Department for Environment, Food and Rural Affairs

Application for consent to release a GMO – organisms other than higher plants

Part A1: Information required under schedule 2 of the Genetically Modified (Deliberate Release) Regulations 2002

Part I

General information

1. The name and address of the applicant and the name, qualifications and experience of the scientist and of every other person who will be responsible for planning and carrying out the release of the organisms and for the supervision, monitoring and safety of the release.

Clinical & Experimental Sciences, LC72 (MP814) South Academic Block, Southampton General Hospital, Tremona Road, Southampton, UK.

Public Health England, Porton Down, Salisbury SP4 0JG, UK

2. The title of the project.

"Experimental challenge of the human nasopharynx with recombinant *Neisseria lactamica* expressing the meningococcal type V autotransporter protein, *Neisseria* Adhesin A (NadA)".

Part II

Information relating to the organisms

Characteristics of the donor, parental and recipient organisms

3. Scientific name and taxonomy.

Donor:

Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Neisseria; *Neisseria meningitidis*

Taxonomy ID: 122586

Recipient:

Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Neisseria; *Neisseria lactamica*

Taxonomy ID: 869214

The purpose of the genetic modification is to construct a strain of the exclusively human, nasopharyngeal commensal bacterium, *Neisseria lactamica* (Nlac) that expresses on its surface the outer membrane protein, *Neisseria* Adhesin A (NadA). NadA is an adhesin protein found in the close relative of Nlac, *Neisseria meningitidis* (Nmen), which is the causative agent of meningococcal disease. The genetically modified organism (GMO) will be used to investigate the role of NadA in the colonisation of the nasopharynx and associated immune responses in a controlled human bacterial challenge. The Experimental Human Challenge group, previously based at the University of Sheffield and now located at the University of Southampton, has conducted two previous human bacterial challenges in adult volunteers, using wild type Nlac strain Y92-1009 [1], [2]. The primary objective of the proposed study is to verify that nasopharyngeal challenge of humans with GM-Nlac is safe. Secondary objectives are determining the impact of NadA expression on the frequency of nasopharyngeal colonisation by GM-Nlac and the type(s) of immune responses generated locally and systemically to these bacteria. Ultimately this strategy may confer benefit as a bacterial medicine expressing genes with therapeutic or prophylactic potential within the human nasopharynx.

There are two GMOs in use in this study. The first is a NadA-expressing *N. lactamica* strain, a derivative of the wild type strain Y92-1009. The GMO has been constructed so that a gene expression cassette, containing an Nlac codon-optimised version of the *nadA* gene (NMB_1994) and a copy of the endogenous *lacZ* gene,

coding for β-D-galactosidase, is stably integrated into the bacterial chromosome at an intergenic locus. The second GMO is the procedural control strain, which has been modified at the same site and using the same protocol as the NadA-expressing strain, but contains a gene expression cassette with only the endogenous *lacZ* gene. The background strain into which these gene expression cassettes were introduced was Y92-1009 *ΔlacZ* (see Item 20b). The GMOs do not contain antibiotic resistance genes.

The NadA protein is a member of the type V autotransporter family of outer membrane proteins. NadA-expression in Nmen is associated with an increased level of adhesion to and uptake by human epithelial cell lines [3]. In a recent survey of European invasive Nmen isolates only 16 of 235 isolates possessed the *nadA* gene [4], suggesting that this is not essential for virulence. Due to the absence of the *nadA* gene in its genome, NadA is not expressed by wild type Nlac. The protein is expressed as a single polypeptide, with three structurally significant domains: (1) the globular head domain, responsible for molecular interaction(s) with the as-yet unidentified epithelial cell receptor(s), (2) the helical 'stalk' domain, which passes through the cytoplasmic membrane and is exposed to the extracellular milieu and (3) the membrane-associated 'pore' domain, which self-assembles into the outer membrane and provides an appropriately-sized channel through which the rest of the polypeptide can pass. All three domains are essential for function.

The NadA protein is one of the 4 strongly immunogenic components of the 4CMenB vaccine against serogroup B meningococcal disease (Bexsero). In wild type Nmen, expression of the *nadA* gene is phase variable, in which DNA replication errors alter the number of repeat sequences immediately upstream of the *nadA* promoter (5'-TAAA-3'), modulating the transcriptional activity of the gene [5]. Changes in the level of gene activity are reflected as increases or decreases in the level of NadA protein expression. In a longitudinal study of nasopharyngeal meningococcal carriage, it was shown that NadA expression in serial Nmen isolates decreased over time, hypothesised to be a result of seroconversion against NadA and the development of an antibody-mediated selective pressure against NadA expression [6]. This hypothesis is partially corroborated by the finding that immunisation with recombinant NadA, prior to attempted Nmen colonisation in a transgenic mouse model, leads to sterilizing immunity, whereby strains expressing a cognate NadA antigen were unable to colonise the murine nasopharynx [7]. In the GMO, *nadA* expression is instead controlled by a hybrid, constitutively active promoter that drives expression of the gene to high levels.

The β-D-galactosidase protein is a glycoside hydrolase enzyme that catalyses the hydrolysis of β-galactosides into monosaccharides through the breaking of a glycosidic bond. Nlac is unique amongst the *Neisseria* in its ability to produce acid from lactose, mediated by its expression of β-D-galactosidase (*lacZ*). In the GMO, the endogenous copy of the *lacZ* gene has been completely removed from the

chromosome (*ΔlacZ*), in order to allow use of the gene as a marker for successful transformation of Nlac. The transformation technology has therefore precluded the requirement for antibiotic resistance markers, and putative successful transformants are screened for on media containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). On X-gal-containing media, bacteria that have β-D-galactosidase activity grow as *blue* colonies, due to the chromogenic degradation of the substrate. The introduced *lacZ* gene is under control of the gene promoter for the Nlac *lst* gene, which codes for α-2, 3-sialyltransferase [8].

4. Usual strain, cultivar or other name.

Donor:

Neisseria meningitidis serogroup B strain: MC58, B: P1.7,16-2: F1-5: ST-74 (cc32)

http://pubmlst.org/perl/bigsdb/bigsdb.pl?page=info&db=pubmlst_neisseria_isolates&id=240

Recipient:

Neisseria lactamica strain: Y92-1009, ND: P1.ND,ND: F4-8: ST-3493 (cc613)

http://pubmlst.org/perl/bigsdb/bigsdb.pl?page=info&db=pubmlst_neisseria_isolates&id=4945

GMO: The GMOs are derivatives of a *ΔlacZ* mutant of *N. lactamica* strain Y92-1009 (see Item 20b). The purpose of the genetic modification is to construct a modified Nlac strain capable of expressing *Neisseria* Adhesin A (NadA) on its surface (strain 4NB1) or to serve as a control strain that has undergone a similar genetic modification, but which does not express heterologous proteins (strain 4YB2).

5. Phenotypic and genetic markers.

Both GMOs are phenotypically β-galactosidase positive and can be readily identified as a strain of *N. lactamica* by mass spectrometry (MALDI-TOF) or through biochemical testing (API NH, Analytical Profile Index, BioMerieux). The GMO strains can be distinguished from wild type Nlac through PCR amplification of the *Neisseria* Heterologous Construct Insertion Site number 1 (NHCIS1) locus, an otherwise intergenic chromosomal sequence located between NLY_27080 and NLY_27100. The gene expression constructs present in both GMOs were targeted to this locus. In wild type strains, NHCIS1 is approximately 2.2 kb in length, whereas in the GMOs the same primers will amplify much larger products (approximately 6.7 kb and 5.4 kb in length from the NadA-expressing and control strains, respectively).

6. The degree of relatedness between the donor and recipient or between parental organisms.

Neisseria lactamica shares approximately 67% of its genetic identity with *Neisseria meningtidis* [9], [10]. Phylogenetic analyses suggest that the two species are functional taxonomic groups that have recently diverged from a common ancestor [11].

7. The description of identification and detection techniques.

Following intranasal inoculation of volunteers, at the appropriate time points throat swabs and nasal washes will be performed. These samples will be used to try and culture viable *Neisseria* species on both GC and chocolate agar plates. Putative *Neisseria* colonies (identified by colony morphology) will be identified as *Neisseria lactamica* in the first instance by metabolism of exogenously applied X-gal, followed by verification with mass spectrometry (MALDI-TOF). The colonies will be specifically identified as the genetically-modified strains by PCR of the NHCIS1 locus. Should MALDI-TOF fail to provide accurate identification, suspected *N. lactamica* isolates will be identified based on a panel of conventional biochemical tests (API NH, bioMerieux). PCR amplification of the NHCIS1 locus from the GMOs will produce products of either 6.7 kb (4NB1) or 5.4 kb,(4YB2) whereas amplification of the same locus from wild type Nlac isolates will produce a 2.2 kb product.

NB: At the time of making this application, it has not been possible to verify the identification of the GMOs by MALDI-TOF, due to the lack of approvals for analysing GMO in the PHE Southampton diagnostic laboratories. Approvals have been applied for, but even in the event that they are not granted in time for the commencement of this project, our alternative identification system provides robust identification of the GMOs.

8. The sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques.

A ‘5041’ profile in the API NH biochemical identification kit gives an excellent score for *N. lactamica*. Both strains of GMO have been tested with this kit and return profiles of ‘5041’. The curated MALDI-TOF peptide fragment database (Bruker Dalatonics) also contains a fingerprint for *N. lactamica*. The combination of microbiological culture, MALDI-TOF mass spectrophotometry, PCR and biochemical identification together demonstrate 100 % specificity.

9. The description of the geographic distribution and of the natural habitat of the organisms including information on natural predators, prey, parasites and competitors, symbionts and hosts.

The exclusive environmental niche of *Neisseria lactamica* is the human nasopharynx. Strains of *Neisseria lactamica* are isolated from humans around the world, suggesting the capacity of this organism to circulate globally. The carriage frequency of *N. lactamica* is greatest in infants, wanes into adolescence and then remains low throughout adulthood [12], [13]. This contrasts with the potential pathogen *Neisseria meningitidis*, which is most frequently isolated from teenagers and is cyclically endemic in between 10-30% of a given adult population [14]. The apparent mutual exclusivity of these species was demonstrated in a human nasopharyngeal challenge experiment, whereby *N. lactamica* was introduced into the nares of healthy, adult volunteers [2]. In individuals colonised by the commensal organism, there was displacement of existing strains of *N. meningitidis* from the nasopharynx, and the acquisition of new meningococcal strains was prevented. Although the exact nature of the relationship between these species has yet to be determined, one interpretation of these findings is that *N. lactamica* and *N. meningitidis* are competing with one another for the same nasopharyngeal niche.

10. The organisms with which transfer of genetic material is known to occur under natural conditions.

Neisseria lactamica is extremely resistant to acquiring new genes through horizontal gene transfer. Despite the natural competence of the organism, a novel transformation system involving hypermethylated DNA had to be developed in order to derive the GMOs. Hypermethylated DNA is DNA in which every deoxycytosine nucleotide residue is methylated (specifically, the DNA exclusively contains 5-methyl-deoxycytosine). Whilst hypermethylation does not change the sequence of the nucleic acid, the chemical composition of the molecule acts to block the otherwise potent restriction endonuclease activities present in Nlac [15]. Without appropriate methylation, DNA taken up by Nlac is degraded before it can undergo recombination with the chromosome and become incorporated into the Nlac genome. No other member of the Neisseriaceae contains a DNA methylase capable of appropriately methylating its chromosomal DNA, so the risk that Nlac will acquire new genes from the nasopharyngeal milieu is negligible.

In the unlikely event of horizontal gene transfer into Nlac over the course of the proposed experiments, the donor genetic material will originate from other species in the human oro-nasopharynx. In the evolutionary history of *N. lactamica* since its speciation, there are relatively few incidences of horizontal gene transfer in general,

but especially from organisms outside of the Neisseriaceae. Notable exceptions to this include the historical acquisition of genes from *Haemophilus influenzae* and most probably a species of *Mycoplasma* [16]. This predilection for *Neisseria*-derived DNA is due to uptake bias in favour of molecules containing Neisserial DNA Uptake Sequences (DUS). DUS are non-palindromic repeat sequences over-represented in the genomes of the Neisseriaceae, and are hypothesised to minimise the risk of *Neisseria* family members taking up DNA molecules containing potentially deleterious sequences [17]. In a previous human challenge experiment using the wild type Nlac strain Y92-1009 from which the GMOs were derived, some volunteers became colonised by the bacteria for 6 months [2]. Analysis of the genomes of serial Y92-1009 isolates taken from these individuals showed no evidence of horizontally acquired DNA, even when there was detectable co-carriage of *N. meningitidis*. In a comparative assessment of transformation efficiencies, using a hypermethylated donor molecule to circumvent restriction endonuclease activity, the GMO strains of Nlac were only as likely to become transformed as the wild type bacterium, indicating that the process of generating the GMOs had not inadvertently selected for an inherently 'more transformable' phenotype (see Figure 7, Item 69a).

Whilst it is refractory to horizontal genetic exchange, *N. lactamica* acts as a reservoir of genes for the pathogenic *Neisseria*. There is evidence of frequent interspecific movement of Nlac genes into Nmen and *N. gonorrhoeae*, with the subsequent generation of hybrid genotypes [18]; [19]; [20];[21].

11. Verification of the genetic stability of the organisms and factors affecting that stability.

Draft assemblies of the whole genome sequences of either GMO were mapped onto a complete, closed genome generated from the DNA of wild type *N. lactamica* strain, Y92-1009 (PacBio). As expected, the alignments (made using progressive Mauve) revealed a modest number of chromosomal rearrangements in the GMO strains as compared to the reference sequence. The size and relative displacement of the chromosomal rearrangements varied greatly. These data are consistent with observations in both *Neisseria gonorrhoeae* [22] and *Neisseria meningitidis* [23], in which chromosomal rearrangements have been intensively studied. The genomes of the *Neisseriaceae* are highly plastic, with relative chromosomal rearrangements commonplace – not only at the species level [24], but also between strains of the same species [25];[26] and between isolates comprising a single bacterial lineage [27]. The *Neisseria* chromosome is highly plastic because it includes a large number of repetitive sequences, such as those shown in Table 1. These sequences not only serve functional roles in the biology of the organism, for example, as a means to modulate gene expression; but also act as focal points for homologous recombination – both with material taken up from outside the cell, and with other

regions of the chromosome containing identical elements. It is therefore unsurprising there have been changes in the genomic architecture of the GMOs relative to the parental strain, given the repeated passages necessary for their derivation.

Repeat type	Repeat Sequence	Value
AT-DUS	'ATGCCGTCTGAA'	1718
AG-DUS	'AGGCCGTCTGAA'	262
AG-mucDUS	'AGGTCGTCTGAA'	45
dRS3	'ATTCCCNNNNNNNNGGGAAT'	454
Correia	'ATAG[CT]GGATTAACAAAAATCAGGAC'	50
	'TATAG[CT]GGATTAAATTTAAACCGGTAC'	1
	'TATAG[CT]GGATTAACAAAAACCGGTAC'	17
	'TATAG[CT]GGATTAAATTTAAATCAGGAC'	17

Table 2: Frequency of repeat sequences in complete, closed *N. lactamica* Y92-1009 genome: AT-DUS, AG-DUS and AG-mucDUS are variant 'dialects' of the *Neisseria* DNA Uptake sequence [28], dRS3 are 'direct repeat sequence 3' elements and Correia refers to inverted repeat sequences also known as Correia repeat enclosed elements (CREE).

Draft assemblies of whole genome sequences were also derived from variants of the GMOs that had been serially passaged *in vitro* for 28 days on TSB agar. Mapping of these assemblies to the PacBio reference sequence showed similar numbers of chromosomal rearrangements. It is important to note however, that the NHCIS1 locus was seemingly unaffected by these stochastic alterations and did not appear at a rearrangement breakpoint. Indeed, the sequences of both the *nadA* and *lacZ* genes were 100% identical in all matched isolates, reflected in the fact that NadA expression levels were unaffected following the period of repeated culture (see Item 28).

In our experimental work, we find that relative to *in vitro* culture, the growth of *N. lactamica* in its biological niche is a stabilising force for its genome. A comparison was made between genomes derived from isolates of wild type *N. lactamica* recovered from experimentally inoculated volunteers and genomes of isolates that had been serially passaged on laboratory agar for identical periods of time. The bacteria isolated from the nasopharynx contained fewer mutations overall, with the main cause of mutation over time attributable to mechanisms of phase variation (manuscript in preparation).

The greatest impact on genetic stability of the GMO is horizontal gene transfer from other bacterial species. We have demonstrated that the GMOs are refractory to

horizontal gene transfer, where repeated attempts to transform the bacteria with genetic material derived from *N. meningitidis* resulted in zero putative transformants (see Figure 6, Item 69). Across evolutionary time there have been very few instances of horizontal gene transfer into *N. lactamica* [16], which is why we had to develop a novel method for the transformation of this bacterium, designed to circumvent the barrier to transformation posed by its suite of restriction endonucleases.

12. The following pathological, ecological and physiological traits:

a. the classification of hazard according to existing Community rules concerning the protection of human health and the environment;

The UK ACDP has not categorised *N. lactamica,* therefore wild type *N. lactamica* should be considered a *group one biological agent* under the European Economic Community (EEC) classification for the protection of workers with biological agents [Directive 2000/54/EC]. Work with GM-*N. lactamica* has been risk assessed by the University of Southampton Genetic Modification & Biosafety Committee (GMBSC) and, following notification of the Health and Safety Executive is routinely handled as activity *class 2*.

b. the generation time in natural ecosystems, and the sexual and asexual reproductive cycle;

Bacteria replicate asexually and undergo binary fission to produce identical daughter cells. No data exists on the generation time of *N. lactamica* in the human nasopharynx, but various investigations on a variety of growth media suggest a generation time longer than that of *E. coli*. The generation time of *N. lactamica* in TSB liquid medium supplemented with 0.2 % yeast extract at 37 ^{o}C was calculated to be *74 minutes* in our hands.

c. information on survivability, including seasonability and the ability to form survival structures, including seeds, spores and sclerotia;

There is a paucity of information on the survivability of *Neisseria lactamica* outside of its biological reservoir. Environmental survival of other species of *Neisseria* has been investigated, with the most work having been performed on the 'pathogenic' *Neisseria*. It is now widely appreciated that *N. gonorrhoeae* can survive on a variety of surfaces for periods of up to 72 h, especially if they are kept moist. Whilst cultured suspensions of the bacteria will expire in the time it takes for the inoculant to dry

(approximately 10 minutes on hard surfaces), there are much higher levels of gonococcal survival in droplets of purulent discharge (systematically reviewed in [29]). Similarly, recovery of viable *Neisseria meningitidis* is possible 24 h after inoculation of bacteria onto solid surfaces, raising the question as to whether fomites of oropharyngeal secretion are a vector for transmission [30]. The composition and moisture content of the external surface modulates the length of survival, with softer and damper materials prolonging environmental survival over dryer and harder ones, as does the temperature to which the bacteria are exposed; for example, meningococci absolutely do not survive storage for 24 h at any non-freezing temperature below 17 ^{o}C. Importantly, the expression of meningococcal capsule had no effect on the survivability of the meningococcus outside of its natural habitat, which implies the survival characteristics of the unencapsulated *Neisseria lactamica* are likely to be similar.

Both the meningococcus and gonococcus have been demonstrated to form biofilm *in vitro* under nutrient starvation (i.e. stress) conditions [31]; [32]. A biofilm is a multicellular bacterial structure in which constituent bacteria have significantly different gene expression profiles to planktonic (i.e. free-floating, individual) cells. Biofilm formation is hypothesised to serve as a persistent ecological reservoir of a diverse range of bacterial species. We have demonstrated *Neisseria lactamica* biofilm formation under nutrient starvation conditions *in vitro* (data not shown). It is plausible, given the autoaggregative phenotype of many strains of *N. lactamica*, that limited multicellularity – perhaps as microclusters [33] is a preferred state of *N. lactamica in vivo*. There is however no evidence to suggest the *in situ* existence of *N. lactamica* biofilms.

d. pathogenicity, including infectivity, toxigenicity, virulence, allergenicity, carrier (vector) of pathogen, possible vectors, host range including non-target organisms and possible activation of latent viruses (proviruses) and ability to colonise other organisms;

Neisseria lactamica is an exclusively human, non-pathogenic commensal bacterium. Although there are a few case reports of *N. lactamica* involvement in disease (see Table 4 in [34]), these are universally secondary complications arising from immunosuppression or trauma, and are exceptionally rare amongst these groups. While most strains of *N. lactamica* contain genes associated with the prophage Pf1, the completely non-pathogenic nature of *N. lactamica* suggests that the prophage is unlikely to be a virulence factor.

e. antibiotic resistance, and potential use of these antibiotics in humans and domestic organisms for prophylaxis and therapy;

The GMOs do not possess any antibiotic resistance genes and remain acutely sensitive to front-line antibiotics used clinically to treat meningococcal disease (see Table 2).

MIC (mg/L)			
	WT	**4NB1**	**4YB2**
Rifampicin	0.38	0.38	0.75
Ciprofloxacin	0.003	0.003	0.008
Ceftriaxone	<.002	0.003	<.002

Table 2: Antimicrobial susceptibility of wild type Y92-1009 (WT), the NadA-expressing, mutant derivative thereof (4NB1) and the Control or 'empty-vector' derivative thereof (4YB2). Minimal Inhibitory Concentration (MIC) breakpoints for pathogenic *Neisseria* species are: rifampicin = >1 mg/L, ciprofloxacin = >0.06 mg/L and ceftriaxone = >0.12 mg/L.

f. involvement in environmental processes including primary production, nutrient turnover, decomposition of organic matter and respiration.

Not applicable. Neither the vector nor the parent/recipient organism is involved in any environmental processes.

13. The sequence, frequency of mobilisation and specificity of indigenous vectors and the presence in those vectors of genes that confer resistance to environmental stresses.

Not applicable. The GMO strains do not contain indigenous vectors. Genetic modification of the GMOs was performed through integration of donor molecules into the bacterial chromosome. Donor molecules contained no sequence to drive replication of the molecule as an extrachromosomal element.

14. The history of previous genetic modifications.

Prior to the targeted genetic alterations to *N. lactamica* described here, there were no previous examples of deliberate genetic modification in any strain of this bacterium published in the literature, indeed there were only reports of repeatedly failed attempts [35]. Recently however, genetic modification of two strains of *N. lactamica* (strains NL-1 and ATCC23970) have been reported [36].

To create a suitable background strain for the construction of the GMOs, the *lacZ* gene, coding for β-D-galactosidase, was completely removed from the genome. Successful transformants (*ΔlacZ*) were screened on the basis of blue/white colony formation on growth medium supplemented with X-gal (see Item 20b.)

Characteristics of the vector

15. The nature and source of the vector.

The GMOs do not contain any extrachromosomal vectors. The GMOs were produced through transformation of *ΔlacZ* Y92-1009 with a hypermethylated PCR product, which was amplified from one of two pUC19-derived plasmids: pUC19NHCIS1::HAEC4:*nadA*-*lacZ* (Figure 2) and pUC19NHCIS1::HAEC4:(Z)-*lacZ* (Figure 3). It is important to note the PCR product contained no plasmid-derived sequences. HAEC4 (Heterologous Antigen Expression Cassette number 4) refers to the specific combination of Nlac-compatible promoter sequences used to drive gene expression. In HAEC4, these promoters are a synthetic hybrid *porA/porB* gene promoter, which is preceded (5') by 200 bp of the Upstream Activation Sequence (UAS) of the meningococcal *porA* gene. Downstream (3') of the hybrid promoter there is a copy of the endogenous Nlac *lst* gene promoter. NB: The *porA*-associated UAS serves to enhance transcription from the native *porA* promoter in the meningococcus. By fusing different lengths of this UAS immediately upstream of the *lst* promoter, we identified maximum enhancement of *lst*-driven reporter gene activity when the length of this sequence was 200 bp (Figure 1).

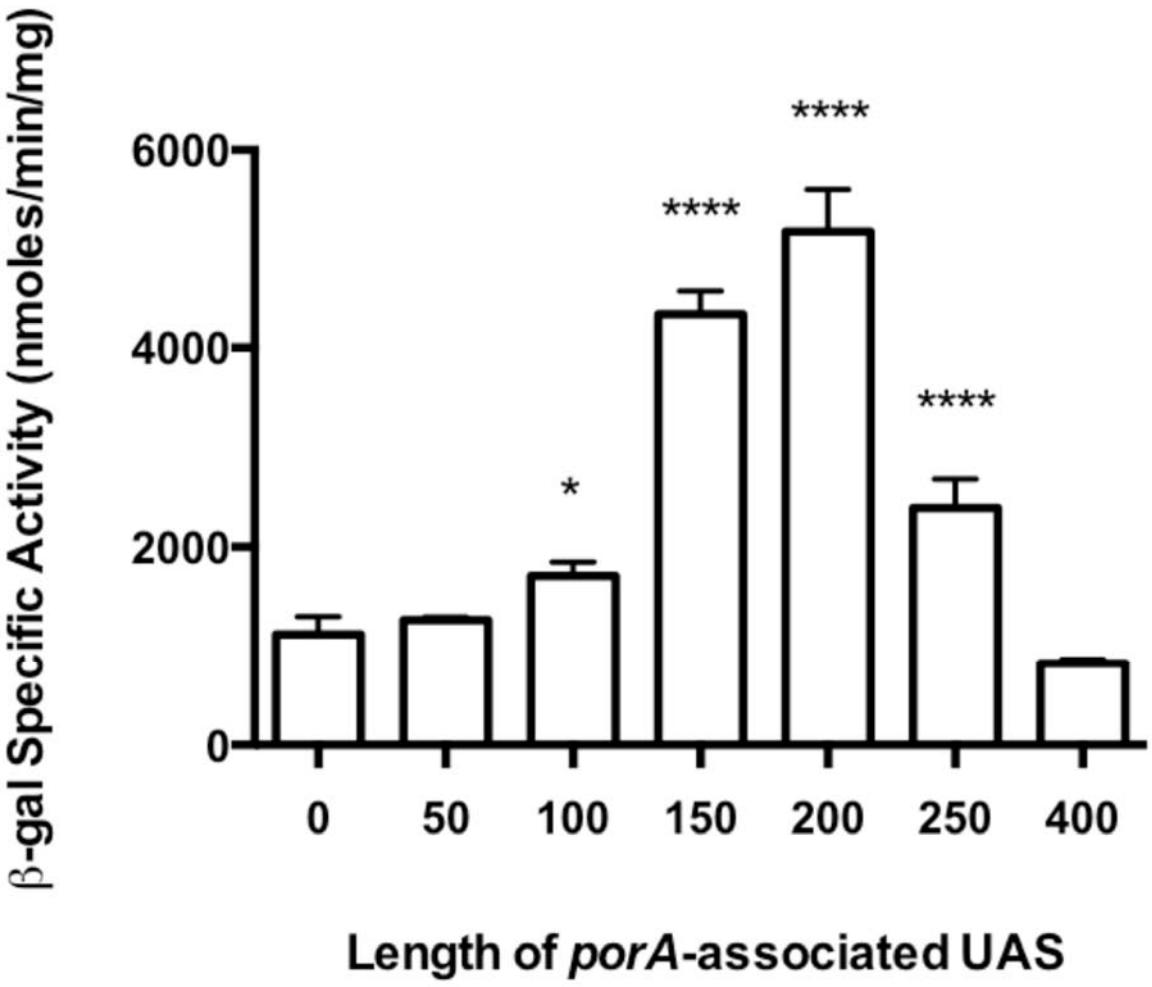


Figure 1: Effect of different lengths of the *porA*–associated UAS on β-galactosidase Specific Activity, expressed from NHCIS1 in *N. lactamica*. Increasing lengths of the UAS were conjugated immediately 5' of the *lst* promoter, which was used to drive expression of the *lacZ* gene. These constructs were targeted to NHCIS1 and used to transform *ΔlacZ N. lactamica*. The Specific Activity of β-galactosidase was measured in lysates of mid-log phase bacteria. *$p \leq 0.05$, ****$p \leq 0.0001$, Dunnett's multiple comparisons test vs. '0' as Control column, (n = 3). Bars represent Mean ± SD, where error bars are not visible, they fall within the line.

In the plasmids used to derive the GMOs, the UAS-enhanced, synthetic hybrid *porA/porB* promoter drives the expression of the *nadA* gene and the *lst* promoter drives expression of *lacZ.* Importantly, in the 'empty-vector' construct amplified from pUC19NHCIS1::HAEC4:(Z)-*lacZ*, there is a short 14 bp linking sequence instead of an open reading frame (ORF). Chromosomal homologous recombination events during the construction of the GMO resulted in replacement of the wild type NHCIS1 locus with homologous sequence flanking either side of the *nadA-lacZ* gene expression cassette.

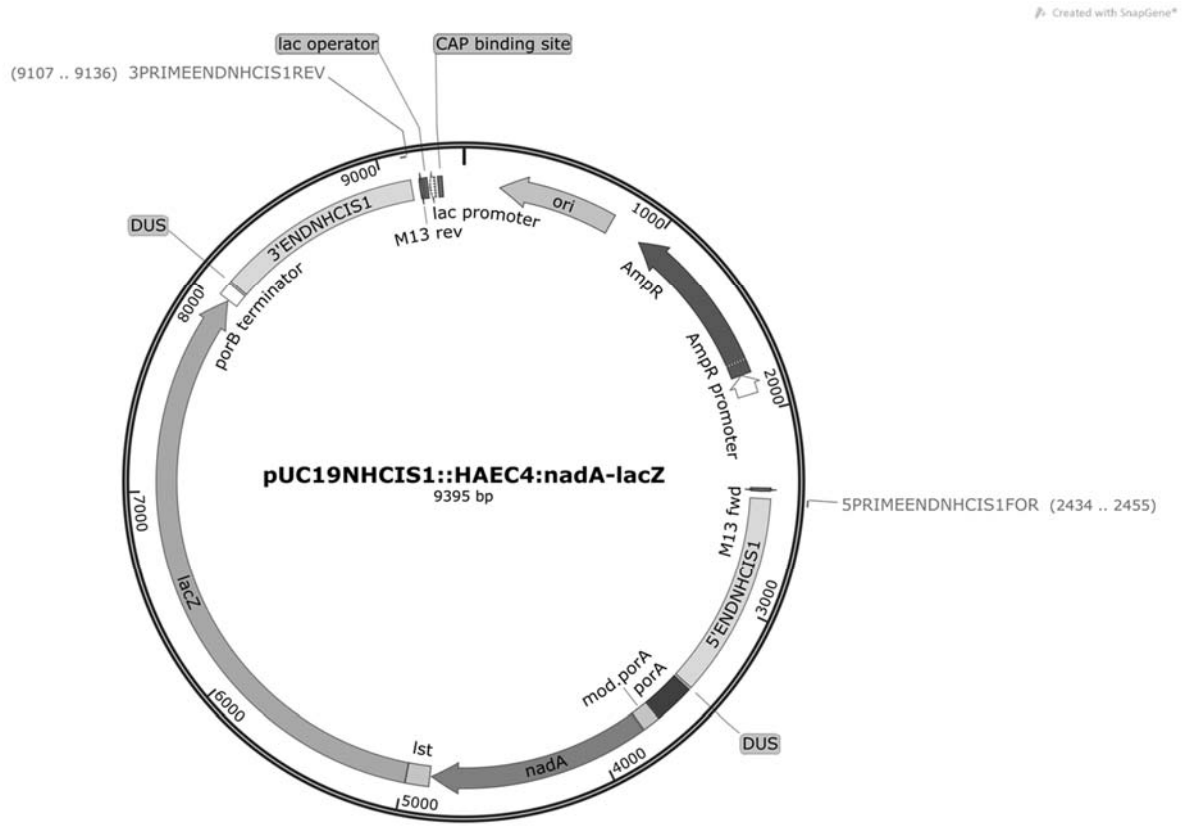


Figure 2: Plasmid map of pUC19NHCIS1::HAEC4:*nadA-lacZ* showing all relevant features. NB: porA refers to the *porA*-associated upstream activator sequence (UAS) and mod.porA refers to the synthetic hybrid *porA*/*porB* gene promoter. The location and span of the primers used to amplify the donor genetic material (NHCIS1::HAEC4:*nadA-lacZ*) using hypermethylated PCR are shown (5PRIMEENDNHCIS1FOR and 3PRIMEENDNHCIS1REV).

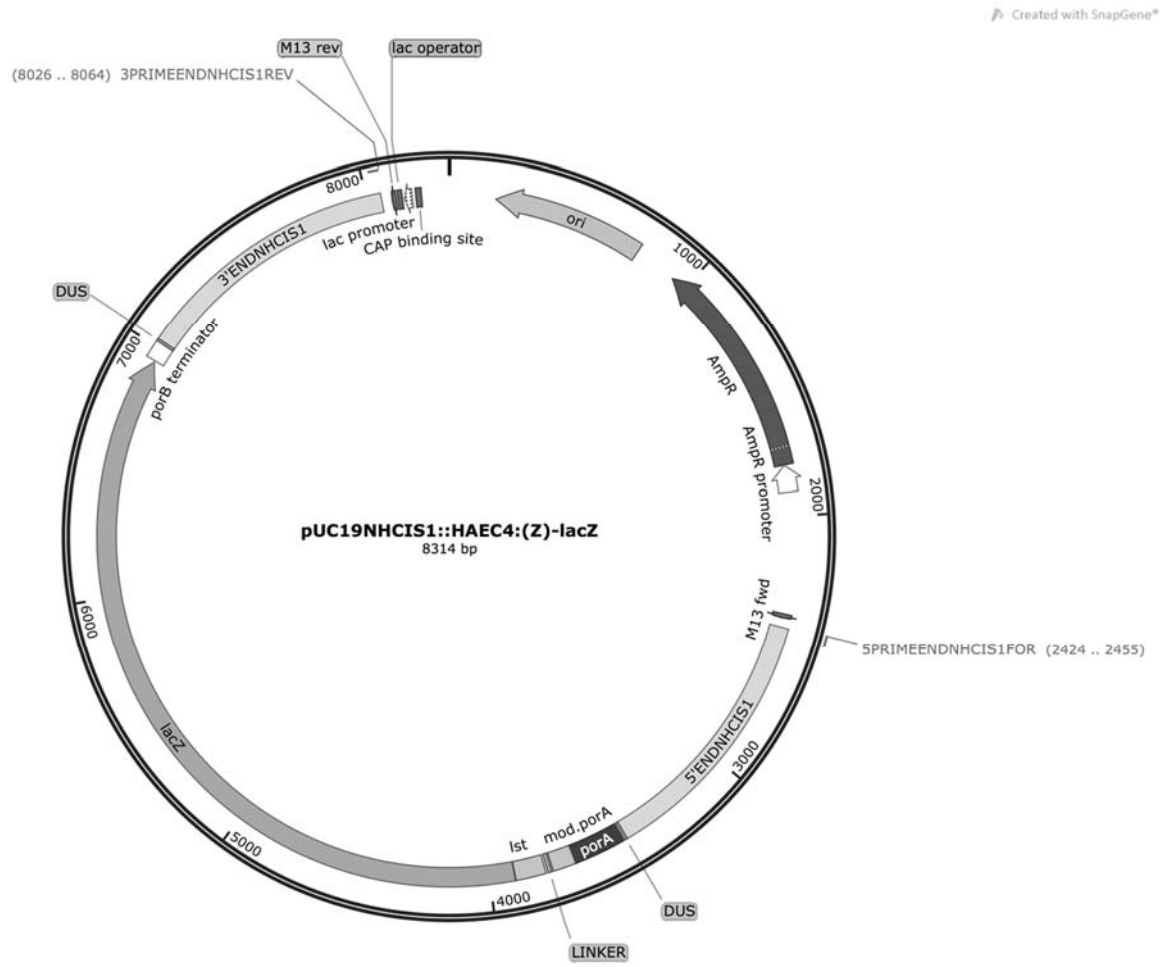


Figure 3: Plasmid map of pUC19NHCIS1::HAEC4:(Z)-*lacZ* showing all relevant features. NB: porA refers to the *porA*-associated upstream activator sequence (UAS) and mod.porA refers to the synthetic hybrid *porA*/*porB* gene promoter. The location and span of the primers used to amplify the donor genetic material (NHCIS1::HAEC4:(Z)-*lacZ*) using hypermethylated PCR are shown (5PRIMEENDNHCIS1FOR and 3PRIMEENDNHCIS1REV).

16. The sequence of transposons, vectors and other non-coding genetic segments used to construct the genetically modified organisms and to make the introduced vector and insert function in those organisms.

The PCR product used to create the GMO is fully described in Item 24. The GMO contains no extrachromosomal vectors.

17. The frequency of mobilisation, genetic transfer capabilities and/or methods of determination of the inserted vector.

The GMOs do not contain any extrachromosomal vectors. The gene expression constructs are stably integrated into the GMO chromosome and maintained as a single copy per bacterium. The gene expression cassettes do not contain sequences that direct their replication outside of the chromosome. For ectopic transformation to occur in the nasopharynx, release of chromosomal DNA from resident GMOs must first occur, followed by uptake by other bacteria of the specific nucleic acid sequence comprising or containing the NHCIS1 locus. Following this, the material must recombine into the genome of its new host in order to be maintained. Each of these are low frequency events, meaning the overall risk of onward transmission occurring *in vivo* is negligible. Though unlikely for the reasons stated, were onward transmission of the gene to occur, the most likely recipients would be other members of the *Neisseria* genus, due to the preponderance of DUS in the GMO chromosome and the predilection for DUS-containing, homotypic DNA observed in *Neisseria* DNA uptake systems [37]. However, as the NadA protein is not required for virulence in *N. meningitidis* [4], the impact of its expression in carriage strains of the *Neisseriaceae* is anticipated to be minimal. Indeed, NadA expression is unlikely to confer an evolutionary advantage to recipient strains over the longer term, as hosts are predicted to seroconvert against the NadA protein (it is, after all, a strongly immunogenic component of the Bexsero vaccine). Because the promoter of the *nadA* gene in this construct is constitutively active, recipient strains will be unable to downregulate its expression through phase variation, which occurs naturally through the course of meningococcal carriage [6]. As such, the NadA protein may instead serve as an evolutionary liability and inadvertently target the recipient strains for destruction by the immune system.

The gene expression construct can be detected by PCR of the NHCIS1 locus, or a PCR that detects the unnaturally close proximity of the *porA* promoter region and *lacZ* coding sequences.

18. The degree to which the vector is limited to the DNA required to perform the intended function.

The gene expression cassettes consist of only the coding sequences of the *nadA* and *lacZ* genes, the sequences of the promoters driving gene expression and two DUS. Collectively, these elements are minimally sufficient to perform the function of the cassette; specifically, the expression of NadA on the bacterial surface (where appropriate) and the production of β-D-galactosidase as a means of screening for putatively transformed *N. lactamica*. The sequences surrounding the gene expression cassette, homologous to the NHCIS1 locus of wild type Y92-1009, are of sufficient length to accurately target the cassette to the correct chromosomal location, and at a high enough frequency to facilitate a screening strategy for successful transformants.

Characteristics of the modified organisms

19. The methods used for the modification.

Standard genetic manipulation methods were used to construct the GMO, including commercial nucleic acid synthesis, isothermal assembly (ligation) of DNA fragments, polymerase chain reaction (PCR, both hypermethylated and non-methylated), bacterial transformation, screening for putative transformants on indicator media and nucleotide sequencing.

20. The methods used -

a. to construct the insert(s) and introduce them into the recipient organism;

Step 1: Construction of plasmid pUC19NHCIS1:

The NHCIS1 locus was PCR amplified from chromosomal DNA isolated from wild type Y92-1009. The primers used were designed to generate overlaps with HincII-digested pUC19 compatible for Isothermal (Gibson) Assembly. Similarly, the terminal ends of HincII-digested pUC19 were extended by PCR amplification using primers designed to generate overlaps with the 5' and 3' ends of the NHCIS1 locus. Isothermal Assembly was used to ligate these fragments and, following transformation into highly competent *E. coli* DH5α and harvest of plasmid from ampicillin resistant colonies, yielded plasmid pUC19NHCIS1.

Step 2: Construction of plasmids pUC19::HAEC4:(Z)-*lacZ* and pUC19::HAEC4:*nadA*-*lacZ*:

The plasmid pUC19NHCIS1 (hereafter, the VECTOR) was PCR amplified using primers designed to simultaneously bifurcate the NHCIS1 sequence and produce Isothermal Assembly-compatible overlaps with the *porA*-associated UAS at one end and the *porB* terminator sequence at the other. An Nlac codon-optimised version of the *nadA* gene, under transcriptional control of the enhanced *porA/porB* hybrid promoter and followed (3') by the *lst* promoter, was synthesised as a gBLOCK (gBLOCK*nadA*, Integrated DNA Technologies). A second gBLOCK was produced whereby the *porA/porB* hybrid promoter was separated from the *lst* promoter by a 14 bp linking sequence, effectively replacing the *nadA* coding sequence with a stretch of non-coding nucleotides (gBLOCK(Z)). Both the wild type Nlac *lacZ* gene and the *porB* transcriptional terminator were PCR amplified from chromosomal DNA, using primers that generated Isothermal Assembly-compatible overlaps with their appropriate partner fragments. These fragments (VECTOR, gBLOCK(*nadA*) OR

gBLOCK(Z), *lacZ* and terminator) were mixed at a ratio of 1:3:3:3 and assembled via Isothermal Assembly. Following transformation of assembled plasmids into highly competent *E. coli* DH5α, transformants were selected for on LB agar supplemented with 100 µg/ml ampicillin and 40 µg/ml X-gal. Ampicillin-resistant, blue colonies were isolated and cultured. Plasmids were harvested from these cultures and where appropriate the *nadA* coding sequence was verified (Source Bioscience). Sequence-verified plasmid was re-designated pUC19::HAEC4:*nadA*-*lacZ*.

Step 3: Hypermethylated PCR amplification of the NHCIS1::HAEC4 cassettes:

The plasmids pUC19NHCIS1::HAEC4:(Z)-*lacZ* and pUC19NHCIS1::HAEC4:*nadA*-*lacZ* were used as templates for hypermethylated PCR amplification of the NHCIS1::HAEC4 gene expression cassettes. Hypermethylated PCR substitutes 5-methyl-deoxycytosine for deoxycytosine in the PCR reaction mix. The PCR requires a low number of cycles to maintain sequence fidelity and an increased extension time per cycle (i.e. 1 minute per kilobase) to allow for the incorporation of the modified nucleotide. The primers used in the reaction annealed to the 5' and 3' termini of the NHCIS1-flanked gene expression cassettes and produced hypermethylated PCR products of the appropriate size. Multiple reactions of the same construct were pooled, supplemented with restriction enzyme-compatible buffer and treated with DpnI to digest the plasmid template. The hypermethylated product was purified by passage through a PCR purification column before the concentration was adjusted to give 0.5 pmol of product per 10 µl of tris-EDTA buffer.

Step 4: Transformation of *ΔlacZ* Y92-1009 *N. lactamica*:

The transformation of *N. lactamica* relies on the natural competence of the bacterium. The *ΔlacZ* mutant derivative of Nlac was grown to OD_{600nm} = 0.3 in Tryptone Soya Broth supplemented with 0.2 % Yeast extract (hereafter, TSB). An aliquot of culture was diluted x100 in fresh TSB and spotted (10 µl) onto Tryptone Soya Broth + 0.2 % Yeast extract agar plates (hereafter, TSA). The spots were allowed to dry in a class 2 Microbiological Safety Cabinet then transferred, right side up, to an incubator for 6 h at 30 ^{o}C, 5 % CO_2. Following this incubation, each spot of nascent bacterial colonies were exposed to 0.5 pmol of the hypermethylated gene expression cassette and returned to the 30 ^{o}C incubator for a further 9-10 h.

b. to delete a sequence.

Step 1: Construction of plasmid pUC19*ΔlacZ*:

Sequences both upstream (1.6 kb) and downstream (1.9 kb) of the endogenous *lacZ* gene from wild type Nlac strain Y92-1009 were amplified from chromosomal DNA, using primers designed to generate Isothermal Assembly-compatible overlaps with

both each other and with HincII-digested pUC19. The overlap between the upstream and downstream DNA fragments was adapted to include a DUS to facilitate bacterial uptake. Similarly, the terminal ends of HincII-digested pUC19 were extended by PCR amplification using primers designed to generate overlaps with the 5' end of the upstream fragment and the 3' end of the downstream fragment. Isothermal Assembly was used to ligate these fragments together and, following transformation into highly competent *E. coli* DH5α and harvest of plasmid from ampicillin resistant colonies, yielded plasmid pUC19*ΔlacZ* .

Step 2: Hypermethylated PCR amplification of the *ΔlacZ* cassette:

See Step 3, Item 20a. The protocol is identical to that described, with the exceptions that the template for hypermethylated PCR was pUC19*ΔlacZ* and that the primers annealed to the 5' and 3' ends of the *ΔlacZ* cassette.

Step 3: Transformation of wild type Y92-1009 *N. lactamica*:

See Step 4, Item 20a. The protocol remains identical to that described, with the exception that transformation was performed into wild type Nlac strain Y92-1009.

21. The description of any insert and/or vector construction.

Full descriptions of the constituent parts of the constructs are detailed in Item 24.

22. The purity of the insert from any unknown sequence and information on the degree to which the inserted sequence is limited to the DNA required to perform the intended function.

The use of a hypermethylated PCR to amplify from the plasmid template means that only known sequences are present in the construct. The primers used did not amplify plasmid-derived sequences. Whole genome sequence analysis has revealed that there are no plasmid-derived sequences present in the chromosome of either GMO.

The gene expression cassette consists of only the coding sequences of the *nadA* and *lacZ* genes, the sequences of the promoters driving gene expression, and two DUS. Collectively, these elements are minimally sufficient to perform the function of the cassette; specifically, the expression of NadA on the bacterial surface and the production of β-D-galactosidase as a means of screening for putatively transformed

N. lactamica. The sequences surrounding the gene expression cassette, homologous to the NHCIS1 locus of wild type Y92-1009, are of sufficient length to accurately target the cassette to the correct chromosomal location, and at a high enough frequency to facilitate a screening strategy for successful transformants.

23. The methods and criteria used for selection

The use of a *ΔlacZ* strain as background for the GMOs enabled exploitation of β-D-galactosidase activity as a screening marker for successfully transformed bacteria.

Step 1: Dilution and plating of putatively transformed *N. lactamica*:

Following incubation with the hypermethylated PCR product, *N. lactamica* were transferred into 1 ml of fresh TSB, then serially diluted x5000 in TSB. One hundred microlitres (100 µl) of the diluted suspension were spread onto multiple TSA plates supplemented with 20 µg/ml X-gal and incubated overnight at 37 ^{o}C, 5 % CO_2 to produce colonies. Blue colonies were isolated and cultured as putative transformants.

Step 2: Verification of *nadA* sequence fidelity:

Chromosomal DNA was extracted from each of the cultures produced from the isolated blue colonies and was used as a template for multiple, high fidelity, low cycle number PCRs of the NHCIS1 locus. The fidelity of the *nadA* coding sequence was determined by sequencing (Source Bioscience).

24. The sequence, functional identity and location of the altered, inserted or deleted nucleic acid segments in question and, in particular, any known harmful sequence.

All sequences in the constructs are known. There are two transformative constructs: NHCIS1::HAEC4:(Z)-*lacZ* and NHCIS1::HAEC4:*nadA*-*lacZ*. There are two genes present in the NHCIS1::HAEC4:*nadA*-*lacZ* cassette: *nadA*, coding for the *Neisseria* Adhesin A (NadA) protein, a type V autotransporter from Nmen strain MC58, and *lacZ*, coding for β-galactosidase. Only the *lacZ* gene is present in the NHCIS1::HAEC4:(Z)-*lacZ* cassette. A schematic of the features included in the *nadA* construct are shown in Figure 4.

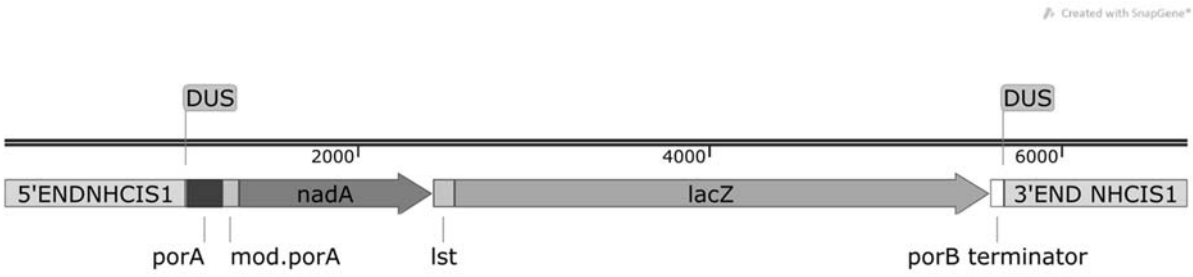


Figure 4: To-scale schematic of the construct used to generate GM-Nlac strain 4NB1 detailing all genes and non-coding features.

The Construct in its entirety (6, 703 bp) consists of:

1. Sequence homologous to the NHCIS1 locus of *N. lactamica* strain Y92-1009 (5'ENDNHCIS1 and 3'ENDNHCIS1). This locus is defined as the 'intergenic' region between NLY_27080 and NLY_27100. In the construct, the locus has been bifurcated and flanks the gene expression cassette.

2. Two *Neisseria* DNA Uptake Sequences (DUS: 5' – GCCGTCTGAA – 3'), included to increase the frequency with which the construct is bound by the Type IV pilus of Nlac and internalised by the bacteria as part of natural competence.

3. A 200 bp upstream activator sequence (UAS) found immediately upstream of the *porA* gene in wild type *N. meningitidis* strains (labelled as 'porA'). This length of sequence has been shown to optimally enhance the activity of promoters when conjugated directly upstream of the -35 RNA Polymerase binding site (see Figure 1, Item 15).

4. A synthetic, hybrid promoter (labelled as 'mod.porA') consisting primarily of sequence derived from the *porA* gene of wild type *N. meningitidis*, but wherein the homopolymeric guanosine nucleotide tract, a feature responsible for the regulation of expression of this gene by phase variation, has been replaced by sequence derived from the non-phase variable *porB* gene of *N. lactamica*.

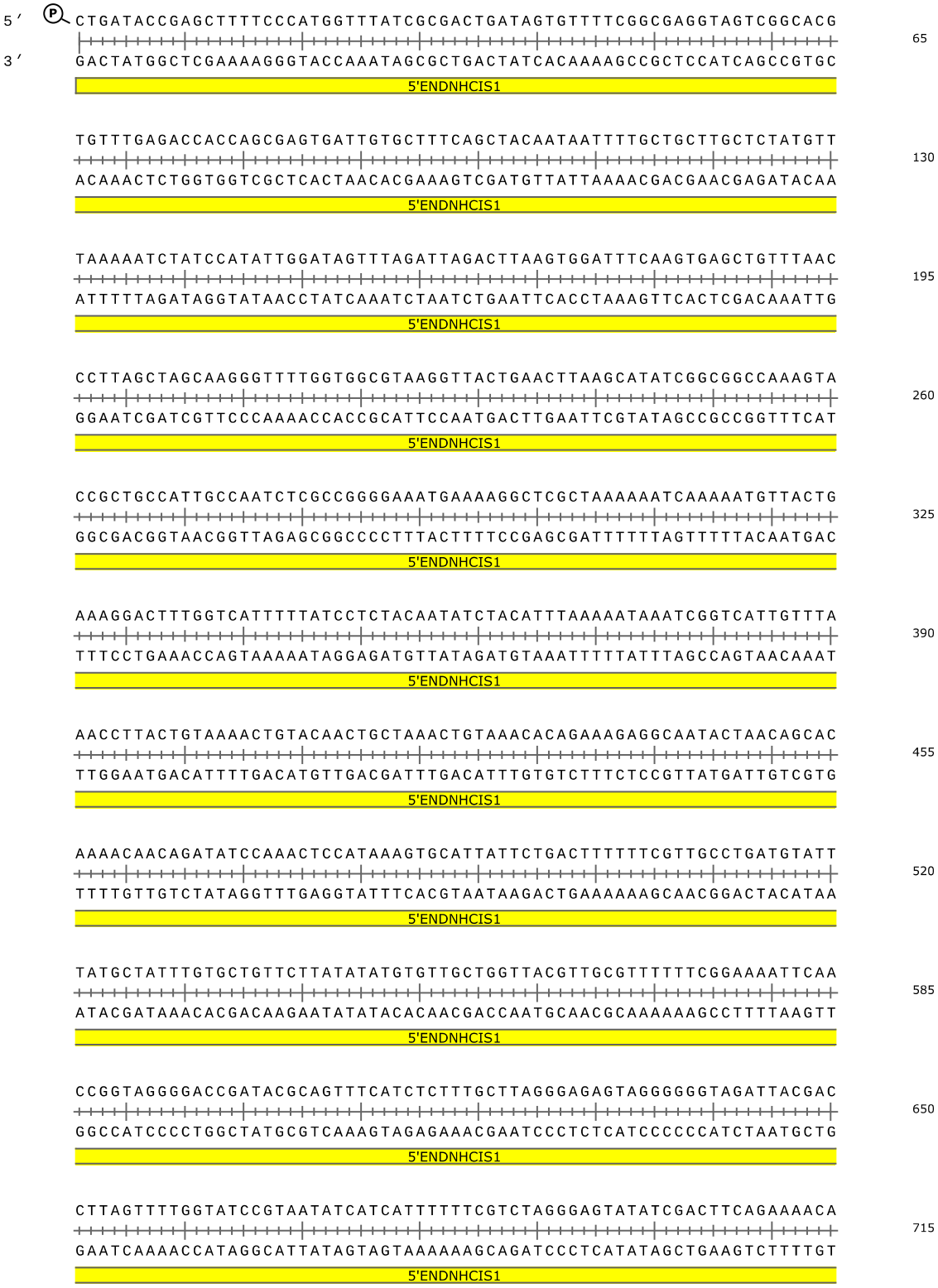
5. A synthetic version of the *nadA* gene (NMB_1994), codon-optimised for expression in *N. lactamica* (CAI: 0.702) but still coding for the same amino acid sequence as the wild type gene present in *N. meningitidis* strain MC58. NB: In the NHCIS1::HAEC4:(Z)-*lacZ* Cassette, this gene is replaced with a 14 bp, non-coding linking sequence (5' – ATCTATTATATAAC – 3').

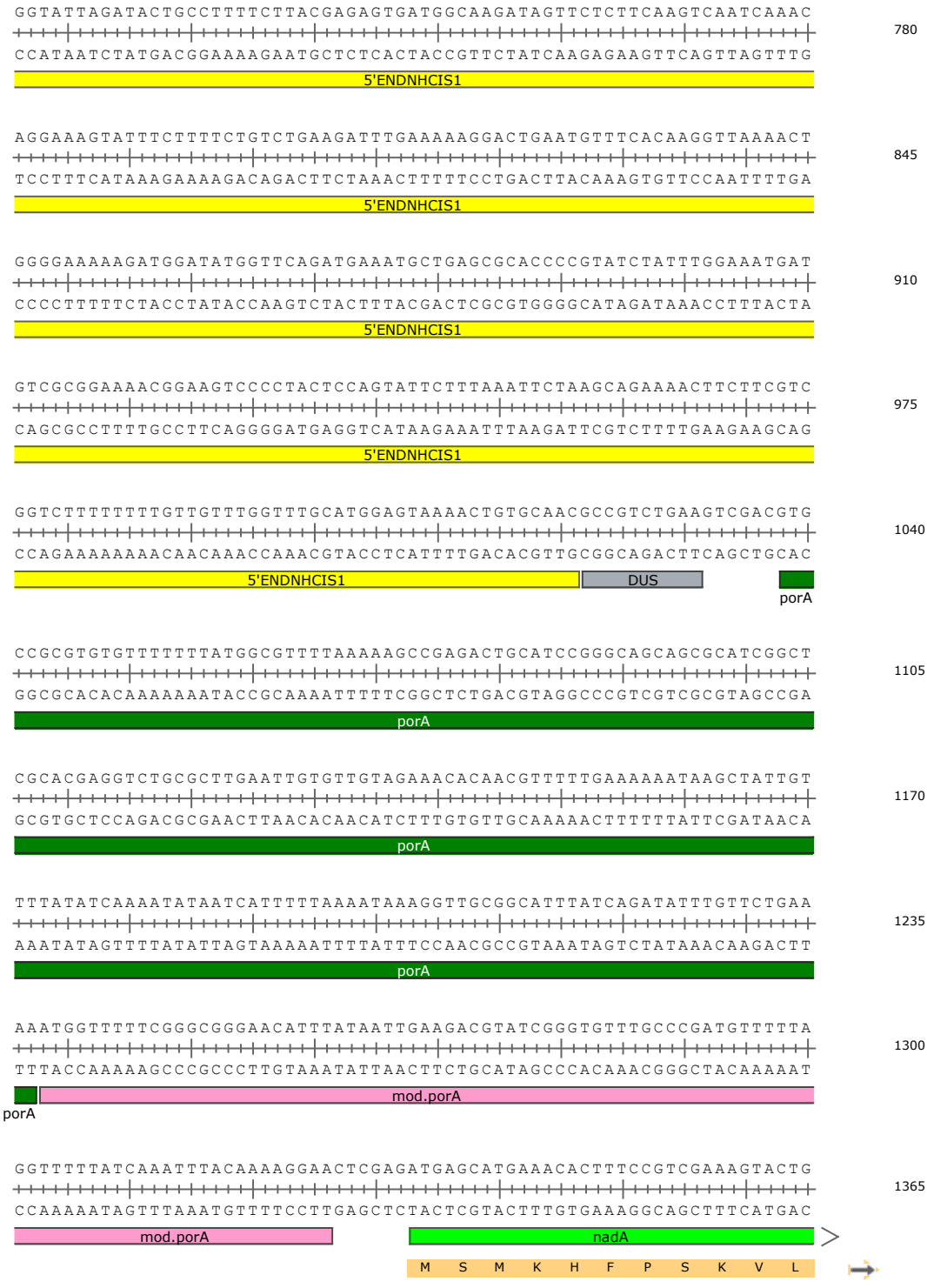
6. The promoter for the *N. lactamica* gene: α-2, 3 sialyltransferase (*lst*)

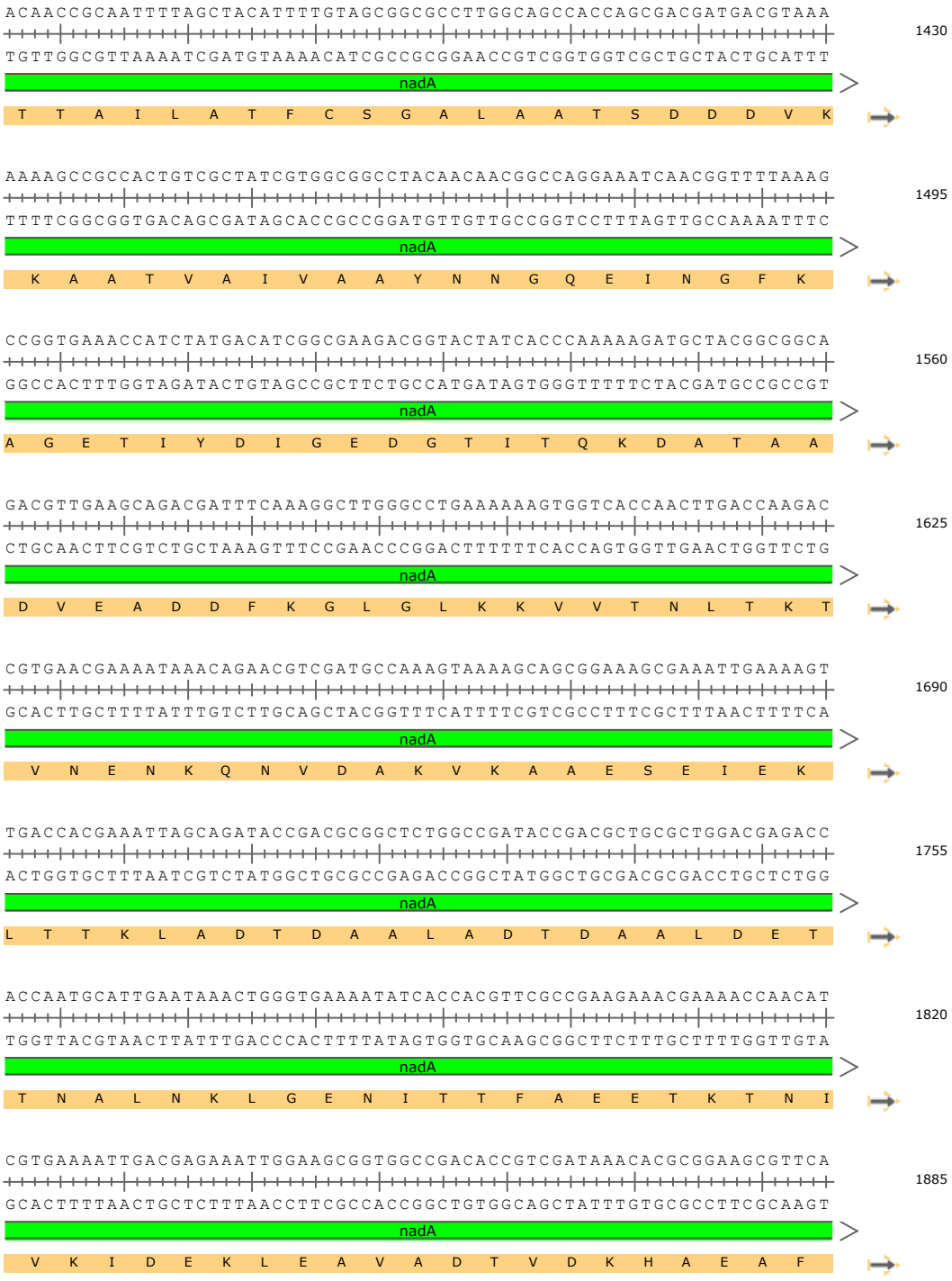
7. A copy of the endogenous *lacZ* gene from wild type *N. lactamica* (*lacZ*), coding for the cytoplasmic enzyme β-galactosidase.
8. A transcriptional terminator (labelled as ‘porB terminator’) derived from the sequence immediately downstream of the *porB* gene from wild type *N. meningitidis* strain MC58.

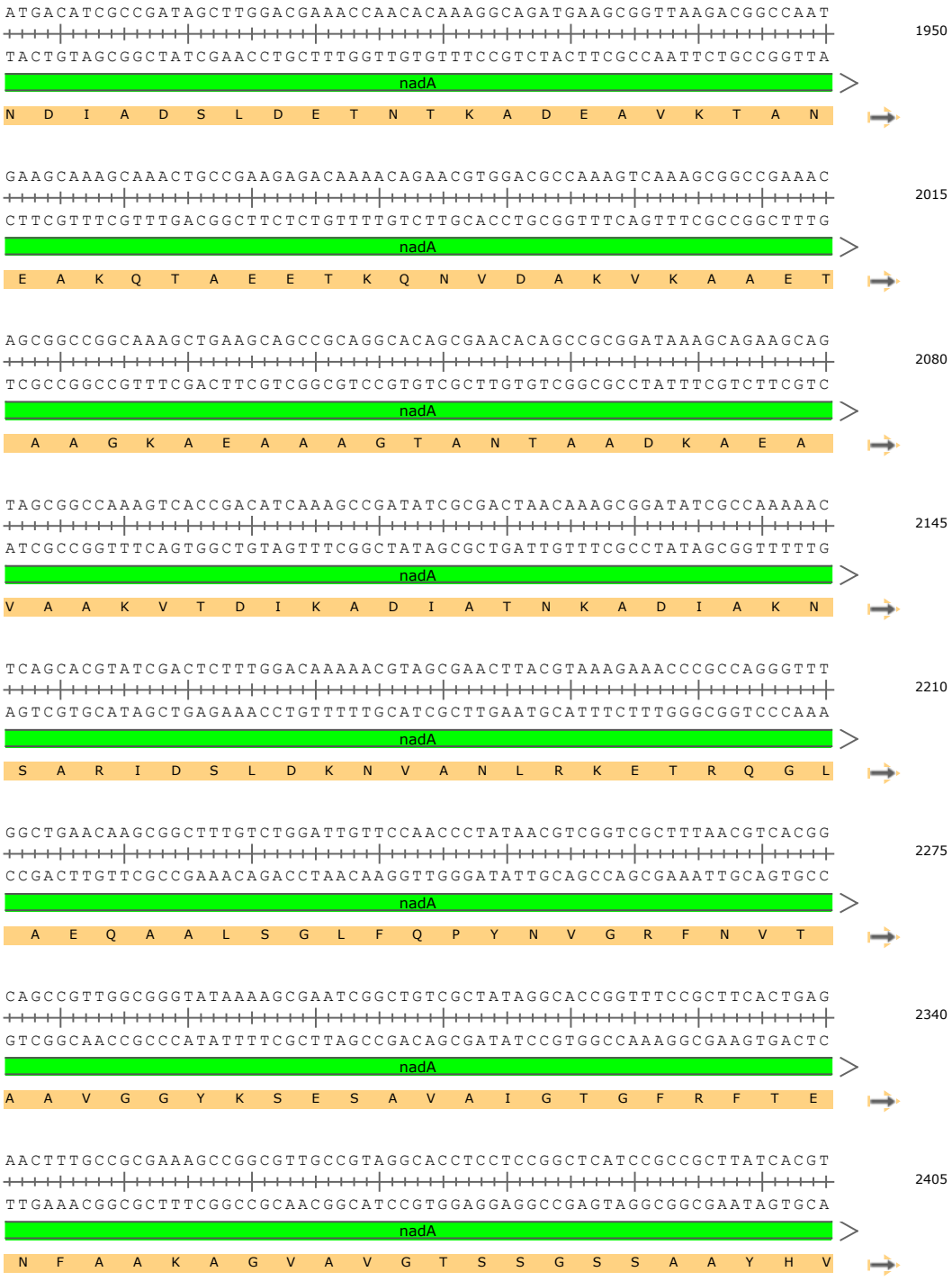
The nucleotide sequence, relevant features and amino acid sequences of the gene products are shown in Figure 5 below:

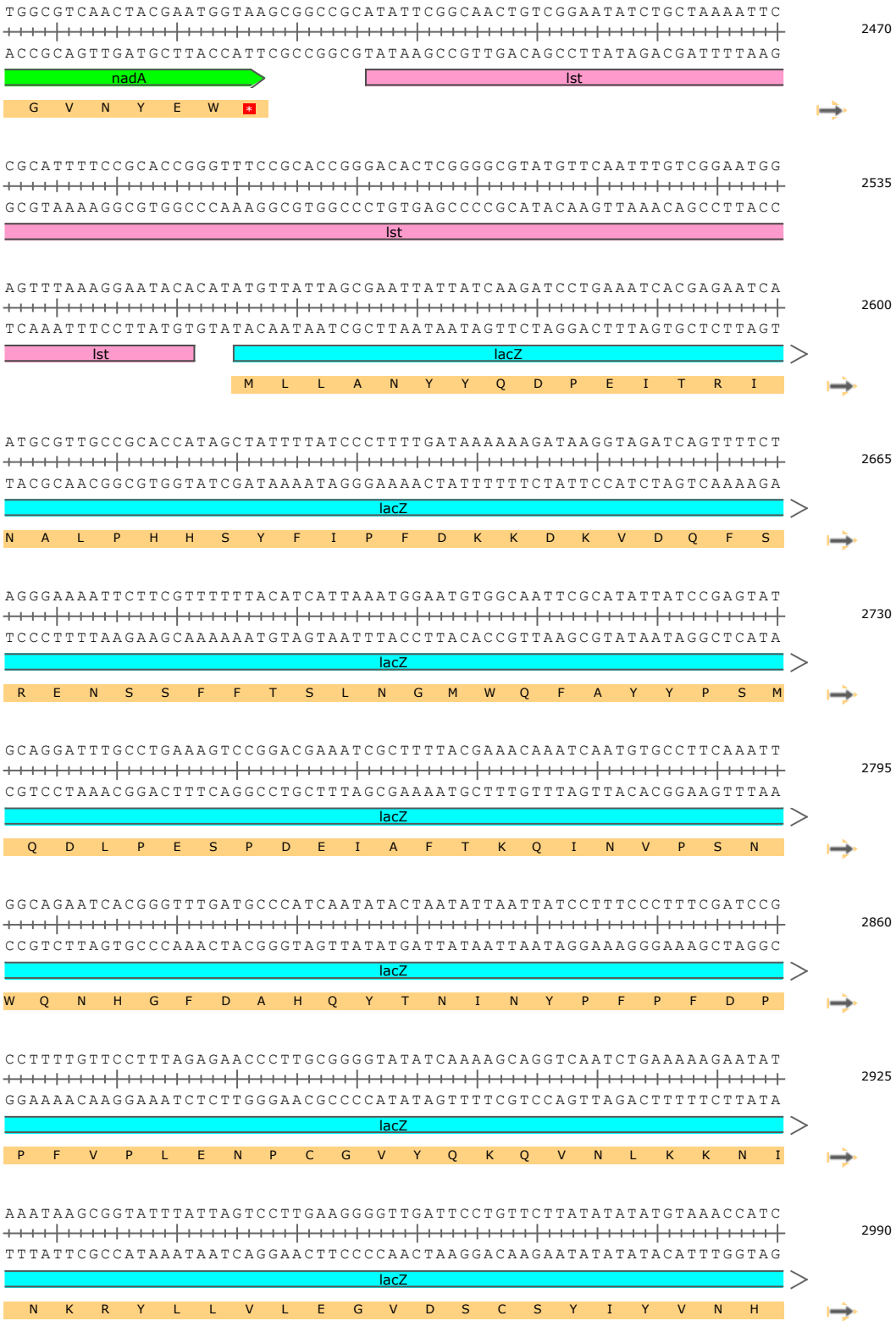
Sequence: NHCIS1-HAEC4-nadA-lacZ.dna (Linear / 6703 bp)
Features: 10 visible, 10 total

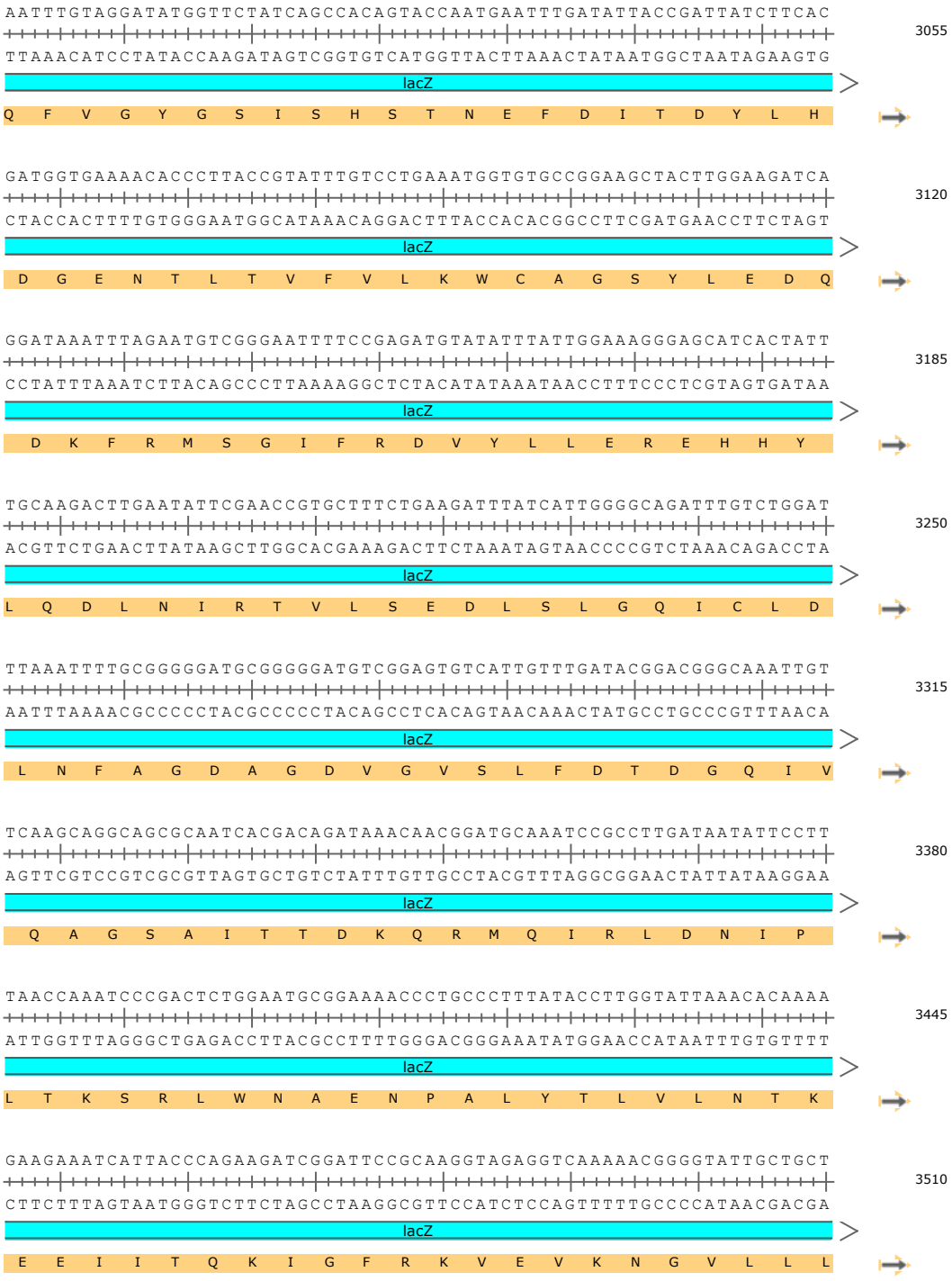

5′
CTGATACCGAGCTTTTCCCATGGTTTATCGCGACTGATAGTGTTTTCGGCGAGGTAGTCGGCACG
65
3′
GACTATGGCTCGAAAAGGGTACCAAATAGCGCTGACTATCACAAAAGCCGCTCCATCAGCCGTGC
5'ENDNHCIS1
TGTTTGAGACCACCAGCGAGTGATTGTGCTTTCAGCTACAATAATTTTGCTGCTTGCTCTATGTT
130
ACAAACTCTGGTGGTCGCTCACTAACACGAAAGTCGATGTTATTAAAACGACGAACGAGATACAA
5'ENDNHCIS1
TAAAAATCTATCCATATTGGATAGTTTAGATTAGACTTAAGTGGATTTCAAGTGAGCTGTTTAAC
195
ATTTTTAGATAGGTATAACCTATCAAATCTAATCTGAATTCACCTAAAGTTCACTCGACAAATTG
5'ENDNHCIS1
CCTTAGCTAGCAAGGGTTTTGGTGGCGTAAGGTTACTGAACTTAAGCATATCGGCGGCCAAAGTA
260
GGAATCGATCGTTCCCAAAACCACCGCATTCCAATGACTTGAATTCGTATAGCCGCCGGTTTCAT
5'ENDNHCIS1
CCGCTGCCATTGCCAATCTCGCCGGGGAAATGAAAAGGCTCGCTAAAAAATCAAAAATGTTACTG
325
GGCGACGGTAACGGTTAGAGCGGCCCCTTTACTTTTCCGAGCGATTTTTTAGTTTTTACAATGAC
5'ENDNHCIS1
AAAGGACTTTGGTCATTTTTATCCTCTACAATATCTACATTTAAAAATAAATCGGTCATTGTTTA
390
TTTCCTGAAACCAGTAAAAATAGGAGATGTTATAGATGTAAATTTTTATTTAGCCAGTAACAAAT
5'ENDNHCIS1
AACCTTACTGTAAAACTGTACAACTGCTAAACTGTAAACACAGAAAGAGGCAATACTAACAGCAC
455
TTGGAATGACATTTTGACATGTTGACGATTTGACATTTGTGTCTTTCTCCGTTATGATTGTCGTG
5'ENDNHCIS1
AAAACAACAGATATCCAAACTCCATAAAGTGCATTATTCTGACTTTTTTCGTTGCCTGATGTATT
520
TTTTGTTGTCTATAGGTTTGAGGTATTTCACGTAATAAGACTGAAAAAAGCAACGGACTACATAA
5'ENDNHCIS1
TATGCTATTTGTGCTGTTCTTATATATGTGTTGCTGGTTACGTTGCGTTTTTTCGGAAAATTCAA
585
ATACGATAAACACGACAAGAATATATACACAACGACCAATGCAACGCAAAAAAGCCTTTTAAGTT
5'ENDNHCIS1
CCGGTAGGGGACCGATACGCAGTTTCATCTCTTTGCTTAGGGAGAGTAGGGGGGTAGATTACGAC
650
GGCCATCCCCTGGCTATGCGTCAAAGTAGAGAAACGAATCCCTCTCATCCCCCCATCTAATGCTG
5'ENDNHCIS1
CTTAGTTTTGGTATCCGTAATATCATCATTTTTTCGTCTAGGGAGTATATCGACTTCAGAAAACA
715
GAATCAAAACCATAGGCATTATAGTAGTAAAAAAGCAGATCCCTCATATAGCTGAAGTCTTTTGT
5'ENDNHCIS1

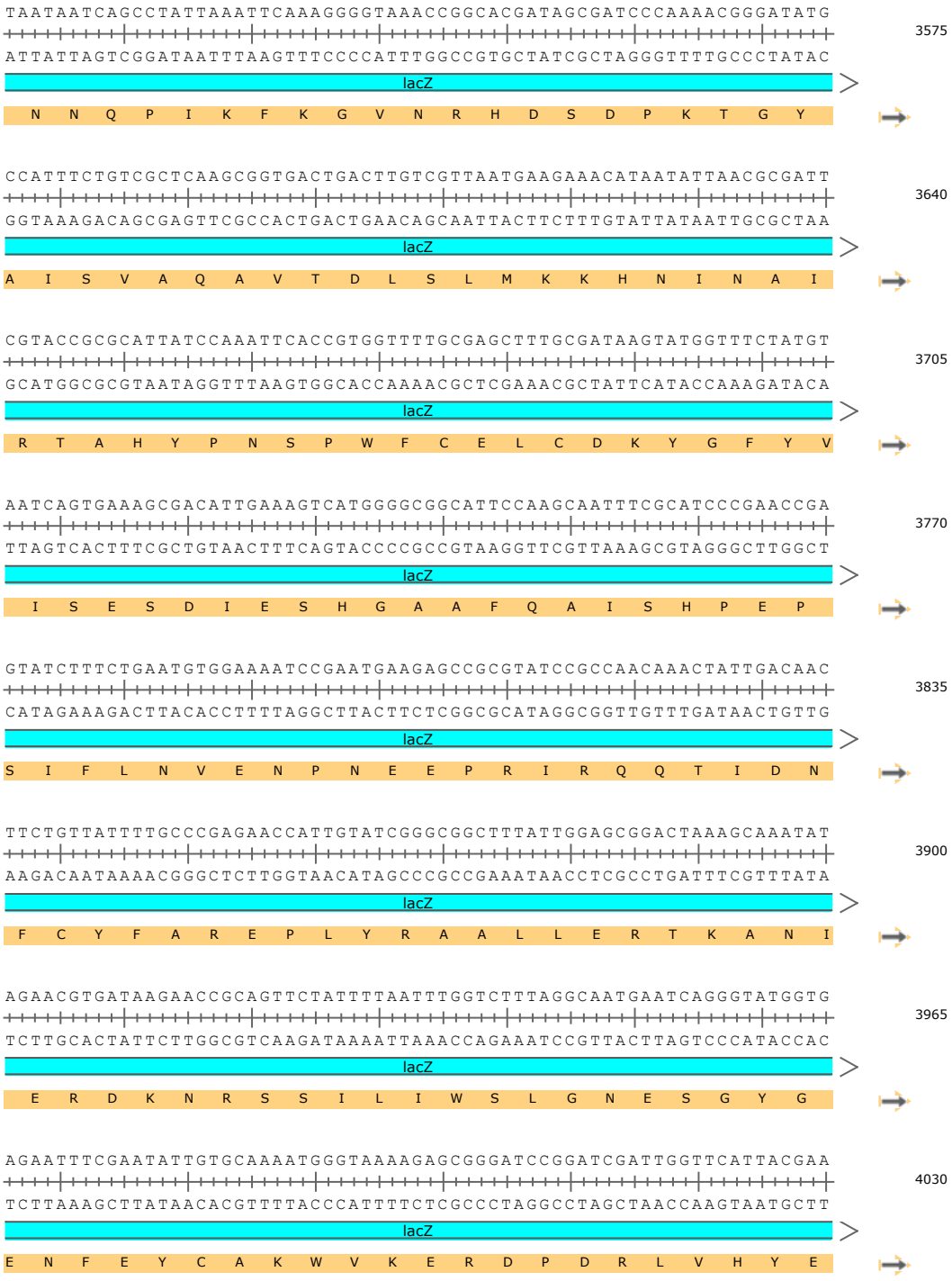

GGTATTAGATACTGCCTTTTCTTACGAGAGTGATGGCAAGATAGTTCTCTTCAAGTCAATCAAAC
CCATAATCTATGACGGAAAAGAATGCTCTCACTACCGTTCTATCAAGAGAAGTTCAGTTAGTTTG
780
5'ENDNHCIS1
AGGAAAGTATTTCTTTTCTGTCTGAAGATTTGAAAAAGGACTGAATGTTTCACAAGGTTAAAACT
TCCTTTCATAAAGAAAAGACAGACTTCTAAACTTTTTCCTGACTTACAAAGTGTTCCAATTTTGA
845
5'ENDNHCIS1
GGGGAAAAAGATGGATATGGTTCAGATGAAATGCTGAGCGCACCCCGTATCTATTTGGAAATGAT
CCCCTTTTTCTACCTATACCAAGTCTACTTTACGACTCGCGTGGGGCATAGATAAACCTTTACTA
910
5'ENDNHCIS1
GTCGCGGAAAACGGAAGTCCCCTACTCCAGTATTCTTTAAATTCTAAGCAGAAAACTTCTTCGTC
CAGCGCCTTTTGCCTTCAGGGGATGAGGTCATAAGAAATTTAAGATTCGTCTTTTGAAGAAGCAG
975
5'ENDNHCIS1
GGTCTTTTTTTTGTTGTTTGGTTTGCATGGAGTAAAACTGTGCAACGCCGTCTGAAGTCGACGTG
CCAGAAAAAAAACAACAAACCAAACGTACCTCATTTTGACACGTTGCGGCAGACTTCAGCTGCAC
1040
5'ENDNHCIS1
DUS
porA
CCGCGTGTGTTTTTTTATGGCGTTTTAAAAAGCCGAGACTGCATCCGGGCAGCAGCGCATCGGCT
GGCGCACACAAAAAAATACCGCAAAATTTTTCGGCTCTGACGTAGGCCCGTCGTCGCGTAGCCGA
1105
porA
CGCACGAGGTCTGCGCTTGAATTGTGTTGTAGAAACACAACGTTTTTGAAAAAATAAGCTATTGT
GCGTGCTCCAGACGCGAACTTAACACAACATCTTTGTGTTGCAAAAACTTTTTTATTCGATAACA
1170
porA
TTTATATCAAAATATAATCATTTTTAAAATAAAGGTTGCGGCATTTATCAGATATTTGTTCTGAA
AAATATAGTTTTATATTAGTAAAAATTTTATTTCCAACGCCGTAAATAGTCTATAAACAAGACTT
1235
porA
AAATGGTTTTTCGGGCGGGAACATTTATAATTGAAGACGTATCGGGTGTTTGCCCGATGTTTTTA
TTTACCAAAAAGCCCGCCCTTGTAAATATTAACTTCTGCATAGCCCACAAACGGGCTACAAAAAT
1300
porA
mod.porA
GGTTTTTATCAAATTTACAAAAGGAACTCGAGATGAGCATGAAACACTTTCCGTCGAAAGTACTG
CCAAAAATAGTTTAAATGTTTTCCTTGAGCTCTACTCGTACTTTGTGAAAGGCAGCTTTCATGAC
1365
mod.porA
nadA
M S M K H F P S K V L

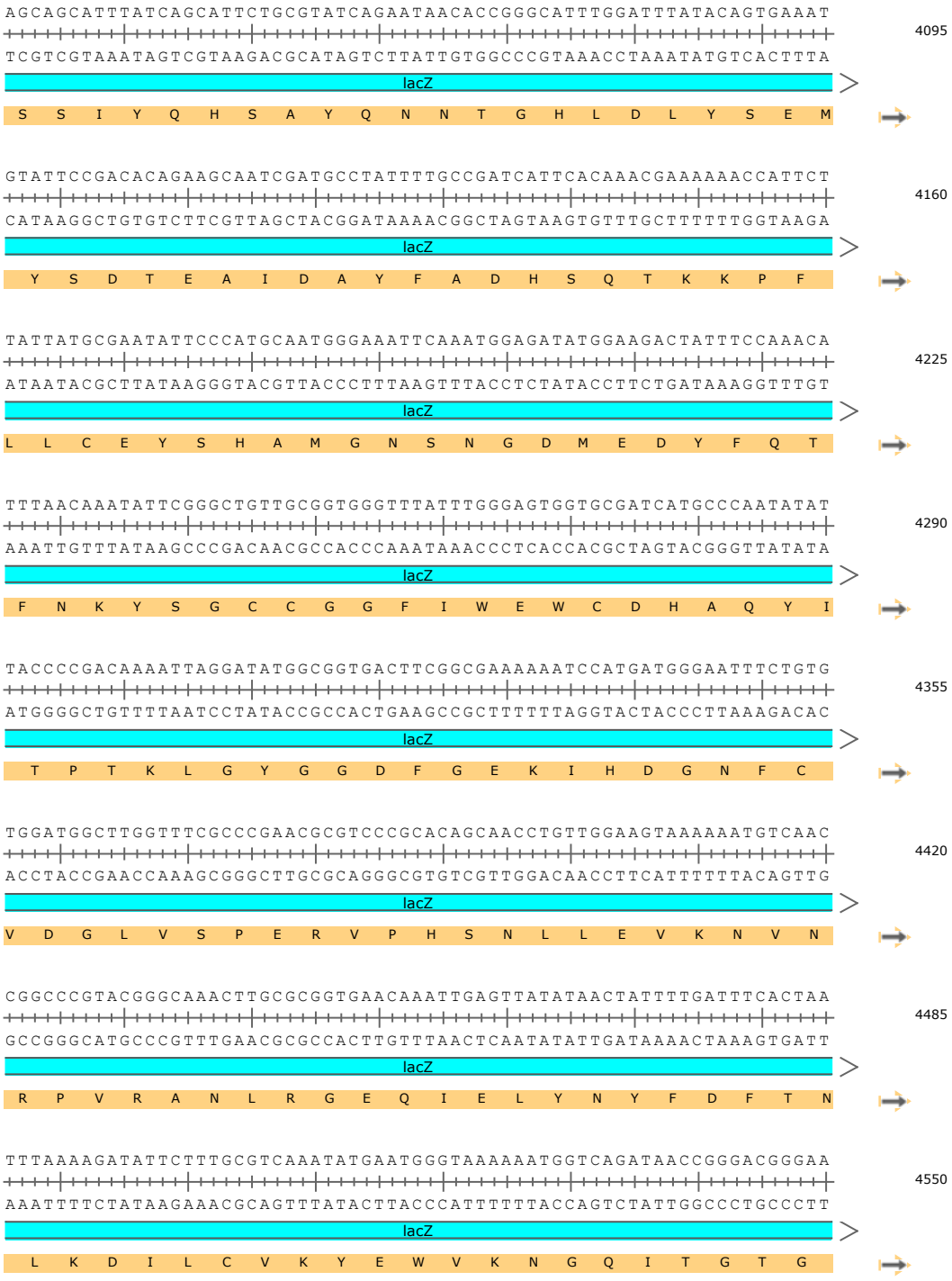

ACAACCGCAATTTTAGCTACATTTTGTAGCGGCGCCTTGGCAGCCACCAGCGACGATGACGTAAA
1430
TGTTGGCGTTAAAATCGATGTAAAACATCGCCGCGGAACCGTCGGTGGTCGCTGCTACTGCATTT
nadA
T T A I L A T F C S G A L A A T S D D D V K
AAAAGCCGCCACTGTCGCTATCGTGGCGGCCTACAACAACGGCCAGGAAATCAACGGTTTTAAAG
1495
TTTTCGGCGGTGACAGCGATAGCACCGCCGGATGTTGTTGCCGGTCCTTTAGTTGCCAAAATTTC
nadA
K A A T V A I V A A Y N N G Q E I N G F K
CCGGTGAAACCATCTATGACATCGGCGAAGACGGTACTATCACCCAAAAAGATGCTACGGCGGCA
1560
GGCCACTTTGGTAGATACTGTAGCCGCTTCTGCCATGATAGTGGGTTTTTCTACGATGCCGCCGT
nadA
A G E T I Y D I G E D G T I T Q K D A T A A
GACGTTGAAGCAGACGATTTCAAAGGCTTGGGCCTGAAAAAAGTGGTCACCAACTTGACCAAGAC
1625
CTGCAACTTCGTCTGCTAAAGTTTCCGAACCCGGACTTTTTTCACCAGTGGTTGAACTGGTTCTG
nadA
D V E A D D F K G L G L K K V V T N L T K T
CGTGAACGAAAATAAACAGAACGTCGATGCCAAAGTAAAAGCAGCGGAAAGCGAAATTGAAAAGT
1690
GCACTTGCTTTTATTTGTCTTGCAGCTACGGTTTCATTTTCGTCGCCTTTCGCTTTAACTTTTCA
nadA
V N E N K Q N V D A K V K A A E S E I E K
TGACCACGAAATTAGCAGATACCGACGCGGCTCTGGCCGATACCGACGCTGCGCTGGACGAGACC
1755
ACTGGTGCTTTAATCGTCTATGGCTGCGCCGAGACCGGCTATGGCTGCGACGCGACCTGCTCTGG
nadA
L T T K L A D T D A A L A D T D A A L D E T
ACCAATGCATTGAATAAACTGGGTGAAAATATCACCACGTTCGCCGAAGAAACGAAAACCAACAT
1820
TGGTTACGTAACTTATTTGACCCACTTTTATAGTGGTGCAAGCGGCTTCTTTGCTTTTGGTTGTA
nadA
T N A L N K L G E N I T T F A E E T K T N I
CGTGAAAATTGACGAGAAATTGGAAGCGGTGGCCGACACCGTCGATAAACACGCGGAAGCGTTCA
1885
GCACTTTTAACTGCTCTTTAACCTTCGCCACCGGCTGTGGCAGCTATTTGTGCGCCTTCGCAAGT
nadA
V K I D E K L E A V A D T V D K H A E A F

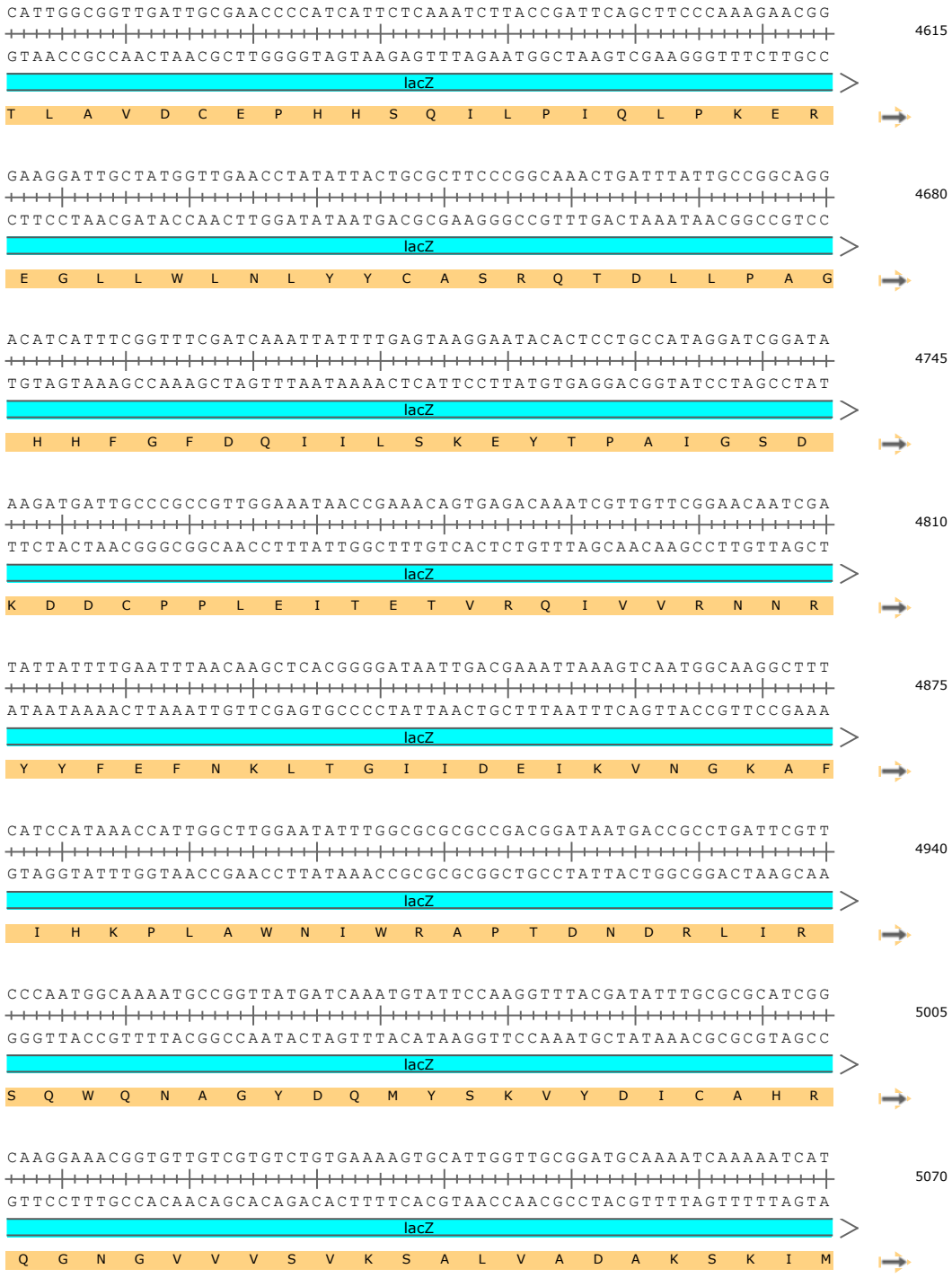

ATGACATCGCCGATAGCTTGGACGAAACCAACACAAAGGCAGATGAAGCGGTTAAGACGGCCAAT
1950
TACTGTAGCGGCTATCGAACCTGCTTTGGTTGTGTTTCCGTCTACTTCGCCAATTCTGCCGGTTA
nadA
N D I A D S L D E T N T K A D E A V K T A N
GAAGCAAAGCAAACTGCCGAAGAGACAAAACAGAACGTGGACGCCAAAGTCAAAGCGGCCGAAAC
2015
CTTCGTTTCGTTTGACGGCTTCTCTGTTTTGTCTTGCACCTGCGGTTTCAGTTTCGCCGGCTTTG
nadA
E A K Q T A E E T K Q N V D A K V K A A E T
AGCGGCCGGCAAAGCTGAAGCAGCCGCAGGCACAGCGAACACAGCCGCGGATAAAGCAGAAGCAG
2080
TCGCCGGCCGTTTCGACTTCGTCGGCGTCCGTGTCGCTTGTGTCGGCGCCTATTTCGTCTTCGTC
nadA
A A G K A E A A A G T A N T A A D K A E A
TAGCGGCCAAAGTCACCGACATCAAAGCCGATATCGCGACTAACAAAGCGGATATCGCCAAAAAC
2145
ATCGCCGGTTTCAGTGGCTGTAGTTTCGGCTATAGCGCTGATTGTTTCGCCTATAGCGGTTTTTG
nadA
V A A K V T D I K A D I A T N K A D I A K N
TCAGCACGTATCGACTCTTTGGACAAAAACGTAGCGAACTTACGTAAAGAAACCCGCCAGGGTTT
2210
AGTCGTGCATAGCTGAGAAACCTGTTTTTGCATCGCTTGAATGCATTTCTTTGGGCGGTCCCAAA
nadA
S A R I D S L D K N V A N L R K E T R Q G L
GGCTGAACAAGCGGCTTTGTCTGGATTGTTCCAACCCTATAACGTCGGTCGCTTTAACGTCACGG
2275
CCGACTTGTTCGCCGAAACAGACCTAACAAGGTTGGGATATTGCAGCCAGCGAAATTGCAGTGCC
nadA
A E Q A A L S G L F Q P Y N V G R F N V T
CAGCCGTTGGCGGGTATAAAAGCGAATCGGCTGTCGCTATAGGCACCGGTTTCCGCTTCACTGAG
2340
GTCGGCAACCGCCCATATTTTCGCTTAGCCGACAGCGATATCCGTGGCCAAAGGCGAAGTGACTC
nadA
A A V G G Y K S E S A V A I G T G F R F T E
AACTTTGCCGCGAAAGCCGGCGTTGCCGTAGGCACCTCCTCCGGCTCATCCGCCGCTTATCACGT
2405
TTGAAACGGCGCTTTCGGCCGCAACGGCATCCGTGGAGGAGGCCGAGTAGGCGGCGAATAGTGCA
nadA
N F A A K A G V A V G T S S G S S A A Y H V

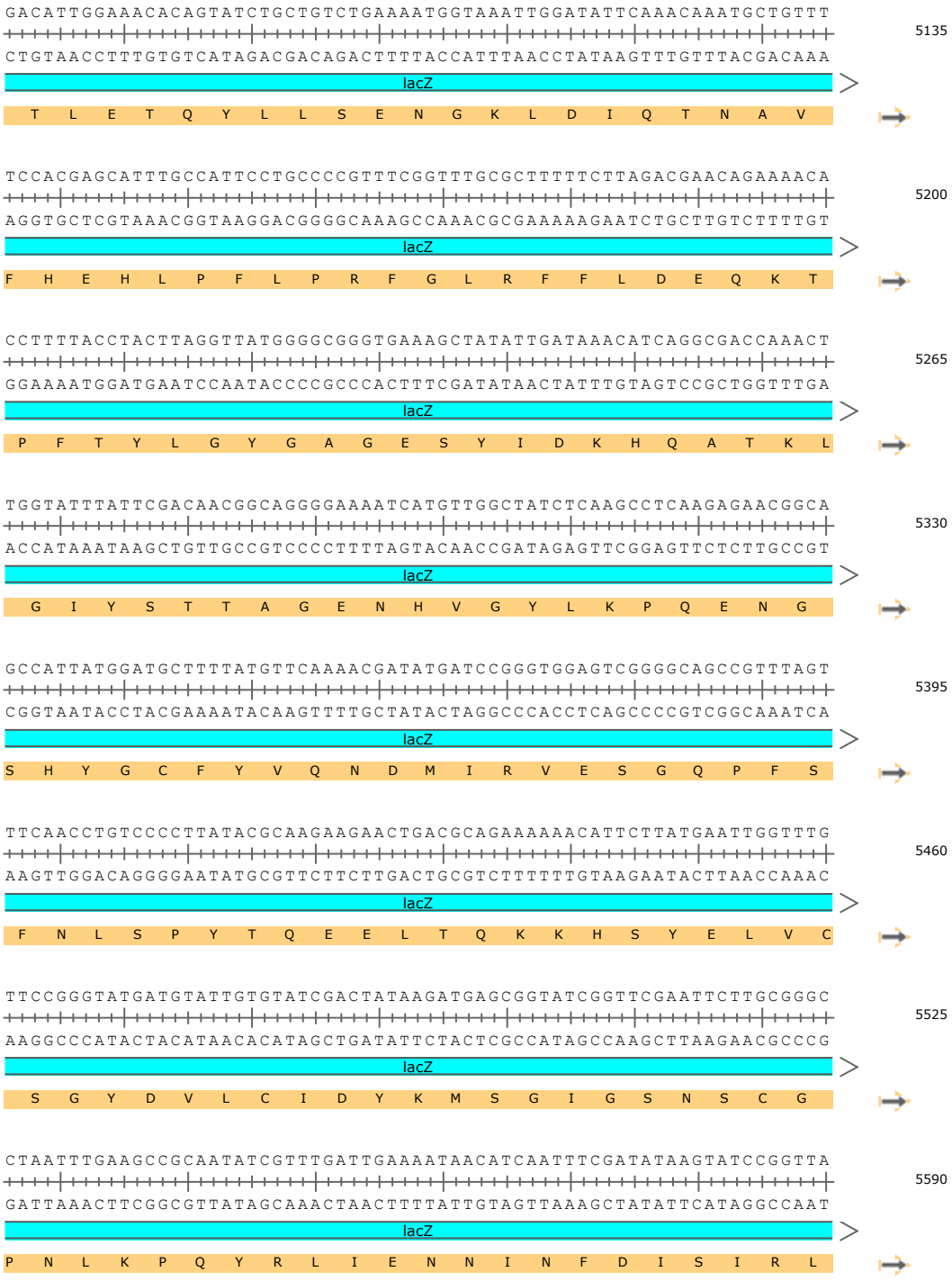

TGGCGTCAACTACGAATGGTAAGCGGCCGCATATTCGGCAACTGTCGGAATATCTGCTAAAATTC
2470
ACCGCAGTTGATGCTTACCATTCGCCGGCGTATAAGCCGTTGACAGCCTTATAGACGATTTTAAG
nadA
lst
G V N Y E W *
CGCATTTTCCGCACCGGGTTTCCGCACCGGGACACTCGGGGCGTATGTTCAATTTGTCGGAATGG
2535
GCGTAAAAGGCGTGGCCCAAAGGCGTGGCCCTGTGAGCCCCGCATACAAGTTAAACAGCCTTACC
lst
AGTTTAAAGGAATACACATATGTTATTAGCGAATTATTATCAAGATCCTGAAATCACGAGAATCA
2600
TCAAATTTCCTTATGTGTATACAATAATCGCTTAATAATAGTTCTAGGACTTTAGTGCTCTTAGT
lst
lacZ
M L L A N Y Y Q D P E I T R I
ATGCGTTGCCGCACCATAGCTATTTTATCCCTTTTGATAAAAAAGATAAGGTAGATCAGTTTTCT
2665
TACGCAACGGCGTGGTATCGATAAAATAGGGAAAACTATTTTTTCTATTCCATCTAGTCAAAAGA
lacZ
N A L P H H S Y F I P F D K K D K V D Q F S
AGGGAAAATTCTTCGTTTTTTACATCATTAAATGGAATGTGGCAATTCGCATATTATCCGAGTAT
2730
TCCCTTTTAAGAAGCAAAAAATGTAGTAATTTACCTTACACCGTTAAGCGTATAATAGGCTCATA
lacZ
R E N S S F F T S L N G M W Q F A Y Y P S M
GCAGGATTTGCCTGAAAGTCCGGACGAAATCGCTTTTACGAAACAAATCAATGTGCCTTCAAATT
2795
CGTCCTAAACGGACTTTCAGGCCTGCTTTAGCGAAAATGCTTTGTTTAGTTACACGGAAGTTTAA
lacZ
Q D L P E S P D E I A F T K Q I N V P S N
GGCAGAATCACGGGTTTGATGCCCATCAATATACTAATATTAATTATCCTTTCCCTTTCGATCCG
2860
CCGTCTTAGTGCCCAAACTACGGGTAGTTATATGATTATAATTAATAGGAAAGGGAAAGCTAGGC
lacZ
W Q N H G F D A H Q Y T N I N Y P F P F D P
CCTTTTGTTCCTTTAGAGAACCCTTGCGGGGTATATCAAAAGCAGGTCAATCTGAAAAAGAATAT
2925
GGAAAACAAGGAAATCTCTTGGGAACGCCCCATATAGTTTTCGTCCAGTTAGACTTTTTCTTATA
lacZ
P F V P L E N P C G V Y Q K Q V N L K K N I
AAATAAGCGGTATTTATTAGTCCTTGAAGGGGTTGATTCCTGTTCTTATATATATGTAAACCATC
2990
TTTATTCGCCATAAATAATCAGGAACTTCCCCAACTAAGGACAAGAATATATATACATTTGGTAG
lacZ
N K R Y L L V L E G V D S C S Y I Y V N H


AATTTGTAGGATATGGTTCTATCAGCCACAGTACCAATGAATTTGATATTACCGATTATCTTCAC
TTAAACATCCTATACCAAGATAGTCGGTGTCATGGTTACTTAAACTATAATGGCTAATAGAAGTG
3055
lacZ
Q F V G Y G S I S H S T N E F D I T D Y L H
GATGGTGAAAACACCCTTACCGTATTTGTCCTGAAATGGTGTGCCGGAAGCTACTTGGAAGATCA
CTACCACTTTTGTGGGAATGGCATAAACAGGACTTTACCACACGGCCTTCGATGAACCTTCTAGT
3120
lacZ
D G E N T L T V F V L K W C A G S Y L E D Q
GGATAAATTTAGAATGTCGGGAATTTTCCGAGATGTATATTTATTGGAAAGGGAGCATCACTATT
CCTATTTAAATCTTACAGCCCTTAAAAGGCTCTACATATAAATAACCTTTCCCTCGTAGTGATAA
3185
lacZ
D K F R M S G I F R D V Y L L E R E H H Y
TGCAAGACTTGAATATTCGAACCGTGCTTTCTGAAGATTTATCATTGGGGCAGATTTGTCTGGAT
ACGTTCTGAACTTATAAGCTTGGCACGAAAGACTTCTAAATAGTAACCCCGTCTAAACAGACCTA
3250
lacZ
L Q D L N I R T V L S E D L S L G Q I C L D
TTAAATTTTGCGGGGGATGCGGGGGATGTCGGAGTGTCATTGTTTGATACGGACGGGCAAATTGT
AATTTAAAACGCCCCCTACGCCCCCTACAGCCTCACAGTAACAAACTATGCCTGCCCGTTTAACA
3315
lacZ
L N F A G D A G D V G V S L F D T D G Q I V
TCAAGCAGGCAGCGCAATCACGACAGATAAACAACGGATGCAAATCCGCCTTGATAATATTCCTT
AGTTCGTCCGTCGCGTTAGTGCTGTCTATTTGTTGCCTACGTTTAGGCGGAACTATTATAAGGAA
3380
lacZ
Q A G S A I T T D K Q R M Q I R L D N I P
TAACCAAATCCCGACTCTGGAATGCGGAAAACCCTGCCCTTTATACCTTGGTATTAAACACAAAA
ATTGGTTTAGGGCTGAGACCTTACGCCTTTTGGGACGGGAAATATGGAACCATAATTTGTGTTTT
3445
lacZ
L T K S R L W N A E N P A L Y T L V L N T K
GAAGAAATCATTACCCAGAAGATCGGATTCCGCAAGGTAGAGGTCAAAAACGGGGTATTGCTGCT
CTTCTTTAGTAATGGGTCTTCTAGCCTAAGGCGTTCCATCTCCAGTTTTTGCCCCATAACGACGA
3510
lacZ
E E I I T Q K I G F R K V E V K N G V L L L


TAATAATCAGCCTATTAAATTCAAAGGGGTAAACCGGCACGATAGCGATCCCAAAACGGGATATG
3575
ATTATTAGTCGGATAATTTAAGTTTCCCCATTTGGCCGTGCTATCGCTAGGGTTTTGCCCTATAC
lacZ
N N Q P I K F K G V N R H D S D P K T G Y
CCATTTCTGTCGCTCAAGCGGTGACTGACTTGTCGTTAATGAAGAAACATAATATTAACGCGATT
3640
GGTAAAGACAGCGAGTTCGCCACTGACTGAACAGCAATTACTTCTTTGTATTATAATTGCGCTAA
lacZ
A I S V A Q A V T D L S L M K K H N I N A I
CGTACCGCGCATTATCCAAATTCACCGTGGTTTTGCGAGCTTTGCGATAAGTATGGTTTCTATGT
3705
GCATGGCGCGTAATAGGTTTAAGTGGCACCAAAACGCTCGAAACGCTATTCATACCAAAGATACA
lacZ
R T A H Y P N S P W F C E L C D K Y G F Y V
AATCAGTGAAAGCGACATTGAAAGTCATGGGGCGGCATTCCAAGCAATTTCGCATCCCGAACCGA
3770
TTAGTCACTTTCGCTGTAACTTTCAGTACCCCGCCGTAAGGTTCGTTAAAGCGTAGGGCTTGGCT
lacZ
I S E S D I E S H G A A F Q A I S H P E P
GTATCTTTCTGAATGTGGAAAATCCGAATGAAGAGCCGCGTATCCGCCAACAAACTATTGACAAC
3835
CATAGAAAGACTTACACCTTTTAGGCTTACTTCTCGGCGCATAGGCGGTTGTTTGATAACTGTTG
lacZ
S I F L N V E N P N E E P R I R Q Q T I D N
TTCTGTTATTTTGCCCGAGAACCATTGTATCGGGCGGCTTTATTGGAGCGGACTAAAGCAAATAT
3900
AAGACAATAAAACGGGCTCTTGGTAACATAGCCCGCCGAAATAACCTCGCCTGATTTCGTTTATA
lacZ
F C Y F A R E P L Y R A A L L E R T K A N I
AGAACGTGATAAGAACCGCAGTTCTATTTTAATTTGGTCTTTAGGCAATGAATCAGGGTATGGTG
3965
TCTTGCACTATTCTTGGCGTCAAGATAAAATTAAACCAGAAATCCGTTACTTAGTCCCATACCAC
lacZ
E R D K N R S S I L I W S L G N E S G Y G
AGAATTTCGAATATTGTGCAAAATGGGTAAAAGAGCGGGATCCGGATCGATTGGTTCATTACGAA
4030
TCTTAAAGCTTATAACACGTTTTACCCATTTTCTCGCCCTAGGCCTAGCTAACCAAGTAATGCTT
lacZ
E N F E Y C A K W V K E R D P D R L V H Y E


AGCAGCATTTATCAGCATTCTGCGTATCAGAATAACACCGGGCATTTGGATTTATACAGTGAAAT
TCGTCGTAAATAGTCGTAAGACGCATAGTCTTATTGTGGCCCGTAAACCTAAATATGTCACTTTA
4095
lacZ
S S I Y Q H S A Y Q N N T G H L D L Y S E M
GTATTCCGACACAGAAGCAATCGATGCCTATTTTGCCGATCATTCACAAACGAAAAAACCATTCT
CATAAGGCTGTGTCTTCGTTAGCTACGGATAAAACGGCTAGTAAGTGTTTGCTTTTTTGGTAAGA
4160
lacZ
Y S D T E A I D A Y F A D H S Q T K K P F
TATTATGCGAATATTCCCATGCAATGGGAAATTCAAATGGAGATATGGAAGACTATTTCCAAACA
ATAATACGCTTATAAGGGTACGTTACCCTTTAAGTTTACCTCTATACCTTCTGATAAAGGTTTGT
4225
lacZ
L L C E Y S H A M G N S N G D M E D Y F Q T
TTTAACAAATATTCGGGCTGTTGCGGTGGGTTTATTTGGGAGTGGTGCGATCATGCCCAATATAT
AAATTGTTTATAAGCCCGACAACGCCACCCAAATAAACCCTCACCACGCTAGTACGGGTTATATA
4290
lacZ
F N K Y S G C C G G F I W E W C D H A Q Y I
TACCCCGACAAAATTAGGATATGGCGGTGACTTCGGCGAAAAAATCCATGATGGGAATTTCTGTG
ATGGGGCTGTTTTAATCCTATACCGCCACTGAAGCCGCTTTTTTAGGTACTACCCTTAAAGACAC
4355
lacZ
T P T K L G Y G G D F G E K I H D G N F C
TGGATGGCTTGGTTTCGCCCGAACGCGTCCCGCACAGCAACCTGTTGGAAGTAAAAAATGTCAAC
ACCTACCGAACCAAAGCGGGCTTGCGCAGGGCGTGTCGTTGGACAACCTTCATTTTTTACAGTTG
4420
lacZ
V D G L V S P E R V P H S N L L E V K N V N
CGGCCCGTACGGGCAAACTTGCGCGGTGAACAAATTGAGTTATATAACTATTTTGATTTCACTAA
GCCGGGCATGCCCGTTTGAACGCGCCACTTGTTTAACTCAATATATTGATAAAACTAAAGTGATT
4485
lacZ
R P V R A N L R G E Q I E L Y N Y F D F T N
TTTAAAAGATATTCTTTGCGTCAAATATGAATGGGTAAAAAATGGTCAGATAACCGGGACGGGAA
AAATTTTCTATAAGAAACGCAGTTTATACTTACCCATTTTTTACCAGTCTATTGGCCCTGCCCTT
4550
lacZ
L K D I L C V K Y E W V K N G Q I T G T G


CATTGGCGGTTGATTGCGAACCCCATCATTCTCAAATCTTACCGATTCAGCTTCCCAAAGAACGG
4615
GTAACCGCCAACTAACGCTTGGGGTAGTAAGAGTTTAGAATGGCTAAGTCGAAGGGTTTCTTGCC
lacZ
T L A V D C E P H H S Q I L P I Q L P K E R
GAAGGATTGCTATGGTTGAACCTATATTACTGCGCTTCCCGGCAAACTGATTTATTGCCGGCAGG
4680
CTTCCTAACGATACCAACTTGGATATAATGACGCGAAGGGCCGTTTGACTAAATAACGGCCGTCC
lacZ
E G L L W L N L Y Y C A S R Q T D L L P A G
ACATCATTTCGGTTTCGATCAAATTATTTTGAGTAAGGAATACACTCCTGCCATAGGATCGGATA
4745
TGTAGTAAAGCCAAAGCTAGTTTAATAAAACTCATTCCTTATGTGAGGACGGTATCCTAGCCTAT
lacZ
H H F G F D Q I I L S K E Y T P A I G S D
AAGATGATTGCCCGCCGTTGGAAATAACCGAAACAGTGAGACAAATCGTTGTTCGGAACAATCGA
4810
TTCTACTAACGGGCGGCAACCTTTATTGGCTTTGTCACTCTGTTTAGCAACAAGCCTTGTTAGCT
lacZ
K D D C P P L E I T E T V R Q I V V R N N R
TATTATTTTGAATTTAACAAGCTCACGGGGATAATTGACGAAATTAAAGTCAATGGCAAGGCTTT
4875
ATAATAAAACTTAAATTGTTCGAGTGCCCCTATTAACTGCTTTAATTTCAGTTACCGTTCCGAAA
lacZ
Y Y F E F N K L T G I I D E I K V N G K A F
CATCCATAAACCATTGGCTTGGAATATTTGGCGCGCGCCGACGGATAATGACCGCCTGATTCGTT
4940
GTAGGTATTTGGTAACCGAACCTTATAAACCGCGCGCGGCTGCCTATTACTGGCGGACTAAGCAA
lacZ
I H K P L A W N I W R A P T D N D R L I R
CCCAATGGCAAAATGCCGGTTATGATCAAATGTATTCCAAGGTTTACGATATTTGCGCGCATCGG
5005
GGGTTACCGTTTTACGGCCAATACTAGTTTACATAAGGTTCCAAATGCTATAAACGCGCGTAGCC
lacZ
S Q W Q N A G Y D Q M Y S K V Y D I C A H R
CAAGGAAACGGTGTTGTCGTGTCTGTGAAAAGTGCATTGGTTGCGGATGCAAAATCAAAAATCAT
5070
GTTCCTTTGCCACAACAGCACAGACACTTTTCACGTAACCAACGCCTACGTTTTAGTTTTTAGTA
lacZ
Q G N G V V V S V K S A L V A D A K S K I M


GACATTGGAAACACAGTATCTGCTGTCTGAAAATGGTAAATTGGATATTCAAACAAATGCTGTTT
CTGTAACCTTTGTGTCATAGACGACAGACTTTTACCATTTAACCTATAAGTTTGTTTACGACAAA
5135
lacZ
T L E T Q Y L L S E N G K L D I Q T N A V
TCCACGAGCATTTGCCATTCCTGCCCCGTTTCGGTTTGCGCTTTTTCTTAGACGAACAGAAAACA
AGGTGCTCGTAAACGGTAAGGACGGGGCAAAGCCAAACGCGAAAAAGAATCTGCTTGTCTTTTGT
5200
lacZ
F H E H L P F L P R F G L R F F L D E Q K T
CCTTTTACCTACTTAGGTTATGGGGCGGGTGAAAGCTATATTGATAAACATCAGGCGACCAAACT
GGAAAATGGATGAATCCAATACCCCGCCCACTTTCGATATAACTATTTGTAGTCCGCTGGTTTGA
5265
lacZ
P F T Y L G Y G A G E S Y I D K H Q A T K L
TGGTATTTATTCGACAACGGCAGGGGAAAATCATGTTGGCTATCTCAAGCCTCAAGAGAACGGCA
ACCATAAATAAGCTGTTGCCGTCCCCTTTTAGTACAACCGATAGAGTTCGGAGTTCTCTTGCCGT
5330
lacZ
G I Y S T T A G E N H V G Y L K P Q E N G
GCCATTATGGATGCTTTTATGTTCAAAACGATATGATCCGGGTGGAGTCGGGGCAGCCGTTTAGT
CGGTAATACCTACGAAAATACAAGTTTTGCTATACTAGGCCCACCTCAGCCCCGTCGGCAAATCA
5395
lacZ
S H Y G C F Y V Q N D M I R V E S G Q P F S
TTCAACCTGTCCCCTTATACGCAAGAAGAACTGACGCAGAAAAAACATTCTTATGAATTGGTTTG
AAGTTGGACAGGGGAATATGCGTTCTTCTTGACTGCGTCTTTTTTGTAAGAATACTTAACCAAAC
5460
lacZ
F N L S P Y T Q E E L T Q K K H S Y E L V C
TTCCGGGTATGATGTATTGTGTATCGACTATAAGATGAGCGGTATCGGTTCGAATTCTTGCGGGC
AAGGCCCATACTACATAACACATAGCTGATATTCTACTCGCCATAGCCAAGCTTAAGAACGCCCG
5525
lacZ
S G Y D V L C I D Y K M S G I G S N S C G
CTAATTTGAAGCCGCAATATCGTTTGATTGAAAATAACATCAATTTCGATATAAGTATCCGGTTA
GATTAAACTTCGGCGTTATAGCAAACTAACTTTTATTGTAGTTAAAGCTATATTCATAGGCCAAT
5590
lacZ
P N L K P Q Y R L I E N N I N F D I S I R L

TAAGCTTTCTGCAAAGATTGGTATCAACAAAAAGCCTGTCGTCAGACAGGCTTTTTTTCTGTTTT
5655
ATTCGAAAGACGTTTCTAACCATAGTTGTTTTTCGGACAGCAGTCTGTCCGAAAAAAAGACAAAA
porB terminator
lacZ
CTGTTTTTAGATGCCGTCTGAATGCTGAAGTAGAAAACCAGCAAGAAGGTAAAAAGAAAGAAGCA
5720
GACAAAAATCTACGGCAGACTTACGACTTCATCTTTTGGTCGTTCTTCCATTTTTCTTTCTTCGT
porB terminator
DUS
3'END NHCIS1
GTTTTTTGGATTTTAGATGTTACCGCAATTGGTTTCCTTTCCTAAAATTTGTTTAAATTATTTGC
5785
CAAAAAACCTAAAATCTACAATGGCGTTAACCAAAGGAAAGGATTTTAAACAAATTTAATAAACG
3'END NHCIS1
AATATTAATATAAACTGGATATTAATGATGAGGATTCAAAAAGGCATACTGAATATAATTTGTAC
5850
TTATAATTATATTTGACCTATAATTACTACTCCTAAGTTTTTCCGTATGACTTATATTAAACATG
3'END NHCIS1
AAAATATTTGCAGTATTTAAAAATGTTGGTTCGTATATGAAAAGTTAAAAATGCCAAAATGTACA
5915
TTTTATAAACGTCATAAATTTTTACAACCAAGCATATACTTTTCAATTTTTACGGTTTTACATGT
3'END NHCIS1
GTTGCTAAACTGTAAAACTGCTAAAGCAACAAAACATAAAAAGGAATGCAGGGATGCGATCACTA
5980
CAACGATTTGACATTTTGACGATTTCGTTGTTTTGTATTTTTCCTTACGTCCCTACGCTAGTGAT
3'END NHCIS1
CATCTTTTTATTCCGTAAGCATTTATGACTTTACGGTCAACTGCTACTCTATGTTTCCAGCTTTT
6045
GTAGAAAAATAAGGCATTCGTAAATACTGAAATGCCAGTTGACGATGAGATACAAAGGTCGAAAA
3'END NHCIS1
CAGCTCCCTATTTTCGAATATTGGACGAGGCATTTTCATCAGTGTCGTAATGCCGACCGAAACCC
6110
GTCGAGGGATAAAAGCTTATAACCTGCTCCGTAAAAGTAGTCACAGCATTACGGCTGGCTTTGGG
3'END NHCIS1
TCACAAACCATATTGGTTCTTGTGGCAGCAACACCTATCCGTTTGTTCAAGCGGCCACAAGAGTA
6175
AGTGTTTGGTATAACCAAGAACACCGTCGTTGTGGATAGGCAAACAAGTTCGCCGGTGTTCTCAT
3'END NHCIS1
ACATGATTGGCTGGTGGCATTGGCTTTAATCTCTTCGATATGAACTCCATTTTTAGCTGCACCTT
6240
TGTACTAACCGACCACCGTAACCGAAATTAGAGAAGCTATACTTGAGGTAAAAATCGACGTGGAA
3'END NHCIS1

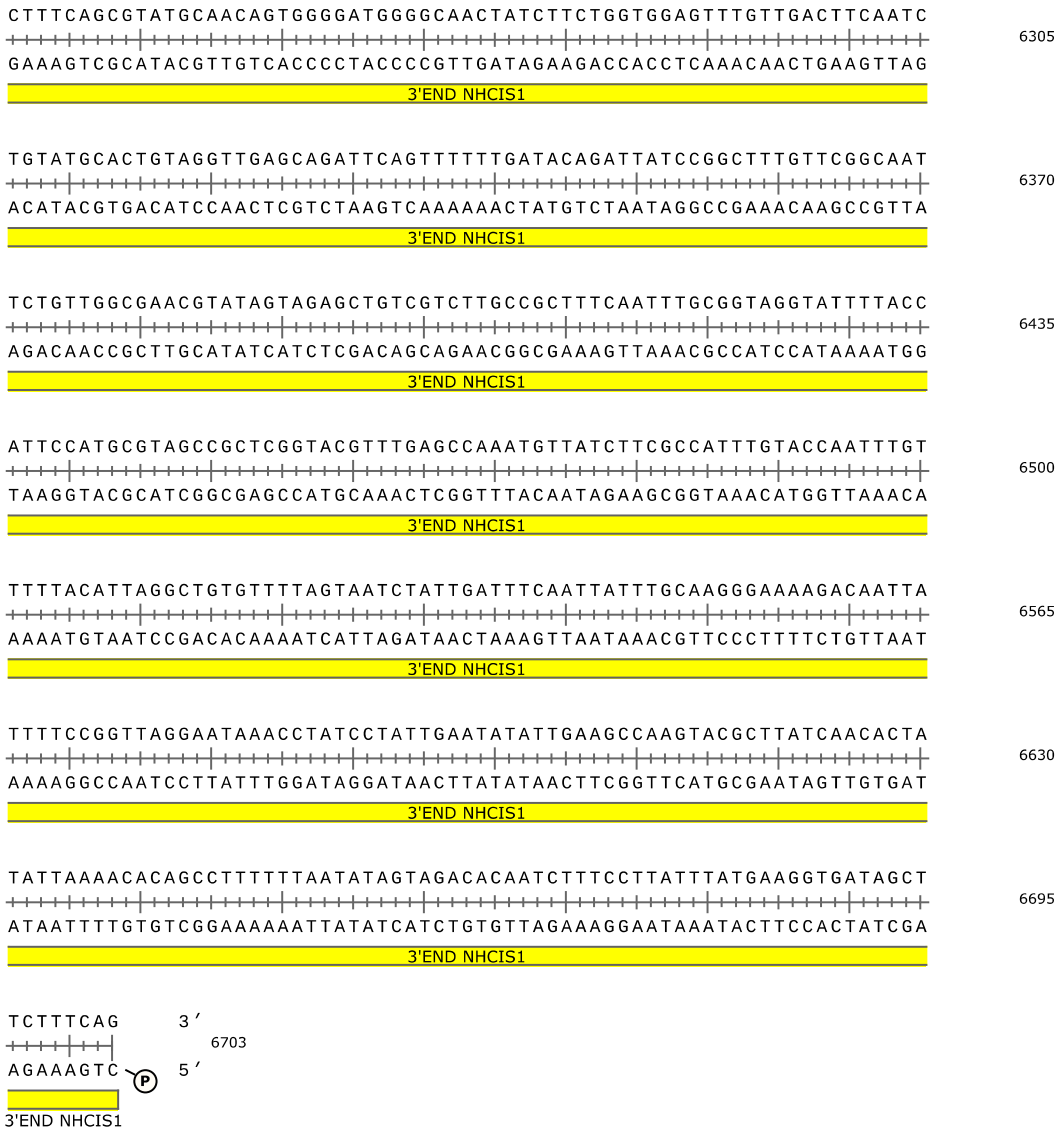


Figure 5: Complete, annotated nucleotide sequence of NHCIS1::HAEC4:*nadA-lacZ* gene expression cassette. NB: In the NHCIS1::HAEC4:(Z)-*lacZ* gene expression cassetee, the *nadA* coding sequence is completely absent and replaced with 14 bp: (5' – ATCTATTATATAAC – 3').

Characteristics of the genetically modified organisms in their final form

25. The description of genetic traits or phenotypic characteristics and in particular any new traits and characteristics that may be expressed or no longer expressed.

The GMOs have been constructed following the deletion of the *lacZ* gene from the wild type *N. lactamica* strain, Y92-1009 and the subsequent insertion of one of two gene expression cassettes (containing either the *lacZ* gene alone or both the *nadA* and *lacZ* genes) into the NHCIS1 locus.

The NadA protein is a member of the type V autotransporter family of outer membrane proteins. NadA-expression in Nmen is associated with an increased level of adhesion to and invasion of human epithelial cell lines. NadA expression is not essential for virulence. The NadA protein is one of the 4 strongly immunogenic components of the 4CMenB vaccine against serogroup B meningococcal disease (Bexsero).

The GMO expresses large amounts of NadA on its surface; significantly more than wild type *N. meningitidis* strain MC58, as measured by flow cytometry (Item 28). Despite this, the *in vitro* growth of the bacterium in rich culture medium (TSB) appears to be unaffected. In semi-defined growth media (modified Frantz) however, the growth characteristics of the GMO differ from the wild type, insofar as the GMO does not autoaggregate like the parental strain. Expression of NadA by the GMO significantly increases the numbers of bacteria binding to the human epithelial cell line, HEp2 (Item 29).

26. The structure and amount of any vector or donor nucleic acid remaining in the final construction of the modified organisms.

The NHCIS1-targeted gene expression cassettes are integrated into the genome in their entirety, replacing the endogenous NHCIS1 locus. As such, the cassette is stably maintained as an inheritable unit in the bacterial genome.

27. The stability of the organism in terms of genetic traits.

Both the NadA-expressing (4NB1) and 'empty vector' (4YB2) GMOs were serially passaged *in vitro* for 28 days on solid media. Each day, ten individual colonies of each GMO were spread onto fresh TSA plates and incubated overnight at 37 ^{o}C, 5 % CO_2. Whole genome sequencing of these strains both at the beginning and end of this process revealed that the NHCIS1-targeted constructs are completely stable, with both the Day 0 and Day 28 versions of each strain containing sequences with 100 % identity to the sequence of the construct present in the plasmids.

This is reflected in the expression levels of the NadA protein (Item 28), which were not significantly changed by the serial passage of the strains. In flow cytometric analysis, using a monoclonal antibody (mAb) raised against recombinant NadA (GSK Novartis) and an Alexafluor488-conjugated secondary antibody, the fluorescence intensity of Nlac strain 4NB1, grown after 28 days of *in vitro* passage, was similar to the fluorescence intensity of the same strain that was cultured from frozen stocks of the GMO (i.e. from Day 0).

28. The rate and level of expression of the new genetic material in the organisms and the method and sensitivity of measurement of that rate and level.

Flow cytometry analysis using a monoclonal antibody (mAb) raised against recombinant NadA (GSK Novartis) shows the NadA adhesin is expressed to a high level on the surface of strain 4NB1. The Mean Fluorescence Intensity of a population of 4NB1 probed with the mAb and an Alexafluor488-conjugated secondary antibody (1646 $\pm$ 252 units, n = 4) is approximately 470 times that of identically treated wild type *N. lactamica* (3.5 $\pm$ 0.27 units, n = 4), and approximately 85 times that of identically treated, acapsulate *N. meningitidis* (MC58 *ΔsiaD*; 19.25 $\pm$ 3.47 units, n = 2). Using the same assay, the fluorescence intensity of a population of strain 4YB2 is similar to that of wild type *N. lactamica* (4.735 units, n = 2), which is similarly unaffected by repeated passage for 28 days (2.975 units, n = 2).

The Specific Activity of β-galactosidase measured in a GM-Nlac strain harbouring the *lacZ* gene under the control of the *lst* promoter in NHCIS1 (1922 $\pm$ 232.5 nmoles/minute/mg protein, n = 3) is not significantly different to that measured in wild type *N. lactamica* cultured under identical conditions (1143 $\pm$ 21.4 nmoles/minute/mg protein, n = 3). Measurements were made using a commercially available β-galactosidase assay kit (Life Technologies).

29. The activity of the gene product.

The NadA protein is an epithelial cell-binding adhesin. Although the receptor for NadA has yet to be elucidated, the expectation was that recombinant expression of the *nadA* gene in *N. lactamica* might increase the propensity of the bacteria to bind to epithelial cells.

N. lactamica wild type strain Y92-1009 and the NadA-expressing GMO were used to infect confluent monolayers of HEp-2 cells at a multiplicity of infection (MOI) of 100. Measurements of the number of bacteria bound to and internalised by the HEp-2 cells were made every 2 h for a total of 6 h. As determined by assessment of bacterial viability, there were significantly more GMO than wild type bacteria bound

to the HEp-2 cells after 4 h of infection ($p \leq 0.0001$, 2-way ANOVA with Tukey's Multiple Comparisons test, n = 4). This implies that the NadA-expressing GMO is binding to a receptor on the surface of the epithelial cell line that the wild type bacteria cannot. Despite significantly increased levels of binding to HEp-2 cells, there was no significant difference between the wild type and GMO bacteria in the number of intracellular bacteria recovered at any time point.

30. The description of identification and detection techniques, including techniques for the identification and detection of the inserted sequence and vector.

Following intranasal inoculation of volunteers, at the appropriate time points throat swabs and nasal washes will be performed. These samples will be used to try and culture viable *Neisseria* species on both GC and chocolate agar plates. Putative *Neisseria* colonies will be identified as *Neisseria lactamica* in the first instance by mass spectrometry (MALDI-TOF) and specifically as the genetically-modified strains by PCR of the NHCIS1 locus. Amplification of the NHCIS1 locus will produce 6.7 kb and 5.4 kb products from strains 4NB1 and 4YB2, respectively. Amplification of the same locus from wild type Nlac isolates will produce a 2.2 kb product. As a backup, PCR amplification of a product exploiting the unnaturally close proximity of the *nadA* and *lacZ* genes would serve to identify the GMO.

31. The sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques.

The microbiological, biochemical and genetic analyses described in Item 30 enable the GMO to be identified with 100 % accuracy.

32. The history of previous releases or uses of the organisms.

This is the first description of the GMO and its first use in an experimental challenge with healthy adult volunteers. No release of this GMO has been made previously. However, the wild type *N.lactamica* strain Y92-1009 has been released in three previous studies conducted by the Experimental Human Challenge Group (two at the University of Sheffield, UK [1];[2] (NCT02249598) and one at the University of Southampton, UK, currently underway). These studies demonstrated no negative impact on human health. We are aware of at least one deliberate release of a GMO organism following intranasal inoculation of human volunteers. A genetically modified *Bordetella pertussis* strain (an attenuated pathogen) was constructed as a vaccine candidate to be administered by the nasal route. In this strain, BPZE1,

dermonecrotic toxin and tracheal cytotoxin were genetically inactivated by allelic exchange of heterogenous genes, and pertussis toxin (PT) was genetically detoxified by two independent mutations, each deactivating the toxic activity of PT, without affecting the immunogenic properties [38]. Investigators then tested BPZE1 for the first time in man in a placebo-controlled, double-blind, dose-escalation trial in which the GMO was inoculated intranasally to healthy young adult male volunteers in a single dose. Volunteers were observed for a short period after inoculation at a facility in Sweden, but then allowed to return home and to live freely in their community and were followed up for immunogenicity blood tests. No therapeutic eradication of the GMO was undertaken [39].

33. In relation to human health, animal health and plant health –

N. lactamica is a human restricted commensal bacterium. There are no anticipated effects on animal or plant health.

a. the toxic or allergenic effects of the non-viable organisms and/or their metabolic products,

The GMOs have not previously been administered to human subjects, so the reactogenic profiles of the GMOs in the human nasopharynx are unknown. However, we do not anticipate the GMO strains to be any more reactogenic than wild type Nlac.

In colonised individuals it is highly likely that GMO will 'bleb' to produce outer membrane vesicles (OMV), which contain Nlac-derived lipooligosaccharide and outer membrane proteins. This process occurs during colonisation with the wild type organism, and native OMV may in fact constitute an immunising agent *in vivo*. In a Phase I safety and immunogenicity study that assessed Nlac-derived OMV as a potential anti-meningococcal vaccine candidate, the reactogenicity profile of a concentrated bolus of deoxycholate-extracted OMV (25 µg) was well tolerated [40]. The rate at which a relatively low-density population of colonising organisms will produce OMV *in situ* is therefore unlikely to provoke a strong reactogenic profile.

b. the comparison of the organisms to the donor, recipient or (where appropriate) parental organism regarding pathogenicity,

The GMOs shows no evidence of increased pathogenicity compared to wild type in a murine model of intraperitoneal (i/p) infection, which is to say that the GMOs showed no evidence of pathogenicity. Wild type *N. lactamica* strain Y92-1009, the NadA-expressing GMO strain, 4NB1 and the transformation Control strain, 4YB2, were

injected i/p at either a low (1.7 x 10^5 CFU) or a high (approx. 2.5 x 10^7 CFU) dose into NIH/OLA mice (10 per group) along with a bolus of holo-human transferrin as an exogenous iron source. An additional transferrin dose was injected after 24 h. Mice were monitored over the course of five days and all remained healthy. This is in contrast to i/p challenge with serogroup B *N. meningitidis* where 1/5 mice survived following a challenge with 2 x 10^6 CFU. There were no survivors following a dose of 2 x 10^7 CFU [41].

c. the capacity of the organisms for colonization

The NadA protein is an epithelial cell-binding adhesin. Although the receptor for NadA has yet to be elucidated, the expectation is that recombinant expression of the *nadA* gene in *N. lactamica* might increase the propensity of the bacteria to bind to human epithelium. The enhanced cell binding phenotype of the NadA-expressing strain is detailed in Item 29.

d. if the organisms are pathogenic to humans who are immunocompetent –

N. lactamica is a human restricted commensal bacterium that is not pathogenic to immunocompetent individuals.

i. diseases caused and mechanisms of pathogenicity including invasiveness and virulence,

ii. communicability,

iii. infective dose,

iv. host range and possibility of alteration,

v. possibility of survival outside of human host,

vi. presence of vectors or means of dissemination,

vii. biological stability,

viii. antibiotic-resistance patterns,

ix. allergenicity, and

x. availability of appropriate therapies.

e. Other product hazards

Part III

Information relating to the conditions of release

The release

34. The description of the proposed deliberate release, including the initial purpose or purposes of the release and any intention to use the genetically modified organisms as or in a product in the future.

Purpose of the deliberate release: In this clinical study we propose to utilise the established human challenge model for inoculation of human volunteers with wild type *Neisseria lactamica* to further study host-pathogen interactions. We will investigate whether experimental colonisation by the NadA expressing GMO results in immunity directed specifically against NadA. This information will be used to (a) understand how mucosal immunity to meningococcal antigens such as NadA (a vaccine component) develops (b) to inform vaccine development by permitting the development of a colonisation model in which the colonisation-protection efficacy of vaccines containing NadA can be tested, and (c) develop potential `bacterial medicine` in which genetically modified commensals are used to deliver immunising or microbiome-modifying gene products into the nasopharynx for health benefit.

The proposed study will follow a protocol entitled `Experimental challenge of the human nasopharynx with recombinant *Neisseria lactamica* expressing the meningococcal type V autotransporter protein *Neisseria* Adhesin A (NadA)`

The co-primary endpoints of the study will be (i) to establish the safety of nasal inoculation of healthy volunteers with a genetically modified strain of *Neisseria lactamica* expressing NadA, and (ii) to assess the NadA specific immunity in healthy volunteers following nasal inoculation with *Neisseria lactamica* expressing NadA.

The future use of the GMO will be (1) to enable investigation of mucosal immunity to a meningococcal antigen during nasal carriage under controlled conditions (2) to test vaccines containing NadA for their ability to protect against colonisation by strains expressing the immunologically cognate antigen, (3) to use *N.lactamica* as the background strain for future bacterial medicines that can introduce a therapeutically beneficial gene into the human nasopharynx (e.g. for microbiome modification).

35. The intended dates of the release and time planning of the experiment including frequency and duration of releases.

The study is expected to commence on the 1st of November 2017 (pending all necessary approvals) and will run for approximately 13 months (with an expected completion date of 30th November 2018). All study participants will have follow-up

visits up to 90 days post challenge. The study will have an enrolment target of 44 adult participants. A target of 22 participants will receive an intranasal 100,000 (10^5) CFU dose of GM *Neisseria lactamica* strain 4NB1 – and in the control arm, 22 participants will receive an intranasal 100,000 (10^5) CFU dose of GM *Neisseria lactamica* strain 4YB2. In this study, all volunteers will be admitted to the NIHR Wellcome Trust Clinical Research Facility for 5 days and will be subject to 24 h clinical observation. They will then be discharged for follow-up as outpatients. The first volunteer to receive each GMO will be challenged individually, followed by a safety review including safety blood results and clinical data at day 7. Providing there are no safety concerns and the investigator signs the safety report form, a second and third volunteer will be challenged. At day 7 of the second and third volunteer a safety review of the first three volunteers will be conducted and providing there are no safety concerns, a fourth and a fifth volunteer will be challenged. After day 7 post challenge of the fifth volunteer a safety report will be written, which will be reviewed within one week by the external safety committee before any further volunteers are challenged. The remaining volunteers will be challenged in groups of a maximum of five. The decision to continue challenging the next volunteer of the following group of five will be taken by the investigator taking into account the advice of the safety committee and will be put in writing before the next volunteer is challenged. It is anticipated that inoculations will continue from November 1st until approximately August 31st 2018, with follow up of the final volunteers being completed by 30th November 2018.

It is anticipated that approximately 50% of volunteers will have evidence of carriage of the GMOs in the nasopharynx by 1 week after inoculation, and those carriers will likely continue to be carriers for at least the 90 days of the study. It is possible there will be onward transmission to close contacts (especially bedroom-sharers) within the first 2 weeks after discharge [42].

All study participants will agree to have 24 h contact with study staff during the four weeks post challenge and to be able to ensure that they are contactable by mobile phone for the duration of the challenge period.

36. The preparation of the site before the release.

All necessary study approvals (DEFRA, National Research Ethics Service, Sponsor (University of Southampton) and University Hospital Southampton R&D) must be in place before the study can commence. The study will be done at the Southampton NIHR Wellcome Trust Clinical Research Facility (HIHR-WTCRF) at University Hospital Southampton according to Good Clinical Practice (GCP) and according to documented legal and local procedures and guidelines prior to study initiation. All site staff will be given study-specific training.

37. The size of the site.

The release will take place in the Southampton NIHR-WTCRF, at University Hospital Southampton, Tremona Rd, Southampton, Grid Reference SU397149. The size of the room in which the release will occur is approximately 15m^2. After 4.5 days of inpatient stay, some volunteers (approximately 50%) will be carrying the organisms in their nose and throat when they are discharged into the wider Southampton community.

Relevant facilities at the Southampton NIHR-WTCRF include patient/volunteer consultation, waiting and recreation areas; 13 consulting rooms (2 configured for infectious participants); a containment level 3 laboratory for microbiological work, 8 in-patient beds; an environmental laboratory with three "containment level 2" environmental chambers; state-of-the-art physiological monitoring and physical and management systems to ensure Regulatory Compliance such as computerised sample inventory, and tracking system (http://www.uhs.nhs.uk/ClinicalResearchinSouthampton/Trials-and-facilities/NIHRWellcome-Trust-Clinical-Research-Facility/Our-facility.aspx). The volunteers will have designated areas including rooms, toilets, shower and recreational room during their stay at the facility. Standard infection control precaution policy will be followed as per NHS and PHE policy.

38. The method or methods to be used for the release.

The inoculation will be done as nose drops (500 µL into each nostril) with the volunteer lying supine with neck extended. The challenge procedure will be carried out in one of the containment level 2 environmental chambers within the NIHR-WTCRF to assure the inoculum will be administered to the volunteer only, without posing any risk of infection to other people or the environment. Staff will wear appropriate personal protective equipment during the challenge procedure. After inoculation the participant will lie supine for 5 minutes. Before and after the challenge they will wash their hands and use an alcohol gel to clean their hands. After the challenge the room will be cleaned following NHS guidelines.

39. The quantity of organisms to be released.

Participants will receive an intranasal 100,000 (10^5) CFU dose of a GMO strain.

40. The disturbance of the site, including the type and method of cultivation, mining, irrigation, or other activities.

Not applicable

41. The worker protection measures taken during the release.

Staff will wear appropriate personal protective equipment during the challenge procedure. Before and after the challenge they will wash their hands and use an alcohol gel to clean their hands. After the challenge the room will be cleaned following NHS guidelines. All procedures to investigate the carriage of the GMO by the participant (throat swabs, nasal washes, nasal swabs, blood tests) will be undertaken in the containment level 2 environmental chambers within the NIHR-WTCRF.

42. The post-release treatment of the site.

For the first 5 days of the release the participants will be observed 24 hours a day as inpatients in the NIHR-WTCRF. There, hospital infection control procedures will be followed and transmission to other humans is extremely unlikely. After discharge into the community, it is anticipated that 50% of the volunteers will be carrying one of the GMO strains in the nose and throat and there is the potential for transmission of that GMO to household contacts especially sleeping partners. To monitor this, any persons who share a bedroom with the participants who are inoculated will be (a) asked for informed consent *a priori*, (b) asked to undergo a throat swab prior to inoculation and 2 weeks after inoculation of the participant. This will be done to monitor the potential for onward transmission into the community. These recommendations were made by our Patient and Public Involvement (PPI) focus group, which advised us on the precautionary measures considered appropriate for incorporation into our protocols.

43. The techniques foreseen for elimination or inactivation of the organisms at the end of the experiment or other purposes of the release.

Whilst admitted to the NIHR-WTCRF, local and site SOPs will be followed for inactivation of the GMO waste prior to disposal. After discharge to the community, participants will follow standard infection control procedures (hand washing, and consent to restrict social activities for 2 weeks after discharge) to minimise onward transmission. In the unexpected event that the GMO causes disease in participants, then treatment with standard antibiotics (e.g. ceftriaxone) will be instituted. In this case, carriers will be treated with the oral drug ciprofloxacin (single dose), which will

eradicate carriage. In the unexpected event that a public health issue arises (i.e. disease caused by the GMO in individuals other than the participants) then the available option is deployment of ring vaccination with the vaccine Bexsero, which contains the cognate NadA antigen and has been shown to prevent meningococcal disease at the population level. It should be re-iterated that disease is highly unlikely given that the GMO does not have the polysaccharide capsule which is the key virulence determinant of *N. meningitidis*.

44. Information on, and the results of, previous releases of the organisms and in particular, releases on a different scale or into different ecosystems.

This is the first use of this GMO in human studies. Work in our laboratories has demonstrated that the GMO is non-virulent in a mouse model (i.e. it did not result in morbidity even after parenteral injection at a high dose, see Item 33b).

The environment (both on the site and in the wider environment}

45. The geographical location and national grid reference of the site or sites onto which the release will be made, or the foreseen areas of use of the product.

The release will take place in the Southampton NIHR Wellcome Trust Clinical Research Facility, at University Hospital Southampton, Tremona Rd, Southampton, Grid Reference SU397149.

Whilst it is anticipated that the majority of study participants will live in the Southampton area throughout the duration of the study, once the volunteers return to their daily lives there is the potential for community spread of the GMO. Although we will require consent *a priori* from the bedroom sharers of participants, and conduct monitoring of *N. lactamica* carriage and potentially offer antibiotic eradication therapy to these individuals (see Item 42), it is not feasible to monitor any further onwards transmission through the wider community. However, as discussed throughout this application, we do not expect the GMO to propagate widely relative to wild type strains of *N.lactamica* because of the immunising nature of NadA, or to pose any greater risk of pathogenesis in humans than the wild type organism, which is to say that we do not expect the GMOs to cause any form of human disease.

46. The physical or biological proximity of the site to humans and other significant biota.

The site is in Southampton, an urban area in southern England.

47. The proximity to significant biotopes, protected areas or drinking water supplies.

The human nasopharynx is the only natural environmental niche of *N.lactamica*; it does not survive elsewhere. The participants, after discharge from hospital, are likely to come into contact with other humans. It is assumed that the transmission of *N.lactamica* will be similar to *N.meningitidis* – about which there is considerable information on transmission. Transmission of *N.meningitidis* between humans occurs with the greatest frequency in household contacts (especially bedroom-sharers), sexual contacts and in people who attend pubs and clubs, or live in University dormitories/halls of residence. Therefore, to limit transmission we will train the participants in infection control, request that they do not attend pubs and clubs in the 2 weeks after discharge (because there is evidence that onward transmission between humans occurs in the first 2 weeks after acquisition [42]) and monitor (and offer eradication treatment) to bedroom contacts.

Public Health England is a collaborator in this study, and the relevant section of the organisation (e.g. the Meningococcal Reference Unit) will be informed that it is underway. Public Health Southampton (now part of Southampton City Council) will be informed of all participants who have been challenged with the GMOs. The participant's GP will also be informed. In addition, any unexpected occurrence of disease in participants will be notified to the data and safety monitoring board (DSMB). If the DSMB considers the event to be causally related to the GMO, the study will be stopped, as per protocol and this information will be passed on immediately to PHE and Public Health Southampton as well as the GP.

48. The climatic characteristics of the region or regions likely to be affected.

Not applicable

49. The geographical, geological and pedological characteristics.

Not applicable

50. The flora and fauna, including crops, livestock and migratory species.

Not applicable

51. The description of target and non-target ecosystems likely to be affected.

The GMOs are limited to human tropism (see Item 47), which limits the impact of the release to modulation of the nasopharyngeal microbiome. The competitive relationship observed between *N. lactamica* and *N. meningitidis* [2], which results in the displacement of the latter by the former, manifested in a subset of study participants as the exquisite replacement of *N. meningitidis* by *N. lactamica* over time. Preliminary microbiome analyses from these individuals showed the incursion of *N. lactamica* into the microbial flora was non-disruptive and that the only significant change to the composition of the microbiome was the exclusion of *N. meningitidis*; all other bacterial genera detected in the analysis were seemingly unaffected by the introduction of *N. lactamica*. The impact of *N. lactamica* on the microbiome is unlikely to be the same in all participants, and we will be collecting samples for a thorough analysis of how the naso/oropharyngeal microbiota changes over time and specifically in response to microbial challenge by *N. lactamica*.

52. The comparison of the natural habitat of the recipient organisms with the proposed site or sites of release.

The natural habitat of *N. lactamica* is humans. Study participants enrolled to the proposed clinical study will be primary recipient of the GMO. As a consequence of the release, the GMO will be released into the community, carried by the participants, who may transmit it to other humans (see Item 47).

All clinical and laboratory waste generated at the release site will be inactivated prior to disposal (according to local GMO standard operating procedures, legal and University policies). All site staff will be trained in infection control procedures.

53. Any known planned developments or changes in land use in the region that could influence the environmental impact of the release.

None known.

Part IV

Information relating to the interactions between the organisms and the environment

Characteristics affecting survival, multiplication and dissemination

54. The biological features which affect survival, multiplication and dispersal.

The GMO is `non-attenuated` (but the wild type is a commensal and non-virulent) and is expected to be able to survive within its biological niche (the human nasopharynx) following colonisation. *N. lactamica* is spread by person to person transmission through exchange of respiratory and throat secretions (saliva) or during close and/or lengthy contact. There is no evidence for airborne transmission of the bacterium through inhalation of respiratory droplets. Following release, there is a chance that close contacts of study participants (i.e. sleeping partners) will also become carriers of the GMO.

Although there is currently no supportive data, it is assumed that the transmission of the GMO will be similar to *N. meningitidis* – about which there is considerable information on transmission. Transmission of *N. meningitidis* between humans occurs with the greatest frequency in household contacts (especially bedroom-sharers), sexual contacts, and in people who attend pubs and clubs, or live in University dormitories/halls of residence. Therefore to limit transmission we will train the participants in infection control, request that they do not attend pubs and clubs in the 2 weeks after discharge (because there is evidence that onward transmission between humans occurs mostly in the first 2 weeks after acquisition [42]) and monitor (and offer eradication treatment) to bedroom contacts.

55. The known or predicted environmental conditions which may affect survival, multiplication and dissemination, including wind, water, soil, temperature and pH.

The GMO is a human-adapted commensal bacterium with no other known reservoir. *N. lactamica* has extremely limited survivability outside of its biological niche. However, the incidence of meningococcal disease peaks during the winter, suggesting an increased rate of *N. meningitidis* transmission during the winter months. However, there has been no data produced to date on whether this can be extrapolated to transmission of *N. lactamica.*

56. The sensitivity to specific agents.

The sensitivity of the wild type and GMO strains to clinically relevant antibiotics has been confirmed (see Item 12e).

Interactions with the environment

57. The predicted habitat of the organism.

The natural habitat of *N. lactamica* is humans. Study participants enrolled to the proposed clinical study will be the primary recipients of the GMO.

58. The studies of the behaviour and characteristics of the organisms and their ecological impact carried out in simulated natural environments, such as microcosms, growth rooms and greenhouses.

Not applicable.

59. The capability of post-release transfer of genetic material-

a. from the genetically modified organisms into organisms in affected ecosystems,

The gene expression cassettes introduced into the GMOs are maintained in single copy in the bacterial chromosome. Following the death or lysis of each *N. lactamica* bacterium, it is therefore possible that a single copy of the *nadA* gene will be released to the extracellular milieu of the nasopharynx. The DNA containing the *nadA* gene could conceivably be taken up into other bacterial residents of the nasopharynx and become integrated into their genome. The propensity of each individual species of bacteria to assimilate the gene is impossible to estimate, but will be affected by a multitude of factors such as the competence of the recipient, the restriction endonuclease activities present in that particular species and the balance of DNA repair systems within each organism, which will determine the recombinogenicity of a given species. The cassette contains no means to initiate its own replication, which means that in order to be maintained there is an absolute requirement for integration of the gene into the recipient's DNA (be this chromosomal or plasmid-based).

The *nadA* gene is closely linked to a Neisserial DNA uptake sequence, which significantly increases the likelihood of naked DNA from the GMO being taken up by other members of the genus *Neisseria*. The system is biased toward *Neisseria* species in the first instance, but even more so toward *Neisseria* species that use the same DUS 'dialect' as *N. lactamica* (DUS vary slightly in sequence between

Neisseria species, with uptake mechanisms biased in favour of their specific DUS subtype, or 'dialect' [28]). Indeed, there is evidence of frequent, interspecific genetic exchange amongst the *Neisseriaceae* [43]. The canonical DUS of *N. lactamica* is also that favoured by the potentially pathogenic *Neisseria* species, *N. meningitidis* and *N. gonnorrhoeae*. As a result, DNA released from the GMOs will be preferentially targeted and bound by the DNA uptake machinery of these bacteria, should they also co-habit the same individual's nasopharynx. It should be noted that, at least in study volunteers, this eventuality is precluded through exclusion of volunteers that test positive for either of these species, or for strains of *N. lactamica*. Following the return of volunteers to the community however, there is no practical way to control for the onward transmission of these organisms, or to determine the microbial flora of close contacts that also become colonised.

Accepting that further dissemination of our Nlac codon-optimised version of *nadA* is possible, it becomes important to consider the impact this will have on recipient organisms:

For those organisms unable to express the gene, perhaps due to a significantly different codon usage bias compared to *N. lactamica*, or the incompatibility of their transcriptional machinery with *Neisseria* promoters, there is likely to be little to no effect on the bacteria themselves. These bacteria will, at worst, act as reservoirs for the *nadA* gene, serving as a potential route of indirect transmission to other (*Neisseria*) species in the future. However, there is no reason why this version of the *nadA* gene is any more likely to be disseminated than naturally occurring alleles in currently circulating strains of *N. meningitidis.* The fact that NadA remains a meningococcal-specific adhesin suggests that the gene is *not* being transmitted between *Neisseria* species and that the gene confers no evolutionary advantage to other members of the genus or species outside of the genus.

In those recipients that are able to express the NadA protein, then the situation is more complicated. Whilst the presence of NadA might enhance the ability of the recipient to colonise a person's nasopharynx in the short term, perhaps by allowing the bacteria to bind the as-yet unidentified NadA receptor on epithelial cells; the constitutive expression of NadA may be detrimental to the longer term survival of the recipient. Because the wild type version of the *nadA* gene is under transcriptional control of a phase variable promoter system, and there is a tendency of meningococcal carriage strains to downregulate NadA expression following the establishment of colonisation [6], it is reasonable to assume that NadA expression constitutes a survival liability to the meningococcus beyond the initial phases of the colonisation process. Acquiring the *nadA* gene from the GMO would therefore mean that recipient organisms constitutively produce the NadA protein, which could act as a target for immunological killing of the bacteria. The only conceivable way to circumvent this detriment would be either for the immunodominant epitopes of NadA to become mutated (assuming a role for acquired immunity in the modulation of

colonisation), or for the gene to become either lost from the genome or truncated/frameshifted. On balance, the expression of NadA from the NHCIS1 cassette most likely confers no survival advantage to bacteria over the longer-term, and colonisation with bacteria that have horizontally acquired the *nadA* gene from the extracellular milieu is likely to be self-limiting (through as-yet poorly defined means).

b. from indigenous organisms to the genetically modified organisms.

The likelihood of the GMOs acquiring genetic material from indigenous organisms is minimal. Although naturally competent, *N. lactamica* strain Y92-1009 is refractory to transformation with DNA from other bacterial species (heterotypic DNA), due to the incompatibility of donated chromosomal DNA with the potent restriction endonuclease activities coded for in the Nlac genome. This incompatibility arises because the majority of other bacteria lack DNA methylases capable of protecting their DNA against the restriction endonuclease activities of Nlac. As a result, most heterotypic DNA taken up by Nlac is degraded before recombination can take place. The GMOs are similarly refractory to transformation with heterotypic DNA. To demonstrate this, nascent colonies of each GMO were exposed to 1 µg of purified chromosomal DNA, extracted from a mutant derivative of *N. meningitidis* strain MC58, in which the *nadA* gene was insertionally inactivated by the kanamycin-resistance conferring gene, *aphA3* (*ΔnadA:aphA3*). Under laboratory conditions optimised for *Neisseria* transformation, the transformation efficiencies of the GMOs with respect to this gene were *zero* (n = 6) (see Figure 6, Item 69). The same purified chromosomal DNA transformed wild type *N. meningitidis* strain MC58 with an average efficiency of 0.06952 transformants/CFU/pmol DNA, demonstrating that the DNA was capable of affecting transformation in compatible strains. It is also important to note that the competence of each GMO was demonstrated through transformation with homotypic DNA (i.e. chromosomal DNA extracted from a strain derived from Y92-1009). Using an equimolar amount of chromosomal DNA purified from an antibiotic resistant mutant derivative of Y92-1009, strain *ΔnlaIII:aphA3*, we observed an average transformation efficiency of 1.88 transformants/CFU/pmol DNA in strain 4NB1.

NB: To produce the GMOs in the first instance required specialised, artificially produced nucleic acids to circumvent the barrier to transformation (see Item 20a: Step 3).

In addition, the genomic integrity of *N. lactamica* Y92-1009 *in vivo* was demonstrated by serial isolates of the wild type strain, taken from participants involved in a longitudinal carriage study. Over the course of 6 months, there was no evidence of the acquisition of exogenous genetic material into the Y92-1009 genome, even in

individuals in whom meningococcal carriage was either present at the time of Nlac inoculation, or transiently afterwards.

60. The likelihood of post-release selection leading to the expression of unexpected or undesirable traits in the genetically modified organisms.

The NadA-expressing GMO does not have a selective or survival advantage in the environment. The role of the NadA adhesin in the bacterium's biological niche is as-yet undefined, but the NadA protein does not bind DNA and will therefore not render the GMO more likely to take up DNA from the environment. Indeed, the GMO has been shown to be no more competent than wild type bacteria in assimilating hypermethylated PCR products into its chromosome. Due to the robust protection of genomic integrity offered by the bacterium's suite of restriction endonucleases, neither GMO is anticipated to assimilate genes coding for new traits (see Item 59b).

The most undesirable trait the GMO could acquire is the ability to synthesise and deposit capsular polysaccharide on its surface, and the most likely source of these genes is *N. meningitidis* (due to the presence of DUS in Nmen chromosomal DNA). The risk of this is mitigated in the first instance by exclusion of volunteers that carry meningococci during the pre-study screening. Subsequent to this, Nlac-colonised individuals are likely to be protected from (re-)acquisition of Nmen by an as-yet undefined mechanism (see Item 68). In the rare event of Nlac and Nmen co-carriage (which occurred transiently in our previous human challenge experiments), the risk of the GMO acquiring capsule genes from Nmen is negligible. We demonstrated this using purified chromosomal DNA from a mutant strain of *N. meningitidis* MC58 that had the capsule synthesis gene, *siaD,* insertionally inactivated by the kanamycin resistance gene, *aphA3* (*ΔsiaD:aphA3*). Under laboratory conditions optimised for *Neisseria* transformation, we exposed nascent colonies of the GMOs to 1 µg of this purified DNA suspension. Whilst the DNA was effective at transforming wild type *N. meningitidis* strain MC58 (0.04 transformants/CFU/pmol DNA), it was incompatible with the recombinogenic processes present in the Nlac strains, in which the transformation efficiency was *zero* (n = 6).

61. The measures employed to ensure and to verify genetic stability, the description of genetic traits which may prevent or minimise dispersal of genetic material and methods to verify genetic stability.

The genetic stability of the GMOs with respect to the uptake of markers from *N. meningitidis* chromosomal DNA is described in Item 59b and Item 60, and shown in Figure 6, Item 69. The NadA-expressing GMO is completely refractory to

transformation with purified DNA extracted from *N. meningitidis* mutants, which given the bias inherent in the *Neisseria* competence machinery for homotypic DNA constitutes the scenario in which there is the greatest likelihood of the GMO becoming transformed. We interpret this as meaning that the likelihood of the GMOs assimilating genetic material from other organisms in the nasopharynx is negligible. In addition, there was concern that repeated selection for (putative) transformants might have inadvertently selected for an inherently 'more transformable' phenotype. Measurement of the transformation efficiencies of the GMOs with hypermethylated PCR constructs, which circumvent the barrier to transformation constituted by the restriction endonuclease activities of NIac, demonstrated that this was not the case, and that the GMOs were only *as likely* as the wild type to take up and assimilate exogenous DNA (see Item 10 and Item 69).

With respect to the stability of the introduced genetic material over time, the GMOs were serially passaged over the course of 28 days *in vitro*. Samples of the original GMOs and the GMOs after serial passage were sent for whole genome sequencing (see Item 11 and Item 27). The fidelity of the gene(s) introduced into the GMOs was maintained over the course of the 28 days of *in vitro* passage, a process shown to permit a larger amount of genome variability, as compared to continuous carriage in the organism's biological niche. In support of the genomic data, the levels of NadA detected on the surface of strain 4NB1 were unchanged after serial passage (see Item 28).

With respect to minimising the dispersal of genetic material, the fact that the gene(s) are maintained in single copy and integrated into the bacterial chromosome reduces the likelihood of spread to other nasopharyngeal inhabitants. As discussed in Item 59a, there remains a possibility that the genes will be assimilated by other bacteria in the same niche, but this is anticipated to be a very low frequency event that is likely to prove a survival disadvantage over the longer term.

In terms of genetic stability the GMOs are anticipated to behave similarly to the wild type strain *in vivo*, insofar as colonisation seems to be a stabilising factor and to reduce the amount of observable mutation/recombination occurring over time. Analysis of the genomic stability of serial GMO isolates taken from volunteers is one of the study objectives, but by its nature can only be performed post-challenge. The identification of GMOs from study participants can be assured through the use of specific PCR, which can be used to detect the unusual proximity of the *porA*-associated UAS and the β-galactosidase genes (*lacZ*) (see Item 7 and Item 8).

62. The routes of biological dispersal, known or potential modes of interaction with the disseminating agent, including inhalation, ingestion, surface contact and burrowing.

The transmission of *N. lactamica* is assumed to be similar to *N. meningitidis* i.e. exclusively respiratory droplet borne and exclusively human to human, though no evidence of the former currently exists for *N. lactamica*. Transmission of *N. meningitidis* between humans occurs with the greatest frequency in household contacts (especially bedroom-sharers), sexual contacts, and in people who attend pubs and clubs, or live in University dormitories/halls of residence. Therefore to limit transmission we will train the participants in infection control, request that they do not attend pubs and clubs in the 2 weeks after discharge (because there is evidence that onward transmission between humans occurs in the first 2 weeks after acquisition [42]) and monitor (and offer eradication treatment) to bedroom contacts. We will additionally ask that volunteers refrain from performing oral sex for the duration of the study, to minimise the risk of disseminating the gene expression construct to *Neisseria gonorrhoeae.*

63. The description of ecosystems to which the organisms could be disseminated.

It can only be transmitted between humans.

64. The potential for excessive population increase of the organisms in the environment.

Although there is a potential competitive advantage over wild type *N. lactamica* (see Item 65) in terms of initial colonisation, the balance of probability is that this colonisation will be short lived. The wild type organism in nature colonises young infants and toddlers, rather than young adults who will be the participants in the proposed experiment. If the GMO is transmitted to this younger age group in the community there is potential that initial colonisation will be achieved to the same degree as wild type, which normally circulates to young children. However this is unlikely to be sustained given that NadA is one component of the Bexsero vaccine, which is now administered in the vaccine schedule to all children in the UK, in a series commencing at age 2 months.

65. The competitive advantage of the organisms in relation to the unmodified recipient or parental organism or organisms.

It is possible that NadA expression by the GMO will confer a competitive advantage over wild-type *N.lactamica*, specifically in terms of the ability of the GMO to colonise the nasopharynx in individuals not currently colonised with a strain of *N. lactamica*. *In vitro* analysis of the GMO's binding characteristics to epithelial cells suggests

there may be an advantage in establishing colonisation (see Item 29). However, the inability of the GMO to down-regulate expression of NadA (see Item 24) suggests that any colonisation may be shorter-lived than that of the wild type bacteria. This is in light of the NadA protein generating sterilising, colonisation-inhibiting immunity in a transgenic mouse model [7], and the fact that NadA expression is naturally down-regulated by phase variation in carriage strains of *N. meningitidis* [6], suggesting continued expression of the protein is a liability.

66. The identification and description of the target organisms if applicable.

Healthy adult humans aged 18-45 years will be the target recipients in the proposed clinical study. Strict inclusion and exclusion criteria will be applied in the research protocol to prevent inoculation of immunosuppressed adults in the clinical study, and to exclude adults who have household contact with immunosuppressed individuals.

67. The anticipated mechanism and result of interaction between the released organisms and the target organisms if applicable.

In the proposed study, the ability of humans to mount an immune response after intranasal exposure to NadA expressed by the GMO will be measured and compared to immune responses in humans infected with a control bacterium that does not express NadA. It is anticipated that the NadA-expressing GMO (4NB1) will colonise the nose and throat of the volunteers, who will then make antibodies against NadA. These antibodies are anticipated to be detectable in the blood by 2 weeks after the start of colonisation. This event will signal that there has been a biologically relevant interaction between the GMO and the human host.

In the proposed study, volunteers will be infected intranasally and then admitted to the Clinical Research Facility of University Hospital Southampton (i.e. they will be inpatients) for 5 days to ensure there is no early effect on health (this is based on a comparison with *Neisseria meningitidis*, the cause of meningococcal disease and the widely accepted view that most meningococcal disease occurs within a few days of intranasal infection of humans by *N. meningitidis*) [44];[45]. They will then be discharged and followed up regularly as outpatients until the 14th and 28th days of infection, when blood and respiratory samples will be taken to monitor immune response. Subsequently they will continue to be followed up as outpatients for 3 months. During this period it is anticipated that colonisation of the volunteers with the GMO will decline.

If, as anticipated, the colonisation of the human volunteers results in no ill-health, it will be possible in the future to conduct further studies to dissect the mechanisms of induction of (local) mucosal immunity within the upper respiratory tract by NadA, and

to measure the effect of NadA expressed by the GMO on the efficiency of colonisation by the GMO. These studies would require accurate power calculations, which will be informed by the results of the current proposed clinical study.

68. The identification and description of non-target organisms which may be adversely affected by the release of the genetically modified organisms, and the anticipated mechanisms of any identified adverse reaction.

Neisseria lactamica is a human-restricted commensal so no effect is anticipated on other higher organisms.

69. The likelihood of post-release shifts in biological interactions or in the host range.

The likelihood of this is small, but two scenarios need to be considered; (a) that the GMO will be further transformed by exogenous nucleic acid that changes the biology of the GMO and (b) that the GMO donates nucleic acid that modifies the biology of other commensals or pathobionts carried by humans infected by the GMO.

(a) that the GMO will be further transformed by exogenous nucleic acid that changes the biology of the GMO:

Horizontal gene transfer with other Neisseria *species:* There is evidence of frequent, interspecific genetic exchange between *Neisseria meningitidis* and *Neisseria lactamica* [18];[19];[20];[21], with genetic material from the latter being readily incorporated into the former and the subsequent generation of hybrid genotypes. Importantly, there is no published evidence of the opposite process, insofar as genetic material from the meningococcus is seemingly unable to transform *N. lactamica* [46]. We have experimental evidence of this for the NadA-expressing GMO; wherein exhaustive attempts to transform the bacteria using purified chromosomal DNA extracted from a panel of meningococcal mutant strains did not result in transformants (Figure 6). DNA was extracted from MC58 mutants in which the *nadA, opc* or *siaD* genes had been insertionally inactivated with the *aphA3* gene (*ΔnadA:aphA3, Δopc:aphA3 and ΔsiaD:aphA3*, respectively). These pools of genetic material were used under laboratory conditions optimised for *Neisseria* transformation to attempt to transform the GMO, which would result in kanamycin-resistant colony formation on selective agar if successful. As a demonstration of the natural competence of the GMO for DNA uptake, the strain was also transformed with chromosomal DNA extracted from a *ΔnlaIII:aphA3* mutant derivative of *N. lactamica* strain Y92-1009, which had been shown in preliminary work to transform *N. lactamica* with high efficiency.

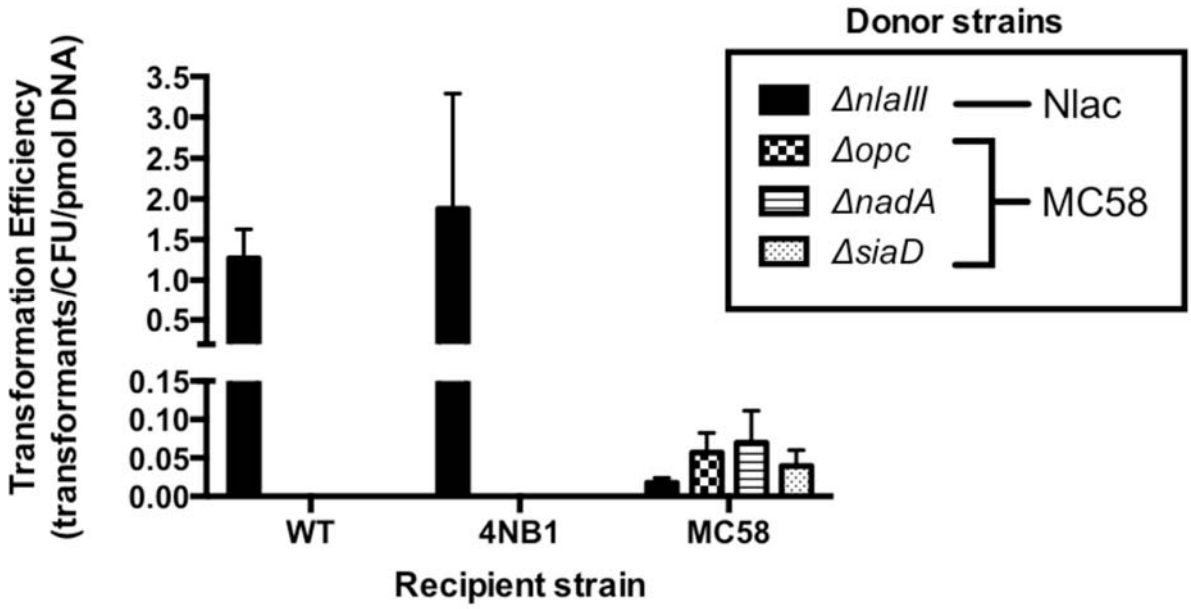


Figure 6: Transformation efficiencies of wild type *N. lactamica* strain Y92-1009, the NadA-expressing derivative thereof, 4NB1 and wild type *N. meningitidis* strain MC58 with purified chromosomal DNA extracted from a panel of *Neisseria* mutants.

The GMO was completely refractory to transformation with purified meningococcal DNA, as was the wild type Nlac strain, Y92-1009. This contrasted with wild type MC58, which was readily transformed by the same material. The reverse was not true however, as wild type MC58 was transformed at low efficiency by purified chromosomal DNA extracted from a mutant strain of Y92-1009, in which the same kanamycin-resistance gene was used to insertionally-inactivate the *nlaIII* gene. Transformation of the meningococcus with this Nlac-derived material represents *de novo* incorporation of heterologous genetic material into the Nmen genome, insofar as there is no homologue of the *nlaIII* gene in MC58. The experiment demonstrates that, although acquisition of new genetic material into the GMO from other *Neisseria* spp is extremely unlikely (a critically important consideration in regards to the potential acquisition of capsule synthesis genes, see below), there is the possibility that other co-carried *Neisseria* spp may be able to acquire the expression construct. However, as discussed in Item 17, the likelihood of other bacteria acquiring the *nadA* gene from the GMO is extremely low, and even then is unlikely to provide a significant evolutionary advantage to the recipient strain(s) over the longer term (see Item 65). Indeed, inappropriate and non-modifiable NadA-expression may represent a liability to the organism in terms of its capacity for long term colonisation (if indeed adaptive serological immunity has any impact on the ability of commensal bacteria to colonise the nasopharynx).

Horizontal gene transfer with non-Neisseria species: The *N. lactamica* chromosome contains regions of 'acquired' DNA, identified as such on the basis of a GC nucleotide content different from the chromosomal average [47]. In its evolutionary

history since speciation, there are relatively few incidences of horizontal gene transfer from organisms outside of the Neisseriaceae. Genes that are 'unique' to *N. lactamica* among the *Neisseria* include: the *lac* operon (*lacZ* and *lacY*), the low GC content of which suggests they may have been acquired from an unrelated oropharyngeal bacterium. In addition, there are four genes putatively involved in phosphorylcholine biogenesis (*licA, licB, licC* and *licD*), which may have been acquired by horizontal exchange from *Haemophilus influenzae.* There are also a small number of adhesins present in *Neisseria lactamica* that are absent from the pathogenic *Neisseria* [16];[11].

Transformability is not self-selecting: The most fundamental difference between Nlac and Nmen is the ability of the meningococcus to synthesise and deposit capsular polysaccharides onto its surface. A genome-based phylogenetic reconstruction provided evidence that *N. meningitidis* emerged as an unencapsulated human commensal from a common ancestor with *Neisseria gonorrhoeae* and *Neisseria lactamica* and consecutively acquired the genes responsible for capsule synthesis via horizontal gene transfer [48]. Since capsule expression is essential for the pathogenesis of meningococcal disease, the ability to produce and deposit capsule therefore represents *the* critical virulence determinant of *Neisseria meningitidis*. Like wild type Y92-1009, the GMO is acapsulate and remains acutely sensitive to killing by human complement (A.R. Gorringe, *personal communication*). We posit that the GMO poses no greater risk to human health than does the wild type Nlac strain, provided that the GMO has no greater propensity to assimilate exogenous DNA from its environment. Inadvertent selection for an inherently 'more-transformable' phenotype could plausibly increase the frequency with which DNA is taken up from the extracellular milieu, increasing the probability of the GMO acquiring genes for the production of a capsule. Our measurements of transformation efficiency using a well-characterised hypermethylated PCR fragment (*ΔnlaIII:aphA3*) show the GMOs have the same propensity to become transformed by exogenous DNA as does the wild type, indicating that there is no increased risk of acquiring (capsule synthesis) genes compared to challenge with wild type Y92-1009 (Figure 7).

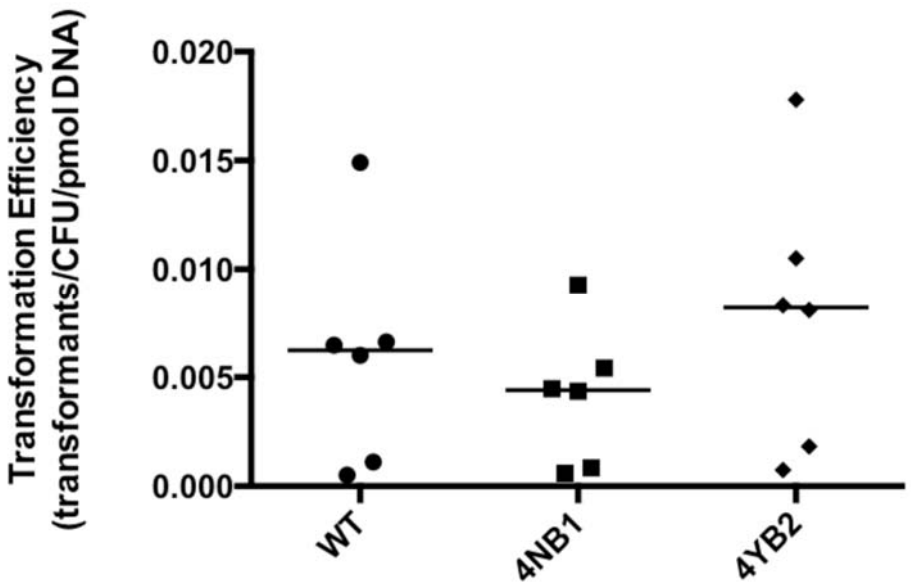


Figure 7: Transformation efficiencies of wild type *N. lactamica* strain Y92-1009 and two genetically modified derivatives thereof, the NadA-expressing strain 4NB1 and the procedural transformation control strain 4YB2; with hypermethylated PCR constructs containing a copy of the *nlaIII* coding sequence insertionally-inactivated with the *aphA3* (kan^R) gene. There are no significant differences in the observed transformation efficiencies between these strains (ANOVA with Tukey's Multiple Comparisons test, n = 6). Bars represent Median.

(b) that the GMO donates nucleic acid that modifies the biology of other commensals or pathobionts carried by humans infected by the GMO:

Neisseria spp demonstrate a significant uptake bias in favour of molecules containing the *Neisseria* DNA Uptake Sequence (DUS), which is a non-palindromic, ten nucleotide repeat sequence over-represented in *Neisseria* genomes (canonically: 5' – GCCGTCTGAA – 3'). Figure 8 shows that inclusion of more than one DUS in a hypermethylated donor DNA molecule is capable of significantly enhancing the efficiency of transformation of *Neisseria lactamica*, although the effect becomes more pronounced as the region of complementarity between the construct and the chromosome increases in length. The finding that multiple DUS increases transformation efficiency is consistent with data published on other *Neisseria* species [49].

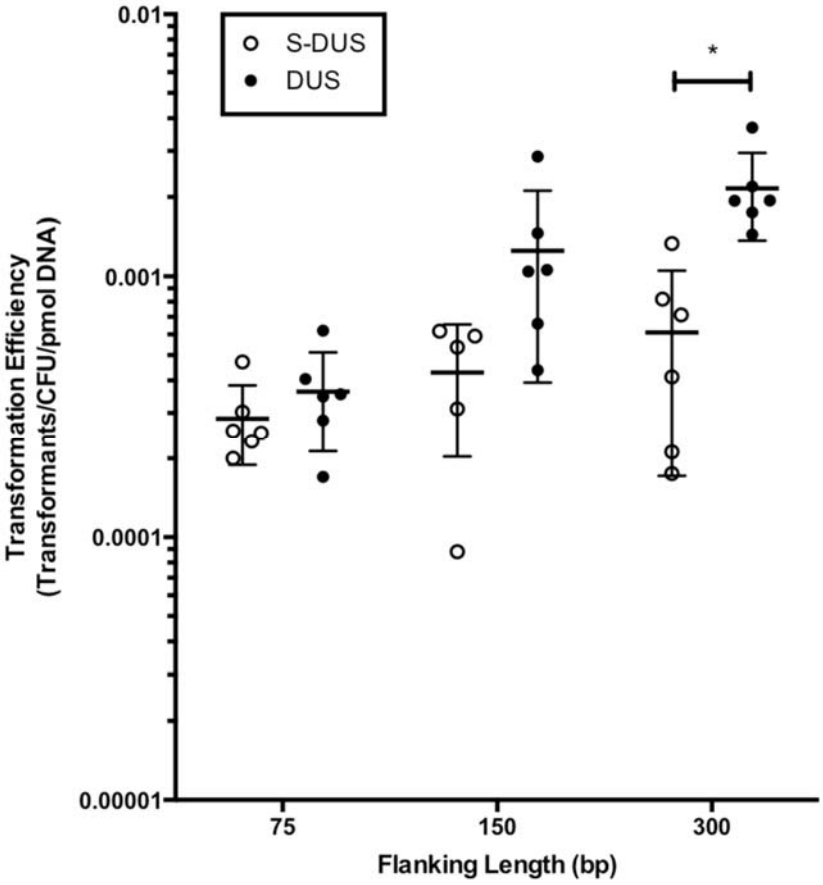


Figure 8: Transformation efficiency of wild type *N. lactamica* strain Y92-1009 with hypermethylated PCR constructs of different lengths and either one (S-DUS, *open circles*) or two (DUS, *closed circles*) DNA Uptake Sequence(s). To produce these pairs of PCR products, either of two forward primers were used with a common reverse primer to amplify the *ΔnlaIII:aphA3* cassette from a plasmid template. One primer contained a DUS at the 5'terminus, whilst the other contained a scrambled DUS (S-DUS) of the same nucleotide content, but in a different sequence (5' to 3'). Fifty micrograms (50 μg) of each of these constructs were used to transform wild type *N. lactamica*, and putative transformants were selected for on kanamycin containing media. *p<0.05, Paired samples t-test (n = 6). Bars represent Mean ± SD.

DUS are hypothesised to act as a conservative, rather than diversifying force, insofar as the competence apparatus of the cell is biased in favour of 'self' DNA, with an evolutionary imperative of promoting 'safe sex' between conspecifics [17]. The construct used to create the NadA-expressing GM-NIac (NHCIS1::HAEC4:*nadA-lacZ*, see Item 24), contains a DUS 301 bp upstream of the *nadA* START codon. It is therefore plausible that, in the context of nasopharyngeal colonisation with this organism, the *nadA* gene may be disseminated to other resident *Neisseria* species following lysis of the GM-NIac strain *in situ*. It is important to note however, that in the Donor chromosome (i.e. serogroup B strain MC58), there is an inverted repeat of the DUS only 262 bp upstream of the *nadA* START codon. As such, the GM-NIac is probably only as likely as *nadA*-containing meningococci to further disseminate the

allele. Remembering that meningococcal pathogenesis is a complex, subtle and polygenic trait, even in the event of the allele escaping into the population of resident *Neisseria meningitidis*, it is unlikely that the addition of a single meningococcal-specific adhesin would significantly enhance the pathogenic potential of an otherwise benign carriage strain.

In summary therefore, the natural DNA uptake mechanisms of *N. lactamica* are heavily biased in favour of genetic exchange with the Neisseriaceae, there are significant and robust barriers to horizontal genetic exchange in *N. lactamica* and the process of creating GM-Nlac does not enrich for inherently 'more transformable' cells. Taken together, and in light of the fact that *Neisseria* pathogenesis is a complex, polygenic trait, we postulate that the introduction of this single meningococcal gene into the genome of *N. lactamica* does not pose significant risk to inoculated volunteers.

70. The known or predicted interactions with non-target organisms in the environment, including competitors, prey, hosts, symbionts, predators, parasites and pathogens.

The only established interaction with non-target organisms in the context of experimental human challenge with *N. lactamica* is the apparent exclusion of *N. meningitidis* from the nasopharynx of Nlac-colonised individuals [2]. The precise mechanisms of this inverse association is still unknown, but is likely to manifest at an ecological level, with the two closely related species competing for the same or extremely similar niche(s). Repeated attempts in our laboratory to identify direct mechanisms of killing of one species by the other have been unsuccessful. Indeed, a recent paper demonstrated co-cultivation of these bacteria in a mixed-species biofilm [36], which corroborates our own, unpublished findings. We aim to shed more light on the impact of experimental challenge with this organism on the nasopharyngeal microbiota, although our preliminary analysis of sample microbiome data suggests the observed replacement phenomenon is exquisite and non-disruptive to other resident bacteria.

71. The known or predicted involvement in biogeochemical processes.

Not applicable.

72. Any other potentially significant interactions with the environment.

Not applicable.

Part V

Information on monitoring, control, waste treatment and emergency response plans

Monitoring techniques

73. Methods for tracing the organisms and for monitoring their effects.

These GMOs have not been used in a clinical study with healthy adult volunteers, or been released in the past. They have been extensively handled in the laboratories at University of Southampton and at Public Health England Porton Down and are easily verified using a GMO-specific PCR. However, the wild type (parent) Nlac strain has been released previously in four clinical studies conducted within the UK, either at Sheffield University or in a current project at University Hospital Southampton in a study approved by the Health Research Authority. All studies include a comprehensive monitoring of the safety of the wild type Nlac strain based on clinical, microbiological, haematological and biochemical measurements. In the proposed study described in this application, clinical study participants will be monitored for signs of infection with the GMO. The results of previous clinical studies using the wild type Nlac strain demonstrated no negative impact on human health. The potential environmental impact of the release of the GMO is thought to be zero.

For the clinical study, exclusion criteria will include current carriage of *Neisseria spp*. (assessed 7 days prior to inoculation), and any condition or medication causing immunosuppression in the volunteer or in their household or occupational contacts. The volunteers will be admitted to the Clinical Research Facility (CRF) of the University Hospital Southampton and will remain an inpatient within the hospital for the first 5 days following the inoculation and will then be discharged and followed up as an outpatient. The volunteers (aged 18-45) will receive an inoculum either of the NadA expressing GMO (4NB1) or of the control GMO (4YB2) (10^5 CFU) intranasally. The inoculum will be given with the volunteer lying supine in a category 2 chamber within the CRF. It is expected that the organism will then colonise the host in approximately 50% of recipients, as this is the colonisation rate observed with the wild type organism. This will be detected by culturing the GMOs from throat swabs and by detecting nucleic acid by PCR of nasal washes and throat swabs. It is predicted that the host will mount an immune response against the GMO without developing symptoms and the response will be detected within the blood (antibodies and possibly specific lymphocytes) and in the upper airway mucosa (salivary antibodies and possibly specific lymphocytes detected in nasal washes). It is predicted that all individuals will exhibit an immune response (IgG antibodies) against whole cell-derived *N. lactamica* outer membrane vesicles but only those individuals infected with the GMO expressing NadA will develop an immune response against NadA. In the unlikely event of development of disease, the GMOs

have confirmed sensitivity to the antimicrobials used to clear carriage (rifampicin and ciprofloxacin) or treat invasive disease (ceftriaxone).

Throat swabs and nasal washes will be taken at Day 0 (challenge), during the 5 day admission, and then at 7, 10, 14, 28 and 90 days post-challenge and at any point they experience any symptoms resulting in the necessity to terminate the study and treat the volunteer with eradicating antibiotics. It is expected that cultivation of the material from throat swabs will yield viable GMOs either 4 days or 7 days after inoculation, as this is what is most commonly observed after inoculation of the wild type organism. During the admission, the potential for environmental shedding of the GMOs will be assessed by measuring colony counts of organisms expired onto a mask worn for 1 hour, and from a 300 L/min air sampler conducted in an environmental chamber at the CRF. This will be done under the guidance of PHE Porton Down scientists (Dr Alan Bennett and team), both at intervals during the admission and at each outpatient visit.

74. Specificity (to identify the organisms and to distinguish them from the donor, recipient or, where appropriate, the parental organisms), sensitivity and reliability of the monitoring techniques.

The GMOs that are cultured from samples from volunteers and environmental collections can be easily confirmed to be the test and control strains through standard diagnostic microbiology techniques. Putative *Neisseria* colonies, identified on the basis of colony morphology, will be discriminated as *N. lactamica* in the first instance by metabolism of X-gal (generation of a blue breakdown product). If necessary (and pending appropriate approvals to analyse GMOs in the PHE Southampton diagnostic laboratories), this identification can be confirmed by MALDI-TOF, or by using a panel of conventional biochemical tests (API, biomerieux). Bacteria will be definitively identified as the target GMO using diagnostic PCR, which exploits the unnaturally close linkage of the *porA* promoter region and *lacZ* genes present in the gene expression construct of NHCIS1.

75. Techniques for detecting transfer of the donated genetic material to other organisms.

During initial screening and immediately before inoculation, nasal washes and throat swabs taken from volunteers will be cultured. As described in Item 74, putative *Neisseria* colonies will be identified as *N. lactamica* in the first instance by metabolism of X-gal to form a blue breakdown product. During this screen, the number of other β-galactosidase positive, non-*Neisseria* organisms will be noted to provide a baseline. If during the course of the study, at any return visit, the number

of β-galactosidase positive, non-*Neisseria* colonies cultured from a volunteer should increase from baseline by more than two fold, then representative samples of each β-galactosidase positive species will be cultivated and used to prepare genomic DNA. Each of these representative DNA samples will then be subjected to the same diagnostic PCR as the putative GMO colonies, to determine whether there has been dissemination of the gene expression cassette. The diagnostic PCR exploits the unnaturally close linkage of the *porA* promoter region and the *lacZ* gene, which due to their proximity we assume will be transferred as a single unit. Whilst we acknowledge that the numbers of β-galactosidase positive, non-*Neisseria* species is liable to fluctuate, it is prudent to set a low threshold for initiating this procedure, as allele escape will need to be reported for consideration by the Data Safety Monitoring Board (DSMB, see Item 76). We acknowledge the limitation of this procedure is that it will only allow us to identify allele escape into a narrow range of other nasopharyngeal commensals, specifically those that can be co-cultured on the media used to sustain the recovered GMOs.

76. Duration and frequency of the monitoring.

Monitoring will take place for the duration of the clinical study. The study is expected to commence on the 1st of November 2017 (pending all necessary approvals) and will run for approximately 13 months (with an expected completion date of 30th of November 2018). All study participants will have follow-up visits up to 90 days post challenge with nasopharyngeal sampling at regular intervals as outpatients. If the participants share a bedroom with someone, that person will undergo a throat swab at baseline and also 14 days after discharge to identify transmission to other humans. If those contacts are found to carry *N.lactamica*, they will be offered ciprofloxacin eradication therapy.

Following challenge with the GMOs, participants will be monitored daily as inpatients for the first 5 days post-challenge. Continuous participant safety monitoring will occur throughout the challenge period through a combination of daily clinical review and monitoring of symptoms in an electronic diary. All study participants will agree to have 24-hour contact with study staff during the 90 days post challenge and to be able to ensure that they are contactable by mobile phone for the duration of the challenge period until the end of the study.

An independent Data Safety Monitoring Board (DSMB) will be established prior to the start of the study. The DSMB will be appointed to provide real-time oversight of safety and trial conduct. The DSMB will have access to data and, if required, will monitor these data and make recommendations to the study investigators on whether there are any ethical or safety reasons why the study should not continue. The DSMB will also be notified immediately if the study team have any concerns regarding the safety of a participant or the general public (e.g. if a participant

develops disease). The outcome of each DSMB review will be communicated directly to the study investigators and documentation of all reviews will be kept in the trial master file. The Chair of the DSMB will also be contacted for advice when the Chief Investigator feels independent advice or review is required.

Control of the release

77. Methods and procedures to avoid and/or minimise the spread of the organisms beyond the site of release or the designated area for use.

Neisseria lactamica carriage is restricted to humans. It is carried exclusively in the nasopharynx and transmits between humans by close contact. There is an inverse relationship between carriage of *N.lactamica* and *N.meningitidis* and the two organisms have similar relationships with humans, except that *N.lactamica* does not cause disease.

Neisseria meningitidis is known to transmit to close contacts of those carrying the organism, particularly those who share households. In a cardinal study undertaken in the UK between 1986 and 1987, 55 cases of meningococcal disease were identified in the South-West of England, an attack rate of 1.54 per 100,000 during the study period. The overall meningococcal carriage rate in 384 close contacts was 18.2% and the carriage rate of the case strain was 11.1%. The carriage rate of indistinguishable strains in household contacts (16.0%) was higher than the carriage rate in contacts living at other addresses (7.0%, $P < 0.05$) [50]. In one Norwegian study of the contacts of index cases of meningococcal disease, there was a high rate of carriage of the pathogenic strain of *N. meningitidis* in patients' household members and kissing contacts, and this supports the practice of giving chemoprophylaxis to these contacts. The prevalence of carriage among other contacts was 2-3 times that found in the general population (0.7%) [51]. After an outbreak of meningococcal disease in Singapore caused by *N.meningitidis* W, associated with the Hajj pilgrimage in 2001, 15% of returning vaccinated pilgrims carried a single serogroup W clone, and 55% of these were still carriers 6 months later. Transmission to 8% of their unvaccinated household contacts occurred within 2 weeks, but no late transmission took place [42].

Of course there is much less information on transmission of *N. lactamica*. Carriage of *N.lactamica* among household contacts of meningococcal disease cases was investigated during a meningococcal epidemic in Auckland, New Zealand. The overall carriage rate for *N. lactamica* was 10.5% (95% CI 7.4-13.5%) with a peak carriage rate in 2-year-olds of 61.5% (95% CI 26.6-88.1%). Factors associated with a significant ($p < 0.05$) increase in the likelihood of carriage included runny nose, the number of people per bedroom and youth. Genetic analysis of isolates revealed a striking correlation of strains within the same household but a high level of diversity

between households, suggesting that household contact is an important factor in acquisition [52].

From these data we conclude that household members, in particular bedroom-sharers of the participants, will be the likeliest persons to be colonised by onward transmission of the GMO. However transmission beyond the household setting will be possible. We know that the wild type strain can persist in the nasopharynx for 6 months, and although onward transmission of *N.meningitidis* is most likely in the first 2 weeks after acquisition [42], transmission of the *N.lactamica* GMO at any time cannot be absolutely prevented. Therefore the action we propose is to limit as much as is reasonable, onward transmission into the community.

To minimise the community spread of the GMO, strict infection control procedures will be enforced during the inpatient stay of the participants in the NIHR CRF. Participants will be trained in infection control, and they will undertake to practice this after discharge at the conclusion of their residential stay in hospital.

Following discharge, the participants will be returning to their community. If they share a bedroom, the bedroom-sharer will be required to provide informed consent *a priori*, and a baseline swab will be taken, plus a throat swab 14 days after discharge of the participant from hospital. The bedroom sharer will also be trained in infection control practice, and will be offered single dose ciprofloxacin eradication therapy if they become colonised at 14 days after discharge of the participant. The participant will be required to avoid pubs and clubs in the 2 weeks after discharge and be asked to refrain from performing oral sex.

One of the exclusion criteria is that participants must be persons who have no contact with immunosuppressed individuals. The purpose of this is to limit the possibility of carriage of the GMO by anyone who might be vulnerable to infections in which *N. lactamica* might become involved.

Public Health Southampton (now part of Southampton City Council) will be informed of all participants who have been challenged with the GMOs. The participants' GP will also be notified. In addition, any unexpected occurrence of disease in participants will be notified to the data and safety monitoring board (DSMB). If the DSMB considers the event to be causally related to the GM, the study will be stopped, per protocol and this information will be passed on immediately to PHE and Public Health Southampton as well as the GP.

78. Methods and procedures to protect the site from intrusion by unauthorised individuals.

The entrance to the Southampton NIHR Clinical Research Facility has an entry code and is manned at a reception desk. The activities at University Hospital Southampton are regularly monitored with onsite security service provisions established.

79. Methods and procedures to prevent other organisms from entering the site.

The infection control procedures instituted at the Southampton NIHR Clinical Research Facility are suitable to prevent nosocomial transmission of exogenous hospital organisms, as well as outward transmission of the GMO.

Waste treatment

80. Type of waste generated.

Relevant clinical waste (blood/saliva/nasal swab samples, tissues, sharps, syringes, disposable clothing, gloves, gowns, masks and aprons).

Laboratory waste (including but not limited to: plastic ware, microbiological waste (agar plates/blood culture bottles, filters), gloves, disposable clothing, paper towels, and clinical samples as described above).

81. Expected amount of waste.

The amount of waste will be typical for the clinical site and laboratory operations amounting to a few clinical waste bags and bins per day. The amount of expected waste will be managed by standard operating procedures currently in place at the site.

82. Description of treatment envisaged.

Waste will be treated according to site standard operating procedures (SOPs) for handling GMO and potentially infectious GMO waste. All GMO activity at site will be inactivated by autoclaving prior to disposal and removal from the site. All associated procedures have been validated, site autoclaves are accredited annually with contracts in place for regular equipment servicing and maintenance.

Post-release GMO shed in the nasopharyngeal samples of study participants will be eliminated according to the SOPs described above.

Emergency response plans

83. Methods and procedures for controlling the organisms in case of unexpected spread.

Within the Southampton NIHR Clinical Research Facility strict precautions are in place to avoid the spread of the GMO from the study participant to others. If the participant sneezes onto a surface at any time, this will be treated as any spillage per SOPs.

Public Health England (PHE) investigators are involved in this study, and the relevant section of the organisation (e.g. the Meningococcal Reference Unit) will be informed that it is underway. Public Health Southampton (now part of Southampton City Council) will be informed of all participants who have been challenged with the GMOs. The participants' GP will also be informed. In addition, any unexpected occurrence of disease in participants will be notified to the data and safety monitoring board. If the DSMB considers the event to be causally related to the GM, the study will be stopped, per protocol and this information will be passed on immediately to PHE and Public Health Southampton as well as the GP.

Unexpected spread within the community would be detected if (a) microbiology laboratories reported to Public Health England that an unusually high number of *N.lactamica* isolates were being detected in routine throat swabs taken from children or adults with suspected sepsis, or (b) disease occurred in individuals other than participants in the study. Of these, (a) would require microbiology laboratories to be made aware of the study and to be asked not to discard non-pathogenic *Neisseria* cultured from routine swabs during the period of the study, and (b) is extremely unlikely because the GMO is non-capsulate and non-virulent in a recognised animal model of *Neisseria* disease, but would be detected in routine speciation of invasive isolates of a patient with sepsis. *Neisseria lactamica* would be such an unusual cause of disease that PHE surveillance would track the occurrence back to this study, via the PHE Meningococcal Reference Unit, who are aware of this study, and hold stock of the wild type organism. If (a) occurs it is unlikely that action would be taken in the absence of any disease caused by the GMO, but public health authorities would have the option of using the same strategy that is used in outbreaks of meningococcal disease, i.e. single dose ciprofloxacin to clear carriage in close contacts or alternatively to vaccinate with the NadA-containing vaccine Bexsero, which has been shown to protect against the occurrence of invasive disease in the case of *N.meningitidis*. If (b) occurs then index cases would be treated with ceftriaxone and contacts would have prophylaxis with ciprofloxacin. Bexsero vaccine would be available if public health authorities deemed it necessary to protect larger populations.

To detect potential disease in participants, they will be instructed to notify the study team of any serious adverse events/reactions following administration of the GMO.

All participants agree to have 24-hour contact with study staff during the 90 days post challenge and to be able to ensure that they, or a friend/family member are contactable by mobile phone for the duration of the challenge period until antibiotic completion. A physician from the clinical team will be on-call 24 hours.

Participants will be issued with a Medic Alert-type card containing information including the antibiotic sensitivity of the GMO, study doctor contact details and instruction for the research team to be contacted immediately in the event of illness/accident.

Potential participants with known antibiotic hypersensitivity or allergy to either of the first-line antibiotics (ciprofloxacin, ceftriaxone) will be excluded from the study.

There are provisions within the protocol and site facilities to allow for admissions of participants as inpatients to University Hospital Southampton in cases of sepsis arising in participants during the study period.

84. Methods, such as eradication of the organisms, for decontamination of the areas affected.

Contaminated areas such as laboratory or clinical area spills may be decontaminated using 2 % Virkon solution and a minimum of 2 mins of contact time. Clinical carriage can be eradicated with single dose oral ciprofloxacin.

85. Methods for disposal or sanitation of plants, animals, soils and any other thing exposed during or after the spread.

Not applicable.

86. Methods for the isolation of the areas affected by the spread.

As this organism exclusively colonises humans, containing onward transmission between individuals could isolate the spread of the GMO. If public health authorities considered this to be necessary the strategy would follow the general approach that is used to control meningococcal disease outbreaks, i.e. use of ciprofloxacin single dose to eradicate carriage in all household or other close contacts of carriers.

87. Plans for protecting human health and the environment in case of the occurrence of an undesirable effect.

If there is detection of asymptomatic carriage of the GMO in the community apparently unconnected with the participant it is unlikely that any action would be taken by public health authorities, but public health authorities would have the option of using the same strategy that is used in outbreaks of meningococcal disease, i.e. single dose ciprofloxacin to clear carriage in close contacts or alternatively (if there is disease caused by the GMO) to vaccinate with the NadA-containing vaccine Bexsero which has been shown to protect against the occurrence of invasive disease in the case of *N.meningitidis*. In the extremely unlikely event of disease occurring in the participants, or in an unconnected person, they would be treated with the intravenous antibiotic ceftriaxone and contacts would have prophylaxis with the oral antibiotic ciprofloxacin, as per clinical guidelines for meningococcal disease. Bexsero vaccine, which contains the same NadA antigen as the GMO would be available if public health authorities deemed it necessary to protect larger populations.

The environment will be unaffected because this organism exclusively colonises humans.

Part VI

A description of the methods used or a reference to standardised or internationally recognised methods used to compile the information required by this schedule, and the name of the body or bodies responsible for carrying out the studies.

The critical steps we have taken and the expertise we have used to compile this application for deliberate release include:

• Construction of the GMO

• Stability and function of the GMO

• Experience of conducting controlled infection studies in healthy adult volunteers

The bodies responsible for carrying out the studies are

- University of Southampton
- University Hospital Southampton
- Public Health England

References:

1. Evans, C.M., et al., *Nasopharyngeal colonization by Neisseria lactamica and induction of protective immunity against Neisseria meningitidis.* Clin Infect Dis, 2011. **52**(1): p. 70-7.
2. Deasy, A.M., et al., *Nasal Inoculation of the Commensal Neisseria lactamica Inhibits Carriage of Neisseria meningitidis by Young Adults: A Controlled Human Infection Study.* Clin Infect Dis, 2015. **60**(10): p. 1512-20.
3. Capecchi, B., et al., *Neisseria meningitidis NadA is a new invasin which promotes bacterial adhesion to and penetration into human epithelial cells.* Mol Microbiol, 2005. **55**(3): p. 687-98.
4. Vogel, U., et al., *Predicted strain coverage of a meningococcal multicomponent vaccine (4CMenB) in Europe: a qualitative and quantitative assessment.* Lancet Infect Dis, 2013. **13**(5): p. 416-25.
5. Metruccio, M.M., et al., *A novel phase variation mechanism in the meningococcus driven by a ligand-responsive repressor and differential spacing of distal promoter elements.* PLoS Pathog, 2009. **5**(12): p. e1000710.
6. Alamro, M., et al., *Phase variation mediates reductions in expression of surface proteins during persistent meningococcal carriage.* Infect Immun, 2014. **82**(6): p. 2472-84.
7. Johswich, K.O., et al., *In vivo adaptation and persistence of Neisseria meningitidis within the nasopharyngeal mucosa.* PLoS Pathog, 2013. **9**(7): p. e1003509.
8. Packiam, M., et al., *Differential expression and transcriptional analysis of the alpha-2,3-sialyltransferase gene in pathogenic Neisseria spp.* Infect Immun, 2006. **74**(5): p. 2637-50.
9. Snyder, L.A. and N.J. Saunders, *The majority of genes in the pathogenic Neisseria species are present in non-pathogenic Neisseria lactamica, including those designated as 'virulence genes'.* BMC Genomics, 2006. **7**: p. 128.
10. Stabler, R.A., et al., *Identification of pathogen-specific genes through microarray analysis of pathogenic and commensal Neisseria species.* Microbiology, 2005. **151**(Pt 9): p. 2907-22.
11. Bennett, J.S., et al., *Independent evolution of the core and accessory gene sets in the genus Neisseria: insights gained from the genome of Neisseria lactamica isolate 020-06.* BMC Genomics, 2010. **11**: p. 652.
12. Cartwright, K.A., et al., *The Stonehouse survey: nasopharyngeal carriage of meningococci and Neisseria lactamica.* Epidemiol Infect, 1987. **99**(3): p. 591-601.
13. Gold, R., et al., *Carriage of Neisseria meningitidis and Neisseria lactamica in infants and children.* J Infect Dis, 1978. **137**(2): p. 112-21.
14. Yazdankhah, S.P. and D.A. Caugant, *Neisseria meningitidis: an overview of the carriage state.* J Med Microbiol, 2004. **53**(Pt 9): p. 821-32.
15. Qiang, B.Q. and I. Schildkraut, *Two unique restriction endonucleases from Neisseria lactamica.* Nucleic Acids Res, 1986. **14**(5): p. 1991-9.
16. van Passel, M.W., et al., *Identification of acquired DNA in Neisseria lactamica.* FEMS Microbiol Lett, 2006. **262**(1): p. 77-84.
17. Treangen, T.J., et al., *The impact of the neisserial DNA uptake sequences on genome evolution and stability.* Genome Biol, 2008. **9**(3): p. R60.

18. Saez-Nieto, J.A., et al., *Neisseria lactamica and Neisseria polysaccharea as possible sources of meningococcal beta-lactam resistance by genetic transformation.* Antimicrob Agents Chemother, 1990. **34**(11): p. 2269-72.
19. Taha, M.K., et al., *Target gene sequencing to characterize the penicillin G susceptibility of Neisseria meningitidis.* Antimicrob Agents Chemother, 2007. **51**(8): p. 2784-92.
20. Karch, A., U. Vogel, and H. Claus, *Role of penA polymorphisms for penicillin susceptibility in Neisseria lactamica and Neisseria meningitidis.* Int J Med Microbiol, 2015. **305**(7): p. 729-35.
21. Maiden, M.C., *Population genomics: diversity and virulence in the Neisseria.* Curr Opin Microbiol, 2008. **11**(5): p. 467-71.
22. Snyder, L.A., J.A. Cole, and M.J. Pallen, *Comparative analysis of two Neisseria gonorrhoeae genome sequences reveals evidence of mobilization of Correia Repeat Enclosed Elements and their role in regulation.* BMC Genomics, 2009. **10**: p. 70.
23. Budroni, S., et al., *Neisseria meningitidis is structured in clades associated with restriction modification systems that modulate homologous recombination.* Proc Natl Acad Sci U S A, 2011. **108**(11): p. 4494-9.
24. Dempsey, J.A., A.B. Wallace, and J.G. Cannon, *The physical map of the chromosome of a serogroup A strain of Neisseria meningitidis shows complex rearrangements relative to the chromosomes of the two mapped strains of the closely related species N. gonorrhoeae.* J Bacteriol, 1995. **177**(22): p. 6390-400.
25. Spencer-Smith, R., et al., *Sequence features contributing to chromosomal rearrangements in Neisseria gonorrhoeae.* PLoS One, 2012. **7**(9): p. e46023.
26. Schoen, C., et al., *Genome flexibility in Neisseria meningitidis.* Vaccine, 2009. **27 Suppl 2**: p. B103-11.
27. Gibbs, C.P. and T.F. Meyer, *Genome plasticity in Neisseria gonorrhoeae.* FEMS Microbiol Lett, 1996. **145**(2): p. 173-9.
28. Frye, S.A., et al., *Dialects of the DNA uptake sequence in Neisseriaceae.* PLoS Genet, 2013. **9**(4): p. e1003458.
29. Goodyear-Smith, F., *What is the evidence for non-sexual transmission of gonorrhoea in children after the neonatal period? A systematic review.* J Forensic Leg Med, 2007. **14**(8): p. 489-502.
30. Tzeng, Y.L., L.E. Martin, and D.S. Stephens, *Environmental survival of Neisseria meningitidis.* Epidemiol Infect, 2014. **142**(1): p. 187-90.
31. Falsetta, M.L., et al., *The Composition and Metabolic Phenotype of Neisseria gonorrhoeae Biofilms.* Front Microbiol, 2011. **2**: p. 75.
32. Neil, R.B. and M.A. Apicella, *Clinical and laboratory evidence for Neisseria meningitidis biofilms.* Future Microbiol, 2009. **4**(5): p. 555-63.
33. Sim, R.J., et al., *Underestimation of meningococci in tonsillar tissue by nasopharyngeal swabbing.* Lancet, 2000. **356**(9242): p. 1653-4.
34. Liu, G., C.M. Tang, and R.M. Exley, *Non-pathogenic Neisseria: members of an abundant, multi-habitat, diverse genus.* Microbiology, 2015. **161**(7): p. 1297-312.
35. O'Dwyer C, A., et al., *Expression of heterologous antigens in commensal Neisseria spp.: preservation of conformational epitopes with vaccine potential.* Infect Immun, 2004. **72**(11): p. 6511-8.

36. Perez-Ortega, J., et al., *Interstrain Cooperation in Meningococcal Biofilms: Role of Autotransporters NalP and AutA.* Front Microbiol, 2017. **8**: p. 434.
37. Elkins, C., et al., *Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence.* J Bacteriol, 1991. **173**(12): p. 3911-3.
38. Mielcarek, N., et al., *Live attenuated B. pertussis as a single-dose nasal vaccine against whooping cough.* PLoS Pathog, 2006. **2**(7): p. e65.
39. Thorstensson, R., et al., *A phase I clinical study of a live attenuated Bordetella pertussis vaccine--BPZE1; a single centre, double-blind, placebo-controlled, dose-escalating study of BPZE1 given intranasally to healthy adult male volunteers.* PLoS One, 2014. **9**(1): p. e83449.
40. Gorringe, A.R., et al., *Phase I safety and immunogenicity study of a candidate meningococcal disease vaccine based on Neisseria lactamica outer membrane vesicles.* Clin Vaccine Immunol, 2009. **16**(8): p. 1113-20.
41. Gorringe, A.R., et al., *Experimental disease models for the assessment of meningococcal vaccines.* Vaccine, 2005. **23**(17-18): p. 2214-7.
42. Wilder-Smith, A., et al., *Persistence of W135 Neisseria meningitidis carriage in returning Hajj pilgrims: risk for early and late transmission to household contacts.* Emerg Infect Dis, 2003. **9**(1): p. 123-6.
43. Marri, P.R., et al., *Genome sequencing reveals widespread virulence gene exchange among human Neisseria species.* PLoS One, 2010. **5**(7): p. e11835.
44. Edwards, E.A., et al., *Immunological investigations of meningococcal disease. III. Brevity of group C acquisition prior to disease occurrence.* Scand J Infect Dis, 1977. **9**(2): p. 105-10.
45. Gilmore, A., et al., *Meningococcal disease at the University of Southampton: outbreak investigation.* Epidemiol Infect, 1999. **123**(2): p. 185-92.
46. H.K., T., et al., *Natural competence for transformation in Neisseria lactamica*, in *14th International Pathogenic Neisseria Conference*. 2004: Milwaukee, Wisconsin USA. p. 241.
47. van Passel, M.W., et al., *An in vitro strategy for the selective isolation of anomalous DNA from prokaryotic genomes.* Nucleic Acids Res, 2004. **32**(14): p. e114.
48. Schoen, C., et al., *Whole-genome comparison of disease and carriage strains provides insights into virulence evolution in Neisseria meningitidis.* Proc Natl Acad Sci U S A, 2008. **105**(9): p. 3473-8.
49. Ambur, O.H., et al., *Restriction and sequence alterations affect DNA uptake sequence-dependent transformation in Neisseria meningitidis.* PLoS One, 2012. **7**(7): p. e39742.
50. Cartwright, K.A., J.M. Stuart, and P.M. Robinson, *Meningococcal carriage in close contacts of cases.* Epidemiol Infect, 1991. **106**(1): p. 133-41.
51. Kristiansen, B.E., Y. Tveten, and A. Jenkins, *Which contacts of patients with meningococcal disease carry the pathogenic strain of Neisseria meningitidis? A population based study.* BMJ, 1998. **317**(7159): p. 621-5.
52. Simmons, G., et al., *Carriage of N. lactamica in a population at high risk of meningococcal disease.* Epidemiol Infect, 2000. **125**(1): p. 99-104.