 3
0.2M glycine buffer
3% sucrose

Filter sterilise buffers.

Protocol (work within a class II containment hood until step 20) for use with either MHB, MC.6 or MC.7 media

Note: All materials that come in direct contact with live *Neisseria meningitidis* must either be decontaminated overnight in 2 % Virkon, or sealed prior to disposal via the clinical waste stream or by autoclaving. Keep a sealable plastic bag inside the hood for disposal of gloves. After use, seal the bag, place inside a clinical waste bag and dispose of the clinical waste at the end of the experiment by taping up the neck of the bag and placing inside a large yellow clinical waste bin. At the end of the experiment disinfect surfaces such as the centrifuge control panel, door handles and pipettes with 70% ethanol.

1. At the end of the day, thaw a stock of *N.lactamica* or *N.meningitidis*. Remove 200 µl using a sterile pipette tip and plate onto the centre of a TSB + 0.2 % yeast extract agar plate.
2. Using a sterile cell spreader, place the spreader on the centre of the plate and rotate the centre of spreader so that the inoculum is spread evenly over the surface.
3. Replace the lid and place in a 37 ^{o}C 5 % CO_2 incubator for 20 minutes with the lid facing up to allow the inoculum to soak into the agar. Also prepare a streak plate to assess purity of culture.
4. Turn plate upside down so that the lid is facing down and culture overnight.
5. At the end of the next day there should be a bacterial lawn covering the plate. Assess streak plate to ensure there is a mono-culture of *N.lactamica*, with no obvious contaminants.
6. Add 3 ml of MC.7 medium to the edge of the bacterial lawn and use a sterile spreader to very gently scrape over the surface of the lawn to liberate the adhered bacteria into the medium. Caution: Aerosol formation.
7. Using a Pasteur pipette, transfer the bacterial suspension from the surface of the plate into a 50ml tube containing a 0.2 µm filter fitted into the cap (Bio-reaction tube).
8. Top the tube up to 30 ml with medium.
9. Incubate at 37 ^{o}C in a 5 % CO_2 shaking incubator at 350 RPM overnight.

10. Use 2 ml of the culture from step 9 to inoculate 150 ml of medium in a 250 ml baffled, vented flask. Use the remainder of the culture to prepare fresh glycerol stocks if required. Caution: Aerosol formation.
11. Incubate for 8 hours. Divide this culture into two other bottles, giving a total of 3 bottles containing 150 ml in each. Culture overnight until an OD_{600nm} of at least 2.0 has been reached.
12. Pour the contents of the bottle into 50 ml tubes. Caution: Aerosol formation. Spray the outside of the tubes with 70 % ethanol to decontaminate any spillages and spray your gloves to do the same. Dispose of gloves, wash hands and put on a fresh pair of gloves.
13. Place tubes in a water bath set to 56 °C and heat kill the bacteria by incubating for 45 minutes. 30 minutes is sufficient, but it will take 10 minutes for the suspension to reach temperature.
14. Centrifuge heat-killed bacteria at 4,500 x *g* for 1 hour at 20 °C to pellet.
15. Pour off supernatant. Pellet may be stored at -20 °C.
16. Measure wet mass of cell pellet and Resuspend in buffer 1 using a buffer:biomass ratio of 5:1 (V/W).
17. Homogenise pellet using a glass homogeniser. Sterilise the glass homogeniser by filling with 70 % ethanol. Leave for 5 minutes and then rinse with sterile water.
18. Centrifuge suspension at 20,000 x *g* for 30 minutes at 4 °C. Sterilise the ultracentrifuge tubes by filling with 70 % ethanol for 5 minutes. Spray caps with 70 % ethanol. Then rinse tubes and caps with sterile water. Do not autoclave the ultracentrifuge tubes and do not expose to pure ethanol.
19. Retain supernatant and resuspend pellet in buffer 1 (a third of the volume used in step 5).
20. Repeat steps 17 and 18.
21. Retain supernatant and combine with that from step 19.
22. Ultracentrifuge suspension at 100,000 x g for 2 hours at 4 °C.
23. Resuspend pellet in 2.5 ml of buffer 2 by flicking and vortexing.
24. Ultracentrifuge suspension at 100,000 x g for 2 hours at 4 °C.
25. Resuspend pellet in 2 ml of buffer 3 and transfer to a sterile universal.

26. Homogenise dOMV suspension by adding 6 sterile glass beads to the suspension and vortexing for approximately 10 minutes.
27. Measure protein concentration of homogenised OMV.
28. Assess OMVs by SDS-PAGE followed by coomassie blue staining or silver staining.
29. Store OMV frozen at -20 °C or -80 °C.

Plasmid Vector Sequences

The following vectors are encompassed by the invention herein and their sequences are provided in the sequence listing as part of the description.

pUC19ΔnlaIII:CLOVER-aphA3 (SEQ ID NO: 21)
pUC19ΔnlaIII:aphA3 (SEQ ID NO: 22)
pUC19NHCIS1::HAEC1:(Z)-lacZ (SEQ ID NO: 23)
pUC19NHCIS1::HAEC2:(Z)-lacZ (SEQ ID NO: 24)
pUC19NHCIS2::HAEC1:(Z)-lacZ (SEQ ID NO: 25)
pUC19NHCIS1::HAEC2:porAplusprom-lacZ (SEQ ID NO: 26)
pUC19NHCIS1::HAEC4:nadA-lacZ (SEQ ID NO: 27)
pUC19NHCIS1::HAEC4:(Z)-lacZ (SEQ ID NO: 28)
pSC101NHCIS1::HAEC4:opcA-lacZ (SEQ ID NO: 29)
pUC19Δnhba::HAEC1:(Z)-lacZ (SEQ ID NO: 30)
pUC19Δnhba (SEQ ID NO: 31)
pSC101ΔlacZ:opcA (SEQ ID NO: 32)
pSC101ΔlacZ-synth.lacZ-3'NHCIS1 (SEQ ID NO: 33)
pSC101NHCIS1::HAEC4:porA(P1.7,16)-lacZ (SEQ ID NO: 34)
pSC101NHCIS1::PVporA(P1.7,16)-lacZ (SEQ ID NO: 35)

CLAIMS

1. A modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

2. The modified *Neisseria lactamica* according to claim 2, wherein expression of the heterologous protein encoded by the recombinant DNA is inducible or constitutive.

3. The modified *Neisseria lactamica* according to claim 1 or 2, wherein the heterologous protein is a *Neisseria meningitidis* protein.

4. The modified *Neisseria lactamica* according to claim 3, wherein the *Neisseria meningitidis* protein is a *Neisseria meningitidis* antigen.

5. The modified *Neisseria lactamica* according to claim 4, wherein *Neisseria meningitidis* antigen is PorA.

6. The modified *Neisseria lactamica* according to claim 1 or 2, wherein the heterologous protein is a eukaryote protein, viral protein, or non-*Neisseria* prokaryote protein.

7. The modified *Neisseria lactamica* according to any preceding claim, wherein the heterologous protein comprises a protein capable of enabling or enhancing *Neisseria lactamica* colonisation of a subject.

8. The modified *Neisseria lactamica* according to claim 7, wherein the colonisation of the subject is nasopharyngeal colonisation of the subject.

9. The modified *Neisseria lactamica* according to any preceding claim, wherein the heterologous protein comprises meningococcal adhesin NadA, or a functional homologue, variant or derivative thereof.

10. The modified *Neisseria lactamica* according to any preceding claim, wherein the heterologous protein comprises *N. meningitidis* PorA, or a functional homologue, variant or derivative thereof.

11. The modified *Neisseria lactamica* according to any preceding claim, wherein the recombinant DNA is stably transformed to provide the modified *Neisseria lactamica*.

12. The modified *Neisseria lactamica* according to any preceding claim, wherein the recombinant DNA is inserted into an intergenic chromosomal sequence.

13. The modified *Neisseria lactamica* according to claim 12, wherein the intergenic chromosomal sequence is:

between genes NLY_27080 and NLY_27100 of *Neisseria lactamica* strain Y92-1009, or an equivalent loci thereof in other *Neisseria lactamica* strains; or

between genes NLY_36160 and NLY_36180 of *Neisseria lactamica* strain Y92-1009, or an equivalent loci thereof in other *Neisseria lactamica* strains.

14. The modified *Neisseria lactamica* according to any preceding claim, wherein the recombinant DNA is inserted into NHCIS1 or NHCIS2, or an equivalent thereof in other *Neisseria lactamica* strains.

15. The modified *Neisseria lactamica* according to any preceding claim, wherein the DNA encoding the heterologous protein is at least 600bp in length.

16. The modified *Neisseria lactamica* according to any preceding claim, wherein the modified *Neisseria lactamica* is a β-galactosidase (*lacZ*) deficient mutant.

17 The modified *Neisseria lactamica* according to any preceding claim, wherein the modified *Neisseria lactamica* does not comprise a functional or non-functional wild-type *lacZ* gene.

18. The modified *Neisseria lactamica* according to any preceding claim, wherein the

recombinant DNA further comprises a selection marker.

19. The modified *Neisseria lactamica* according to claim 18, wherein the selection marker comprises a *Neisseria lactamica* β-galactosidase (*lacZ*) gene or a non-*Neisseria lactamica* β-galactosidase (*lacZ*) gene.

20. The modified *Neisseria lactamica* according to claim 18 or 19, wherein the selection marker does not comprise an antibiotic resistance marker/gene.

21. The modified *Neisseria lactamica* according to any preceding claim, wherein the recombinant DNA encodes a promoter.

22. The modified *Neisseria lactamica* according to any preceding claim, wherein the recombinant DNA encodes two promoters.

23. The modified *Neisseria lactamica* according to claim 22, wherein a first promotor promotes expression of the heterologous protein and a second promoter promotes expression of the selection marker, or vice versa.

24. The modified *Neisseria lactamica* according to claim 22 or 23, wherein the first promoter comprises the promoter from the α-2,3 sialyltransferase (*lst)* gene.

25. The modified *Neisseria lactamica* according to any of claims 22 to 24, wherein the second promotor comprises the promotor for the gene coding for Porin B (*porB*).

26. The modified Neisseria lactamica according to claim 22, wherein the first and/or second promoter comprise a hybrid *porA*/*porB* promoter.

27. The modified *Neisseria lactamica* according to any preceding claim, wherein the modified *Neisseria lactamica* does not comprise wild type *lacZ* gene sequence, or substantial parts thereof.

28. A method of prophylactic treatment for pathogenic infection of a subject comprising nasopharyngeal inoculation of a modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

29. The method of prophylactic treatment according to claim 26, wherein the pathogenic infection is meningococcal infection.

30. A method of reducing or preventing meningococcal colonisation of a subject comprising nasopharyngeal inoculation of a modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

31. A method of preventing or reducing meningococcal colonisation of a subject comprising nasopharyngeal inoculation of a wild-type *Neisseria lactamica*.

32. A method of modifying the microbiome of a subject comprising nasopharyngeal inoculation of a modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

33. A method of modifying the microbiome of a subject comprising nasopharyngeal inoculation of wild-type *Neisseria lactamica*.

34. A modified *Neisseria lactamica* for use for the prophylactic treatment of meningococcal infection of a subject, wherein the prophylactic treatment comprises nasopharyngeal inoculation of the modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

35. A wild-type *Neisseria lactamica* for use for the prophylactic treatment of meningococcal infection of a subject, wherein the prophylactic treatment comprises nasopharyngeal inoculation of the wild-type *Neisseria lactamica*.

36. A modified *Neisseria lactamica* for use for reducing or preventing colonisation of *Neisseria meningitidis* in a subject, the use comprising nasopharyngeal inoculation of the modified *Neisseria lactamica*, wherein the modified *Neisseria lactamica* is transformed with recombinant DNA encoding a heterologous protein.

37. A wild-type *Neisseria lactamica* for use for reducing or preventing colonisation of *Neisseria meningitidis* in a subject, the use comprising nasopharyngeal inoculation of the modified wild-type *Neisseria lactamica*.

38. The method of prophylactic treatment for pathogenic infection according to claim 28 or claim 29, or the method of reducing or preventing meningococcal colonisation according to claim 30, or the method of modifying the microbiome of a subject according to claim 32, or the modified *Neisseria lactamica* for use according to claim 34 or 36, wherein the modified *Neisseria lactamica* is provided in a suspension comprising between about 2 x 10^2 per ml and about 2 x 10^8 per ml of the modified *Neisseria lactamica*; or

the method of preventing or reducing meningococcal colonisation of a subject according to claim 21, or method of modifying the microbiome of a subject according to claim 33, or the wild-type *Neisseria lactamica* for use according to 35 or 37, wherein the wild type *Neisseria lactamica* is provided in a suspension comprising between about 2 x 10^2 per ml and about 2 x 10^8 per ml of the wild type *Neisseria lactamica*.

39. The method or use according to claim 36, wherein the suspension of modified or wild-type *Neisseria lactamica* may comprise a pharmaceutically acceptable carrier.

40. The method or use according to claim 39, wherein the pharmaceutically acceptable carrier comprises glycerol.

41. A nucleic acid for mutagenesis of *Neisseria lactamica* comprising one or more Heterologous Antigen Expression Cassettes (HAEC), wherein the HAEC comprises:

-a heterologous nucleic acid sequence;

-a first promoter upstream of the heterologous nucleic acid

sequence;

-a second promoter downstream of the heterologous nucleic acid sequence; and

-a selection marker downstream of the second promoter;

wherein the HAEC is flanked by a sequence homologous to a region of *Neisseria lactamica* chromosome.

42. A nucleic acid for mutagenesis of *Neisseria lactamica* comprising one or more Heterologous Antigen Expression Cassettes (HAEC), wherein the HAEC comprises:

-a heterologous nucleic acid sequence;

-a selection marker; and

-a promoter upstream of the heterologous nucleic acid sequence and selection marker;

wherein the HAEC is flanked by a sequence homologous to a region of *Neisseria lactamica* genome.

43. The nucleic acid according to claim 41 or claim 42, wherein the nucleic acid is a cloning vector.

44. The nucleic acid according to claim 41 or claim 42, wherein the nucleic acid is a PCR product.

45. The nucleic acid according to any one of claims 41 to 44, wherein the nucleic acid comprises a hypermethlyated PCR product.

46. The nucleic acid according to any one of claims 41 to 45, wherein nucleic acid is devoid of one or more restriction enzyme recognition sites selected from NlaI, NlaII, Drg, NlaIII, NlaIV and NgoMIV recognition sites, or combinations thereof.

47. The nucleic acid according to any one of claims 41 to 46, wherein the heterologous expression construct comprises the sequence of HAEC1 (see Figure 11), HAEC2 (see Figure 12), HAEC3 (see Figure 25), or HAEC4 (see Figure 26).

48. The nucleic acid according to any one of claims 41 to 47, wherein the HAEC encodes an antigen.

49. The nucleic acid according to any one of claims 41 or 43 to 48, wherein the first promoter comprises the promoter from the sialyltransferase gene and the second promotor comprises the promotor for the gene coding for Porin B (*porB*).

50. The nucleic acid according to any one of claims 41 or 43 to 48, wherein the first and/or second promoter comprises a hybrid *porA/porB* promoter.

51. The nucleic acid according to any one of claims 41 to 50, wherein the selection marker comprises a *Neisseria lactamica* β-galactosidase (*lacZ*) gene.

52. The nucleic acid according to any one of claims 41 to 51, wherein the selection marker does not comprise an antibiotic resistance marker.

53. The nucleic acid according to any one of claims 41 to 52, wherein the nucleic acid comprises a canonical Neisserial DNA Uptake Sequence (DUS) or a canonical AT-flanked Neisserial DNA Uptake Sequence (AT-DUS).

54. The nucleic acid according to any one of claims 41 to 53, wherein the nucleic acid comprises pNHCIS(X)::HAEC(Y):(Z)-*lacZ*.

55. The nucleic acid according to any one of claims 41 to 54, wherein the nucleic acid comprises any one cloning vector selected from
pUC19NHCIS1::HAEC1:(Z)-*lacZ*;
pUC19NHCIS2::HAEC1:(Z)-*lacZ*;
pNHCIS2::HAEC1:(Z)-*lacZ*;
pUC19NHCIS1::HAEC2:*porA*plusprom-*lacZ*;
pUC19NHCIS1::HAEC4:*nadA*-*lacZ*;
pUC19NHCIS1::HAEC4:*nadA*-*lacZ*;

pSC101NHCIS1::HAEC4:*opcA-lacZ*;

pUC19*Δnhba*::HAEC1:(Z)-*lacZ;*

pUC19*Δnhba*;

pSC101::*ΔlacZ:opcA*;

pSC101::*ΔlacZ*-synth.*lacZ*-3'ENDNHCIS1;

pSC101NHCIS1::HAEC4:*porA*(P1.7,16)-*lacZ*; or

pSC101NHCIS1::PV*porA*(P1.7,16)-*lacZ.*

56. A method of mutagenesis of *Neisseria lactamica* comprising transformation of *Neisseria lactamica* with the nucleic acid according to claims 41 to 55.

57. The method of mutagenesis according to claim 56, wherein the nucleic acid is hypermethylated nucleic acid.

58. The method of mutagenesis according to claim 56 or 57, further comprising screening for successful transformants by the use of the selection marker comprising a β-galactosidase (*lacZ*) gene.

59. An outer membrane vesicle (OMV) vaccine, wherein the OMV is an OMV of the modified *Neisseria lactamica* according to any one of claims 1 to 27.

60. The outer membrane vesicle (OMV) vaccine according to claim 59, wherein the OMV comprises a protein, or a variant or part thereof, which is heterologous to the modified *Neisseria lactamica*, optionally wherein the protein is PorA.

61 A composition comprising the OMV according to claim 59 or 60; or the modified *Neisseria lactamica* according to any one of claims 1 to 27, or a composition comprising wild-type *N. lactamica*.

62. The composition according to claim 61, wherein the composition is a pharmaceutically acceptable composition.

63. The OMV according to claim 59 or 60; or the modified *Neisseria lactamica* according to any one of claims 1 to 27; or the composition according to claim 61 or 62, for use in a vaccine.

64. A method of vaccination of a subject for the prevention of infection or colonisation of a pathogen comprising the administration of the OMV according to claim 59 or 60; or the modified *Neisseria lactamica* according to any one of claims 1 to 27; or the composition according to claim 61 or 62.

65. The use of claim 63 or the method of claim 64, wherein the vaccination is for prevention of infection or colonisation of *Neisseria meningitidis* in the subject.

66. A modified *Neisseria lactamica*, wild type *Neisseria lactamica*; composition; nucleic acid; method of mutagenesis; method of reducing or preventing colonisation; method of prophylactic treatment; method of modifying a microbiome; or uses, substantially as described herein, optionally with reference to the accompanying figures.

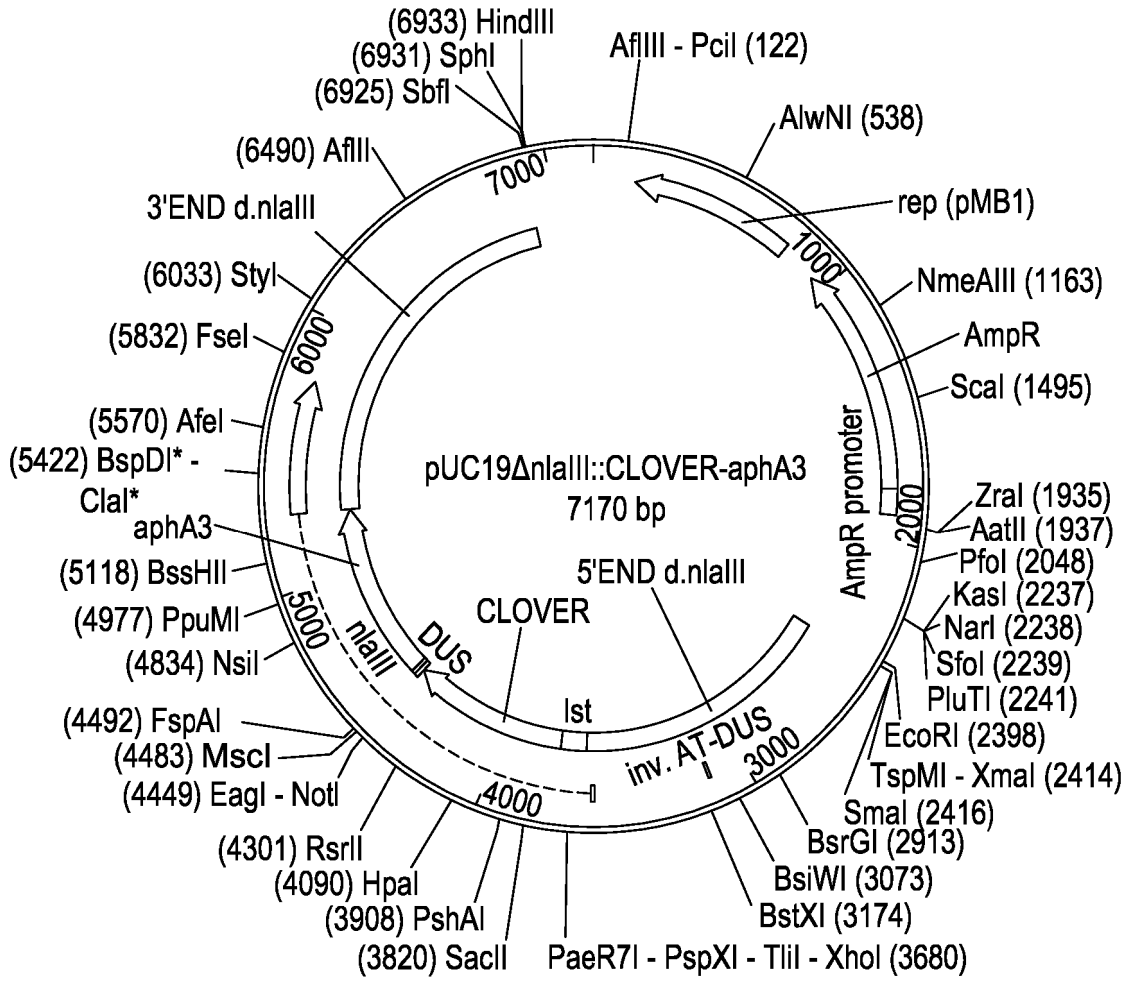
(6933) HindIII
(6931) SphI
(6925) SbfI
(6490) AflII
3'END d.nlaIII
(6033) StyI
(5832) FseI
(5570) AfeI
(5422) BspDI* - ClaI*
aphA3
(5118) BssHII
(4977) PpuMI
(4834) NsiI
(4492) FspAI
(4483) MscI
(4449) EagI - NotI
(4301) RsrII
(4090) HpaI
(3908) PshAI
(3820) SacII
PaeR7I - PspXI - TliI - XhoI (3680)
BstXI (3174)
BsiWI (3073)
BsrGI (2913)
SmaI (2416)
TspMI - XmaI (2414)
EcoRI (2398)
PluTI (2241)
SfoI (2239)
NarI (2238)
KasI (2237)
PfoI (2048)
AatII (1937)
ZraI (1935)
AmpR promoter
ScaI (1495)
AmpR
NmeAIII (1163)
rep (pMB1)
AlwNI (538)
AflIII - PciI (122)
pUC19ΔnlaIII::CLOVER-aphA3
7170 bp
5'END d.nlaIII
CLOVER
DUS
nlaIII
lst
inv. AT-DUS
1000
2000
3000
4000
5000
6000
7000

Figure 1

5' -

ATGGTTTCGAAAGGGGAAGAACTGTTTACAGGCGTGGTACCAATCCTCGTGGAACTGGATGGCGATGTCAACGGCCATAAATTC

TCTGTCCGCGGCGAAGGTGAAGGCGATGCAACCAATGGCAAATTGACGTTGAAATTCATTTGCACCACCGGCAAACTGCCCGTTC

CGTGGCCGACGCTGGTCACAACCTTCGGATATGGCGTCGCCTGCTTCTCGCGCTACCCGGATCACATGAAACAACACGACTTCTT

CAAATCCGCAATGCCGGAAGGTTATGTTCAGGAACGTACTATTTCGTTCAAGGATGACGGCACCTACAAAACCCGCGCCGAAGTC

AAATTTGAAGGCGATACCTTAGTTAACCGTATTGAATTGAAAGGCATTGACTTTAAAGAAGACGGCAACATCCTGGGCCACAAAT

TAGAATACAACTTCAACTCGCATAACGTCTATATCACAGCCGATAAGCAGAAAAATGGCATAAAAGCCAACTTCAAAATTCGCCA

CAACGTTGAGGATGGTAGCGTTCAGTTGGCTGACCACTATCAACAAAACACTCCCATCGGCGACGGTCCGGTACTGTTGCCCGAC

AACCATTACCTTTCCCACCAGTCCGCCCTGTCCAAAGATCCGAATGAAAAACGCGACCACATGGTTCTGCTGGAGTTCGTCACTGC

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TTTCCGGCTTTATCGACTTGGGCCGCAGCGGGCGCGCAGATAAATGGTACGACATTGCGTTCTGCGTCCGCAGCATCCGTGAAG

ATATCGGTGAAGAACAATACGTCGAGCTCTTTTTCGACTTGCTGGGTATTAAACCGGATTGGGAAAAAATCAAATATTATATCCT

GTTGGATGAATTATTCTAG - 3'

Figure 2 (SEQ ID NO: 3)

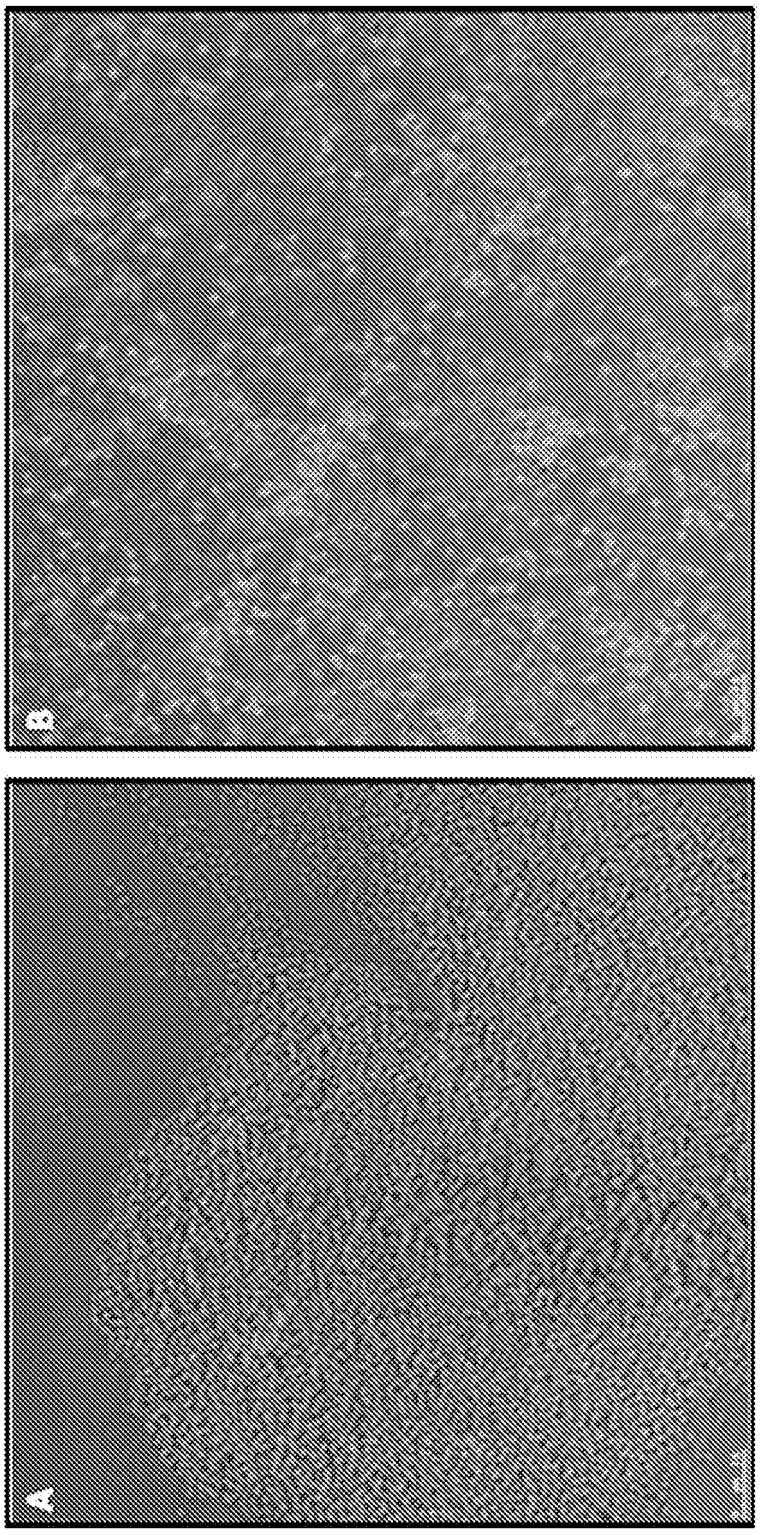


Figure 3

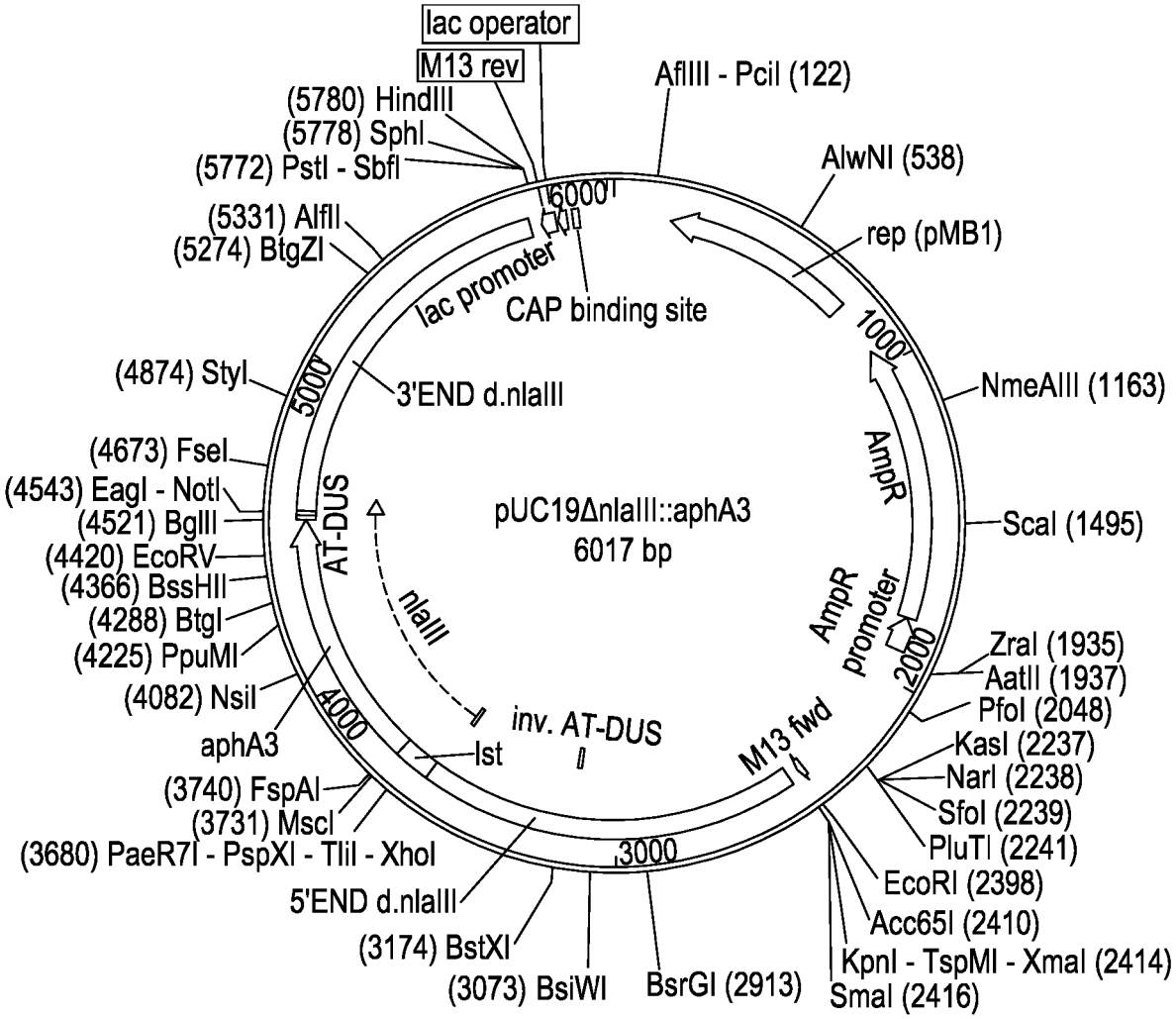

lac operator
M13 rev
(5780) HindIII
(5778) SphI
(5772) PstI - SbfI
(5331) AlfI
(5274) BtgZI
(4874) StyI
(4673) FseI
(4543) EagI - NotI
(4521) BglII
(4420) EcoRV
(4366) BssHII
(4288) BtgI
(4225) PpuMI
(4082) NsiI
aphA3
(3740) FspAI
(3731) MscI
(3680) PaeR7I - PspXI - TliI - XhoI
5'END d.nlaIII
(3174) BstXI
(3073) BsiWI
BsrGI (2913)
SmaI (2416)
KpnI - TspMI - XmaI (2414)
Acc65I (2410)
EcoRI (2398)
PluTI (2241)
SfoI (2239)
NarI (2238)
KasI (2237)
PfoI (2048)
AatII (1937)
ZraI (1935)
ScaI (1495)
NmeAIII (1163)
rep (pMB1)
AlwNI (538)
AflIII - PciI (122)
lac promoter
CAP binding site
3'END d.nlaIII
AT-DUS
nlaIII
inv. AT-DUS
lst
M13 fwd
AmpR
AmpR promoter
pUC19ΔnlaIII::aphA3
6017 bp
1000
2000
3000
4000
5000
6000

Figure 1

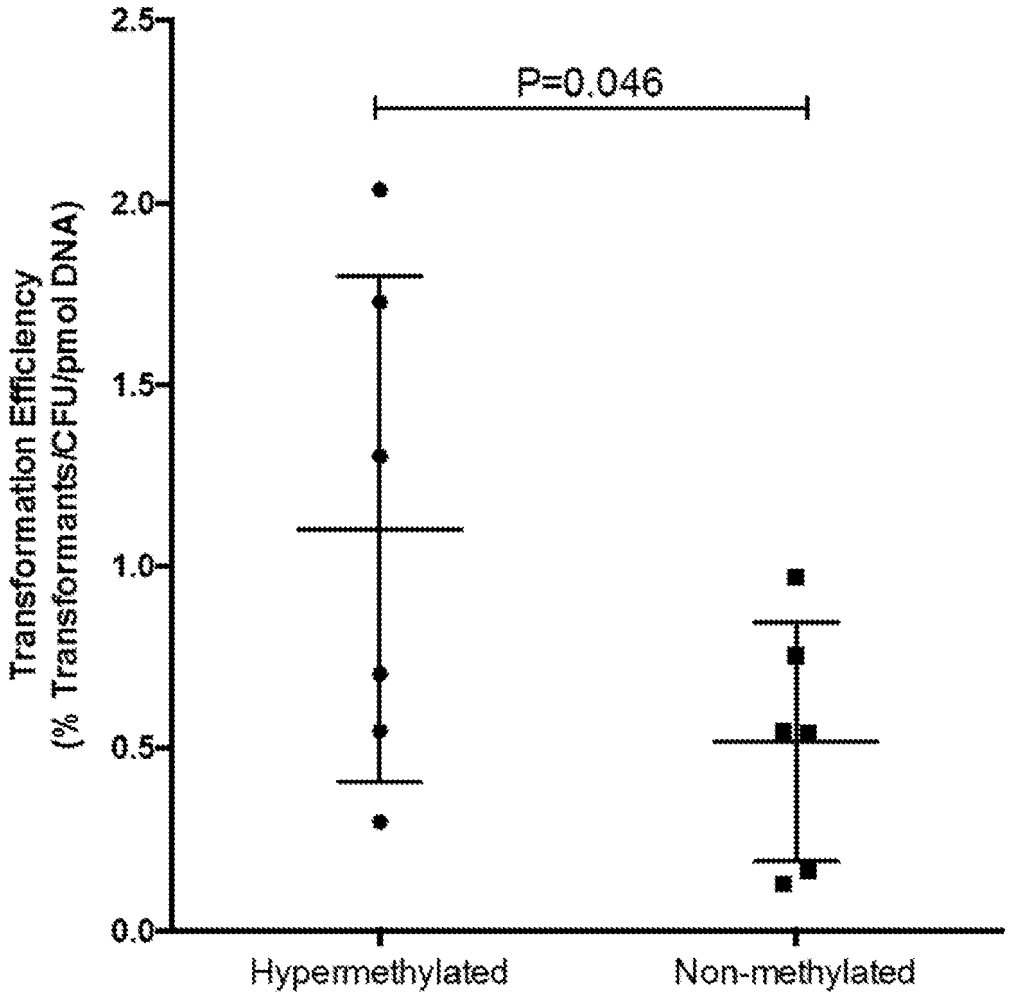

P=0.046
Transformation Efficiency
(% Transformants/CFU/pmol DNA)
2.5
2.0
1.5
1.0
0.5
0.0
Hypermethylated
Non-methylated

Figure 5

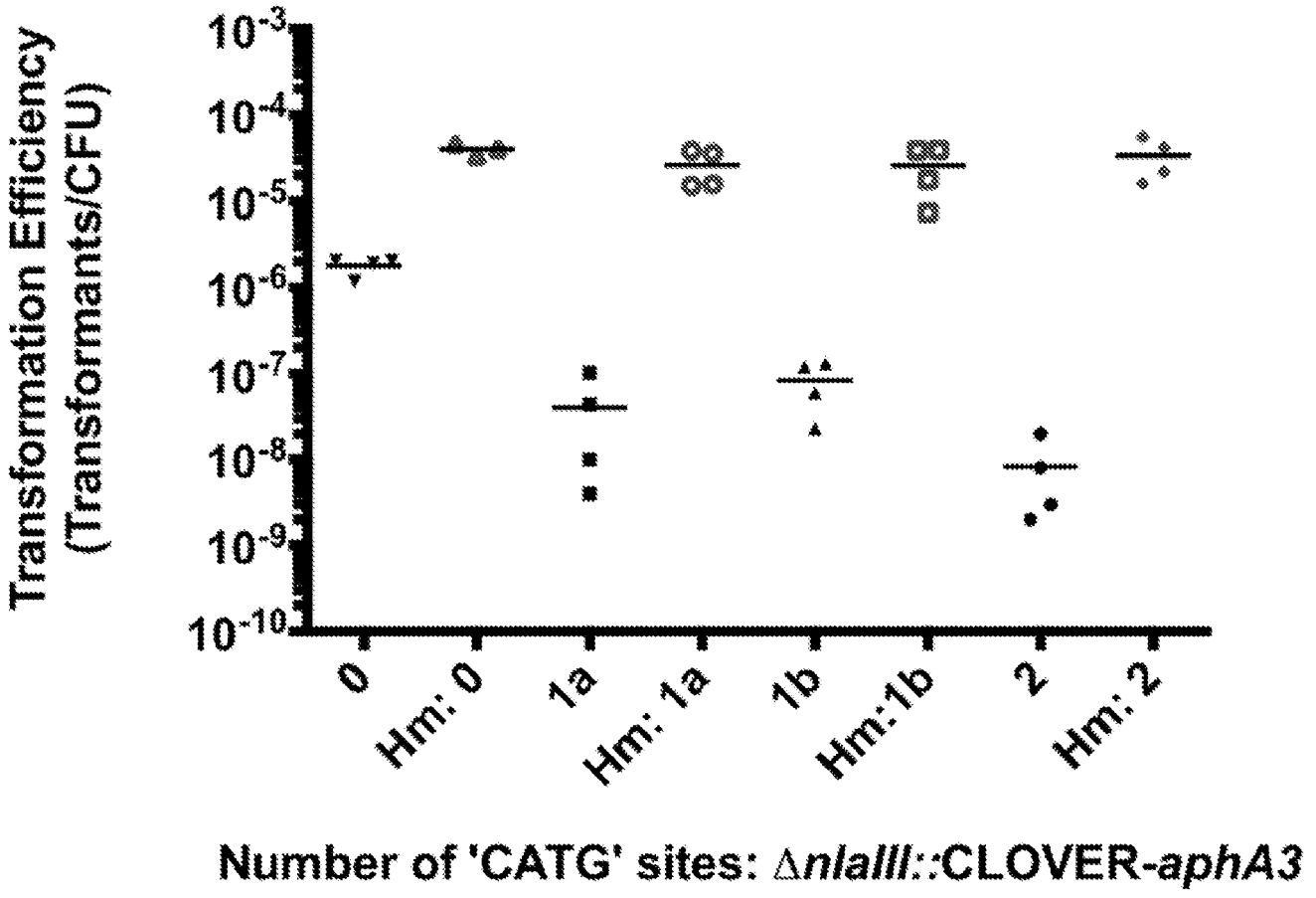
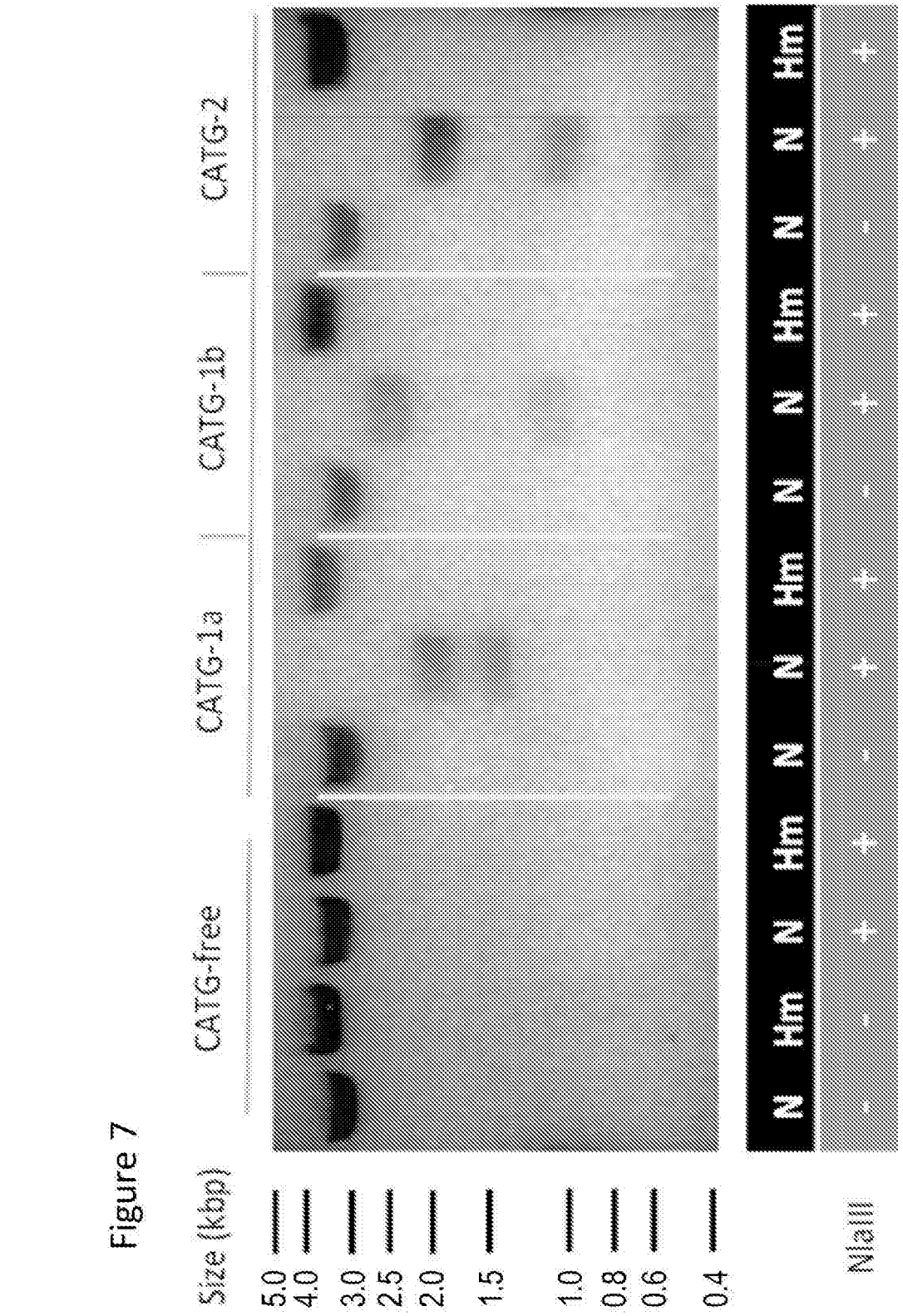
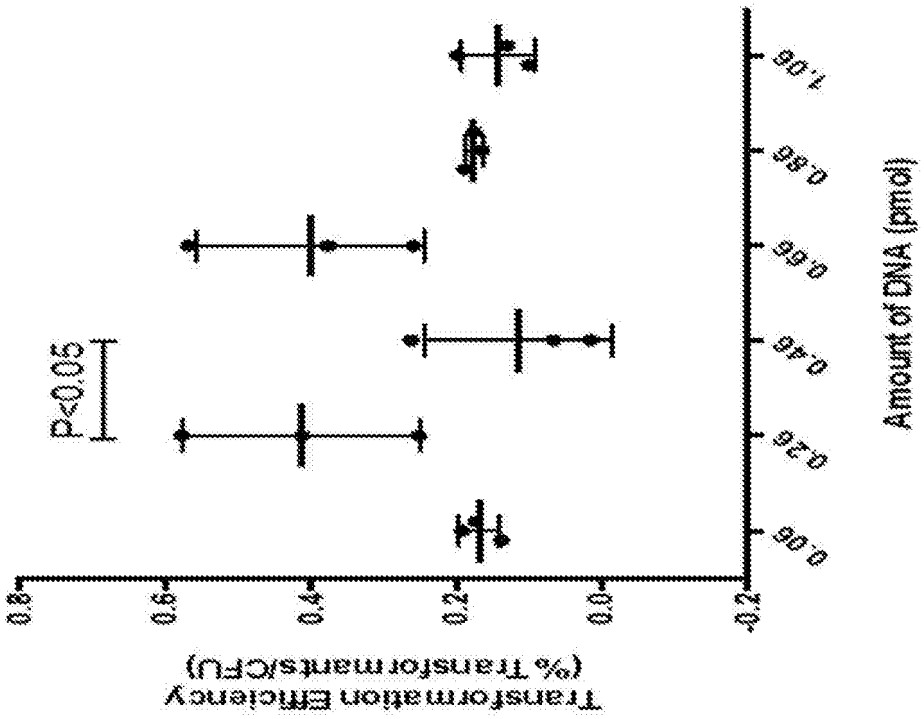

Transformation Efficiency
(Transformants/CFU)
10-3
10-4
10-5
10-6
10-7
10-8
10-9
10-10
0
Hm: 0
1a
Hm: 1a
1b
Hm:1b
2
Hm: 2
Number of 'CATG' sites: ΔnlaIII::CLOVER-aphA3

Figure 6

Figure 7


CATG-free
CATG-1a
CATG-1b
CATG-2
Size (kbp)
5.0
4.0
3.0
2.5
2.0
1.5
1.0
0.8
0.6
0.4
N Hm N Hm N N Hm N N Hm N N
- - + + - + + - + + - +
NlaIII


Transformation Efficiency
(% Transformants/CFU)
0.8
0.6
0.4
0.2
0.0
-0.2
P<0.05
0.0
0.2
0.4
0.6
0.8
1.0
Amount of DNA (pmol)

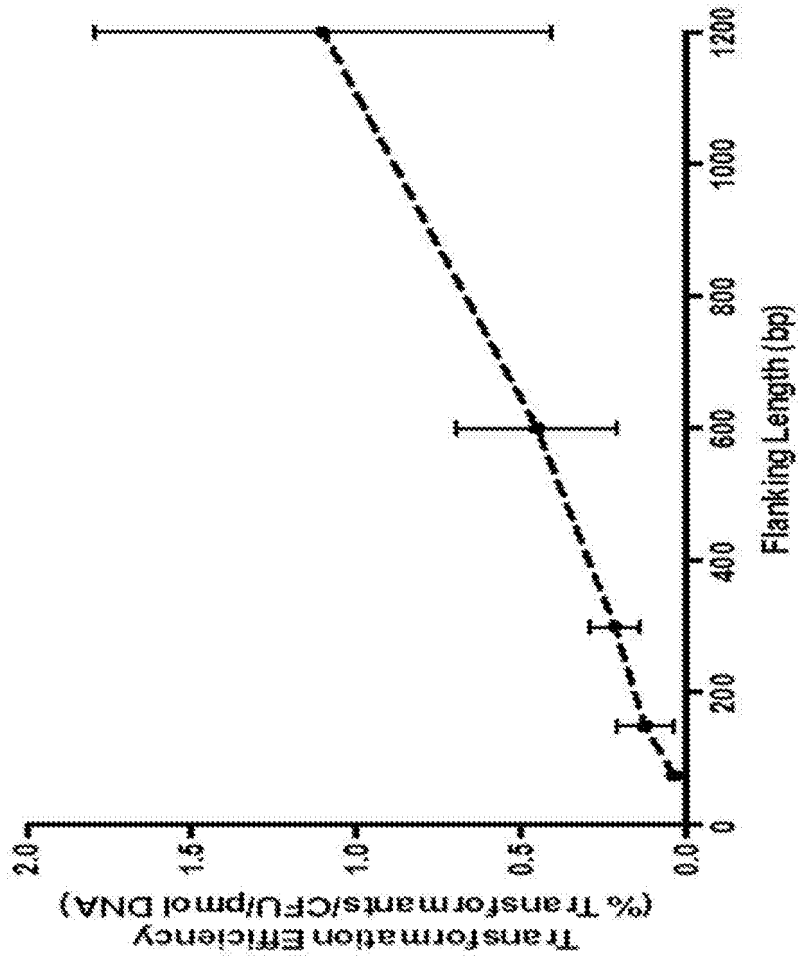

Transformation Efficiency
(% Transformants/CFU/pmol DNA)
2.0
1.5
1.0
0.5
0.0
0
200
400
600
800
1000
1200
Flanking Length (bp)

Figure 8

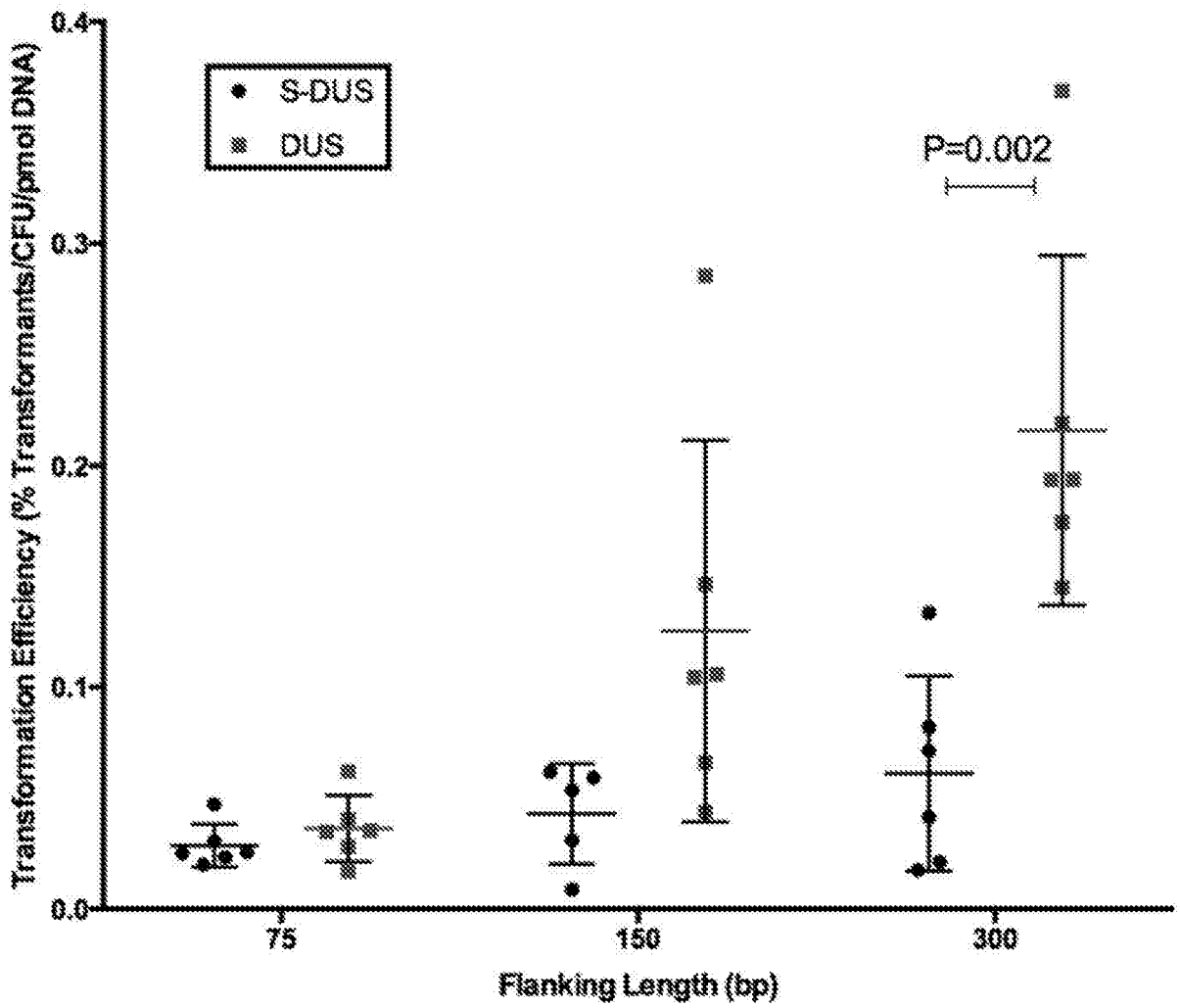
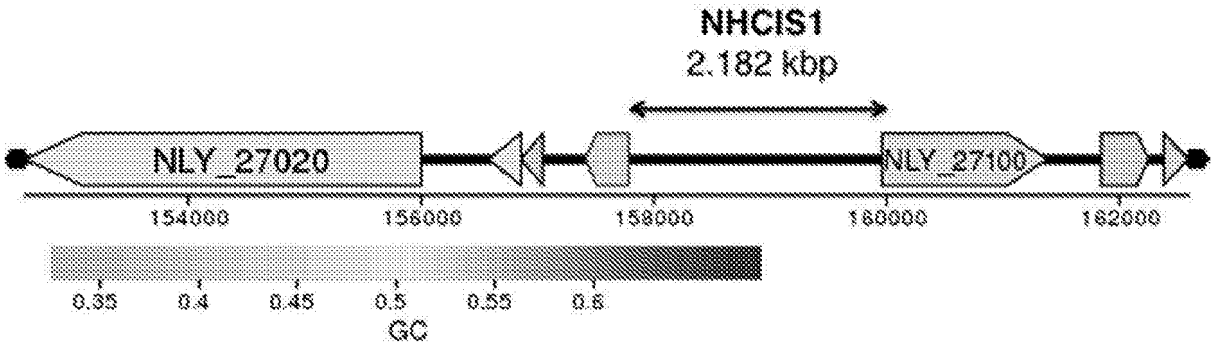

S-DUS
DUS
P=0.002
Transformation Efficiency (% Transformants/CFU/pmol DNA)
0.4
0.3
0.2
0.1
0.0
75
150
300
Flanking Length (bp)

Figure 9



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AGTACCGCTGCCATTGCCAATCTCGCCGGGGAAATGAAAAGGCTCGCTAAAAAATCAAAAATGTTACTGAAAGGACTTTGGTCAT
TTTTATCCTCTACAATATCTACATTTAAAAATAAATCGGTCATTGTTTAAACCTTACTGTAAAACTGTACAACTGCTAAACTGTAAAC
ACAGAAAGAGGCAATACTAACAGCACAAAACAACAGATATCCAAACTCCATAAAGTGCATTATTCTGACTTTTTTCGTTGCCTGAT
GTATTTATGCTATTTGTGCTGTTCTTATATATGTGTTGCTGGTTACGTTGCGTTTTTTCGGAAAATTCAACCGGTAGGGGACCGATA
CGCAGTTTCATCTCTTTGCTTAGGGAGAGTAGGGGGGTAGATTACGACCTTAGTTTTGGTATCCGTAATATCATCATTTTTTCGTCT
AGGGAGTATATCGACTTCAGAAAACAGGTATTAGATACTGCCTTTTCTTACGAGAGTGATGGCAAGATAGTTCTCTTCAAGTCAAT
CAAACAGGAAAGTATTTCTTTTCTGTCTGAAGATTTGAAAAAGGACTGAATGTTTCACAAGGTTAAAACTGGGGAAAAAGATGGA
TATGGTTCAGATGAAATGCTGAGCGCACCCCGTATCTATTTGGAAATGATGTCGCGGAAAACGGAAGTCCCCTACTCCAGTATTC
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3'NHCIS1: (SEQ ID NO: 5)

5'TGCTGAAGTAGAAAACCAGCAAGAAGGTAAAAAGAAAGAAGCAGTTTTTTGGATTTTAGATGTTACCGCAATTGGTTTCCT
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TGTACAAAATATTTGCAGTATTTAAAAATGTTGGTTCGTATATGAAAAGTTAAAAATGCCAAAATGTACAGTTGCTAAACTGTAAA
ACTGCTAAAGCAACAAAACATAAAAAGGAATGCAGGGATGCGATCACTACATCTTTTTATTCCGTAAGCATTTATGACTTTACGGT
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GGCATTGGCTTTAATCTCTTCGATATGAACTCCATTTTTAGCTGCACCTTCTTTCAGCGTATGCAACAGTGGGGATGGGGCAACTA
TCTTCTGGTGGAGTTTGTTGACTTCAATCTGTATGCACTGTAGGTTGAGCAGATTCAGTTTTTTGATACAGATTATCCGGCTTTGTT
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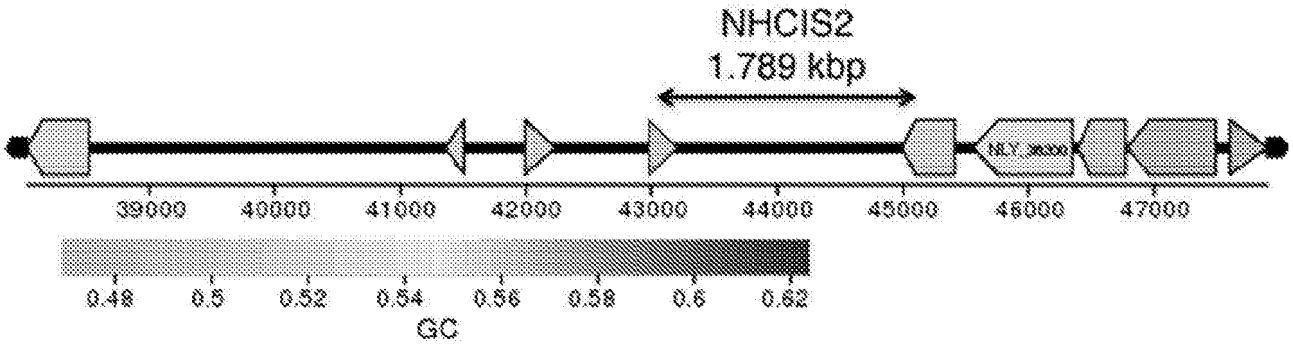
Figure 10



5' NHCIS2: (SEQ ID NO: 6)

5'*CGGGCCGGAAGACGATTATATGAATGACGATCATCTGGCTTTTTTCCGCGAATTGCTGGTAAAAATGCAAGACGAACTCAT CGAAAATGCTTCCGCTACGACAGGGCATCTCCAAGAACATGAATCAGCCCCCGATCCTGCCGACCGTGCCACACAGGAAGAAGAG TACGCATTGGAACTCCGTACCCGCGATCGGGAACGAAAACTTCTCAGTAAAATACAGGCGACCATCCGCAATATTGACAGGGGCG ATTATGGATTCTGCGCCGATACGGGAGAACCTATCGGGCTGAAGCGGCTGTTGGCGCGCCCGACCGCCACTTTGTCCGTAGAGGC GCAAGAGCGTCGTGAGAGAATGAAAAAACAGTTTGCCGACTAA*TAGCGGCAAACGAAA**ATGCCGTCTGAA**GCCCCGAGT**TTCA GACGGCAT**ATTCACAAAGGCGCACCAGCCAGAGGAGAAGAGGAAGGGATTTTTGGAGGCGGCGCAGCATTTGGCGGAAATAA AAAACCTTATCTGACAGGGATATGACGAATTTCCCCAAAAAATCCCGCTGAAAGTGTTGACCGCCTCCGTCTTCGGGCGTATAGTT CGGTTCTTCGCTGCCGACGAAGCGGCGGAATGAAACGGACAAGTATATCACGGTTTGCAGGATGTTTGACGCATCGGCCGTACA TACCGACAGTTTCAAACGCTCTTTAACAAAACAGATTACCGATAAGTGTGAGTGCCTCGGGCCTCACACTGTTTGAAAGACAGAC AAGATAATGTTTTGAACATTGTCCTGTCGGTTTCTTTGAAGCAGACCAGAAGTTAAAAAGTTAGAGATTGAACATAAGAGTTTGA TCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGGACGGCAGCGGGGTAGTGCTTGCACTACTGCCGGCG AGTGGCGAACGGGTGAGTAACATATCGGAACGTACCGGGCAGTGGGGGATAACTGATCGAAAGATCAGCTAATACCGCATATTT TCTGAGGAAGAAAGCAGGGG**3'**

3' NHCIS2: (SEQ ID NO: 7)

5'ACCATTTGGCCTTGCGCTATCCGAGCGGCCGATATCTGATTAGCTTGTTGGCGGGGTAAGGGCCCACCAAGGCGACGATC AGTAGCGGGTCTGAGAGGACGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTT TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTCTGAAGAAGGCCTTCGGGTTGTAAAGGACTTTTGTCGGGGAAGA AAAGGCTGTTGCTAATATCAGCGGCTGATGACGGTACCCGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATA CGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGGGCGCAGACGGTTACTTAAGCAGGATGTGAAATCCCCGGGC TCAACCCGGGAATTGCGTTCTGAACTGGGTGGCTAGAGTGTGTCAGAGGGAGGTAGAATTCCACGTGTAGCAGTGAAATGCGTA GAGATGTGGAGGAATACCGATGGCGAAGGCAGCCTCCTGGGATAACACTGACGTTCATGTCCGAAAGCGTGGGTAGCAAACAG GATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCGATTAGCTGTTGGGCAGCCTGACTGCTTGGTAGCGAAGCTAACGCGT GAAATCGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTG GATTAATTCGATGCAACGCGAAGAACCTTACCCGGTTTTGACATGTACGGAATCCTCCGGAGACGGAGGAGTGCCTTCGGGAGC CGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATT AGTTGCCATCATTCAGTTGGGCACTCTAATGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGAC**3'**

Figure 11

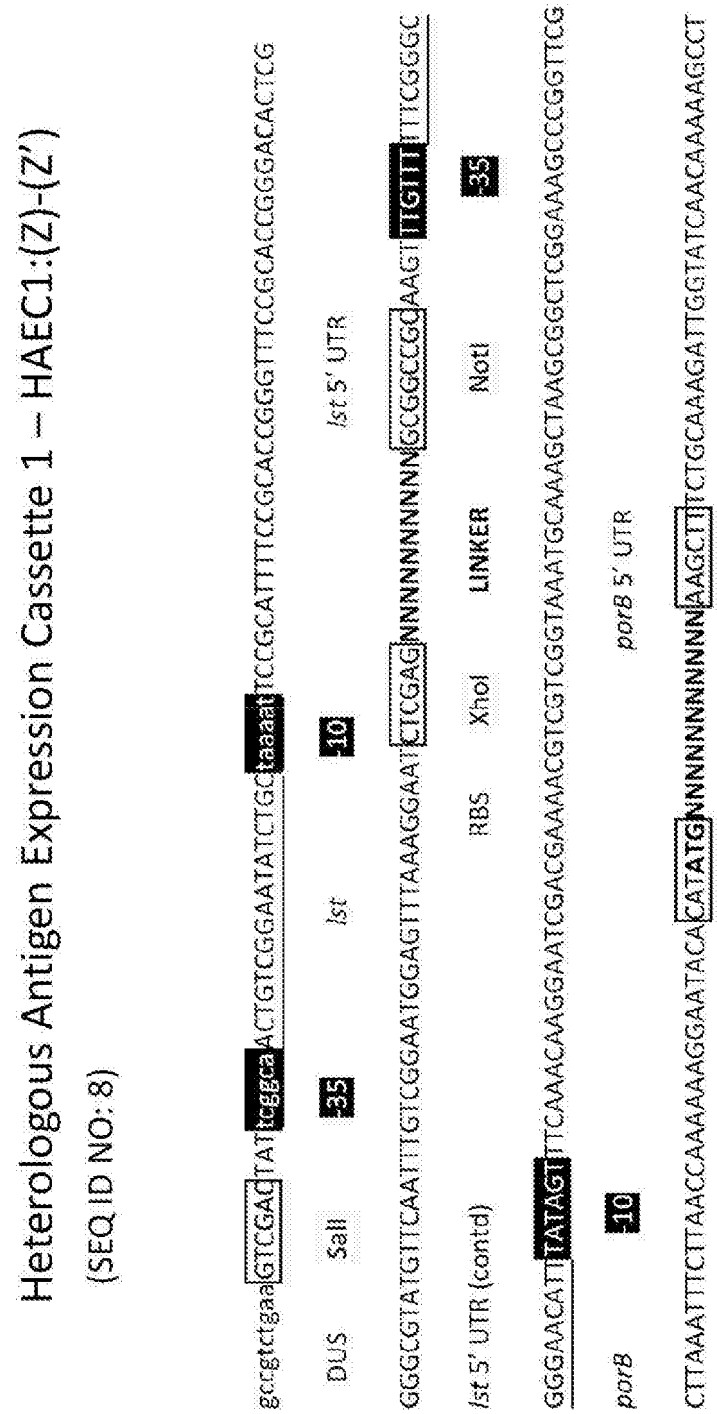

Heterologous Antigen Expression Cassette 1 – HAEC1:(Z)-(Z')
(SEQ ID NO: 8)
gcrgtctgaaGTCGACTATtcggcaACTGTCGGAATATCTGCtaaaatTCCGCATTTTCCGCACCGGGTTTCCGCACCGGGACACTCG
DUS SalI -35 10 lst lst 5' UTR
GGGCGTATGTTCAATTTGTCGGAATGGAGTTTAAAGGAATCTCGAGNNNNNNNNNNGCGGCCGCAAGTTTGTTTTCGGGC
lst 5' UTR (contd) RBS XhoI LINKER NotI -35
GGGAACATTTATAGTTTCAAACAAGGAATCGACGAAAACGTCGTCGGTAAATGCAAAGCTAAGCGGCTCGGAAAGCCCGGTTCG
porB 10 porB 5' UTR
CTTAAATTTCTTAACCAAAAAAGGAATACACATATGNNNNNNNNNNAAGCTTTCTGCAAAGATTGGTATCAACAAAAAGCCT
porB 5' UTR (contd) RBS NdeI LINKER HindIII porB TERMINATOR
GTCGTCAGACAGGCTTTTTTTCTGTTTTCTGTTTTTAGatgccgtctgaa
porB TERMINATOR contd. AT-DUS

Figure 12

Heterologous Antigen Expression Cassette 2 – HAEC2:(Z)-(Z')

(SEQ ID NO: 9)

gccgtctgaaGTCGACAGTTGTTTTCGGGCGGGAACATTTATAGTTTCAAACAAGGAATCGACGAAAACGTCGTCGGTAAATGC

DUS SalI -35 *porB* -10 *porB* 5' UTR

AAAGCTAAGCGGCTCGGAAAGCCCGGTTCGCTTAAATTTCTTAACCAAAAAAGGAATCTCGAGNNNNNNNNNNNNGCGGCCGC

porB 5' UTR (contd) RBS XhoI **LINKER** NotI

ATATtcggcaACTGTCGGAATATCTGCtaaaatTCCGCATTTTCCGCACCGGGTTTCCGGCACCGGGACACTCGGGGCGTATGTTCA

-35 *lst* -10 *lst* 5' UTR

ATTTGTCGGAATGGAGTTTAAAGGAATACACATATGNNNNNNNNNNAAGCTTTCTGCAAAGATTGGTATCAACAAAAAGCCT

lst 5' UTR (contd) RBS NdeI **LINKER** HindIII *porB* TERMINATOR

GTCGTCAGACAGGCTTTTTTTCTGTTTTTCTGTTTTTAGatgccgtctgaa

porB TERMINATOR contd. AT-DUS

Figure 13

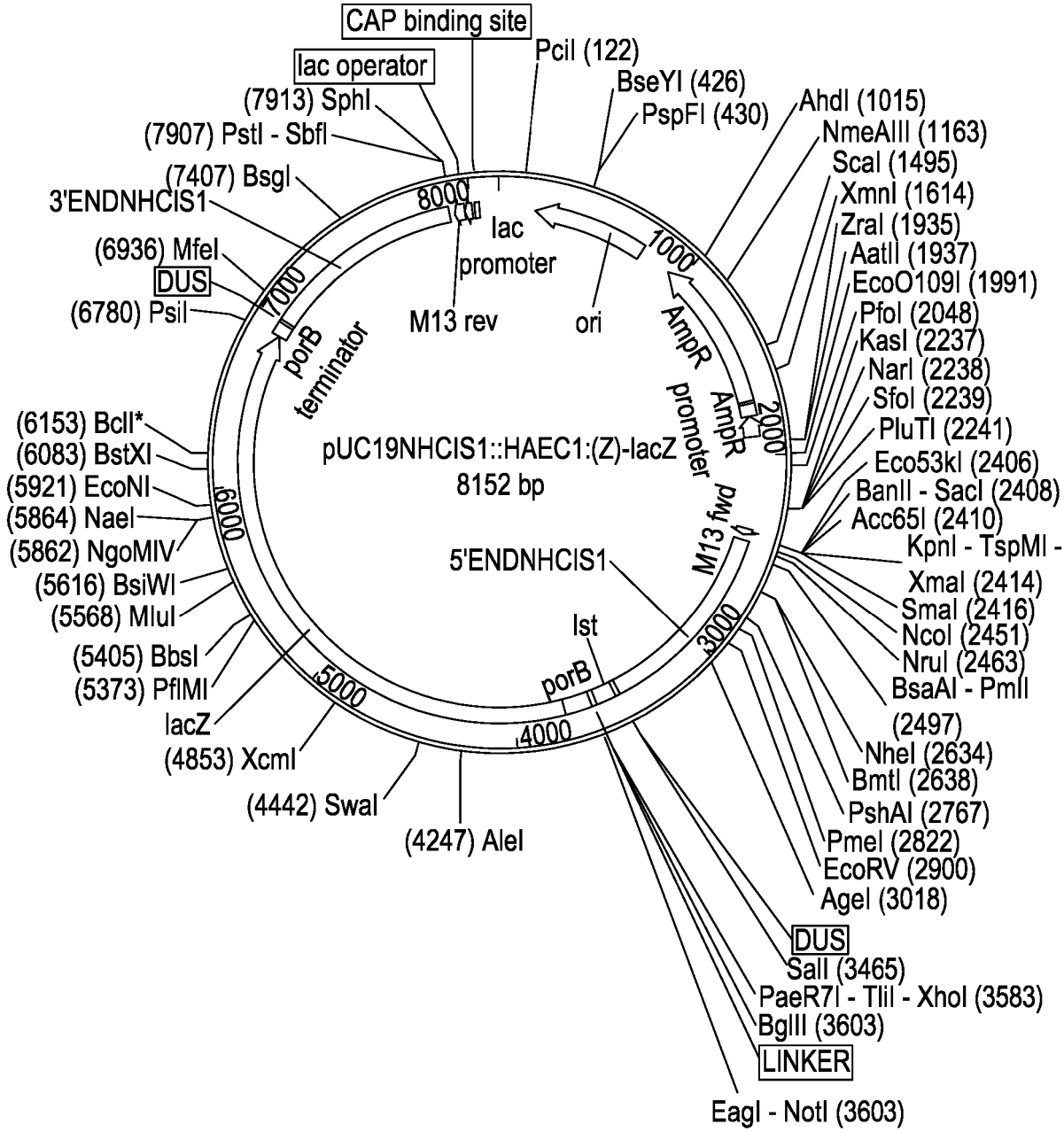

CAP binding site
lac operator
(7913) SphI
(7907) PstI - SbfI
(7407) BsgI
3'ENDNHCIS1
(6936) MfeI
DUS
(6780) PsiI
(6153) BclI*
(6083) BstXI
(5921) EcoNI
(5864) NaeI
(5862) NgoMIV
(5616) BsiWI
(5568) MluI
(5405) BbsI
(5373) PflMI
lacZ
(4853) XcmI
(4442) SwaI
(4247) AleI
PciI (122)
BseYI (426)
PspFI (430)
AhdI (1015)
NmeAIII (1163)
ScaI (1495)
XmnI (1614)
ZraI (1935)
AatII (1937)
EcoO109I (1991)
PfoI (2048)
KasI (2237)
NarI (2238)
SfoI (2239)
PluTI (2241)
Eco53kI (2406)
BanII - SacI (2408)
Acc65I (2410)
KpnI - TspMI -
XmaI (2414)
SmaI (2416)
NcoI (2451)
NruI (2463)
BsaAI - PmlI
(2497)
NheI (2634)
BmtI (2638)
PshAI (2767)
PmeI (2822)
EcoRV (2900)
AgeI (3018)
DUS
SalI (3465)
PaeR7I - TliI - XhoI (3583)
BglII (3603)
LINKER
EagI - NotI (3603)
8000
1000
2000
3000
4000
5000
6000
7000
lac
promoter
M13 rev
ori
AmpR
AmpR
promoter
M13 fwd
porB
terminator
pUC19NHCIS1::HAEC1:(Z)-lacZ
8152 bp
5'ENDNHCIS1
lst
porB

Figure 14

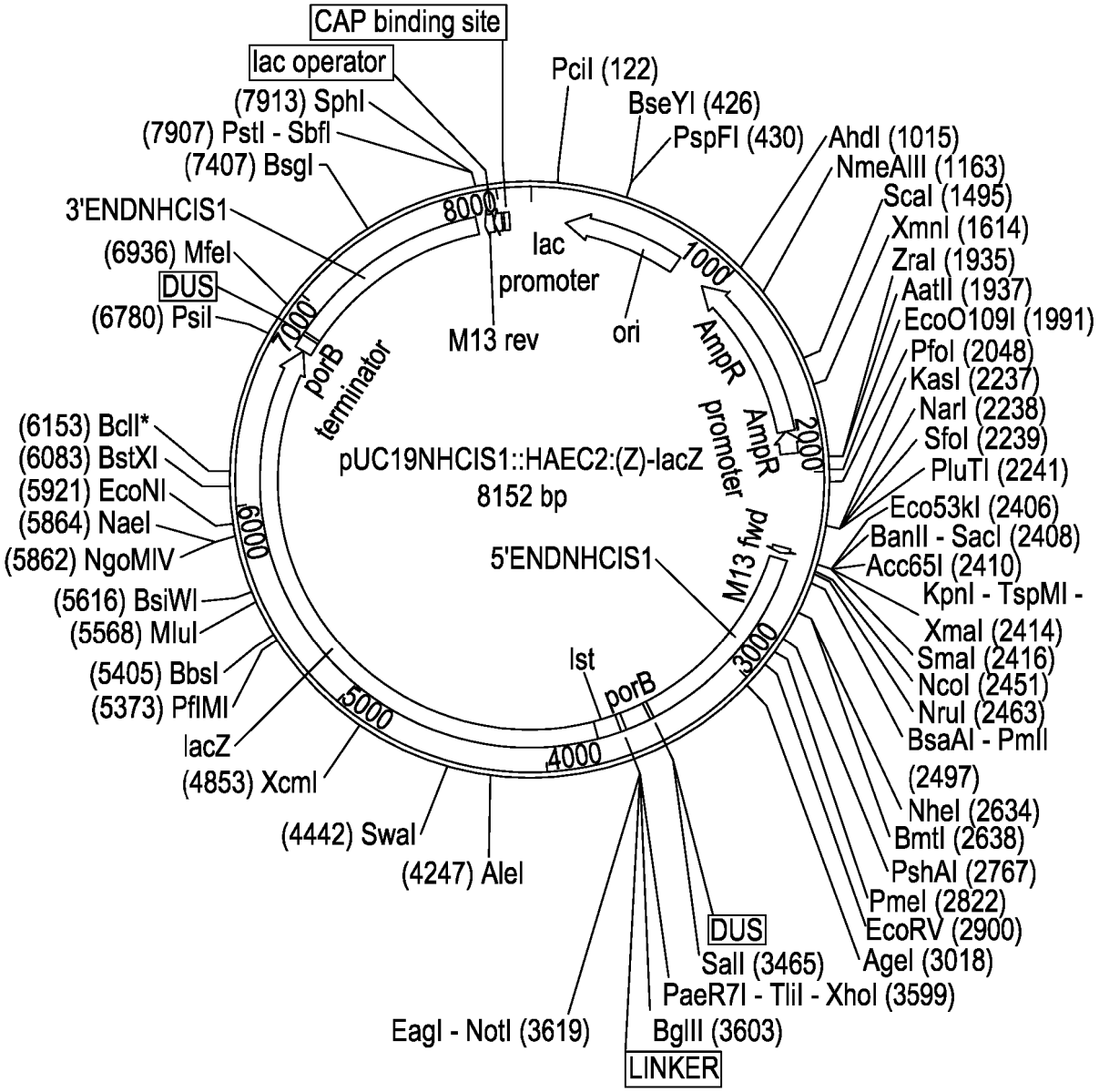

CAP binding site
lac operator
(7913) SphI
(7907) PstI - SbfI
(7407) BsgI
3'ENDNHCIS1
(6936) MfeI
DUS
(6780) PsiI
(6153) BclI*
(6083) BstXI
(5921) EcoNI
(5864) NaeI
(5862) NgoMIV
(5616) BsiWI
(5568) MluI
(5405) BbsI
(5373) PflMI
lacZ
(4853) XcmI
(4442) SwaI
(4247) AleI
EagI - NotI (3619)
BglII (3603)
LINKER
DUS
SalI (3465)
PaeR7I - TliI - XhoI (3599)
AgeI (3018)
EcoRV (2900)
PmeI (2822)
PshAI (2767)
BmtI (2638)
NheI (2634)
BsaAI - PmlI (2497)
NruI (2463)
NcoI (2451)
SmaI (2416)
XmaI (2414)
KpnI - TspMI - Acc65I (2410)
BanII - SacI (2408)
Eco53kI (2406)
PluTI (2241)
SfoI (2239)
NarI (2238)
KasI (2237)
PfoI (2048)
EcoO109I (1991)
AatII (1937)
ZraI (1935)
XmnI (1614)
ScaI (1495)
NmeAIII (1163)
AhdI (1015)
PspFI (430)
BseYI (426)
PciI (122)
lac promoter
M13 rev
ori
AmpR
AmpR promoter
M13 fwd
5'ENDNHCIS1
lst
porB
porB terminator
pUC19NHCIS1::HAEC2:(Z)-lacZ
8152 bp
1000
2000
3000
4000
5000
6000
7000
8000

Figure 15

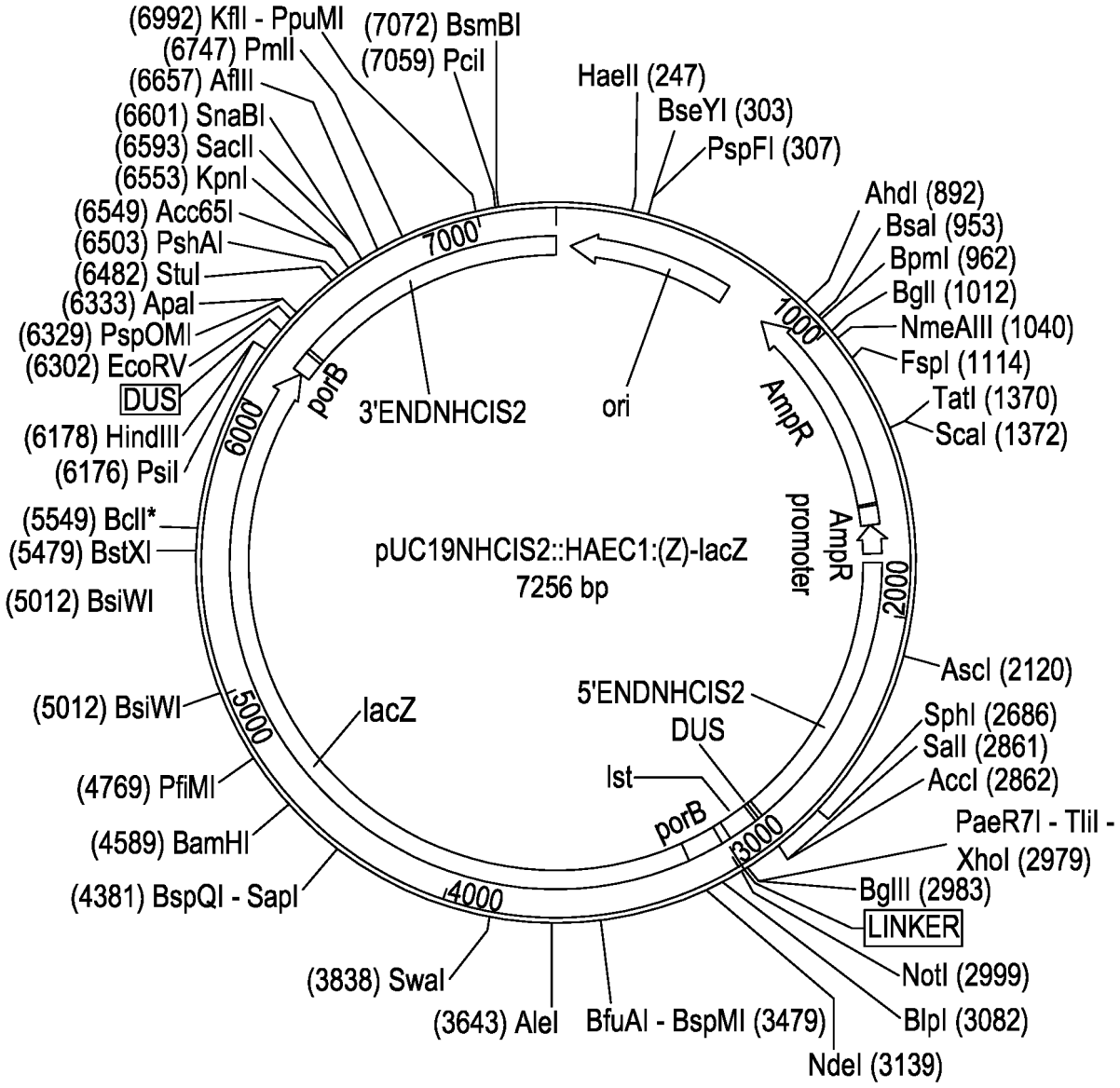

(6992) KflI - PpuMI
(6747) PmlI
(6657) AflII
(6601) SnaBI
(6593) SacII
(6553) KpnI
(6549) Acc65I
(6503) PshAI
(6482) StuI
(6333) ApaI
(6329) PspOMI
(6302) EcoRV
DUS
(6178) HindIII
(6176) PsiI
(5549) BclI*
(5479) BstXI
(5012) BsiWI
(5012) BsiWI
(4769) PfiMI
(4589) BamHI
(4381) BspQI - SapI
(3838) SwaI
(3643) AleI
BfuAI - BspMI (3479)
NdeI (3139)
BlpI (3082)
NotI (2999)
LINKER
BglII (2983)
XhoI (2979)
PaeR7I - TliI -
AccI (2862)
SalI (2861)
SphI (2686)
AscI (2120)
(7072) BsmBI
(7059) PciI
HaeII (247)
BseYI (303)
PspFI (307)
AhdI (892)
BsaI (953)
BpmI (962)
BglI (1012)
NmeAIII (1040)
FspI (1114)
TatI (1370)
ScaI (1372)
7000
1000
2000
3000
4000
5000
6000
porB
3'ENDNHCIS2
ori
AmpR
promoter
AmpR
pUC19NHCIS2::HAEC1:(Z)-lacZ
7256 bp
lacZ
5'ENDNHCIS2
DUS
lst
porB

Figure 16

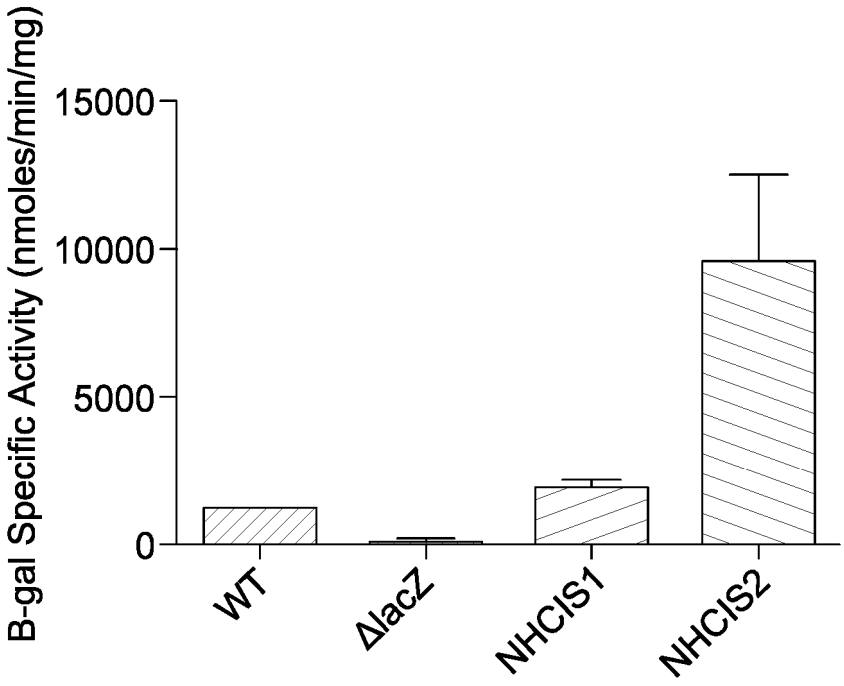

B-gal Specific Activity (nmoles/min/mg)
15000
10000
5000
0
WT
ΔlacZ
NHCIS1
NHCIS2

Figure 17

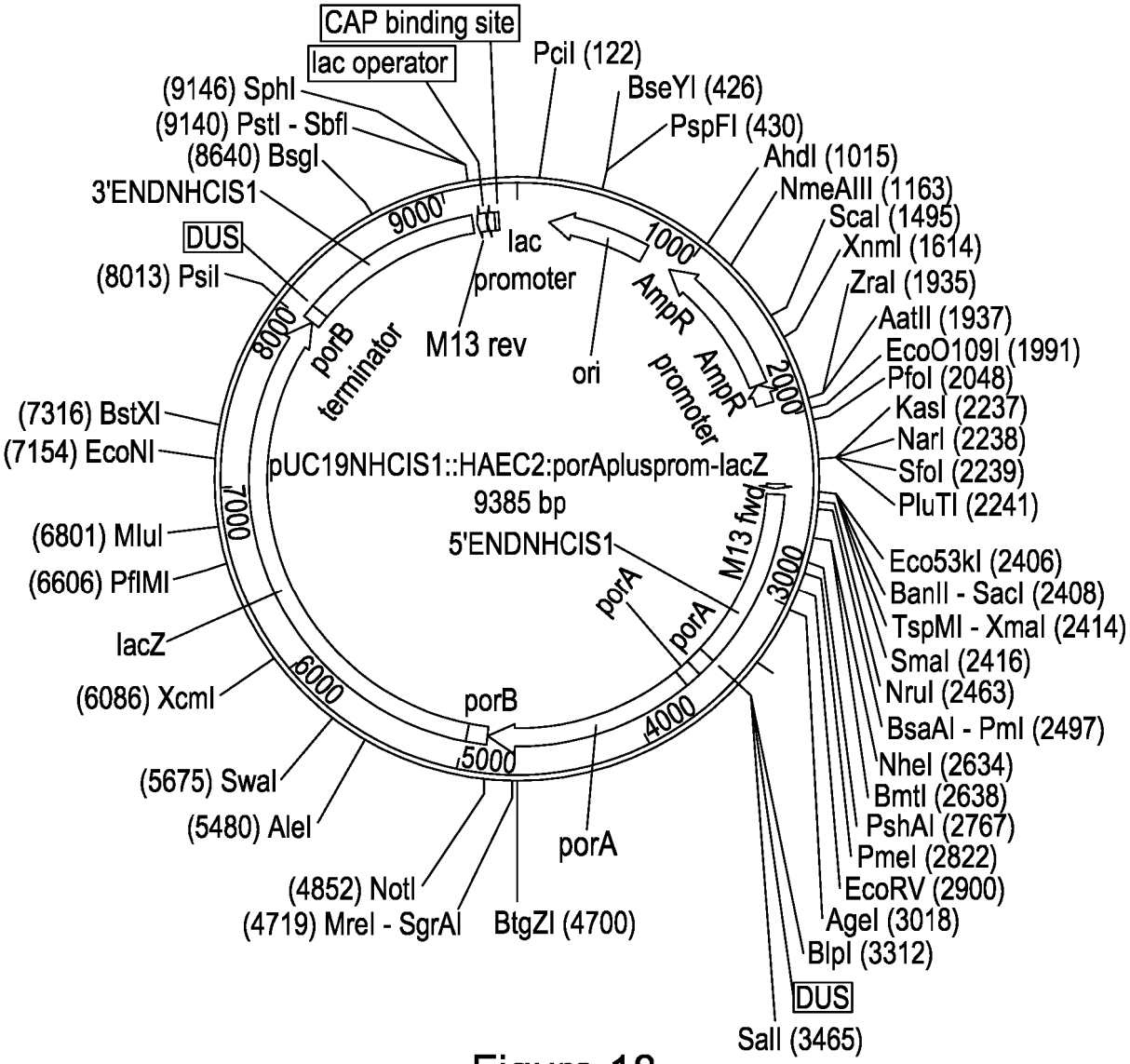
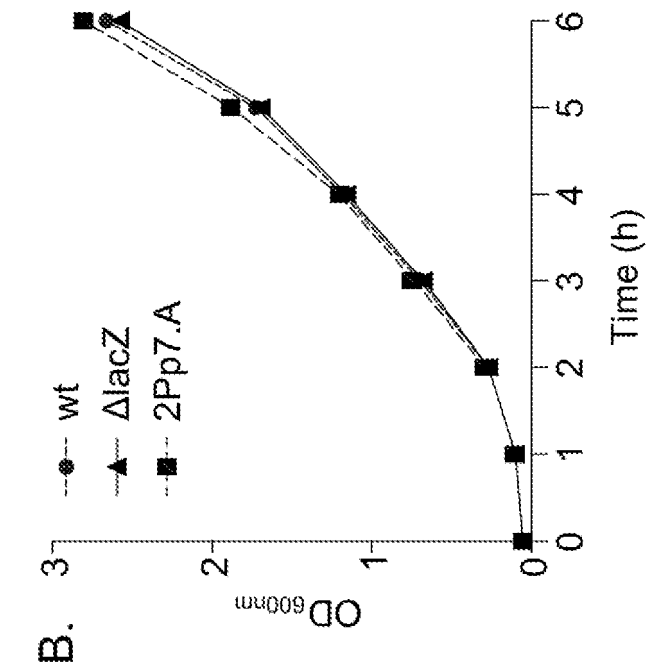
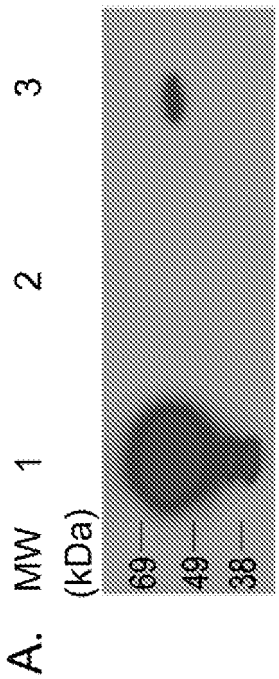

CAP binding site
lac operator
PciI (122)
BseYI (426)
PspFI (430)
AhdI (1015)
NmeAIII (1163)
ScaI (1495)
XnmI (1614)
ZraI (1935)
AatII (1937)
EcoO109I (1991)
PfoI (2048)
KasI (2237)
NarI (2238)
SfoI (2239)
PluTI (2241)
Eco53kI (2406)
BanII - SacI (2408)
TspMI - XmaI (2414)
SmaI (2416)
NruI (2463)
BsaAI - PmlI (2497)
NheI (2634)
BmtI (2638)
PshAI (2767)
PmeI (2822)
EcoRV (2900)
AgeI (3018)
BlpI (3312)
DUS
SalI (3465)
(9146) SphI
(9140) PstI - SbfI
(8640) BsgI
3'ENDNHCIS1
DUS
(8013) PsiI
(7316) BstXI
(7154) EcoNI
(6801) MluI
(6606) PflMI
lacZ
(6086) XcmI
(5675) SwaI
(5480) AleI
(4852) NotI
(4719) MreI - SgrAI
BtgZI (4700)
porA
porB
5'ENDNHCIS1
M13 fwd
porA
porA
porB
terminator
M13 rev
lac
promoter
ori
AmpR
AmpR
promoter
pUC19NHCIS1::HAEC2:porAplusprom-lacZ
9385 bp
1000
2000
3000
4000
5000
6000
7000
8000
9000

Figure 18





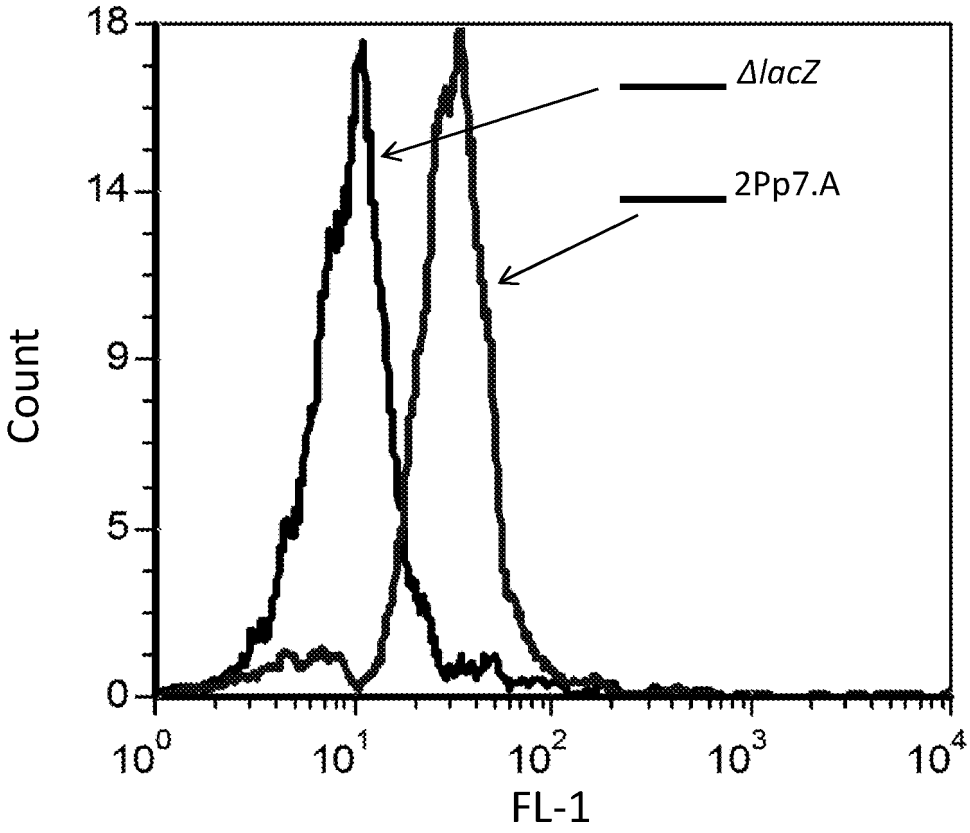
KEY:

Lane 1 = wt *N. men* (H44/76)

Lane 2 = *ΔlacZ N. lac*

Lane 3 = *ΔlacZ* NHCIS1:*porA-lacZ N. lac* (2Pp7.A)

Figure 19



anti-PorA 1,7 (SM300) – alexafluor488

Figure 20

NHCIS1::X (where X represents one of the *lst* promoter-derived constructs detailed below):

lst:*lacZ* (SEQ ID NO: 10)

GTCGACtcggcaactgtcggaatatctgctaaaaattccgcattttccgcaccgggtttccgcaccgggacactcggggcgtatgttcaatttg
tcggaatggagtttaaaggaataca**CAT**

lst(50): *lacZ* (SEQ ID NO: 11)

GTCGACTCATTTTTAAAATAAAGGTTGCGGCATTTATCAGATATTTGTTCTGAAAAtcggcaactgtcggaatatctg
ctaaaaattccgcattttccgcaccgggtttccgcaccgggacactcggggcgtatgttcaatttgtcggaatggagtttaaaggaataca**CAT**

lst(100): *lacZ* (SEQ ID NO: 12)

GTCGACAAACACAACGTTTTTGAAAAAATAAGCTATTGTTTTATATCAAAATATAATCATTTTTAAAATAAAG
GTTGCGGCATTTATCAGATATTTGTTCTGAAAAtcggcaactgtcggaatatctgctaaaaattccgcattttccgcaccgggtttc
cgcaccgggacactcggggcgtatgttcaatttgtcggaatggagtttaaaggaataca**CAT**

lst(150): *lacZ* (SEQ ID NO: 13)

GTCGACGGCAGCAGCGCATCGGCTCGCACGAGGTCTGCGCTTGAATTGTGTTGTAGAAACACAACGTTTTT
GAAAAAATAAGCTATTGTTTTATATCAAAATATAATCATTTTTAAAATAAAGGTTGCGGCATTTATCAGATAT
TTGTTCTGAAAAtcggcaactgtcggaatatctgctaaaaattccgcattttccgcaccgggtttccgcaccgggacactcggggcgtatgtt
caatttgtcggaatggagtttaaaggaataca**CAT**

lst(200): *lacZ* (SEQ ID NO: 14)

GTCGACGTGCCGCGTGTGTTTTTTTATGGCGTTTTAAAAAGCCGAGACTGCATCCGGGCAGCAGCGCATCG
GCTCGCACGAGGTCTGCGCTTGAATTGTGTTGTAGAAACACAACGTTTTTGAAAAAATAAGCTATTGTTTTAT
ATCAAAATATAATCATTTTTAAAATAAAGGTTGCGGCATTTATCAGATATTTGTTCTGAAAAtcggcaactgtcggaa
atatctgctaaaaattccgcattttccgcaccgggtttccgcaccgggacactcggggcgtatgttcaatttgtcggaatggagtttaaaggaata
ca**CAT**

lst(250): *lacZ* (SEQ ID NO: 15)

GTCGACGAGCTAAGGCGAGGCAACGCCGTACTTGTTTTTGTTAATCCACTATAAAGTGCCGCGTGTGTTTTT
TTATGGCGTTTTAAAAAGCCGAGACTGCATCCGGGCAGCAGCGCATCGGCTCGCACGAGGTCTGCGCTTGA
ATTGTGTTGTAGAAACACAACGTTTTTGAAAAAATAAGCTATTGTTTTATATCAAAATATAATCATTTTTAAAA
TAAAGGTTGCGGCATTTATCAGATATTTGTTCTGAAAAtcggcaactgtcggaatatctgctaaaattccgcattttccgcacc
gggtttccgcaccgggacactcggggcgtatgttcaatttgtcggaatggagtttaaaggaataca**CAT**

lst(400):*lacZ* (SEQ ID NO: 16)

GTCGACATTCGGCTTGATTTCGATACACCCGACACACGCAGGAAATTATAGTGGATTAATAAAAATCAGGAC
AAGGCGACGAAGCCGAAGACAGTACAGATAGTACGAAACCGATTCACTTGGTGCTTCAGCACCTTAGAGAA
TCGTTCTCTTTGAGCTAAGGCGAGGCAACGCCGTACTTGTTTTTGTTAATCCACTATAAAGTGCCGCGTGTGT
TTTTTTATGGCGTTTTAAAAAGCCGAGACTGCATCCGGGCAGCAGCGCATCGGCTCGCACGAGGTCTGCGCT
TGAATTGTGTTGTAGAAACACAACGTTTTTGAAAAAATAAGCTATTGTTTTATATCAAAATATAATCATTTTTA
AAATAAAGGTTGCGGCATTTATCAGATATTTGTTCTGAAAAtcggcaactgtcggaatatctgctaaaattccgcattttccg
caccgggtttccgcaccgggacactcggggcgtatgttcaatttgtcggaatggagtttaaaggaataca**CAT**

Figure 21

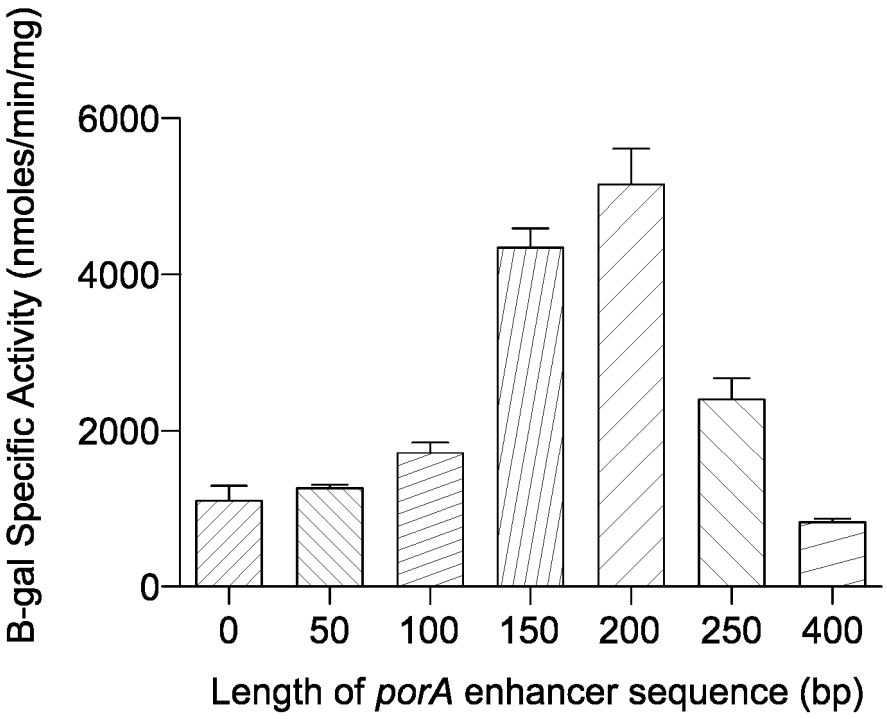

B-gal Specific Activity (nmoles/min/mg)
6000
4000
2000
0
0
50
100
150
200
250
400
Length of porA enhancer sequence (bp)

Figure 22

Figure 23 (SEQ ID NO: 17)

lst(200+5): *lacZ*

GTCGACGTGCCGCGTGTGTTTTTTTATGGCGTTTTAAAAAGCCGAGACTGCATCCGGGCAGCAGCGCATCG

SalI

GCTCGCACGAGGTCTGCGCTTGAATTGTGTTGTAGAAACACAACGTTTTTGAAAAAATAAGCTATTGTTTTAT

porA enhancer sequence

ATCAAAATATAATCATTTTTAAAATAAAGGTTGCGGCATTTATCAGATATTTGTTCTGAAAATGCATTcggcaac

NsiI -35

tgtcggaatatctgcaaaaattccgcattttccgcaccgggtttccgcaccgggacactcggggcgtatgttcaatttgtcggaatggagtttaa

porB -10 *porB* 5′ UTR

aggaatacaCATATG

NdeI

START codon...

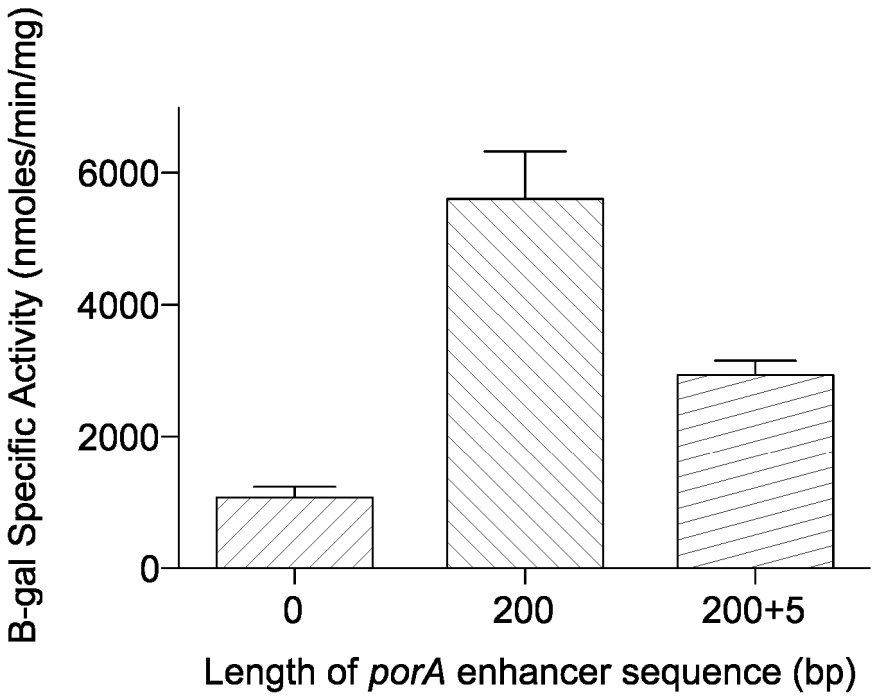

B-gal Specific Activity (nmoles/min/mg)
6000
4000
2000
0
0
200
200+5
Length of porA enhancer sequence (bp)

Figure 24

Figure 25

Heterologous Antigen Expression Cassette 3 – HAEC3:(Z)-(Z')

(SEQ ID NO: 18)

GTCGACGAGCTAAGGCGAGGCAACGCCGTACTTGTTTTTGTTAATCCACTATAAAGTGCCGCGTGTGTTTTTTTATGGCGTTTTAA

SalI

AAAGCCGAGACTGCATCCGGGCAGCAGCGCATCGGCTCGCACGAGGTTCTGCGCTTGAATTGTGTTGTAGAAACACAACGTTTTT

porA enhancer sequence (250 bp)

GAAAAAATAAGCTATTGTTTTATATCAAAATATAATCATTTTTAAAATAAAGGTTGCGGCATTTATCAGATATTTGTTCTGAAAAAT

GGTTttcgggcgggaacattTATAATTGAAGACGTATCGGGTGTTTGCCCGATGTTTTTAGGTTTTTATCAAATTTACAAAAGGAACTC

-35 *porB* -10 *porA* 5' UTR RBS

GAGNNNNNNNNNNGCGGCCGCAAGTTTGTTTACTGTCGGAATATCTGCTATAGTTTCAAACAAGGAATCGACGAAAACGTC

XhoI LINKER NotI -35 *lst* -10

GTCGGTAAATGCAAAGCTAAGCGGCTCGGAAAGCCCGGTTCGCTTAAATTTCTTAACCAAAAAAGGAATACACATATGNNNNN

porB 5' UTR RBS NdeI

NNNNNAAGCTTTCTGCAAGATTGGTATCAACAAAAAGCCTGTCGTCAGACAGGCTTTTTTTCTGTTTTCTGTTTTTAGatgccgtctg

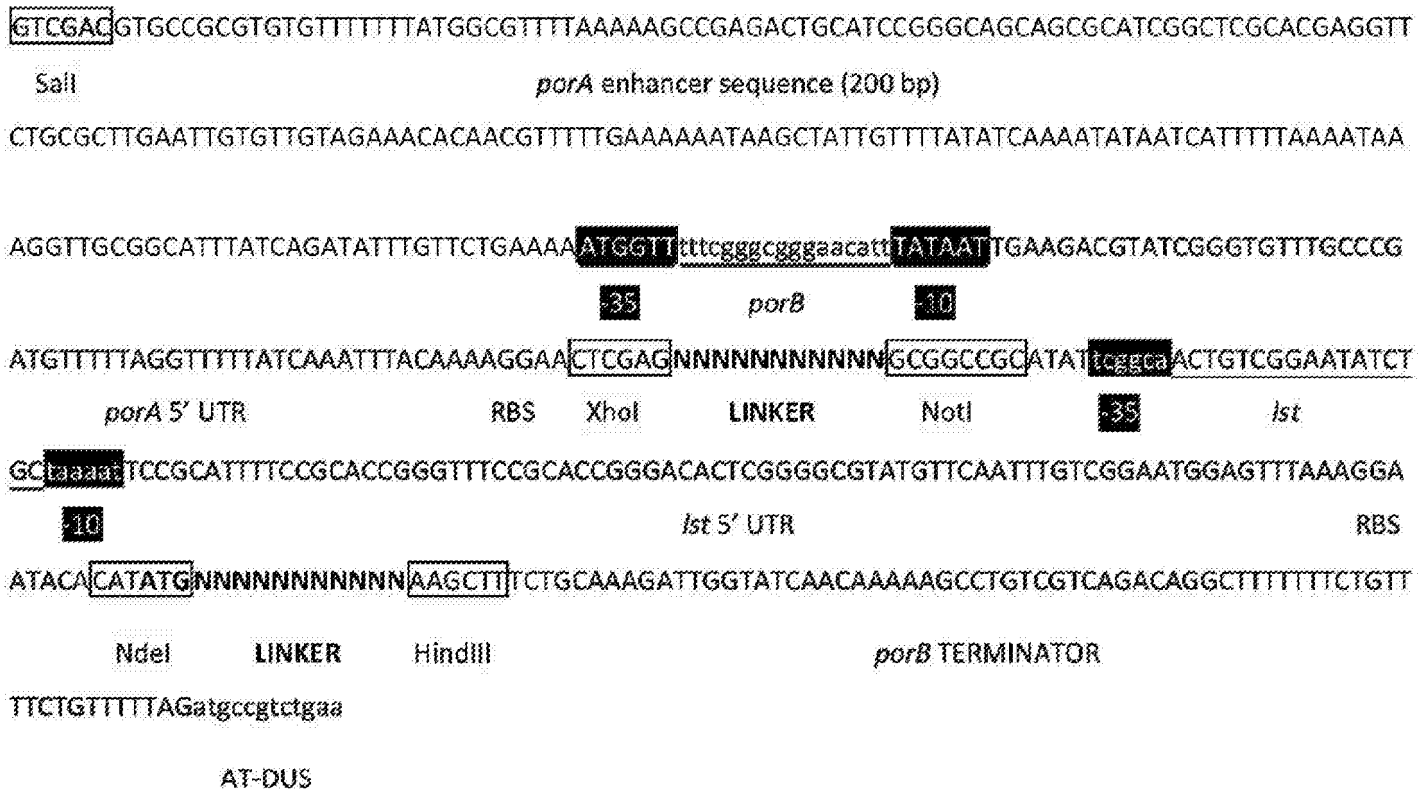
LINKER HindIII *porB* TERMINATOR AT-DUS

aa

Figure 26

Heterologous Antigen Expression Cassette 4 – HAEC4:(Z)-(Z')

(SEQ ID NO: 19)



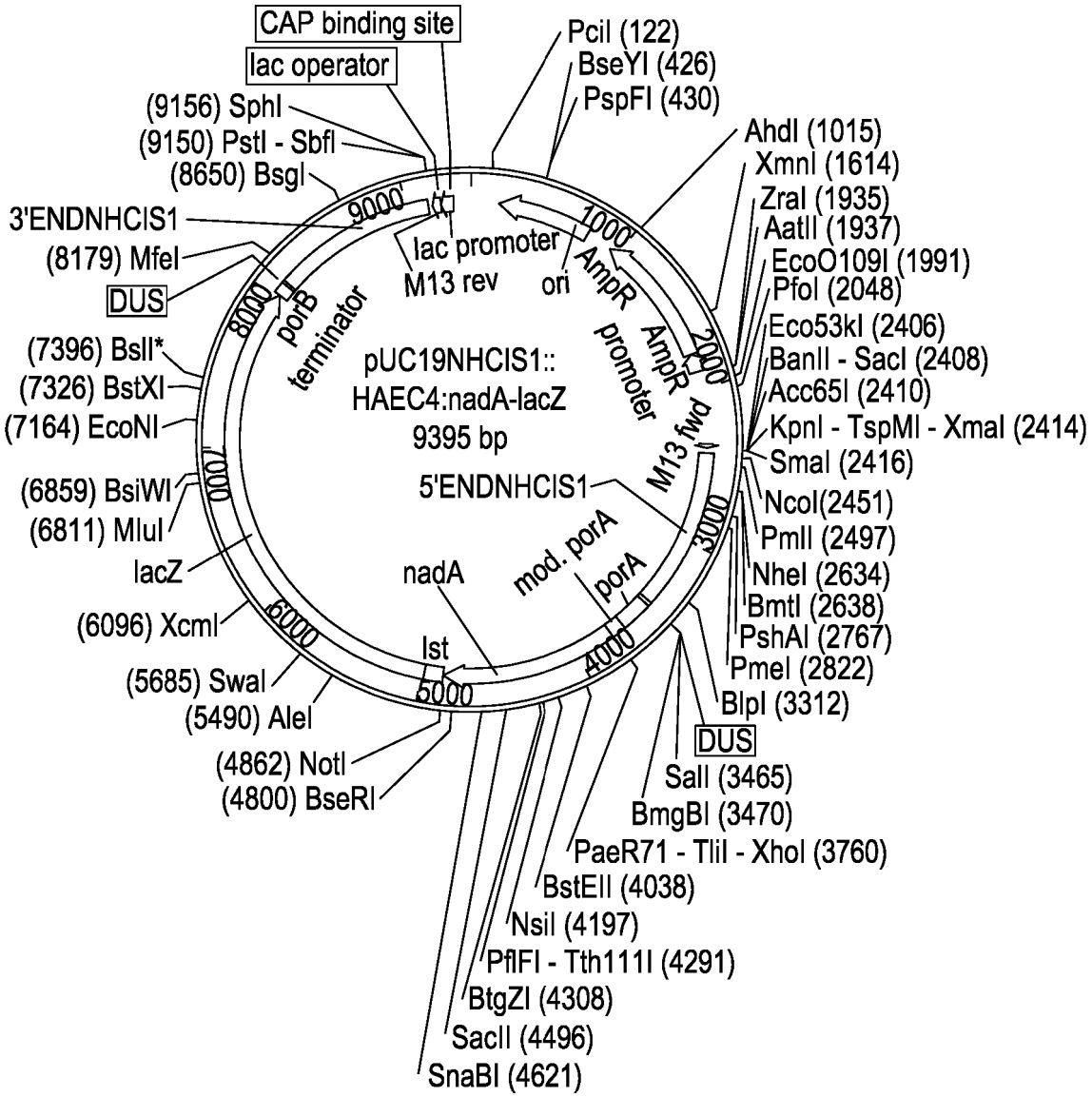

CAP binding site
lac operator
(9156) SphI
(9150) PstI - SbfI
(8650) BsgI
3'ENDNHCIS1
(8179) MfeI
DUS
(7396) BsII*
(7326) BstXI
(7164) EcoNI
(6859) BsiWI
(6811) MluI
lacZ
(6096) XcmI
(5685) SwaI
(5490) AleI
(4862) NotI
(4800) BseRI
SnaBI (4621)
SacII (4496)
BtgZI (4308)
PflFI - Tth111I (4291)
NsiI (4197)
BstEII (4038)
PaeR71 - TliI - XhoI (3760)
BmgBI (3470)
SalI (3465)
DUS
BlpI (3312)
PmeI (2822)
PshAI (2767)
BmtI (2638)
NheI (2634)
PmlI (2497)
NcoI(2451)
SmaI (2416)
KpnI - TspMI - XmaI (2414)
Acc65I (2410)
BanII - SacI (2408)
Eco53kI (2406)
PfoI (2048)
EcoO109I (1991)
AatII (1937)
ZraI (1935)
XmnI (1614)
AhdI (1015)
PspFI (430)
BseYI (426)
PciI (122)
9000
1000
2000
3000
4000
5000
6000
7000
8000
lac promoter
M13 rev
ori
AmpR
AmpR promoter
M13 fwd
5'ENDNHCIS1
mod. porA
porA
nadA
lst
porB terminator
pUC19NHCIS1::
HAEC4:nadA-lacZ
9395 bp

Figure 27

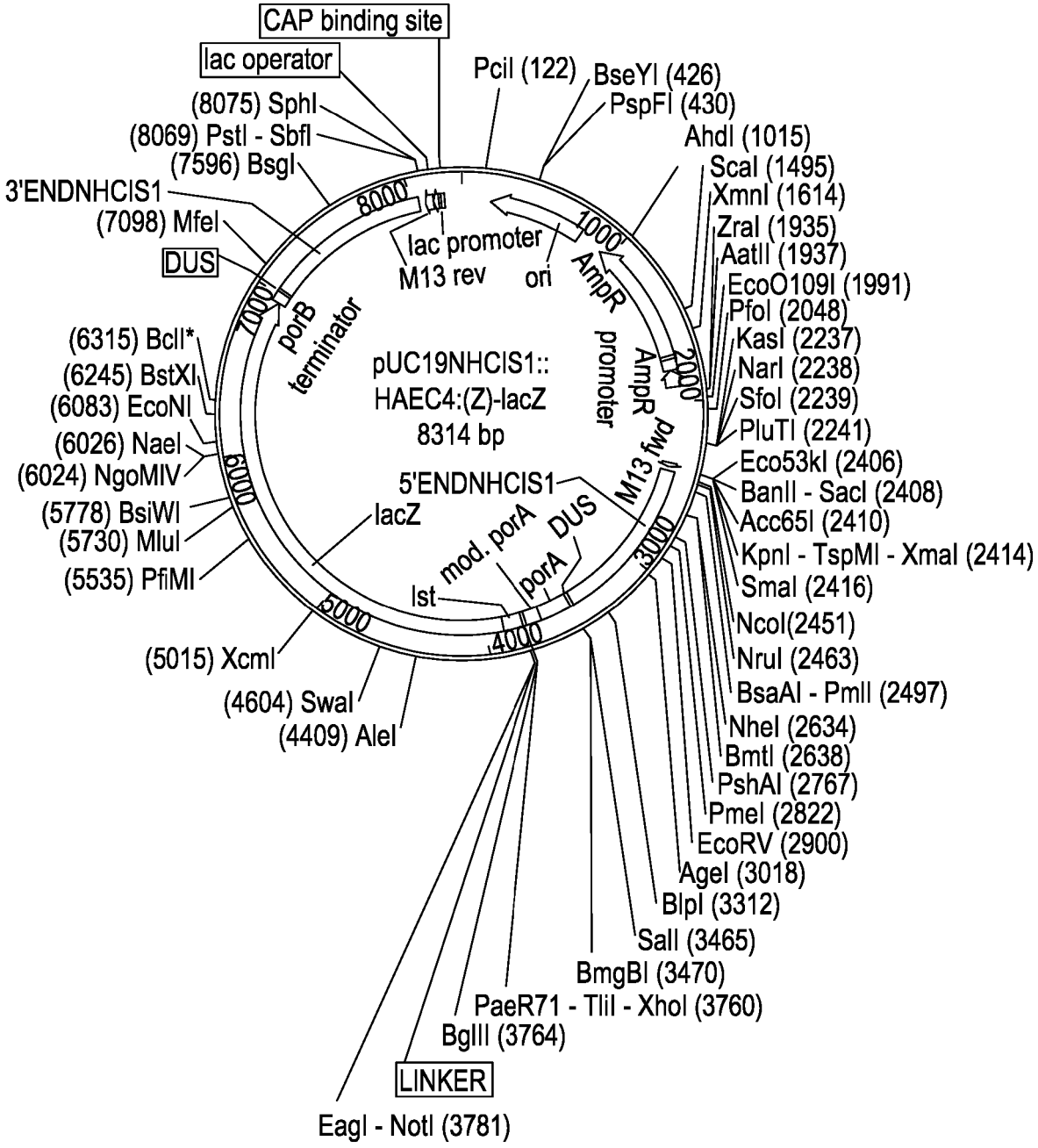

CAP binding site
lac operator
(8075) SphI
(8069) PstI - SbfI
(7596) BsgI
3'ENDNHCIS1
(7098) MfeI
DUS
(6315) BclI*
(6245) BstXI
(6083) EcoNI
(6026) NaeI
(6024) NgoMIV
(5778) BsiWI
(5730) MluI
(5535) PflMI
(5015) XcmI
(4604) SwaI
(4409) AleI
PciI (122)
BseYI (426)
PspFI (430)
AhdI (1015)
ScaI (1495)
XmnI (1614)
ZraI (1935)
AatII (1937)
EcoO109I (1991)
PfoI (2048)
KasI (2237)
NarI (2238)
SfoI (2239)
PluTI (2241)
Eco53kI (2406)
BanII - SacI (2408)
Acc65I (2410)
KpnI - TspMI - XmaI (2414)
SmaI (2416)
NcoI(2451)
NruI (2463)
BsaAI - PmlI (2497)
NheI (2634)
BmtI (2638)
PshAI (2767)
PmeI (2822)
EcoRV (2900)
AgeI (3018)
BlpI (3312)
SalI (3465)
BmgBI (3470)
PaeR71 - TliI - XhoI (3760)
BglII (3764)
LINKER
EagI - NotI (3781)
8000
1000
2000
3000
4000
5000
6000
7000
lac promoter
M13 rev
ori
AmpR
promoter
AmpR
M13 fwd
porB
terminator
pUC19NHCIS1::
HAEC4:(Z)-lacZ
8314 bp
5'ENDNHCIS1
lacZ
mod. porA
DUS
porA
lst

Figure 28

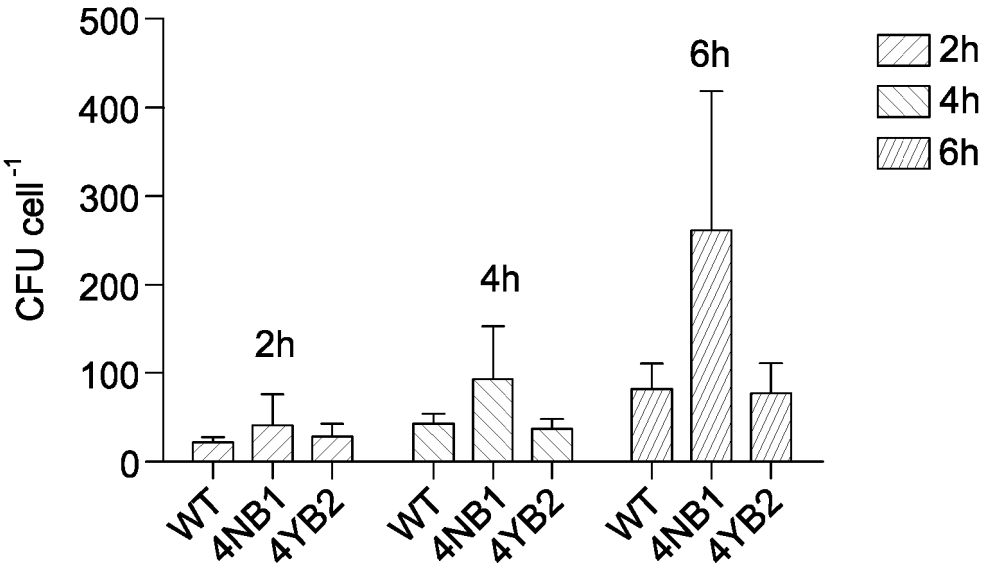

500
400
300
200
100
0
CFU cell-1
2h
4h
6h
WT
4NB1
4YB2
WT
4NB1
4YB2
WT
4NB1
4YB2
2h
4h
6h

Figure 29

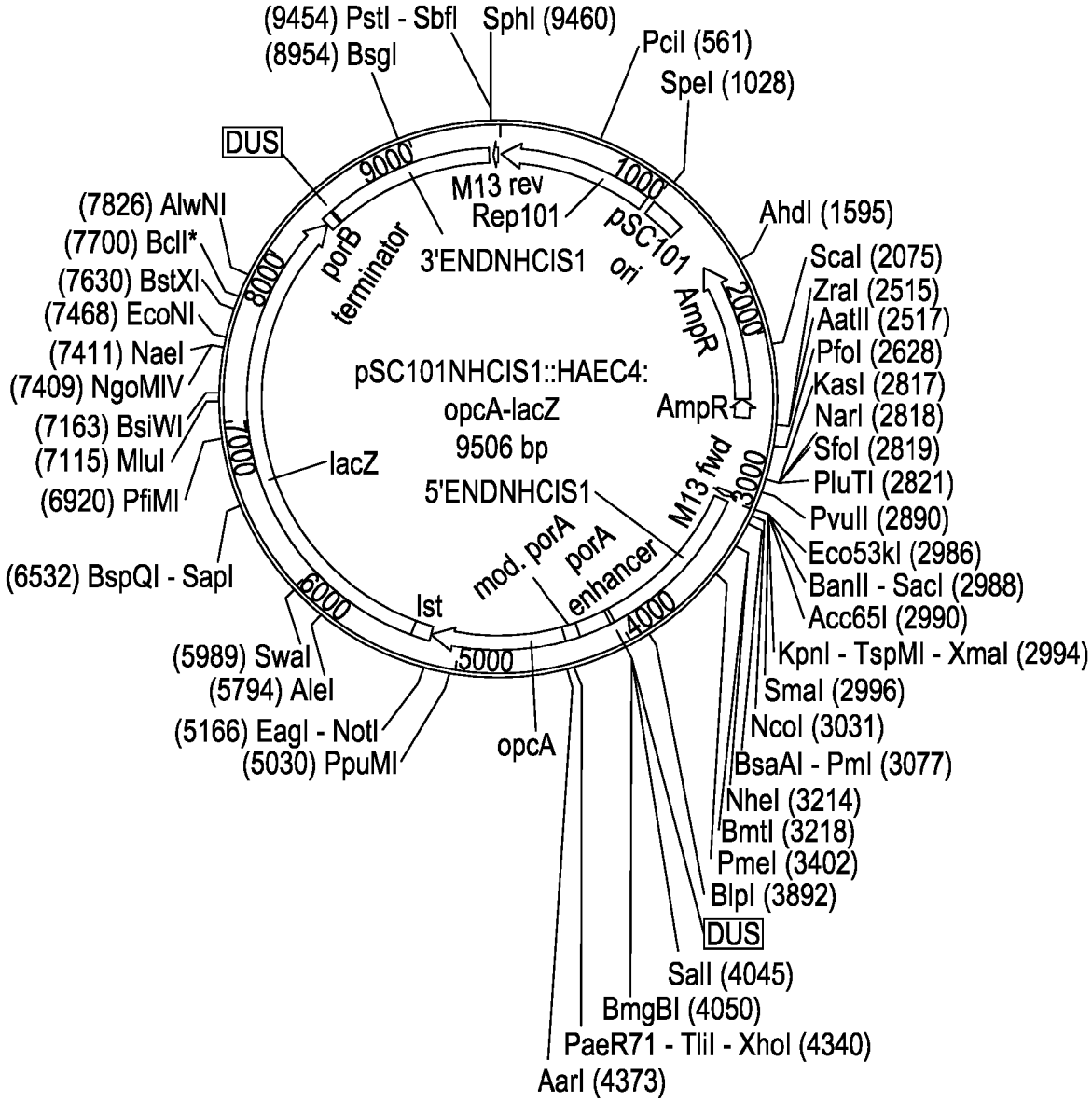

(9454) PstI - SbfI
(8954) BsgI
SphI (9460)
PciI (561)
SpeI (1028)
DUS
9000
M13 rev
Rep101
1000
pSC101
ori
AhdI (1595)
(7826) AlwNI
(7700) BclI*
(7630) BstXI
(7468) EcoNI
(7411) NaeI
(7409) NgoMIV
(7163) BsiWI
(7115) MluI
(6920) PfiMI
porB
terminator
3'ENDNHCIS1
8000
2000
AmpR
ScaI (2075)
ZraI (2515)
AatII (2517)
PfoI (2628)
KasI (2817)
NarI (2818)
SfoI (2819)
PluTI (2821)
PvuII (2890)
Eco53kI (2986)
BanII - SacI (2988)
Acc65I (2990)
KpnI - TspMI - XmaI (2994)
SmaI (2996)
NcoI (3031)
BsaAI - PmlI (3077)
NheI (3214)
BmtI (3218)
PmeI (3402)
BlpI (3892)
DUS
SalI (4045)
BmgBI (4050)
PaeR71 - TliI - XhoI (4340)
AarI (4373)
pSC101NHCIS1::HAEC4:
opcA-lacZ
9506 bp
AmpR
7000
lacZ
5'ENDNHCIS1
M13 fwd
3000
(6532) BspQI - SapI
6000
lst
mod. porA
porA
enhancer
4000
5000
(5989) SwaI
(5794) AleI
(5166) EagI - NotI
(5030) PpuMI
opcA

Figure 30

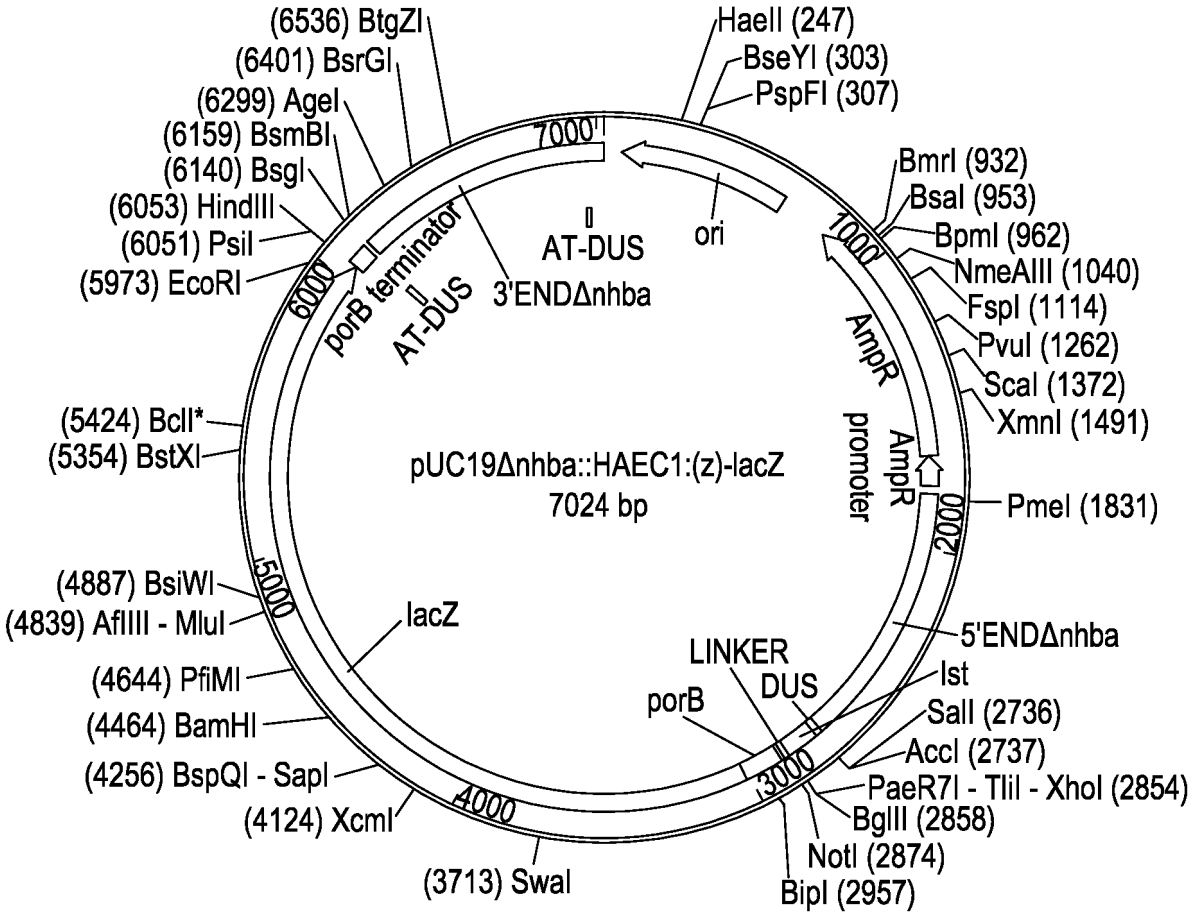

pUC19Δnhba::HAEC1:(z)-lacZ
7024 bp
7000
1000
2000
3000
4000
5000
6000
ori
AT-DUS
3'ENDΔnhba
porB terminator
AT-DUS
AmpR
promoter
AmpR
lacZ
LINKER
DUS
porB
5'ENDΔnhba
(6536) BtgZI
(6401) BsrGI
(6299) AgeI
(6159) BsmBI
(6140) BsgI
(6053) HindIII
(6051) PsiI
(5973) EcoRI
(5424) BclI*
(5354) BstXI
(4887) BsiWI
(4839) AflIII - MluI
(4644) PfiMI
(4464) BamHI
(4256) BspQI - SapI
(4124) XcmI
(3713) SwaI
HaeII (247)
BseYI (303)
PspFI (307)
BmrI (932)
BsaI (953)
BpmI (962)
NmeAIII (1040)
FspI (1114)
PvuI (1262)
ScaI (1372)
XmnI (1491)
PmeI (1831)
Ist
SalI (2736)
AccI (2737)
PaeR7I - TliI - XhoI (2854)
BglII (2858)
NotI (2874)
BipI (2957)

Figure 31

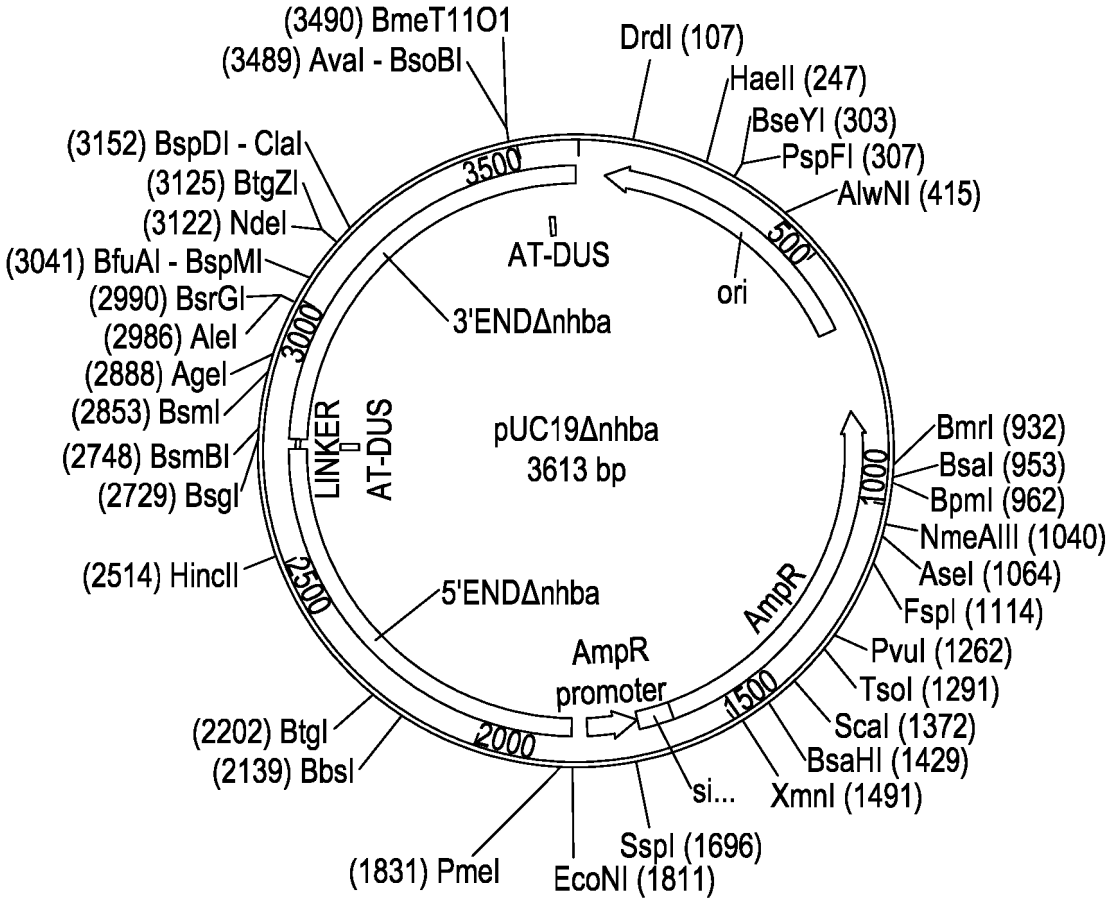

(3490) BmeT11O1
(3489) AvaI - BsoBI
DrdI (107)
HaeII (247)
BseYI (303)
PspFI (307)
AlwNI (415)
(3152) BspDI - ClaI
(3125) BtgZI
(3122) NdeI
(3041) BfuAI - BspMI
(2990) BsrGI
(2986) AleI
(2888) AgeI
(2853) BsmI
(2748) BsmBI
(2729) BsgI
(2514) HincII
(2202) BtgI
(2139) BbsI
(1831) PmeI
EcoNI (1811)
SspI (1696)
XmnI (1491)
BsaHI (1429)
ScaI (1372)
TsoI (1291)
PvuI (1262)
FspI (1114)
AseI (1064)
NmeAIII (1040)
BpmI (962)
BsaI (953)
BmrI (932)
3500
500
1000
1500
2000
2500
3000
AT-DUS
ori
3'ENDΔnhba
LINKER
AT-DUS
pUC19Δnhba
3613 bp
5'ENDΔnhba
AmpR
promoter
AmpR
si...

Figure 32

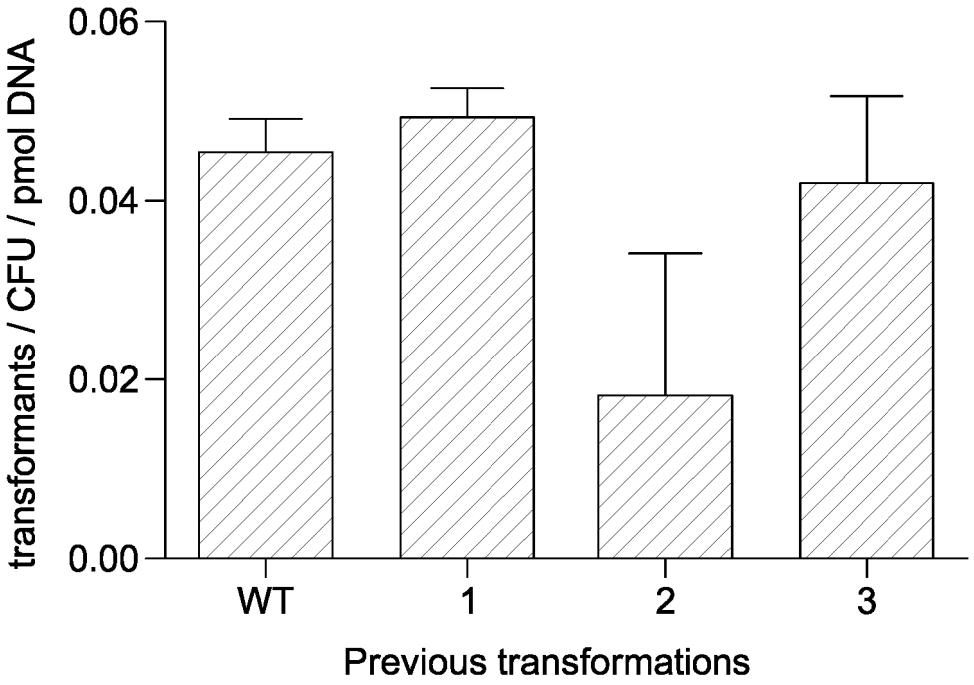

0.06
0.04
0.02
0.00
transformants / CFU / pmol DNA
WT
1
2
3
Previous transformations

Figure 33

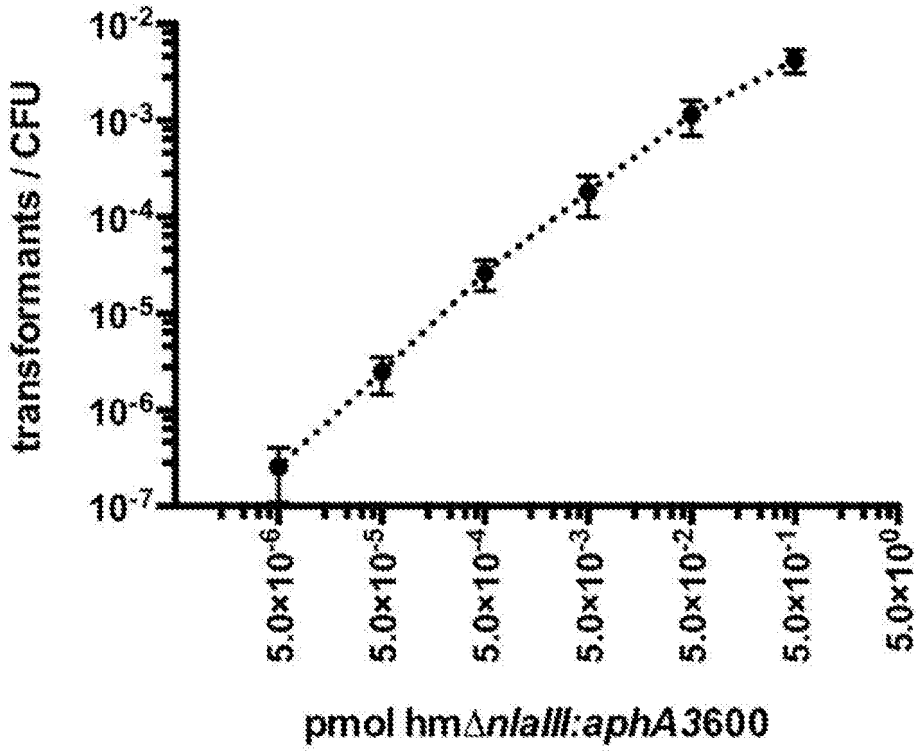

transformants / CFU
10-2
10-3
10-4
10-5
10-6
10-7
5.0×10-6
5.0×10-5
5.0×10-4
5.0×10-3
5.0×10-2
5.0×10-1
5.0×100
pmol hmΔnlaIII:aphA3600

Figure 34

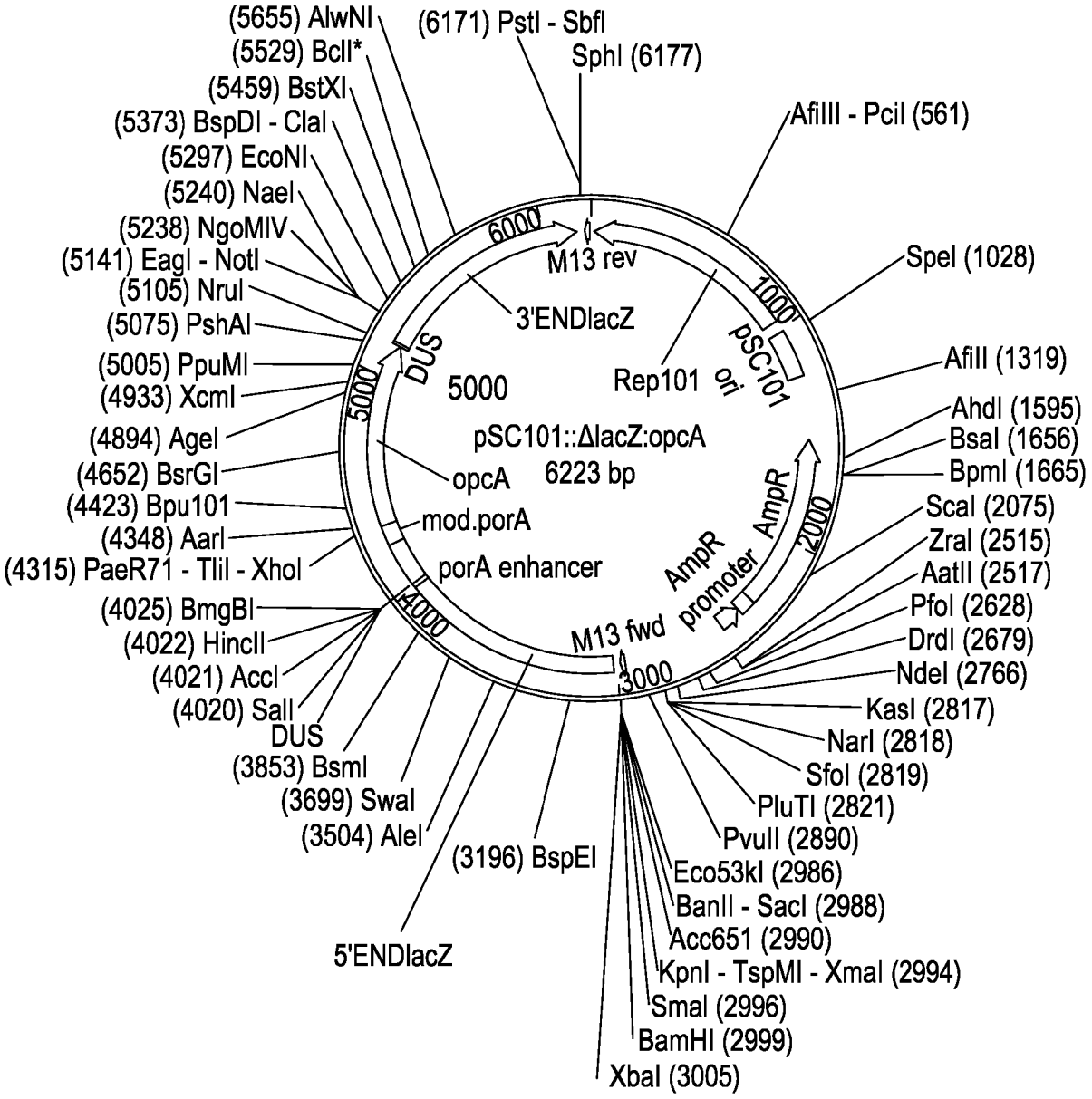

(5655) AlwNI
(6171) PstI - SbfI
(5529) BclI*
SphI (6177)
(5459) BstXI
AfiIII - PciI (561)
(5373) BspDI - ClaI
(5297) EcoNI
(5240) NaeI
(5238) NgoMIV
SpeI (1028)
(5141) EagI - NotI
(5105) NruI
(5075) PshAI
AfiII (1319)
(5005) PpuMI
(4933) XcmI
AhdI (1595)
(4894) AgeI
BsaI (1656)
(4652) BsrGI
BpmI (1665)
(4423) Bpu101
ScaI (2075)
(4348) AarI
ZraI (2515)
(4315) PaeR71 - TliI - XhoI
AatII (2517)
(4025) BmgBI
PfoI (2628)
(4022) HincII
DrdI (2679)
(4021) AccI
NdeI (2766)
(4020) SalI
KasI (2817)
DUS
NarI (2818)
(3853) BsmI
SfoI (2819)
(3699) SwaI
PluTI (2821)
(3504) AleI
PvuII (2890)
(3196) BspEI
Eco53kI (2986)
BanII - SacI (2988)
5'ENDlacZ
Acc651 (2990)
KpnI - TspMI - XmaI (2994)
SmaI (2996)
BamHI (2999)
XbaI (3005)
6000
M13 rev
1000
3'ENDlacZ
pSC101 ori.
DUS
5000
5000
Rep101
pSC101::ΔlacZ:opcA
opcA
6223 bp
mod.porA
AmpR
promoter
AmpR
porA enhancer
2000
4000
M13 fwd
3000

Figure 35

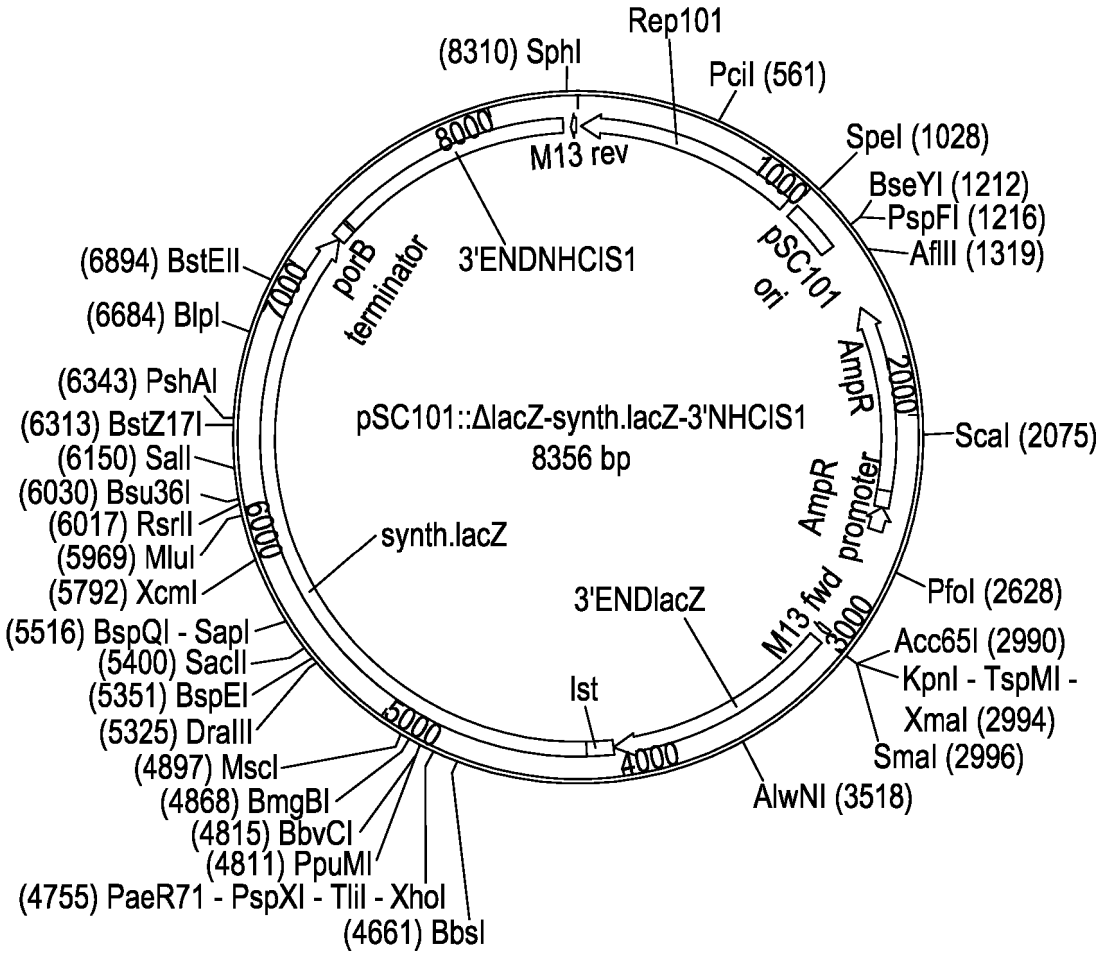

Rep101
(8310) SphI
PciI (561)
SpeI (1028)
BseYI (1212)
PspFI (1216)
AflII (1319)
ScaI (2075)
PfoI (2628)
Acc65I (2990)
KpnI - TspMI -
XmaI (2994)
SmaI (2996)
AlwNI (3518)
Ist
3'ENDlacZ
M13 fwd
AmpR promoter
AmpR
2000
1000
pSC101 ori
3'ENDNHCIS1
M13 rev
8000
porB terminator
7000
(6894) BstEII
(6684) BlpI
(6343) PshAI
(6313) BstZ17I
(6150) SalI
(6030) Bsu36I
(6017) RsrII
(5969) MluI
(5792) XcmI
(5516) BspQI - SapI
(5400) SacII
(5351) BspEI
(5325) DraIII
(4897) MscI
(4868) BmgBI
(4815) BbvCI
(4811) PpuMI
(4755) PaeR71 - PspXI - TliI - XhoI
(4661) BbsI
6000
5000
4000
3000
synth.lacZ
pSC101::ΔlacZ-synth.lacZ-3'NHCIS1
8356 bp

Figure 36

>Coding sequence of synth.*lacZ* (CAI: 0.687) (SEQ ID NO: 20)

ATGCTGTTGGCAAACTACTACCAAGACCCGGAAATTACCCGCATCAACGCACTCCCGCAtCATTCCTACTTCA
TCCCATTCGACAAgAAAGACAAAGTTGACCAATTTTCGCGCGAgAAcTCCAGCTTCTTCACATCCCTGAACG
GCATGTGGCAgTTCGCtTACTACCCTTCTATGCAAGACcTGCCGGAATCTCCCGAtGAgATtGCGTTCACTAAA
CAgATaAACGTACCGAGCAACTGGCAaAACCATGGCTTcGACGCGCAcCAgTACACCAACATcAACTACCCGT
TtCCATTtGACCCtCCgTTCGTACCCCTGGAAAATCCGTGTGGTGTCTACCAgAAACAaGTTAACCTGAAgAAA
AACATCAACAAACGTTAccTcCTGGTtCTGGAgGGcGTgGACagcTGCagcTACATCTAcGTgAAtCACCAGTTcG
TtGGTTACGGCAGCATTTCCCATtcCACgAACGAGTTcGAcATCACTGAcTACTTGCATGACGGcGAgAATACg
CTCACtGTCTTCGTAtTGAAGTGGTGCGCgGGCtcCTAtCTGGAgGAcCAAGACAAgTTCCGCATGTCCGGTAT
CTTtCGGGACGTcTACTTGcTcGAgCGCGAACAcCATTACCTGCAGGATCTCAACATCCGCACtGTcCTGAGCG
AgGACCTcagcCTcGGCCAaATcTGCCTTGAcCTGAACTTCGCCGGTGAcGCCGGCGACGTgGGTGTcagTCTcT
TCGACACCGAtGGCCAGATCGTgCAgGCCGGTTCCGCCATtACTACgGACAAgCAgCGTATGCAgATTCGtCTG
GAcAAcATcCCgTTGACTAAAAGtCGTttgTGGAAcGCCGAgAAtCCGGCATTGTAcACgcTtGTTCTGAATACC
AAAGAgGAgATTATcACgCAAAAAATtGGCTTtCGtAAAGTTGAAGTGAAgAATGGCGTGcTGTTGCTGAAcA
ACCAaCCGATCAAgTTtAAGGGtGTTAAtCGCCATGActcCGACCCTAAAACGGGGTACGCTATTTCCGTCGCC
CAAGCCGTCACGGACCTGTCACTGATGAAAAAACACAATATCAACGCGATTCGCACTGCACATTATCCGAAT
TCCCCCTGGTTCTGCGAACTGTGTGACAAATATGGGTTTTACGTGATCAGTGAAAGCGACATTGAATCACAC
GGTGCAGCCTTCCAGGCTATCTCCCATCCGGAACCGTCAATTTTCCTTAACGTGGAAAACCCCAACGAAGAA
CCGCGGATCCGCCAACAAACAATCGACAACTTTTGCTACTTCGCTCGTGAACCGTTGTATCGTGCGGCACTG
CTGGAACGTACCAAAGCCAACATTGAACGTGACAAAAACCGCTCTTCCATTTTGATTTGGTCTTTGGGCAAC
GAGAGCGGCTACGGCGAAAACTTCGAATACTGCGCAAAATGGGTTAAAGAACGCGATCCTGATCGTTTGGT
CCACTACGAATCAAGCATCTATCAGCATAGCGCATACCAAAATAACACCGGTCATTTGGATCTATACAGTGAA
ATGTACTCCGATACGGAAGCCATTGATGCCTACTTTGCAGACCACAGCCAGACCAAAAAACCGTTCCTGCTA
TGTGAATACAGCCACGCCATGGGCAATTCCAACGGTGACATGGAAGATTACTTTCAAACCTTTAACAAATAC
TCCGGCTGTTGCGGCGGTTTCATCTGGGAATGGTGTGACCACGCACAATATATCACCCCGACGAAATTGGGC
TACGGTGGCGACTTTGGAGAGAAAATCCATGATGGCAATTTCTGTGTCGATGGGTTGGTTAGCCCTGAACG
CGTACCCCACTCGAATCTGTTGGAGGTTAAGAACGTTAACCGCCCGGTCCGCGCTAACCTGAGGGGTGAAC
AAATAGAATTGTACAACTACTTCGATTTCACCAACTTAAAAGACATCTTGTGCGTAAAATACGAATGGGTCAA
AAATGGTCAAATTACTGGCACCGGTACACTGGCGGTCGACTGCGAACCCCACCACTCCCAGATTTTGCCTAT
cCAaCTGCCGAAGGAGCGTGAgGGTcTcTTgTGGcTtAAtCTGTACTATTGtGCCagcCGTCAGACcGACCTGCT
CCCTGCgGGCCACCACTTtGGCTTtGACCAgATcATCCTGTCAAAAGAGTAtACCCCCGCGATTGGCAGCGAC
AAgGACGAcTGtCCaCCtCTGGAGATCACtGAgACCGTCCGCCAGATTGTGGTcCGTAAtAACCGTTACTACTT
CGAgTTCAAtAAATTGACTGGtATTATCGATGAGATcAAgGTGAACGGtAAAGCCTTTATtCAcAAACCGCTCG
CCTGGAAcATCTGGCGtGCCCCCACCGAcAAcGAtCGTTTGATcCGCTCACAGTGGCAgAACGCGGGCTACG
AcCAgATGTACTCTAAAGTcTAtGACATCTGtGCACACCGCCAgGGCAAtGGcGTCGTTGTCTCGGTAAAGTCG
GCGCTCGTCGCAGACGCCAAATCGAAgATtATGACGCTGGAgACCCAATACTTGCTCagcGAgAACGGCAAA
CTGGACATCCAgACCAACGCaGTGTTTCAtGAACAcCTCCCGTTtTTaCCACGCTTTGGCCTcCGtTTCTTtCTG
GATGAgCAAAAgACCCCGTTCACTTAtCTGGGCTAcGGCGCCGGCGAgTCTTACATCGACAAgCAcCAAGCCA
CgAAATTGGGCATcTAcTCCACCACCGCCGGCGAgAACCATGTcGGtTAccTgAAACCGCAGGAAAATGGTTC
CCAcTACGGcTGTTTcTAcGTGCAgAAtGAcATGATtCGCGTAGAAAGCGGCCAACCcTTCTCCTTTAAttTaagc
CCGTACACCCAgGAAGAgTTGACCCAAAAgAAaCACTCCTACGAgCTCGTCTGcagcGGATAcGACGTCctcTG
CATTGAtTAcAAAATGTCTGGcATTGGCTCCAACAGCTGTGGCCCCAACcTGAAACCTCAgTACCGCCTCATC
GAgAACAAtATtAAcTTtGAcATTTCCATTCGCCTCTAG

Figure 37 SEQ ID NO: 20

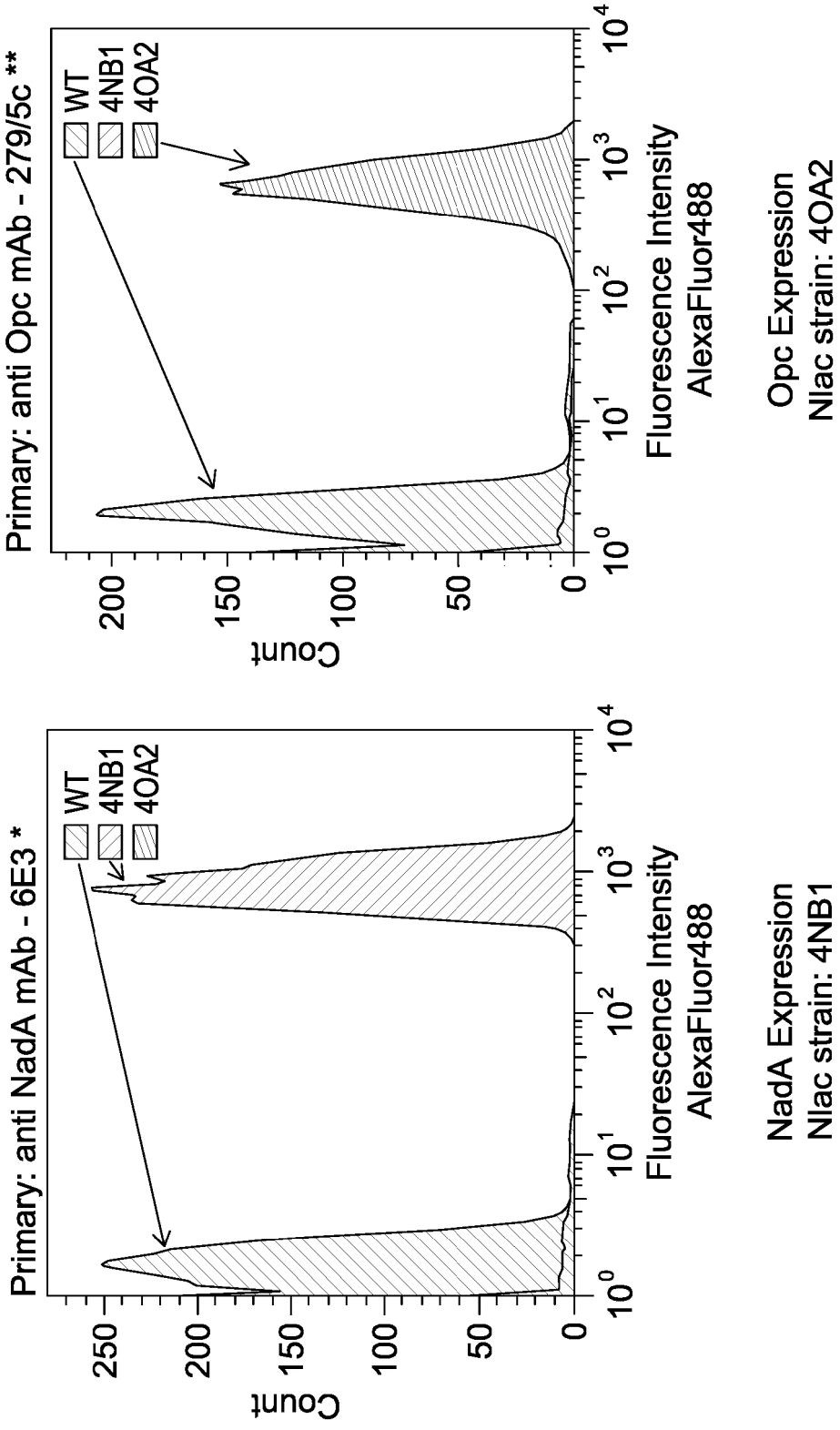
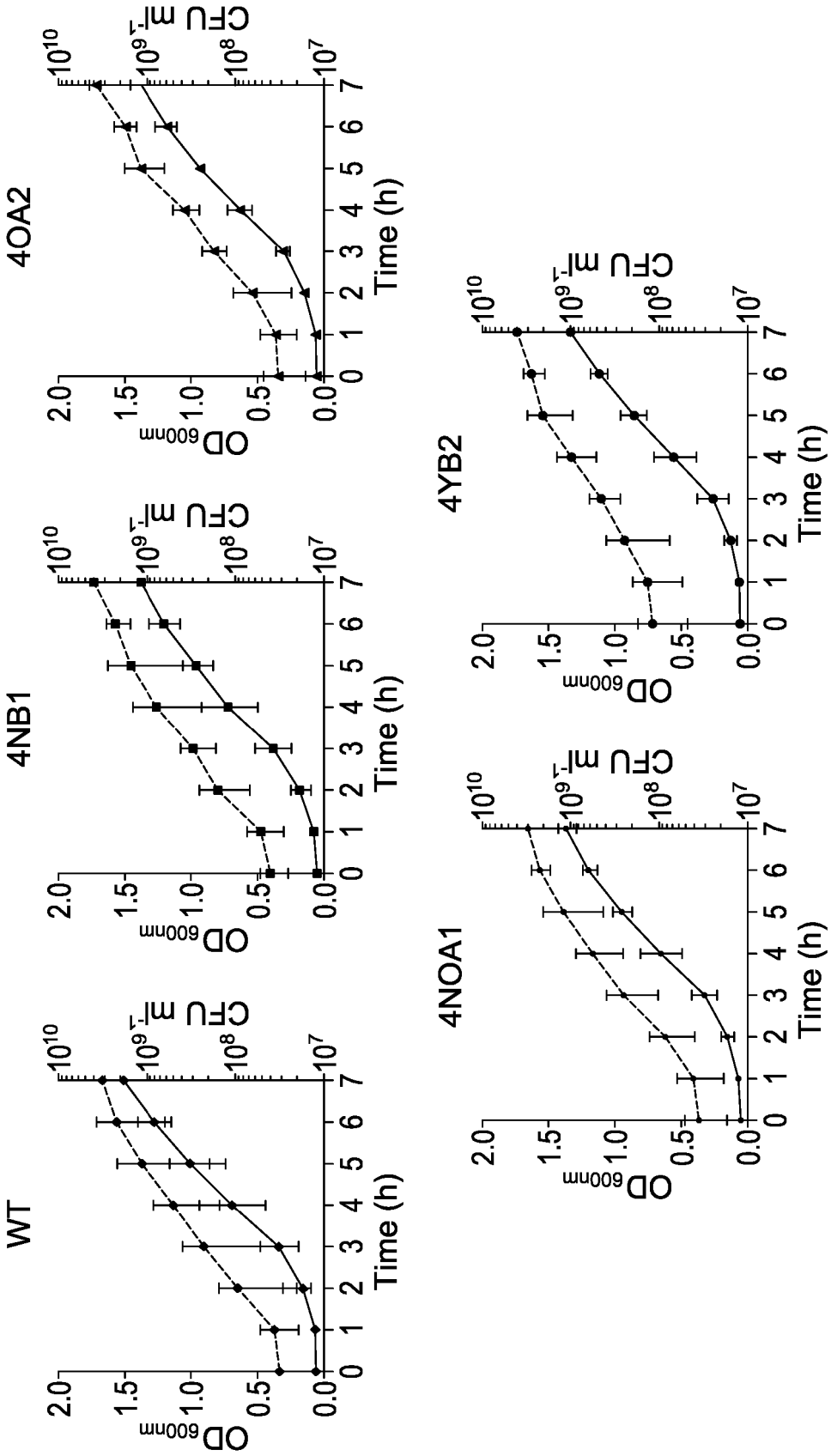

Primary: anti NadA mAb - 6E3 *
WT
4NB1
4OA2
Count
Fluorescence Intensity
AlexaFluor488
NadA Expression
Nlac strain: 4NB1
Primary: anti Opc mAb - 279/5c **
WT
4NB1
4OA2
Count
Fluorescence Intensity
AlexaFluor488
Opc Expression
Nlac strain: 4OA2

Figure 38



Points represent Mean ± SD, where no error bars are visible they fall within the points
1-way ANOVA of AUC w Tukey's multiple comparisons test, ns n = 4.

Figure 39

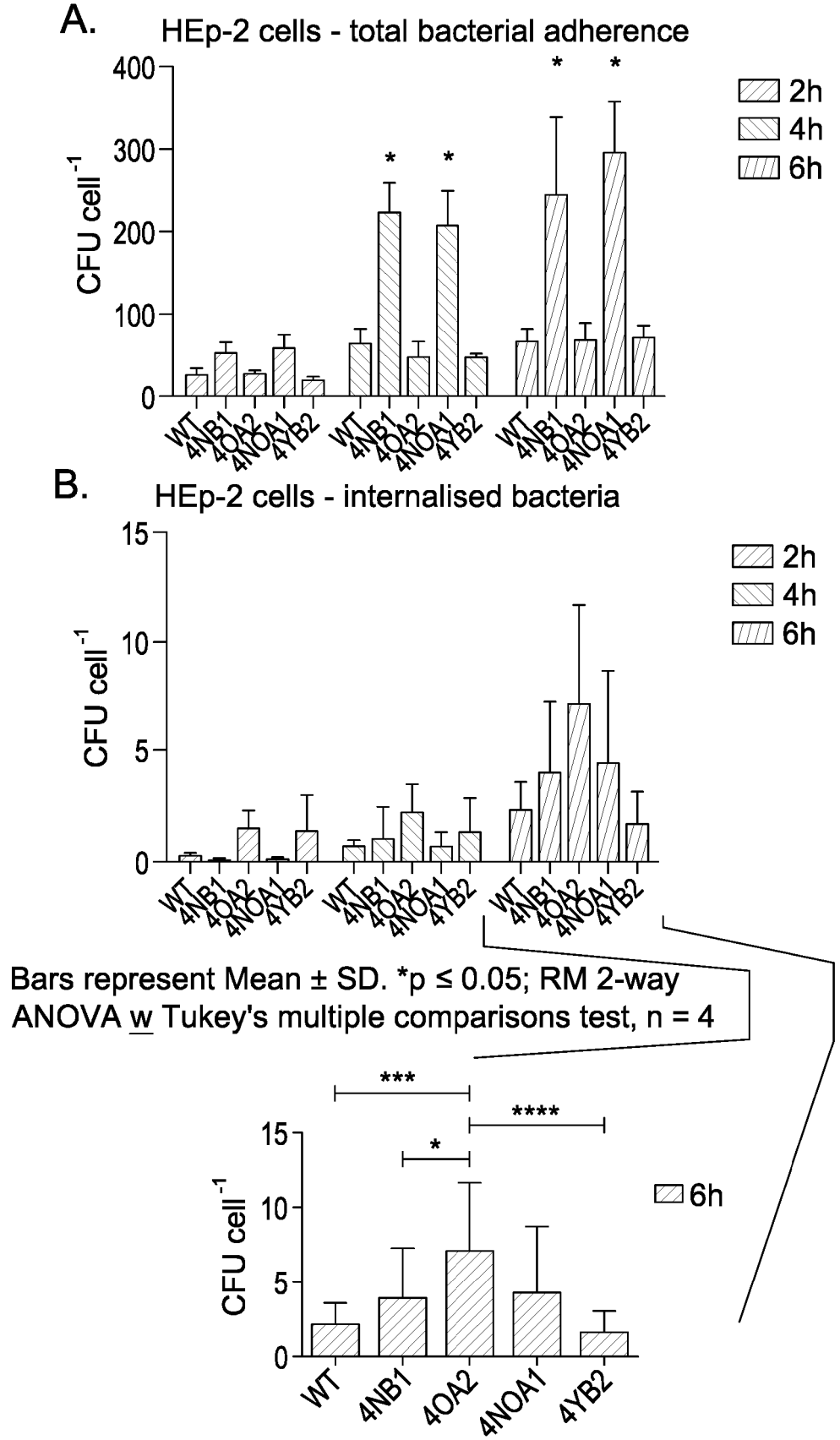

A.
HEp-2 cells - total bacterial adherence
CFU cell-1
400
300
200
100
0
2h
4h
6h
WT
4NB1
4OA2
4NOA1
4YB2
B.
HEp-2 cells - internalised bacteria
CFU cell-1
15
10
5
0
2h
4h
6h
Bars represent Mean ± SD. *p ≤ 0.05; RM 2-way ANOVA w Tukey's multiple comparisons test, n = 4
***
****
*
CFU cell-1
15
10
5
0
6h
WT
4NB1
4OA2
4NOA1
4YB2
Bars represent Mean ± SD. *p ≤ 0.05; ***p ≤ 0.001; ****p ≤ 0.0001; RM 2-way ANOVA w Tukey's multiple comparisons test, n = 4

Figure 40

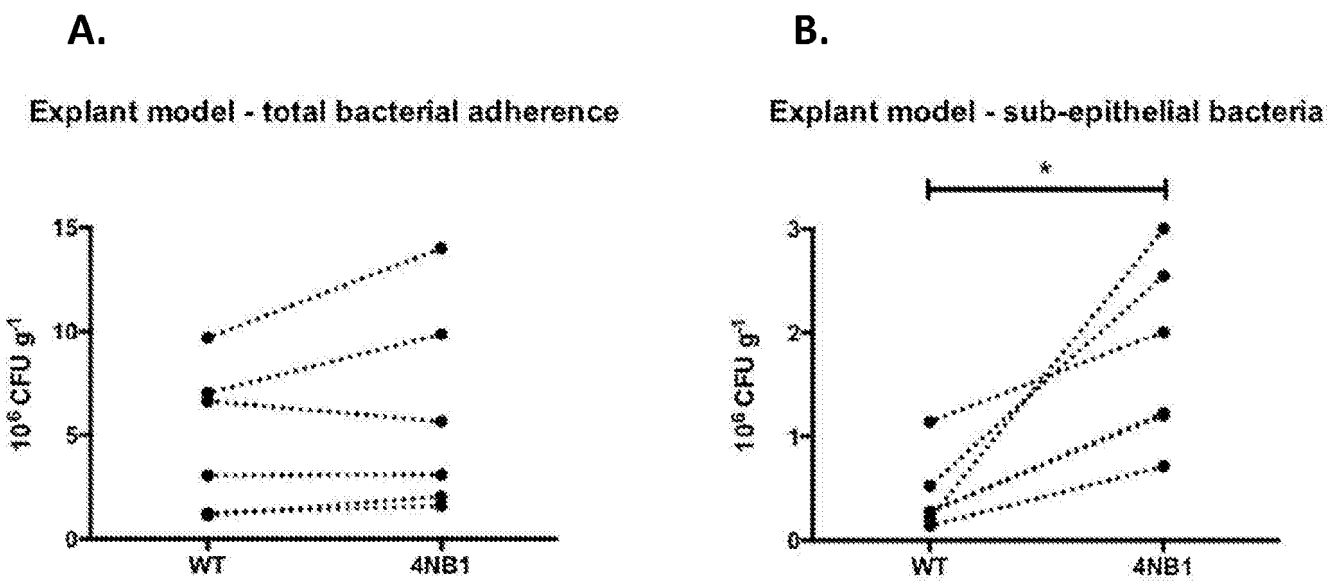

A.
Explant model - total bacterial adherence
15
10
5
0
10^6 CFU g^{-1}
WT
4NB1
B.
Explant model - sub-epithelial bacteria
*
3
2
1
0
10^6 CFU g^{-1}
WT
4NB1

Figure 41

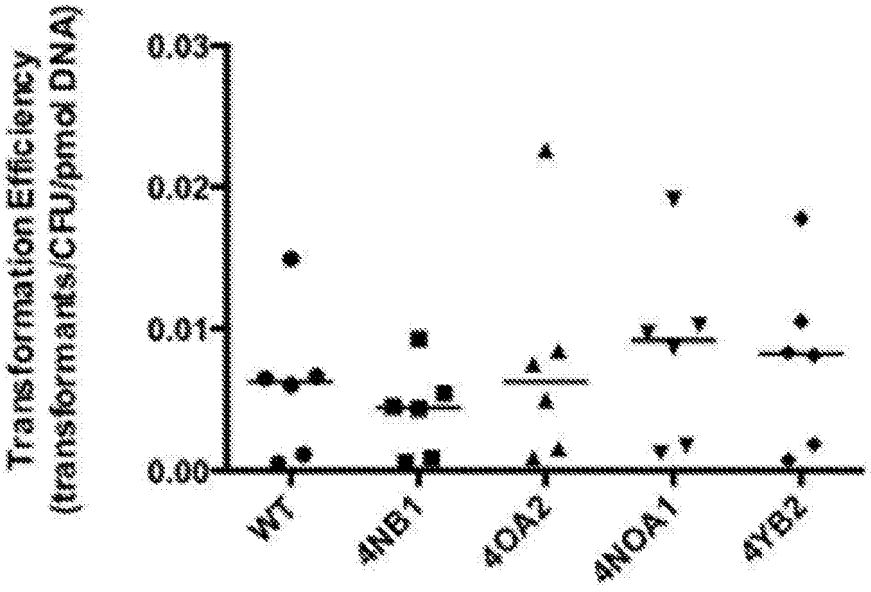
Table 1. E-test results: MIC vs. (GM)-*N. lactamica* strains

	WT	4NB1	4OA2	4NOA1	4YB2
Rif	0.38	0.38	0.38	0.38	0.75
Cip	0.003	0.003	0.002	0.003	0.008
Ceftri	<.002	0.003	<.002	0.003	<.002

Table 2. MIC breakpoints for pathogenic *Neisseria* species

	Breakpoint MIC (mg/L)			Reference
Antibiotic	*R >*	*I*	*S ≤*	
Rif	1	-	1	*Taha et al. Antimicrob Agents Chemother. 2010 Sep;54(9):3651-8.*
Cip	0.06	0.06	0.03	*BSAC Standardized Disc Susceptibility Testing Method v .12 (2013)*
Ceftri	0.12	-	0.12	*BSAC Standardized Disc Susceptibility Testing Method v .12 (2013)*

Figure 42



RM 1-way ANOVA with Tukey's Multiple Comparisons test, ns n = 6.

Figure 43

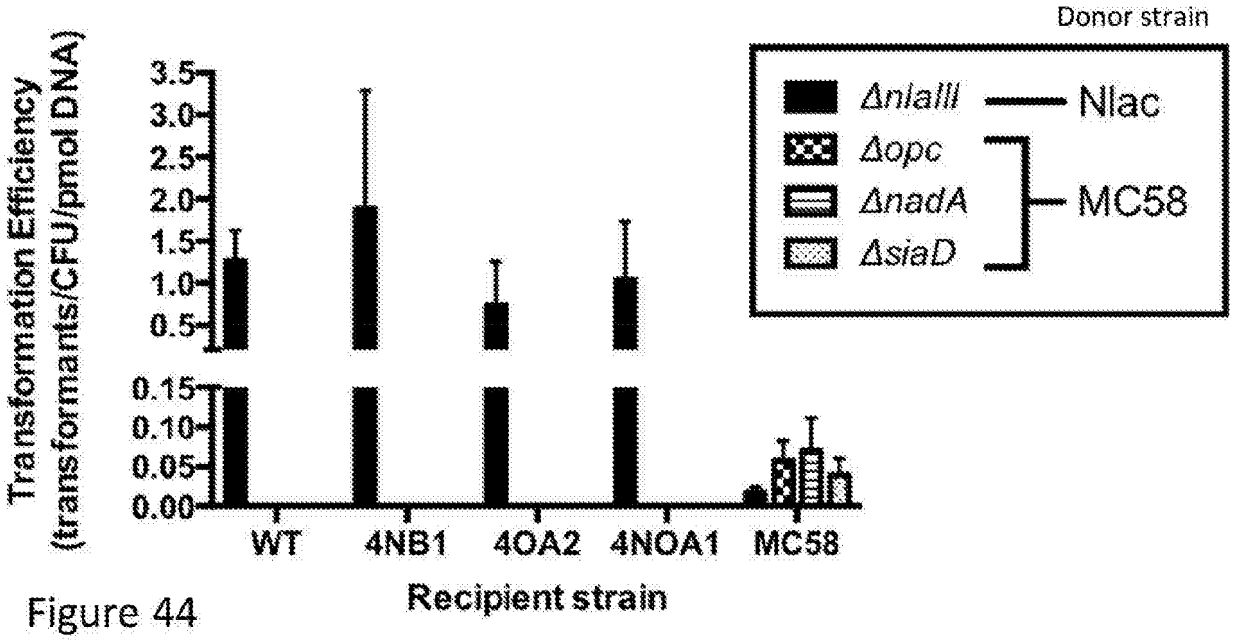


Figure 44

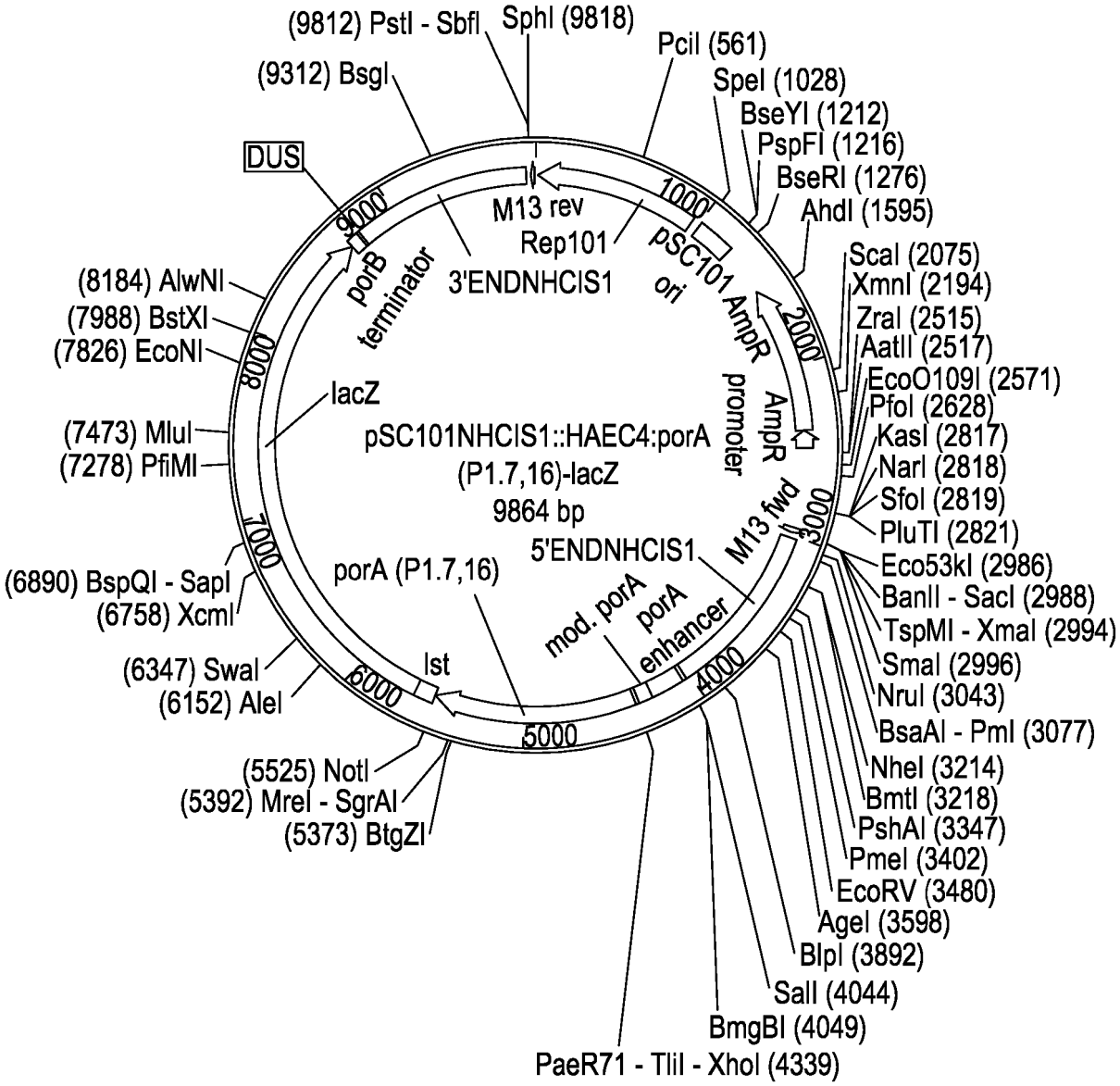

(9812) PstI - SbfI
SphI (9818)
PciI (561)
(9312) BsgI
SpeI (1028)
BseYI (1212)
PspFI (1216)
DUS
BseRI (1276)
AhdI (1595)
M13 rev
Rep101
pSC101 ori
ScaI (2075)
XmnI (2194)
(8184) AlwNI
porB terminator
3'ENDNHCIS1
ZraI (2515)
(7988) BstXI
AatII (2517)
(7826) EcoNI
AmpR promoter
AmpR
EcoO109I (2571)
PfoI (2628)
lacZ
KasI (2817)
(7473) MluI
pSC101NHCIS1::HAEC4:porA
NarI (2818)
(7278) PfiMI
(P1.7,16)-lacZ
SfoI (2819)
9864 bp
M13 fwd
PluTI (2821)
5'ENDNHCIS1
Eco53kI (2986)
(6890) BspQI - SapI
porA (P1.7,16)
BanII - SacI (2988)
(6758) XcmI
mod. porA
porA enhancer
TspMI - XmaI (2994)
SmaI (2996)
(6347) SwaI
lst
NruI (3043)
(6152) AleI
BsaAI - PmI (3077)
NheI (3214)
(5525) NotI
BmtI (3218)
(5392) MreI - SgrAI
PshAI (3347)
(5373) BtgZI
PmeI (3402)
EcoRV (3480)
AgeI (3598)
BlpI (3892)
SalI (4044)
BmgBI (4049)
PaeR71 - TliI - XhoI (4339)

Figure 45

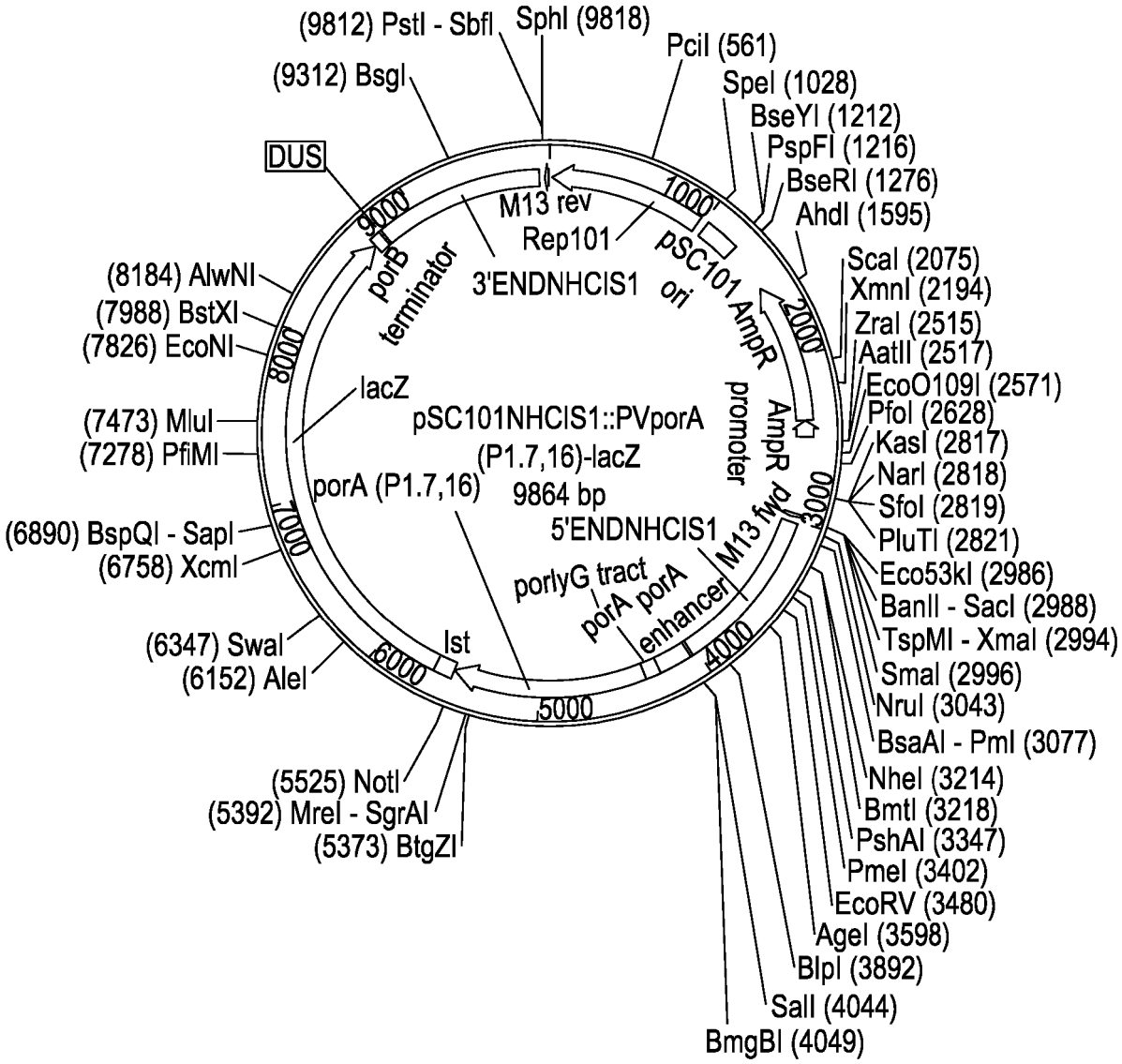

(9812) PstI - SbfI
SphI (9818)
PciI (561)
(9312) BsgI
SpeI (1028)
BseYI (1212)
PspFI (1216)
DUS
BseRI (1276)
AhdI (1595)
M13 rev
Rep101
pSC101 ori
ScaI (2075)
XmnI (2194)
(8184) AlwNI
3'ENDNHCIS1
porB terminator
(7988) BstXI
ZraI (2515)
(7826) EcoNI
AatII (2517)
AmpR promoter
AmpR
EcoO109I (2571)
lacZ
PfoI (2628)
(7473) MluI
pSC101NHCIS1::PVporA
KasI (2817)
(7278) PfiMI
(P1.7,16)-lacZ
NarI (2818)
porA (P1.7,16)
9864 bp
SfoI (2819)
(6890) BspQI - SapI
5'ENDNHCIS1
PluTI (2821)
(6758) XcmI
porIyG tract
M13 rev
Eco53kI (2986)
porA porA enhancer
BanII - SacI (2988)
(6347) SwaI
lst
TspMI - XmaI (2994)
(6152) AleI
SmaI (2996)
NruI (3043)
BsaAI - PmlI (3077)
(5525) NotI
NheI (3214)
(5392) MreI - SgrAI
BmtI (3218)
(5373) BtgZI
PshAI (3347)
PmeI (3402)
EcoRV (3480)
AgeI (3598)
BlpI (3892)
SalI (4044)
BmgBI (4049)

Figure 46

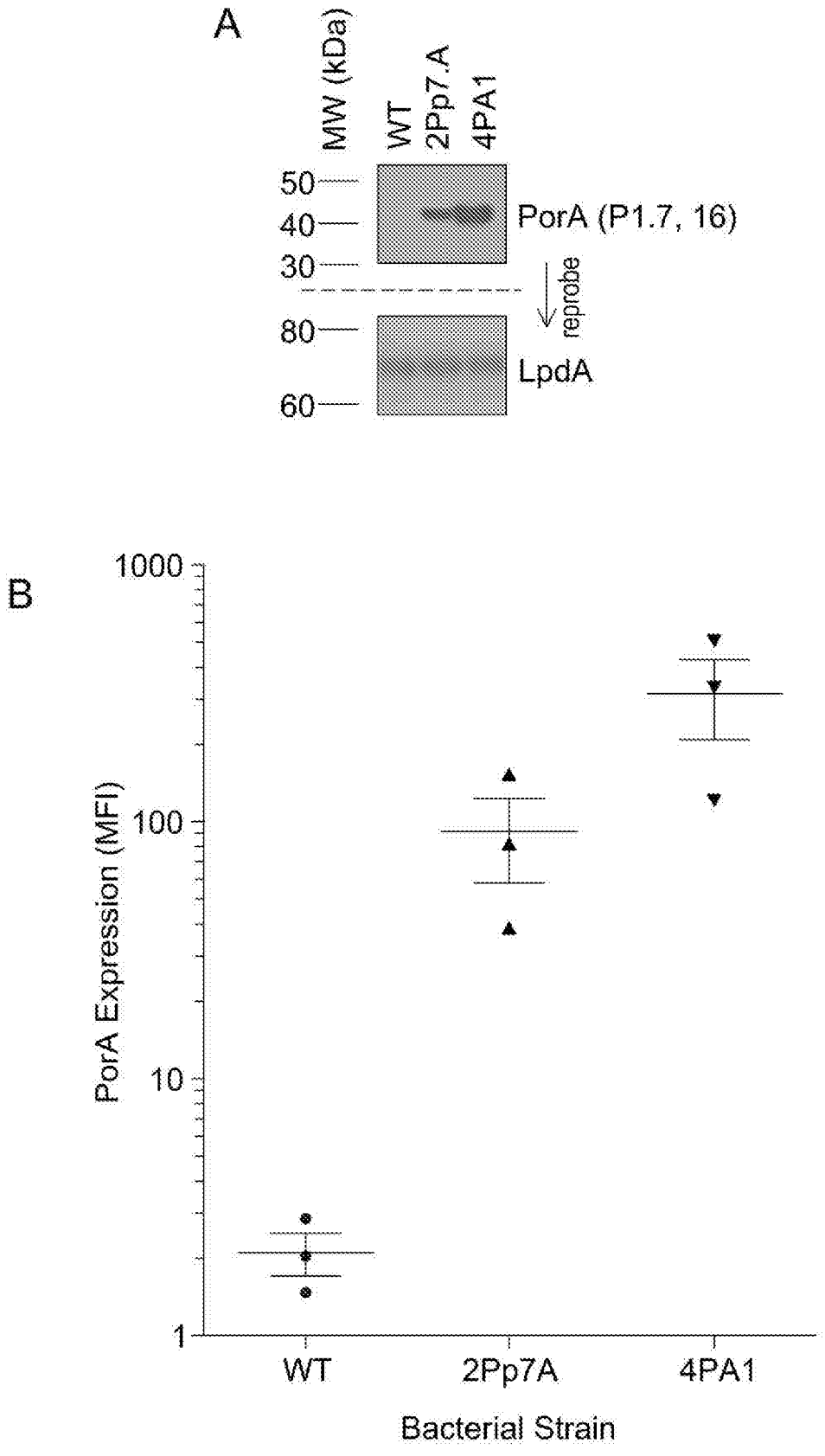

A
MW (kDa)
WT
2Pp7.A
4PA1
50
40
30
80
60
PorA (P1.7, 16)
reprobe
LpdA
B
1000
100
10
1
PorA Expression (MFI)
WT
2Pp7A
4PA1
Bacterial Strain

Figure 47

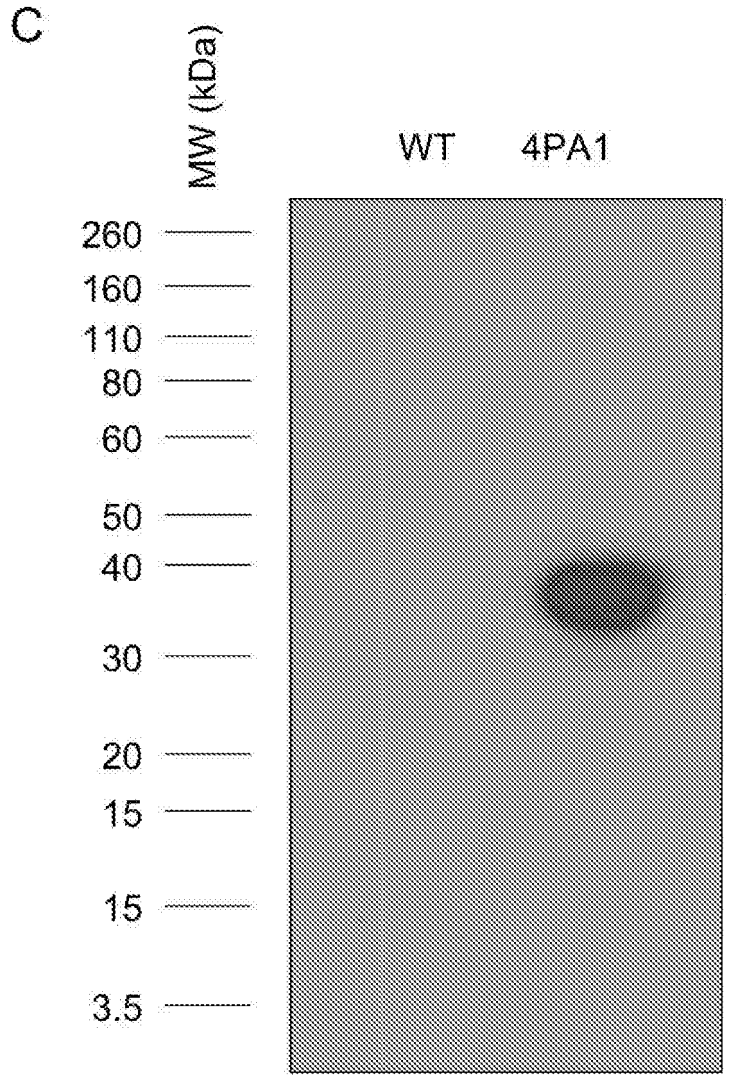


Figure 47 continued

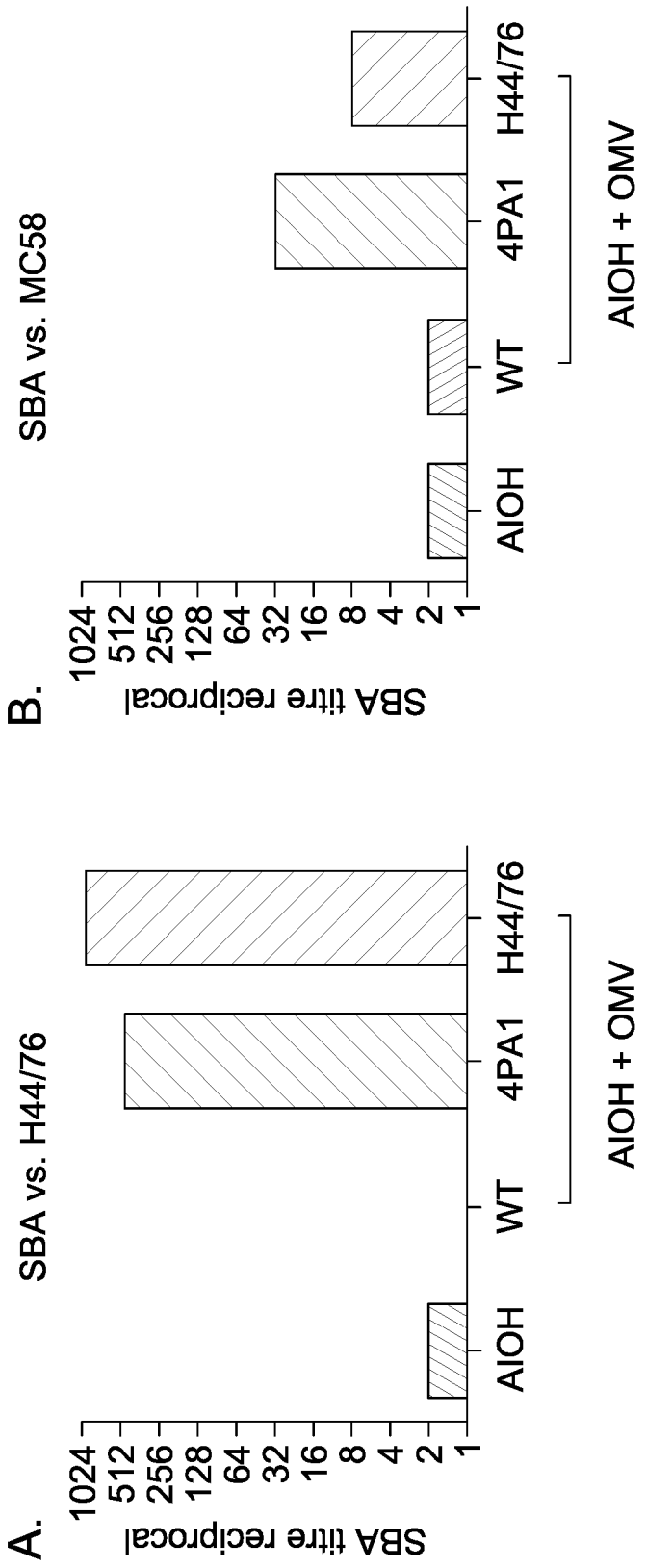

A.
SBA vs. H44/76
SBA titre reciprocal
1024
512
256
128
64
32
16
8
4
2
1
AIOH
WT
4PA1
H44/76
AIOH + OMV
B.
SBA vs. MC58
SBA titre reciprocal
1024
512
256
128
64
32
16
8
4
2
1
AIOH
WT
4PA1
H44/76
AIOH + OMV

Figure 48

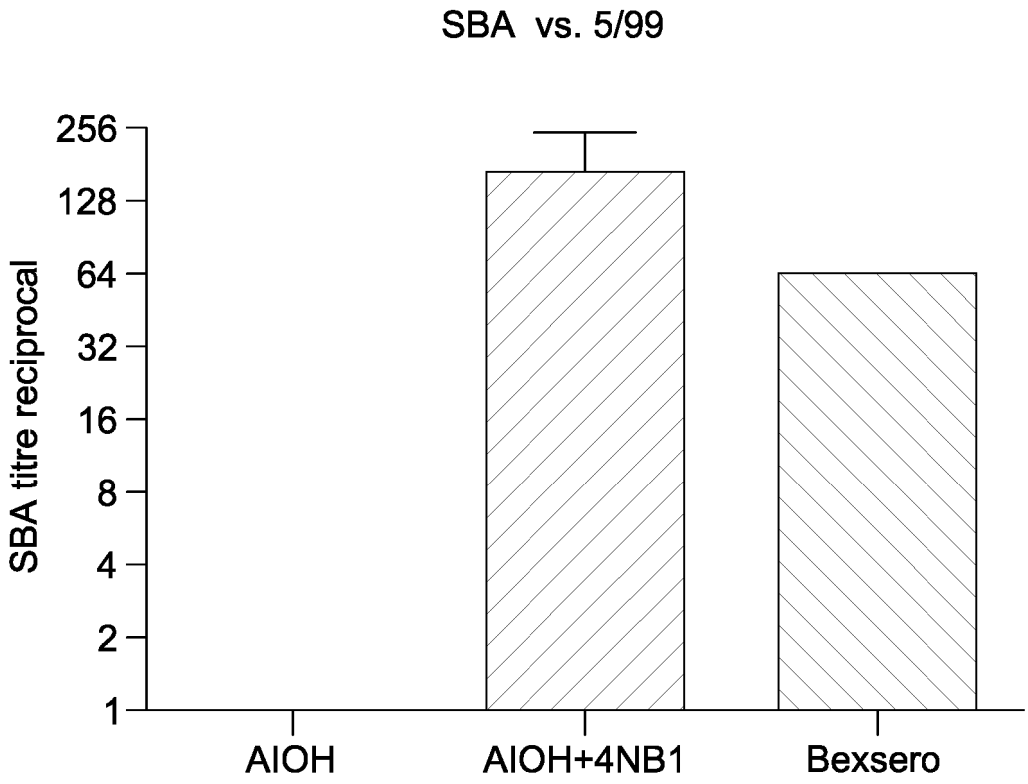

SBA vs. 5/99
SBA titre reciprocal
256
128
64
32
16
8
4
2
1
AlOH
AlOH+4NB1
Bexsero

Figure 49

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/053944

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/74 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 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 practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, COMPENDEX, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/077143 A1 (HEALTH PROT AGENCY [GB]; GORRINGE ANDREW [GB]; VAUGHAN THOMAS [GB]) 30 June 2011 (2011-06-30) the whole document -----	1-12,14, 15,18, 21-23, 25, 28-44, 46,48, 53,56, 59-66
X	WO 00/50074 A2 (MICROBIOLOGICAL RES AUTHORITY [GB]; IMP COLLEGE SCHOOL OF SCIENCE [GB]) 31 August 2000 (2000-08-31) the whole document, in particular the claims, especially claims 33, 34 ----- -/--	1-4,6-8, 11,20, 28-39, 41-43, 59-66

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

[X] See patent family annex.

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"E" earlier application or patent but published on or after the international filing date

"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
2 March 2017	15/03/2017

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	Bassias, Ioannis

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2016/053944

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/053944

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	New England Biolabs, Inc., I Schildkraut, New England Biolabs, Inc.: "pEGsph502[NEB808](ATCC® 69045TM)", ATCC , 16 November 1993 (1993-11-16), XP002767256, Retrieved from the Internet: URL:https://www.lgcstandards-atcc.org/Products/All/69045.aspx?&p=1&rel=generalinformation#generalinformation [retrieved on 2017-02-13] the whole document	1,2,6, 11,15, 18,21-23
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, US, vol. 72, no. 11, 1 November 2004 (2004-11-01), pages 6511-6518, XP008044838, ISSN: 0019-9567, DOI: 10.1128/IAI.72.11.6511-6518.2004	1-66
A	WO 03/051379 A1 (HEALTH PROT AGENCY [GB]; FOSTER KEITH ALAN [GB]; GORRINGE ANDREW RICHA) 26 June 2003 (2003-06-26)	59-66
A	Jay R. Laver ET AL: "Neisserial Molecular Adaptations to the Nasopharyngeal Niche" In: "ADVANCES IN MICROBIAL PHYSIOLOGY.", 1 January 2015 (2015-01-01), ACADEMIC PRESS, LONDON., GB, XP055345238, ISSN: 0065-2911 vol. 66, pages 323-355, DOI: 10.1016/bs.ampbs.2015.05.001,	1-66

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2016/053944

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011077143 A1	30-06-2011	NONE	
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		JP 2005514388 A	19-05-2005
		US 2005013831 A1	20-01-2005
		WO 03051379 A1	26-06-2003