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 organisms (Deliberate Release) Regulations 2002

Part I: General information

1. The name and address of the applicant responsible for planning and carrying out the release of the organisms and for the supervision, monitoring and safety of the release.

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United Kingdom Health Security Agency, Porton Down, Salisbury SP4 0JG, UK

2. The title of the project.

Safety, colonisation and immunogenicity following nasal inoculation with genetically modified *Neisseria lactamica* expressing Factor H binding protein and *Neisseria* adhesin A

STUDY SUMMARY.

The purpose of the genetic modification is to construct 4 strains of the exclusively human, nasopharyngeal commensal bacterium, *N. lactamica*, so that each strain expresses on its surface:

(i) the type V meningococcal autotransporter, *Neisseria* Adhesin A (NadA)
(ii) one of four different variants of the meningococcal outer membrane protein, Factor H Binding Protein (FHbp), specifically variants 1.4, 1.13, 2.19 and 3.45, each of which has been specifically mutated to disrupt its binding interaction with human complement component, Factor H. These variants are FHbp1.4(H248L), FHbp1.13(H248L), FHbp2.19(L130R G133D) and FHbp3.45(E254A).

GM *N. lactamica* strain	NadA	FHbp 1.4 (H248L)	FHbp 1.13 (H248L)	FHbp 2.19 (L130R G133D)	FHbp 3.45 (E254A)
N. lactamica 4NFA313_H	✓	✓			
N. lactamica 4NFB313_H	✓		✓		
N. lactamica 4NFC313	✓			✓	
N. lactamica 4NFD313_E	✓				✓

Table 1: Expression of meningococcal antigens by each of the 4xrNlac GM-*N.lactamica* strains

The genetically modified organisms (GMOs) will ultimately be used to investigate whether an episode of nasopharyngeal GMO colonisation following controlled infection with a multi-strain challenge agent, comprised of all 4 GMOs (hereafter, 4xrNlac) elicits an immune response (or combination of immune responses) that leads to a given participant becoming 'protected from colonisation with 4xrNlac', following experimental rechallenge. We define a participant as becoming 'protected from colonisation' if they: (i) become refractory to a further episode of nasopharyngeal GMO colonisation following experimental rechallenge with 4xrNlac, or (ii) are still able to become colonised by the bacteria comprising 4xrNlac after rechallenge, but wherein the duration for which they are colonised, or the size of the bacterial population resident on the nasopharyngeal epithelium is significantly reduced, relative to their first episode of colonisation. The primary outcome measure of the intended study is to observe and compare the proportion of participants who become colonised by one or more of the bacterial strains comprising 4xrNlac following challenge and, where appropriate, rechallenge. If a 'protected from colonisation following homologous rechallenge' phenotype is evident, then it implies 4xrNlac might have utility in preventing natural acquisition of strains of *N. meningitidis* circulating in the community, on the basis that *N. lactamica* and *N. meningitidis* are closely related and share several similar and immunologically cross-reactive surface structures. Indeed, the purpose of the genetic modification is to further enhance the structural similarity between these organisms without increasing the pathogenic potential of the recipient species.

The impact of reducing the meningococcal carriage frequency would most plausibly be to break meningococcal transmission chains, lowering the frequency with which susceptible individuals become exposed to the meningococcus, and therefore reducing the incidence of invasive meningococcal disease (IMD) in the community. To realise this vision, and with a view to informing the rational design of future vaccines capable of generating so-called 'herd protection' against *N. meningitidis*, we will collect longitudinal measurements of serological and cell-mediated adaptive immune responses to look for correlations and to subsequently disambiguate the mechanisms that underpin this protected phenotype.

Meningococcal vaccine antigens for heterologous expression.

All genes coding for meningococcal vaccine antigens have been targeted to the *Neisseria* Heterologous Construct Insertion Site number 1 (NHCIS1) locus, an otherwise intergenic chromosomal sequence located between NLY_27080 and NLY_27100 in the chromosome of *N. lactamica* strain, Y92-1009. The same locus has previously been targeted with constructs that yielded two strains of genetically modified *N. lactamica* (GM-*N. lactamica*) that either: (i) express NadA alone (strain **4NB1**), or (ii) did not express any heterologous antigens, but retained all other genetic modifications present in 4NB1 and was wild type with respect to gene content (strain **4YB2**). Strains 4NB1 and 4YB2 were deliberately released in a previous controlled human infection model experiment (CHIME) (DEFRA REF: 17/R50/01), see Item 44 for further details.

NadA is an adhesin protein expressed in a small subset of meningococcal strains. Due to the absence of the *nadA* gene in its genome, NadA is not expressed by wild type *N. lactamica*. In a recent survey of European invasive *N. meningitidis* isolates, only 16 of 235 *N. meningitidis* isolates possessed the *nadA* gene [1], suggesting the gene product is dispensible for pathogenesis. NadA-expression in *N. meningitidis* is associated with an increased level of adhesion to and uptake by human epithelial cell lines [2]. The NadA 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.

NadA is one of the four strongly immunogenic protein components of the anti-serogroup B meningococcal disease vaccine, Bexsero (4CMenB) [1]. In wild type *N. meningitidis*, 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 [3]. 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 *N. meningitidis* 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 [4]. This hypothesis is partially corroborated by the finding that immunisation with recombinant NadA, prior to attempted *N. meningitidis* colonisation in a transgenic mouse model, leads to sterilising immunity, whereby strains expressing a cognate NadA antigen were unable to colonise the murine nasopharynx [5]. In each of the 4xrNlac strains discussed herein, *nadA* expression is instead controlled by a hybrid, constitutively active promoter that drives expression of the gene to high levels.

FHbp is an approximately 27 kDa outer membrane-anchored lipoprotein originally discovered using reverse vaccinology [6] and later shown to be responsible for protection of the meningococcus against complement-mediated killing through its ability to bind the human negative complement regulator, Factor H [7] through molecular mimicry of host carbohydrates [8]. Unlike NadA, FHbp expression is widespread among circulating strains of the meningococcus, although invasive disease isolates that lack FHbp expression have been characterised [9]. The amino acid sequence of FHbp is highly variable, phylogenetically resolving into three variant groups (var1, var2 and var3) or two subfamilies (1 and 2/3), depending on the classification system (herein, we refer exclusively to the variant group system of classification). Antibodies directed against one subfamily are poorly cross-reactive with the other, and there is only limited cross-reactivity of antibodies targeting epitopes from variant groups 2 and 3. FHbp is present in both the 4CMenB (Bexsero) and Trumenba anti-serogroup B invasive meningococcal disease vaccines, generating multiple antibody clones which, whilst not necessarily bactericidal on their own, can work synergistically to trigger C1q-mediated killing of the meningococcus [10].

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*

NB: also referred to as ‘meningococcus’, the causative agent of invasive meningococcal disease (IMD).

Taxonomy ID: 122586

Recipient:

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

Taxonomy ID: 869214

4. Usual strain, cultivar or other name.

Donor:

It is important to note that the genes encoding each heterologous antigen for expression in GM-*N. lactamica* are rationally-designed nucleotide sequences, codon-

optimised either for high level expression in *N. lactamica* (i.e. the *nadA* gene) or to maximise nucleotide sequence diversity from the other three *fhbp* alleles in use in this project. Also note that the nucleotide sequence of each of the *fhbp* genes has been intentionally mutated so that the expressed proteins have specific amino acid substitutions, which prevent each FHbp variant protein from binding human complement Factor H. The nucleic acids that were used to create the constructs that ultimately modified the chromosome of the Recipient strain were originally synthesised in a laboratory and were therefore **not** cloned from any bacterial donor. The strains listed as 'Donor' strains below are therefore only representative strains, in whose genomes are encoded a gene that, when expressed, results in expression of a wild type version of the protein of interest.

1. *nadA*

   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

2. *fhbp1.4*

   Neisseria meningitidis serogroup B strain: M15 240727, B: P1.7-2,4:F3-3: ST-303 (cc41/44)

https://pubmlst.org/bigsdb?page=info&db=pubmlst_neisseria_isolates&id=39362

3. *fhbp1.13*

   Neisseria meningitidis serogroup B strain: M17 240102, B: P1.22,9:F5-12:ST-12991 (cc269)

https://pubmlst.org/bigsdb?page=info&db=pubmlst_neisseria_isolates&id=53286

4. *fhbp2.19*

   Neisseria meningitidis serogroup B strain: M16 240369, B: P1.22,9:F5-5:ST-1163 (cc269)

https://pubmlst.org/bigsdb?page=info&db=pubmlst_neisseria_isolates&id=44766

5. *fhbp3.45*

   Neisseria meningitidis serogroup B strain: M15 240917, B: P1.22,14:F5-5:ST-213 (cc213)

https://pubmlst.org/bigsdb?page=info&db=pubmlst_neisseria_isolates&id=41500

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:

All GMOs are derivatives of a *ΔlacZ* mutant derivative of *N. lactamica* strain Y92-1009 (see Item 20b). The purpose of the genetic modification is to construct four modified *N. lactamica* strains, each one capable of expressing *Neisseria* Adhesin A (NadA) and one of four different variants of mutagenised FHbp on its surface, such that the heterologous FHbp no longer binds to human complement Factor H (see Item 25, Figure 12).

5. Phenotypic and genetic markers.

All 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 *N. lactamica* through PCR amplification of NHCIS1. The gene expression constructs present in all four GMOs were targeted to this locus (Item 24). 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 10 kb in length).

Additionally, each GMO contains a rationally-designed, synthetic *fhbp* gene that is maximally divergent from the other *fhbp* alleles encoded into the other strains. Each of these genes is under transcriptional control of a synthetic, hybrid promoter, consisting of the meningococcal *porA* gene promoter, enhanced by its native upstream activation sequence (UAS) and in which the 17 bp nucleotide sequence that separates the -35 and -10 boxes of the promoter-proper have been replaced with the concomitant nucleotide sequence from the endogenous *N. lactamica porB* gene promoter. The fusion of this promoter sequence to each synthetic gene creates unique PCR targets that should not exist anywhere else in nature.

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* [11], [12]. Phylogenetic analyses suggest that the two species are functional taxonomic groups that have recently diverged from a common ancestor [13].

7. The description of identification and detection techniques.

Following intranasal inoculation of participants, at the appropriate time points, throat swabs and nasal washes will be performed. These samples will be processed to elute collected microorganisms into suspension (where appropriate), and aliquots of these eluates will be used to culture viable *Neisseria* species on GC agar, supplemented with both 1% (v/v) Vitox supplement and 40 μg/ml X-Gal. Systematic cultivation of laboratory isolates shows that only a subset of Neisseria species, specifically *Neisseria meningitidis, Neisseria gonorrhoeae, Neisseria lactamica* and *Neisseria bacilliformis*, are capable of growth on this supplemented GC medium, and that only *Neisseria lactamica* grows as blue-coloured colonies.

Putative *Neisseria lactamica* colonies will be identified by colony morphology and colony colour (blue) in the first instance, followed by biochemical characterisation if required (API NH, bioMerieux). Should biochemical characterisation prove inconclusive, the GMOs can be identified by PCR of the NHCIS1 locus.

Total genomic DNA will be extracted from the remaining volume of each sample eluate, and the number of genome copies of each of the four GM-*N. lactamica* strains will be enumerated using bespoke qPCR assays. These assays specifically amplify unique sections of each rationally-designed *fhbp* gene, coded within the modified NHCIS1 locus.

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*. 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, qPCR, 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 [14], [15]. 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 [16]. The apparent mutual exclusivity of these species was demonstrated in a CHIME, whereby *N. lactamica* was introduced into the nares of healthy, adult participants [17]. 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 (for empirical evidence of this, see Item 25, Figure 14). Despite the natural competence of the organism, a novel transformation system involving hypermethylated DNA was developed 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 nucleotide sequence of the nucleic acid, the chemical composition of the molecule acts to block the otherwise potent restriction endonuclease activities present in *N. lactamica* [18]. Without appropriate methylation, DNA taken up by *N. lactamica* is much more likely to be degraded before it can undergo recombination with the chromosome and become incorporated into the *N. lactamica* genome. No other member of the Neisseriaceae contains a DNA methylase capable of appropriately methylating its chromosomal DNA, so the risk that *N. lactamica* will acquire new genes from the nasopharyngeal milieu is negligible.

N. lactamica as recipient of genetic material.

In the event of horizontal gene transfer into *N. lactamica* 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* [19]. 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 (see Item 11, Table 2), and are hypothesised to minimise the risk of *Neisseria* family members taking up DNA molecules containing potentially deleterious sequences [20]. In a previous CHIME using the wild type *N. lactamica* strain Y92-1009 (from which the GMOs were derived), some participants became colonised by the bacteria for 6 months [17]. 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* [21].

N. lactamica as donor of genetic material.

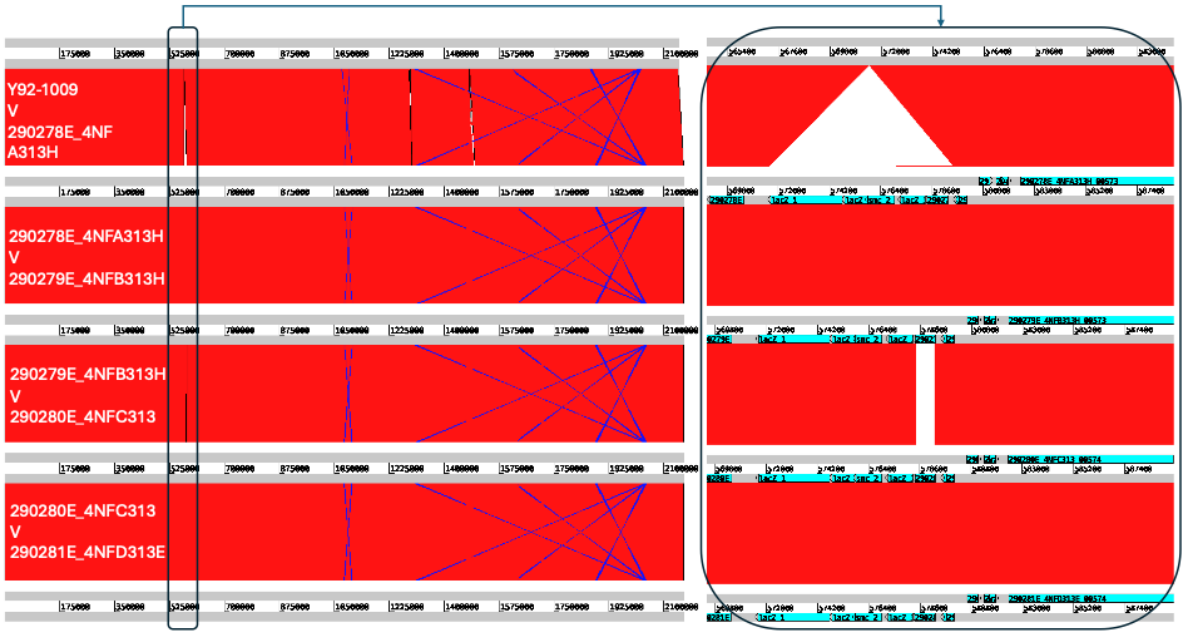
Whilst it is extremely resistant to the incorporation of exogenous genetic material, *N. lactamica* acts as a reservoir of genes for the pathogenic *Neisseria*. There is evidence of frequent interspecific movement of *N. lactamica* genes into *N. meningitidis* and *N. gonorrhoeae*, with the subsequent generation of hybrid genotypes [22-25].

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 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 either heterologous antigen-coding 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 [26]. However, as neither the NadA nor FHbp proteins are required for virulence in *N. meningitidis* [1], the impact of their expression in carriage strains of the *Neisseriaceae* is anticipated to be minimal. Indeed, expression of either heterologous antigen is unlikely to confer an evolutionary advantage to recipient strains: In the short term, the site-directed mutagenesis of each *fhbp* allele ensures that putative recipients will not experience gain-of-function with regard to binding of human complement Factor H (see Item 25, Figure 12), and over the longer term, human hosts are predicted to seroconvert against the antigens (which are, after all, strongly immunogenic components of the Bexsero and Trumenba vaccines). Importantly, because the promoters of the *nadA* and *fhbp* genes are constitutively active in these constructs, recipient strains will be unable to downregulate the expression of the heterologous genes through phase variation, which occurs naturally through the course of meningococcal carriage (and presumably through the carriage of other commensal Neisseria) [4]. As such, the heterologous antigens may instead serve as a survival liability and inadvertently target the recipient strains for destruction by the immune system.

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

Assemblies of the closed whole genome sequences of all four GMOs (long and short read hybrid sequencing, MicrobesNG) were mapped onto a complete, closed genome generated from the DNA of wild type *N. lactamica* strain, Y92-1009 (PacBio). GMO genomes were then compared pairwise for nucleotide identity (Figure 1).

Figure 1: Comparative analysis of GM-*N. lactamica* genomes to Y92-1009 using the Artemis Comparison Tool (ACT). Re



d blocks denote regions of shared nucleotide identity based on bit scores from blastn using default parameters. Regions where sequence alignments are lower than the maximum bit score are highlighted as white segments. These either represent regions missing from the WT chromosome (as shown in top right, representing the NHCIS1 locus and heterologous gene expression cassette) or low nucleotide similarity (middle right, representing a comparison between genes in the heterologous antigen expression cassette coding for a variant 1 FHbp (i.e. FHbp1.13(H248L) in 4NFB313_H) and a variant 2 FHbp (i.e. FHbp2.19(L130R G133D) in 4NFC313), which are phylogenetically distinct from one another). Diagonal lines denote putative translocations. Given the similarity in location across all five genomes, those in blue are likely to be misalignments at positions of repetitive DNA sequence (e.g. IS elements).

These data are consistent with observations in both *Neisseria gonorrhoeae* [27] and *Neisseria meningitidis* [28], 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 [29], but also between strains of the same species [30, 31] and between isolates comprising a single bacterial lineage [32]. The *Neisseria* chromosome is highly plastic because it includes many repetitive sequences, such as those shown in Table 2. These sequences not only serve functional roles in the biology of the organism, for example, 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 (see below).

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 [33], dRS3 are 'direct repeat sequence 3' elements and Correia refers to inverted repeat sequences also known as Correia repeat enclosed elements (CREE).

In our previous application to conduct a Deliberate Release of genetically modified *N. lactamica*, we disclosed that chromosomal rearrangements like those reported above were observed following 28 days of daily serial passage of our GMOs on bacterial culture medium. Importantly, we showed that the NHCIS1 locus was unaffected by these stochastic alterations and did not appear at a rearrangement breakpoint. Indeed, the sequences of all heterologous coding sequences were 100% identical in all matched isolates after this period of repeated passage, and NadA expression levels were unaffected.

In stark contrast to *in vitro* culture, we have observed that 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 [21].

In light of these findings, and given that: (i) in the strains under consideration in this application, we have also targeted our genetic modifications to NHCIS1, (ii) to produce our challenge inoculum, we will use the Master stocks of each strain to generate seed colonies, meaning there is almost no opportunity for genetic drift to occur *in vitro*, and (iii) post production of the challenge inoculum-proper, the bacteria within will only be used for nasal challenge of participants, and those that successfully colonise a participant will be subject only to the genome-stabilising selective pressures and evolutionary bottlenecks associated with *in vivo* carriage; we have foregone daily serial passage of these mutants.

The greatest impact on genetic stability of the GMO is theoretically horizontal gene transfer from other bacterial species. We have demonstrated that the GMOs are extremely resistant to horizontal gene transfer, where repeated attempts to transform the bacteria with genetic material derived from *N. meningitidis* resulted in zero putative transformants (see Item 25, Figure 14). Across evolutionary time there have been very few instances of horizontal gene transfer into *N. lactamica* [19], 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 [34]). 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 [35]. 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 [35].

Both the meningococcus and gonococcus have been demonstrated to form biofilm *in vitro* under nutrient starvation (i.e. stress) conditions [36, 37]. 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 [38] is a preferred state of *N. lactamica in vivo*. However, there is 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 [39]), 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 or which might be useful for the clearance of nasopharyngeal carriage of the GMO (see Table 3).

Strain	MIC (mg/L)			
	Benzylpenicillin Lot: 1010280580 Exp 28/09/25	Ceftriaxone Lot: 1010212950 27/08/2024	Rifampicin Lot: 1009548680 Exp: 21/08/24	Ciprofloxacin Lot: 101477600 Exp: 23/01/26
N. lactamica 4YB2	0.25	< 0.002	0.5	0.006
N. lactamica 4NFA313_H	0.25	< 0.002	0.5	0.006
N. lactamica 4NFB313_H	0.25	< 0.002	0.5	0.006
N. lactamica 4NFC313	0.25	< 0.002	0.5	0.006
N. lactamica 4NFD313_E	0.25	< 0.002	0.5	0.006

Table 3: Antimicrobial susceptibility of the genetically-modified but wild type with respect to gene content Y92-1009 derivative, 4YB2 and the NadA- and FHbp variant-expressing GM-*N. lactamica* strains comprising the proposed challenge inoculum (4NFA313_H, 4NFB313_H, 4NFC313 and 4NFD313_E). Minimal Inhibitory Concentration (MIC) breakpoints for *Neisseria meningitidis* are: benzylpenicillin = 0.25 mg/L, 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 which 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 by our research group, 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 [40].

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 of *N. lactamica* strain, Y92-1009 (Item 20b). Removal of the *lacZ* gene enabled its use as a genetic marker of successful transformation through blue/white screening on X-gal-containing medium.

A previous CHIME using GM-*N. lactamica* that expressed either NadA (strain 4NB1), or contained a gene expression cassette without a coding sequence (i.e. a wild type-

equivalent, but genetically modified control) (strain 4YB2) has been conducted at the University of Southampton (DEFRA REF: 17/R50/01), see Item 44 for further details.

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 a stepwise process of multiple transformations using hypermethylated DNA constructs, which were targeted either directly into the NHCIS1 region of the chromosome of *ΔlacZ* Y92-1009 (see Item 14) or targeted to sequences introduced into the NHCIS1 locus by previous transformations (see Item 24). Each nucleic acid construct was maintained in *E. coli* as part of a plasmid with a pSC101-derived origin of replication/*rep101* gene, and a pUC19-derived ampicillin resistance gene (Figure 2). Each plasmid provided the template for hypermethylated amplification of the target sequences. The hypermethylated PCR amplicons served as the donor material for incorporation into the *N. lactamica* chromosome and contained no sequences amplified from the plasmid backbone.

In the plasmids used to maintain the gene expression constructs in *E. coli*, the *porA*-associated-UAS-enhanced, synthetic hybrid *porA/porB* promoter drives the expression of both the $fhbp_{x.yz}$ (where x.yz represents one of 1.4, 1.13, 2.19 or 3.45) and *nadA* genes, whilst a copy of the endogenous *N. lactamica lst* promoter drives expression of both the *lacZ* and Synth.*lacZ* genes (Figure 2). Chromosomal homologous recombination events during the construction of the GMOs resulted in replacement of the wild type NHCIS1 locus with homologous sequence flanking either side of the gene expression cassette, the content of which varied depending upon the different stages of construction (see Item 24).

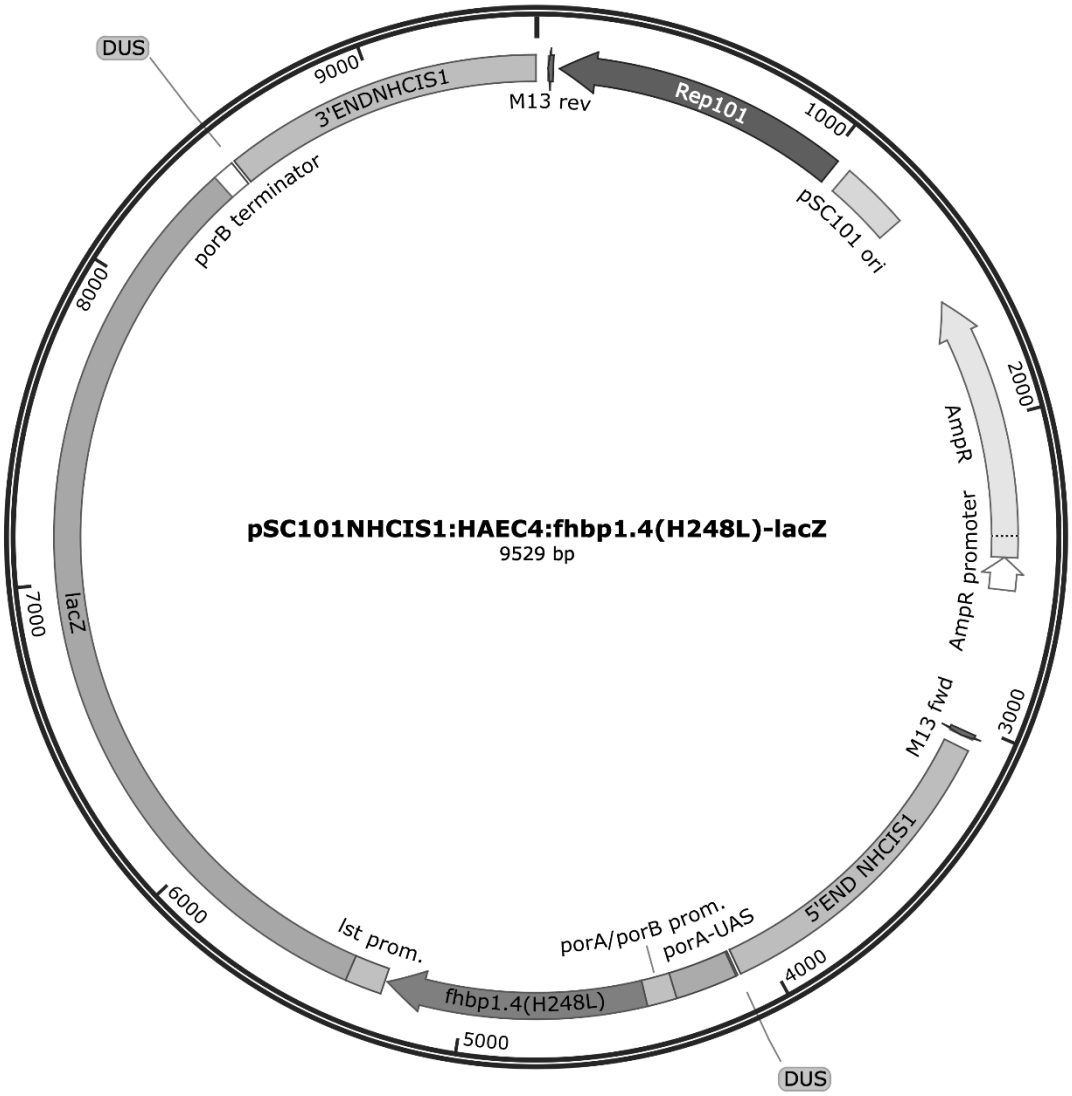


Figure 2: Representative plasmid map containing 'transformative construct 1' (see Item 24) in a pSC101/pUC19 hybrid backbone. All relevant features of this plasmid are shown, which was used to maintain 'transformative construct 1' at high fidelity in *E. coli* and to serve as template for hypermethylated PCR in the generation of donor nucleic acids.

Note: HAEC4 (Heterologous Antigen Expression Cassette number 4) refers to the specific combination of *N. lactamica*-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 (see Figure 3). Downstream (i.e. 3') of the hybrid promoter and the coding sequence whose expression it drives, there is a copy of the endogenous *N. lactamica lst* gene promoter.

Note: 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 3).

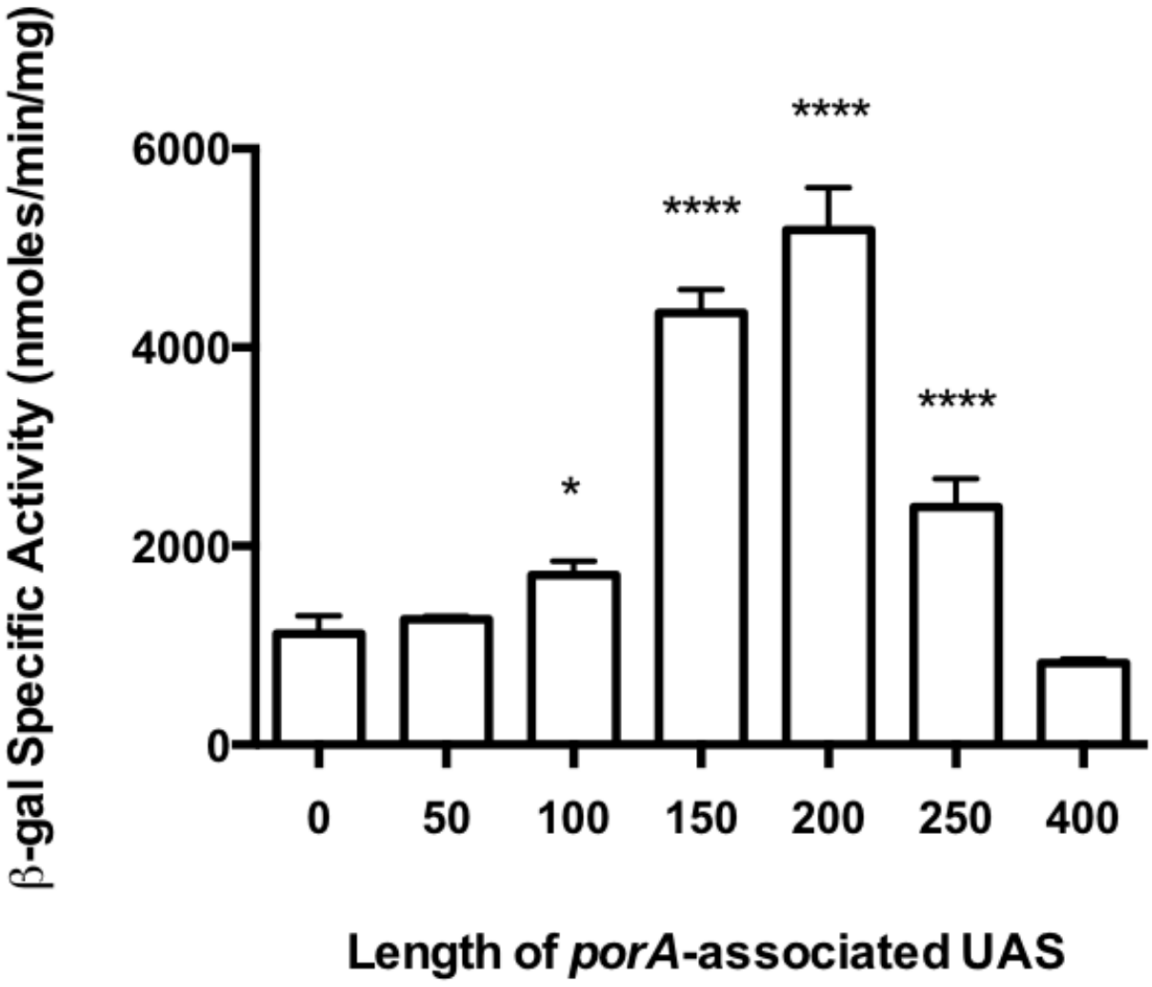


Figure 3: Effect of different lengths of the *porA*–associated UAS on β-galactosidase Specific Activity, expressed from NHCIS1 in *N. lactamica*. Increasing lengths of the UAS were conjugated immediately 5' of the *lst* promoter, which was used to drive expression of the *lacZ* gene. These constructs 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.

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 products used to create the GMOs are 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.

The gene expression construct can be detected by any one of multiple different PCRs: (i) PCR of the NHCIS1 locus, which if inherited intact as part of a larger DNA uptake event, will produce an amplicon much larger than the same PCR conducted using chromosomal DNA from wild type Y92-1009 as the template (i.e. approximately 2 kb); (ii) a PCR that detects the unnaturally close proximity of the *porA* promoter region and *lacZ* coding sequences; (iii) a PCR that detects the unnatural fusion of *lst* promoter sequences with the *lacZ* coding sequence; and (iv) PCR of the *fhbp* coding sequences, which through their rational design are unlikely to yield amplicons of an appropriate size from any other natural genomic source.

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

The gene expression cassettes present in each GMO (which essentially represents a composite of three separate transformation events – see Item 24) consist of: (i) the coding sequences of the rationally-designed *nadA* and *fhbp*$_{x.yz}$ genes; (ii) two copies of a meningococcal *porA*-UAS-enhanced, *porA/porB* hybrid promoter, each of which drives expression of one of the two heterologous antigen genes; (iii) two 1000bp remnants of the coding sequence for the endogenous *N. lactamica lacZ* gene, which flank either end of the *nadA* gene and its promoter; (iv) multiple Neisseria DNA Uptake Sequences (DUS), to enhance pilus-mediated natural competence of the organism; (v) a rationally designed, synthetic version of the complete *lacZ* coding sequence (Synth.*lacZ*), designed to be as divergent as possible at the nucleotide sequence level from the 1000bp remnants of the endogenous *lacZ* coding sequence, but which codes for a fully functioning, wild type B-galactosidase protein; and (vi) two copies of the endogenous, *N. lactamica lst* gene promoter, to drive relatively low level, constitutive expression of the two different *lacZ* genes. Collectively, these elements are minimally sufficient to perform the function of the cassette; specifically, the expression of mutated, human complement Factor H non-binding FHbp$_{x.yz}$ and NadA on the bacterial surface, and the production of β-D-galactosidase as a means of screening for putatively transformed *N. lactamica*.

The sequences flanking either end of the gene expression cassette, homologous to the NHCIS1 locus of the chromosome of wild type Y92-1009, are of sufficient length to accurately target the cassette to the correct chromosomal location 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), site-directed mutagenesis, bacterial transformation, screening for putative transformants on indicator media and nucleotide sequencing.

20. The methods used:

a. to construct inserts and introduce them into the recipient organism;

In general, each rationally designed coding sequence and its promoter were synthesised as 1 or more large, double stranded DNA fragment(s) (gBLOCKS, Integrated DNA Technologies), flanked at either end by 30-50 bp of nucleotide sequence identical to that which would become, in the finished, circularised plasmid, contiguous neighbouring sequence. These additional nucleotides served as isothermal assembly-compatible overhangs, which facilitated the use of this technology to assemble separate nucleic acids into complete plasmids. Where sequences were needed for a new plasmid/expression construct and had already been synthesised or cloned from another source, these sequences were amplified using high fidelity PCR and extended primers, the result of which were amplicons with isothermal assembly-compatible sequence overlaps.

To illustrate the methodology for construction of the inserts and their insertion into the recipient strain of *N. lactamica*, we will focus on engineering the bacterium to express FHbp in the first instance:

Step 1: Plasmid engineering.

The first part of the stepwise generation of these mutants was to introduce one copy of each of the rationally designed *fhbp*$_{x.yz}$ genes, under transcriptional control of the *porA/porB* hybrid promoter, and a copy of the endogenous *N. lactamica lacZ* gene, under transcriptional control of the *lst* gene promoter, into the NHCIS1 locus of *ΔlacZ N. lactamica*. To do this, each *fhbp*$_{x.yz}$ gene, coding for a wild type (i.e. fully functional) version of each FHbp variant, along its promoter, were synthesised as described above. Each fragment was assembled into a plasmid backbone consisting of Rep101, a pSC101 origin of replication, a pUC19-derived ampicillin resistance gene (*ampR*), the endogenous *N. lactamica lacZ* coding sequence under transcriptional control of the *lst* gene promoter, and the NHCIS1 flanking sequences. This plasmid was transformed into competent *E. coli* DH5α for maintenance, amplification and sequencing. To introduce specific amino acid mutations into the coding sequences of the *fhbp*$_{x.yz}$ genes, the whole plasmid was amplified using high-fidelity PCR and primers that included the altered nucleotide sequences. Plasmids were re-circularised from the amplification product using KLD reagent as part of a

site-directed mutagenesis kit (New England Biosciences, UK). Plasmids containing the altered nucleotides sequences were isolated and verified by Sanger sequencing.

Note: For each FHbp variant, multiple amino acid substitutions have been reported in the literature as disrupting the interaction of FHbp with human complement Factor H but maintaining the immunogenicity of the protein [41-44]. Because this project required heterologous antigen expression, which is never guaranteed to work despite the close evolutionary relationship between the donor (*N. meningitidis*) and recipient (*N. lactamica*) organisms, it was prudent to survey whether these mutations were permissive for expression in the *N. lactamica* context. Additionally, it has been shown that the N-terminal domain of some variant group 2 FHbps (including FHbp2.19) are significantly less thermally stable than those in variant groups 1 and 3 [45]. The N-terminal domain of FHbp2.19 undergoes thermal unfolding at near-physiological temperatures, reducing the likelihood that potentially immunogenic structures will be encountered and responded to *in vivo*. By substituting 2 amino acid residues crucial for the thermostability of variant group 1 FHbps into the N-terminal domain of FHbp2.19 (L130R and G133D), the authors demonstrated a synergistic increase in thermostability, resulting in an increase of the modified N-terminal domain unfolding temperature by 21 ^{o}C [45]. Subsequently, the same group showed that the modified protein, FHbp2.19 (L130R G133D) was unable to bind human complement Factor H and was able to elicit higher anti-FHbp2.19 IgG titres in immunised mice when compared to immunisation with wild type FHbp2.19 [46]. Considering these findings, the decision was made to adopt the modified, more thermostable (ts) version of FHbp2.19, FHbp2.19 (L130R G133D) as the background upon which to explore further amino acid substitutions designed to disrupt the binding of human complement Factor H.

Therefore, the following site-specific mutations were introduced into the relevant *fhbp* coding sequences:

1. *fhbp1.4*: R41S, E92K, S223R, H248L
2. *fhbp1.13*: R41S, E92K, S223R, H248L
3. *fhbp2.19*: (L130R G133D), K218N, G219S
4. *fhbp3.45*: D217A, T227A, E254A

Step 2: Hypermethylated PCR amplification of the NHCIS1::HAEC4 cassettes.

Each of the plasmids containing a mutated *fhbp*$_{x.yz}$ coding sequence and the *lacZ* gene, flanked by NHCIS1 sequences, were used as templates for hypermethylated PCR amplification of the NHCIS1::HAEC4 gene expression cassettes. Primers were designed to hybridise to NHCIS1 sequences present at the 5' and 3' termini of the gene expression cassette.

NB: 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. In our hands,

Phusion DNA polymerase is permissive for hypermethylated PCR, whilst other high fidelity DNA polymerases (e.g. Q5 DNA polymerase, New England Biosciences UK) are not. Multiple reactions of the same construct were pooled after thermocycling, supplemented with restriction enzyme-compatible buffer and treated with DpnI to digest the plasmid template. The hypermethylated product was purified, and the degraded remnants of the plasmid discarded, by passage through a PCR purification column. The concentration of hypermethylated DNA (hmDNA) was adjusted to give 0.5 pmol of product per 10 µl of Tris-EDTA buffer.

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

The transformation of *N. lactamica* relies on the natural competence of the bacterium, mediated by expression of the type IV pilus. The *ΔlacZ* mutant derivative of *N. lactamica* 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, colony side up, to an incubator for 6 h at 30 °C, 5 % CO_2. Following this incubation, each spot of nascent bacterial colonies was exposed to 0.5 pmol of the hypermethylated gene expression cassette and returned to the 30 °C incubator for a further 9-10 h. In this instance, putative transformants of *N. lactamica* were screened for based on growth as *blue* colonies on X-gal-containing medium (see Item 23).

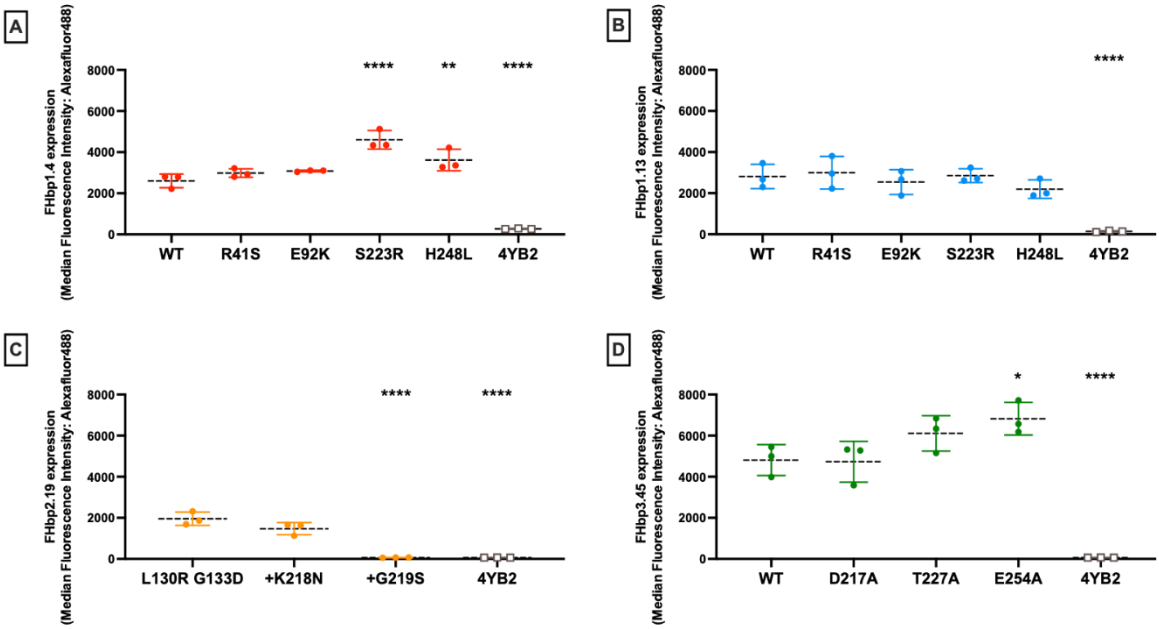


Figure 4: Specific amino acid substitutions in the coding sequence of $FHbp_{x.yz}$ are variably permissive for expression on the bacterial surface. GM-*N. lactamica* strains engineered to express one of: (i) the wild type (WT) version of FHbp: 1.4 (Panel A, red), 1.13 (Panel B, blue) or 3.45 (Panel D, green); (ii) mutagenised versions of each respective WT FHbp

variant, wherein the indicated amino acid residue substitutions have been made; (iii) a mutant version of FHbp 2.19 (Panel C, gold), wherein amino acid residue substitutions L130R and G133D have been made to the N terminal domain to increase its thermostability; or (iv) mutagenised versions of thermostabilised FHbp2.19, wherein the indicated amino acid substitutions have been made *in addition to* L130R and G133D; or the genetically-modified but wild type-equivalent strain, 4YB2 (grey), were grown to log phase and prepared for flow cytometry. Approximately one hundred million (10^8) colony-forming units of each strain were incubated with either mouse-derived, anti-FHbp variant 1 monoclonal antibody (JAR4) at a dilution of 1:500 (Panels A and B), or mouse-derived, anti-FHbp variant 2/3 monoclonal antibody (JAR11) at a dilution of 1:1000 (Panels C and D) in 5% FBS-dH_2O for 30 minutes at RT. After washing, samples were labelled by incubation with a 1:250 dilution of polyclonal, goat-derived, anti-mouse IgG conjugated to Alexafluor488 in 5% FBS-dH_2O for 30 minutes at RT in the dark (ALL Panels). After washing, all samples were fixed by incubation in 3.75% formaldehyde, 1% methanol for 15 minutes at RT in the dark, followed by resuspension into 5% FBS-dH_2O. The fluorescence intensity of each sample was measured using a BD FACSAria flow cytometer, and the Median Fluorescence Intensity of gated 'singlet' events for each population are reported. *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$ Repeated Measures 1-way ANOVA with Dunnett's test vs. panel-specific MFI of 'WT' as Control. Dotted bars represent Mean ± standard deviation (SD) (n=3).

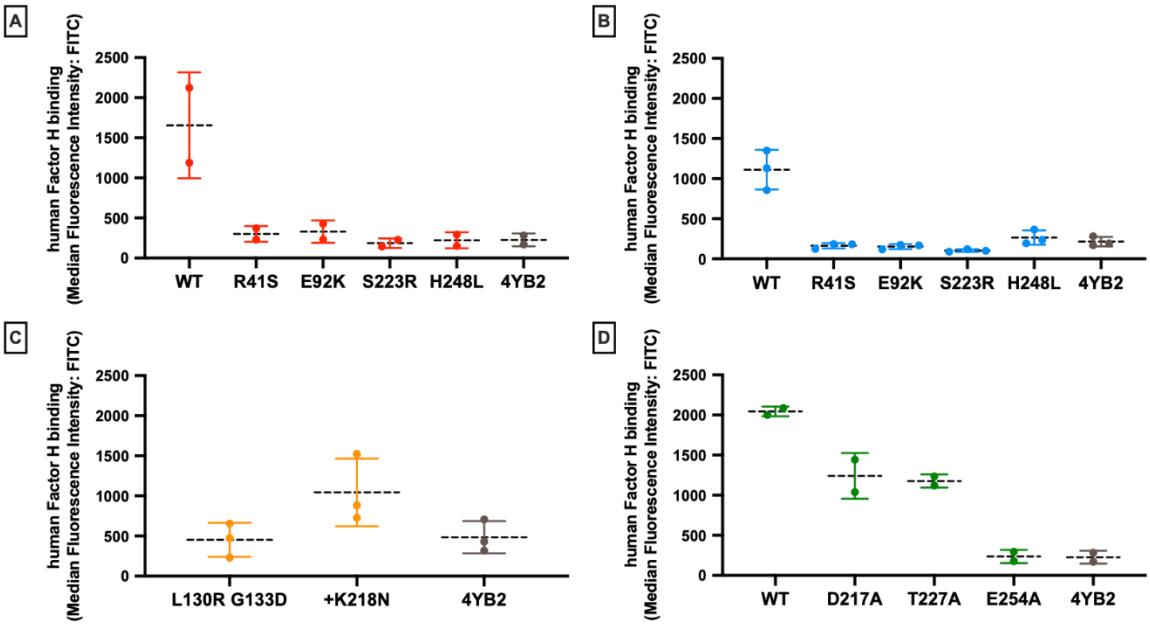


Figure 5: Specific amino acid substitutions in the coding sequence of FHbp$_{x.yz}$ are variably effective at disrupting the interaction between the bacterial protein and human complement Factor H. GM-*N. lactamica* strains engineered to express one of: (i) the wild type (WT) version of FHbp: 1.4 (Panel A, red), 1.13 (Panel B, blue) or 3.45 (Panel D, green); (ii) mutagenised versions of each respective WT FHbp variant, wherein the indicated amino acid residue substitutions have been made; (iii) a mutant version of FHbp 2.19 (Panel C, gold), wherein amino acid residue substitutions L130R and G133D have been made to the N terminal domain to increase its thermostability; or (iv) mutagenised versions of thermostabilised FHbp2.19, wherein the indicated amino acid substitutions have been made *in addition to* L130R and G133D; or the genetically-modified but wild type-equivalent strain, 4YB2 (grey), were grown to log phase and prepared for flow cytometry. Approximately one hundred million (10^8) colony-forming units of each strain were incubated with human Factor H (hfH) at either 20 µg ml^{-1}

(Panels A, B and D) or 40 µg ml^{-1} (Panel C) in 5% FBS-dH_2O for 30 minutes at RT. After washing, hfH bound to the bacterial surface was labelled by incubation with a 1:25 dilution of anti-human Factor H monoclonal antibody OX-24 directly conjugated to FITC [OX-24-FITC] in 5% FBS-dH_2O for 30 minutes at RT in the dark. After washing, all samples were fixed by incubation in 3.75% formaldehyde, 1% methanol for 15 minutes at RT in the dark, followed by resuspension into 5% FBS-dH_2O. The fluorescence intensity of each sample was measured using a BD FACSAria flow cytometer, and the Median Fluorescence Intensity of gated 'singlet' events for each population are reported. Dotted bars represent Mean ± standard deviation (SD) (n>2).

Note: Figures 4 and 5 above constituted an interim analysis of FHbp surface expression and human complement Factor H binding, performed to determine which of the multiple mutated versions of each FHbp variant to take forward and serve as the background for chromosomal integration of the *nadA* and Synth.*lacZ* genes (see Item 25). Given that further mutation of the FHbp2.19 (L130R G133D) coding sequence had either completely disrupted surface expression (i.e. G219S, Figure 4) or served to restore some Factor H binding activity (i.e. K218N, Figure 5), the thermostabilised, non-Factor H binding double mutant was the only appropriate candidate to represent the variant group 2 FHbps in our panel. Whilst all three potential mutations of FHbp3.45 were permissive for surface expression in *N. lactamica* (Panel D, Figure 4), only one mutation was able to completely abrogate human Factor H binding (i.e. E254A, Figure 5). This again made it the only suitable candidate to represent the variant group 3 FHbps in our panel. Because all the amino acid substitutions made to the variant group 1 FHbp coding sequences were both permissive for surface expression and abrogated human Factor H binding, the decision was made to proceed using the H248L-mutated versions of these proteins, on the basis that H248 of both variant group 1 FHbps superimposed over E254 of FHbp3.45 when the structures of these proteins were overlaid. The nature of the disruption of the interaction between FHbp and human complement Factor H was therefore posited to be most similar between the H248L and E254A mutants, ultimately providing parity between these strains as constituents of 4xrNlac.

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 *N. lactamica* 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 2, 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 3, Item 20a. The protocol remains identical to that described, with the exception that transformation was performed into wild type *N. lactamica* strain Y92-1009, and putative transformants were selected for because of growth as *white* colonies on X-gal-containing medium (see Item 23).

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 any of the four GMOs.

The components of the gene expression cassettes present in each GMO are described in Item 18. Collectively, these elements are minimally sufficient to perform the function of the cassette; specifically, the expression of mutated, human complement Factor H non-binding $FHbp_{x.yz}$ and NadA on the bacterial surface, and the production of β-D-galactosidase as a means of screening for putatively transformed *N. lactamica*.

The sequences flanking either end of the gene expression cassette, homologous to the NHCIS1 locus of the chromosome 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 presence of *lacZ* in the *N. lactamica* genome enabled the exploitation of β-D-galactosidase activity as a screening marker for successfully transformed bacteria. In our stepwise transformation process, we were either adding in or insertionally-inactivating versions of the *lacZ* gene, which meant successful transformation led to growth on X-gal-containing media as either blue or white colonies, respectively. Successful transformations into a *lacZ*-deficient background would result in putative transformants growing as blue colonies on X-gal-containing medium, whilst successful transformations into a strain capable of expressing β-D-galactosidase would result in putative transformants growing as white colonies on X-gal-containing medium.

Specifically, the transformations to: (i) integrate *fhbp*$_{x.yz}$ and *lacZ* into the NHCIS1 locus of the *⊿lacZ* background and (ii) integrate the Synth.*lacZ* gene into the junction between the 3' remnant of the copy of the endogenous *N. lactamica lacZ* gene and the 3' half of the sequence homologous to NHCIS1 in the strains transformed with both *fhbp*$_{x.yz}$ and *nadA*, resulted in the growth of putative transformants as blue colonies; whilst the transformation to integrate the *nadA* gene into the copy of the endogenous *N. lactamica lacZ* gene in NHCIS1, resulted in the growth of putative transformants as white colonies.

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

Following incubation with the appropriate hypermethylated PCR product (see Item 20), *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. Dependent upon the specific transformation and the *lacZ* sufficiency or deficiency of the background strain, either blue or white colonies (as appropriate) were isolated and cultured as putative transformants.

Step 2: Verification of sequence fidelity:

Chromosomal DNA was extracted from each of the cultures produced from the isolated colonies and was used as a template for multiple, high fidelity, low cycle number PCRs of either the *fhbp*$_{x.yz}$ or *nadA* genes (as appropriate). The fidelity of the coding sequences and their promoters were determined by sequencing these amplification products (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 six transformative constructs:

1. NHCIS1::HAEC4:*fhbp*$_{1.4}$(H248L)-*lacZ*
2. NHCIS1::HAEC4:*fhbp*$_{1.13}$(H248L)-*lacZ*
3. NHCIS1::HAEC4:*fhbp*$_{2.19}$(L130R G133D)-*lacZ*
4. NHCIS1::HAEC4:*fhbp*$_{3.45}$(E254A)-*lacZ*
5. ∆*lacZ*::*nadA*
6. 3'END*lacZ*:Synth.*lacZ*:3'ENDNHCIS1

For each of constructs 1 through 4, there are two genes present: *fhbp*$_{x.yz}$ (where x.yz represents one of: 1.4, 1.13, 2.19 or 3.45) and *lacZ*, coding for β-D-galactosidase. Each *fhbp*$_{x.yz}$ gene codes for one mutated variant of the meningococcal outer membrane lipoprotein, Factor H binding protein (FHbp) (NB: the letter-number-letter combination(s) shown in brackets after the gene name denote: (i) the single letter designation of the amino acid present in the wild type version of the appropriate protein, (ii) its position in the amino acid sequence relative to the lipidated, N-terminal Cys residue of the protein (i.e. residue number 1), and (iii) the single letter designation of the amino acid present in the mutant version of the appropriate protein). A representative schematic of the features included in constructs 1 through 4 is shown in Figure 6.

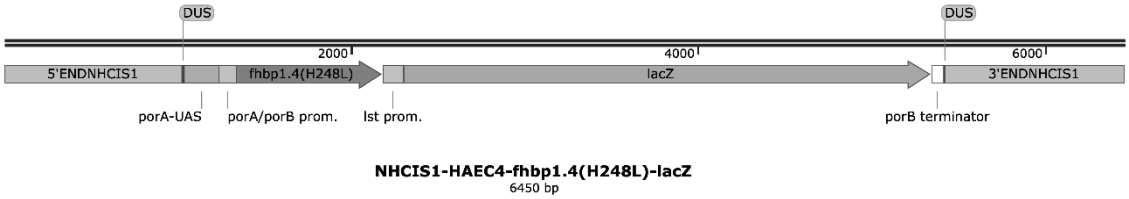


Figure 6: To-scale schematic of transformative construct 1, used to generate GM-*N. lactamica* strain 4FA313_(H248L) from a Y92-1009 ∆*lacZ* background, detailing all genes and non-coding features. Note that transformative constructs 2 through 4 are otherwise identical to that shown, with the exception that each contains a different *fhbp* coding sequence (see below). Transformation of the Y92-1009 ∆*lacZ* strain with constructs 2, 3 and 4 generated GM-*N. lactamica* strains 4FB313_(H248L), 4FC313_(L130R G133D) and 4FD313_(E254A), respectively. Each of the strains expressing mutant FHbp variants became the background for subsequent transformation with ∆*lacZ::nadA*

The NHCIS1::HAEC4:*fhbp1.4*(H248L)-*lacZ* construct in its entirety (6,450 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.

   5'ENDNHCIS1 nucleotide sequence (5' →3'):

CTGATACCGAGCTTTTCCCATGGTTTATCGCGACTGATAGTGTTTTCGGCGAGGTAGTCGGCACGTGT
TTGAGACCACCAGCGAGTGATTGTGCTTTCAGCTACAATAATTTTGCTGCTTGCTCTATGTTTAAAAA

TCTATCCATATTGGATAGTTTAGATTAGACTTAAGTGGATTTCAAGTGAGCTGTTTAACCCTTAGCTA
GCAAGGGTTTTGGTGGCGTAAGGTTACTGAACTTAAGCATATCGGCGGCCAAAGTACCGCTGCCATTG
CCAATCTCGCCGGGGAAATGAAAAGGCTCGCTAAAAAATCAAAAATGTTACTGAAAGGACTTTGGTCA
TTTTTATCCTCTACAATATCTACATTTAAAAATAAATCGGTCATTGTTTAAACCTTACTGTAAAACTG
TACAACTGCTAAACTGTAAACACAGAAAGAGGCAATACTAACAGCACAAAACAACAGATATCCAAACT
CCATAAAGTGCATTATTCTGACTTTTTTCGTTGCCTGATGTATTTATGCTATTTGTGCTGTTCTTATA
TATGTGTTGCTGGTTACGTTGCGTTTTTTCGGAAAATTCAACCGGTAGGGGACCGATACGCAGTTTCA
TCTCTTTGCTTAGGGAGAGTAGGGGGGTAGATTACGACCTTAGTTTTGGTATCCGTAATATCATCATT
TTTTCGTCTAGGGAGTATATCGACTTCAGAAAACAGGTATTAGATACTGCCTTTTCTTACGAGAGTGA
TGGCAAGATAGTTCTCTTCAAGTCAATCAAACAGGAAAGTATTTCTTTTCTGTCTGAAGATTTGAAAA
AGGACTGAATGTTTCACAAGGTTAAAACTGGGGAAAAAGATGGATATGGTTCAGATGAAATGCTGAGC
GCACCCCGTATCTATTTGGAAATGATGTCGCGGAAAACGGAAGTCCCCTACTCCAGTATTCTTTAAAT
TCTAAGCAGAAAACTTCTTCGTCGGTCTTTTTTTTGTTGTTTGGTTTGCATGGAGTAAAACTGTGCAA
C

3’ENDNHCIS1 nucleotide sequence (5’→3’):

TGCTGAAGTAGAAAACCAGCAAGAAGGTAAAAAGAAAGAAGCAGTTTTTTGGATTTTAGATGTTACCG
CAATTGGTTTCCTTTCCTAAAATTTGTTTAAATTATTTGCAATATTAATATAAACTGGATATTAATGA
TGAGGATTCAAAAAGGCATACTGAATATAATTTGTACAAAATATTTGCAGTATTTAAAAATGTTGGTT
CGTATATGAAAAGTTAAAAATGCCAAAATGTACAGTTGCTAAACTGTAAAACTGCTAAAGCAACAAAA
CATAAAAAGGAATGCAGGGATGCGATCACTACATCTTTTTATTCCGTAAGCATTTATGACTTTACGGT
CAACTGCTACTCTATGTTTCCAGCTTTTCAGCTCCCTATTTTCGAATATTGGACGAGGCATTTTCATC
AGTGTCGTAATGCCGACCGAAACCCTCACAAACCATATTGGTTCTTGTGGCAGCAACACCTATCCGTT
TGTTCAAGCGGCCACAAGAGTAACATGATTGGCTGGTGGCATTGGCTTTAATCTCTTCGATATGAACT
CCATTTTTAGCTGCACCTTCTTTCAGCGTATGCAACAGTGGGGATGGGGCAACTATCTTCTGGTGGAG
TTTGTTGACTTCAATCTGTATGCACTGTAGGTTGAGCAGATTCAGTTTTTTGATACAGATTATCCGGC
TTTGTTCGGCAATTCTGTTGGCGAACGTATAGTAGAGCTGTCGTCTTGCCGCTTTCAATTTGCGGTAG
GTATTTTACCATTCCATGCGTAGCCGCTCGGTACGTTTGAGCCAAATGTTATCTTCGCCATTTGTACC
AATTTGTTTTTACATTAGGCTGTGTTTTAGTAATCTATTGATTTCAATTATTTGCAAGGGAAAAGACA
ATTATTTTCCGGTTAGGAATAAACCTATCCTATTGAATATATTGAAGCCAAGTACGCTTATCAACACT
ATATTAAAACACAGCCTTTTTTAATATAGTAGACACAATCTTTCCTTATTTATGAAGGTGATAGCTTC
TTTCAG

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 *N. lactamica* 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-UAS’). 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 Item 15, Figure 3).

   ***porA*-associated UAS nucleotide sequence (5’→3’):**

GTGCCGCGTGTGTTTTTTTATGGCGTTTTAAAAAGCCGAGACTGCATCCGGGCAGCAGCGCATCGGCT
CGCACGAGGTCTGCGCTTGAATTGTGTTGTAGAAACACAACGTTTTTGAAAAAATAAGCTATTGTTTT
ATATCAAAATATAATCATTTTTAAAATAAAGGTTGCGGCATTTATCAGATATTTGTTCTGAAAA

4. A synthetic, hybrid promoter (labelled as 'porA/porB prom.') consisting primarily of sequence derived from the *porA* gene of wild type *N. meningitidis*, but wherein the homopolymeric guanosine nucleotide tract that separates the -10 and -35 boxes of that promoter has been replaced by the 17bp, non-phase variable sequence that separates the -10 and -35 boxes of the *N. lactamica porB* promoter (lower case lettering, below).

   ***porA/porB* hybrid promoter nucleotide sequence (5'→3'):**

ATGGTTtttcgggcgggaacattTATAATTGAAGACGTATCGGGTGTTTGCCCGATGTTTTTAGGTTT
TTATCAAATTTACAAAAGGAACTCGAG

5. A rationally-designed, synthetic version of the *fhbp$_{1.4}$*_(H248L) gene, coding for a mutated version of FHbp variant 1.4 wherein Histidine residue 248 of the wild type protein is replaced with a Leucine residue (lower case lettering in nucleotide sequence and **bold** text in amino acid sequence, below), so as to disrupt its interaction with human complement Factor H.

   ***fhbp$_{1.4}$*_(H248L) nucleotide sequence (5'→3'):**

ATGACGCGATCGAAACCCGTTAATCGCACTGCCTTTTGCTGTCTGAGCTTGACAGCCGCACTAATTCT
TACGGCTTGCTCATCCGGCGGTGGAGGGGTGGCCGCGGATATCGGCGCAGGTCTGGCTGACGCCTTGA
CCGCGCCGCTAGATCATAAGGACAAATCCCTTCAATCGCTGACCTTGGATCAGTCTGTCCGGAAGAAC
GAAAAACTAAAGCTTGCAGCTCAAGGAGCGGAGAAAACATATGGGAATGGCGACTCACTGAACACTGG
TAAGTTGAAAAATGATAAGGTATCCCGTTTCGACTTTATAAGGCAGATTGAAGTTGATGGACAACTAA
TCACCCTTGAGAGCGGGGAATTTCAGGTTTACAAACAATCTCATTCTGCCCTGACGGCTCTGCAGACC
GAGCAAGTGCAGGATTCTGAACATTCCGGTAAGATGGTTGCGAAACGGCAATTCCGGATCGGCGACAT
TGCCGGAGAGCACACGTCTTTTGATAAGTTGCCGGAAGGGGGTCGGGCAACCTACCGCGGCACGGCGT
TCGGTTCAGACGATGCTTCCGGTAAACTAACATATACCATCGACTTTGCGGCAAAGCAGGGCCATGGG
AAAATTGAACACCTTAAGTCTCCGGAGTTGAATGTGGATCTGGCCGCCTCCGATATCAAACCCGATAA
AAAGCGGCATGCCGTCATTTCCGGCTCTGTGTTGTACAACCAAGCCGAAAAAGGATCCTATTCTCTGG
GTATCTTCGGCGGTCAGGCTCAAGAGGTTGCGGGCTCCGCAGAAGTTGAGACGGCGAATGGTATTCGG
ttgATCGGTTTGGCCGCGAAGCAGTAA

   Translated amino acid sequence – FHbp1.4 (H248L):

MTRSKPVNRTAFCCLSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKN
EKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTALQT
EQVQDSEHSGKMVAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDASGKLTYTIDFAAKQGHG
KIEHLKSPELNVDLAASDIKPDKKRHAVISGSVLYNQAEKGSYSLGIFGGQAQEVAGSAEVETANGIR
LIGLAAKQ*

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

   ***lst* promoter nucleotide sequence (5'→3'):**

ATATCGGCAACTGTCGGAATATCTGCTAAAATTCCGCATTTTCCGCACCGGGTTTCCGCACCGGGACA
CTCGGGGCGTATGTTCAATTTGTCGGAATGGAGTTTAAAGGAATACACAT

7. A copy of the endogenous *lacZ* gene from wild type *N. lactamica* (*lacZ*), coding for the cytoplasmic enzyme β-D-galactosidase.

   ***lacZ* nucleotide sequence (5'→3'):**

ATGTTATTAGCGAATTATTATCAAGATCCTGAAATCACGAGAATCAATGCGTTGCCGCACCATAGCTA
TTTTATCCCTTTTGATAAAAAAGATAAGGTAGATCAGTTTTCTAGGGAAAATTCTTCGTTTTTTACAT
CATTAAATGGAATGTGGCAATTCGCATATTATCCGAGTATGCAGGATTTGCCTGAAAGTCCGGACGAA
ATCGCTTTTACGAAACAAATCAATGTGCCTTCAAATTGGCAGAATCACGGGTTTGATGCCCATCAATA
TACTAATATTAATTATCCTTTCCCTTTCGATCCGCCTTTTGTTCCTTTAGAGAACCCTTGCGGGGTAT
ATCAAAAGCAGGTCAATCTGAAAAAGAATATAAATAAGCGGTATTTATTAGTCCTTGAAGGGGTTGAT
TCCTGTTCTTATATATATGTAAACCATCAATTTGTAGGATATGGTTCTATCAGCCACAGTACCAATGA
ATTTGATATTACCGATTATCTTCACGATGGTGAAAACACCCTTACCGTATTTGTCCTGAAATGGTGTG
CCGGAAGCTACTTGGAAGATCAGGATAAATTTAGAATGTCGGGAATTTTCCGAGATGTATATTTATTG
GAAAGGGAGCATCACTATTTGCAAGACTTGAATATTCGAACCGTGCTTTCTGAAGATTTATCATTGGG
GCAGATTTGTCTGGATTTAAATTTTGCGGGGGATGCGGGGGATGTCGGAGTGTCATTGTTTGATACGG
ACGGGCAAATTGTTCAAGCAGGCAGCGCAATCACGACAGATAAACAACGGATGCAAATCCGCCTTGAT
AATATTCCTTTAACCAAATCCCGACTCTGGAATGCGGAAAACCCTGCCCTTTATACCTTGGTATTAAA
CACAAAAGAAGAAATCATTACCCAGAAGATCGGATTCCGCAAGGTAGAGGTCAAAAACGGGGTATTGC
TGCTTAATAATCAGCCTATTAAATTCAAAGGGGTAAACCGGCACGATAGCGATCCCAAAACGGGATAT
GCCATTTCTGTCGCTCAAGCGGTGACTGACTTGTCGTTAATGAAGAAACATAATATTAACGCGATTCG
TACCGCGCATTATCCAAATTCACCGTGGTTTTGCGAGCTTTGCGATAAGTATGGTTTCTATGTAATCA
GTGAAAGCGACATTGAAAGTCATGGGGCGGCATTCCAAGCAATTTCGCATCCCGAACCGAGTATCTTT
CTGAATGTGGAAAATCCGAATGAAGAGCCGCGTATCCGCCAACAAACTATTGACAACTTCTGTTATTT
TGCCCGAGAACCATTGTATCGGGCGGCTTTATTGGAGCGGACTAAAGCAAATATAGAACGTGATAAGA
ACCGCAGTTCTATTTTAATTTGGTCTTTAGGCAATGAATCAGGGTATGGTGAGAATTTCGAATATTGT
GCAAAATGGGTAAAAGAGCGGGATCCGGATCGATTGGTTCATTACGAAAGCAGCATTTATCAGCATTC
TGCGTATCAGAATAACACCGGGCATTTGGATTTATACAGTGAAATGTATTCCGACACAGAAGCAATCG
ATGCCTATTTTGCCGATCATTCACAAACGAAAAAACCATTCTTATTATGCGAATATTCCCATGCAATG
GGAAATTCAAATGGAGATATGGAAGACTATTTCCAAACATTTAACAAATATTCGGGCTGTTGCGGTGG
GTTTATTTGGGAGTGGTGCGATCATGCCCAATATATTACCCCGACAAAATTAGGATATGGCGGTGACT
TCGGCGAAAAAATCCATGATGGGAATTTCTGTGTGGATGGCTTGGTTTCGCCCGAACGCGTCCCGCAC
AGCAACCTGTTGGAAGTAAAAAATGTCAACCGGCCCGTACGGGCAAACTTGCGCGGTGAACAAATTGA
GTTATATAACTATTTTGATTTCACTAATTTAAAAGATATTCTTTGCGTCAAATATGAATGGGTAAAAA
ATGGTCAGATAACCGGGACGGGAACATTGGCGGTTGATTGCGAACCCCATCATTCTCAAATCTTACCG
ATTCAGCTTCCCAAAGAACGGGAAGGATTGCTATGGTTGAACCTATATTACTGCGCTTCCCGGCAAAC
TGATTTATTGCCGGCAGGACATCATTTCGGTTTCGATCAAATTATTTTGAGTAAGGAATACACTCCTG
CCATAGGATCGGATAAAGATGATTGCCCGCCGTTGGAAATAACCGAAACAGTGAGACAAATCGTTGTT
CGGAACAATCGATATTATTTTGAATTTAACAAGCTCACGGGGATAATTGACGAAATTAAAGTCAATGG
CAAGGCTTTCATCCATAAACCATTGGCTTGGAATATTTGGCGCGCGCCGACGGATAATGACCGCCTGA
TTCGTTCCCAATGGCAAAATGCCGGTTATGATCAAATGTATTCCAAGGTTTACGATATTTGCGCGCAT
CGGCAAGGAAACGGTGTTGTCGTGTCTGTGAAAAGTGCATTGGTTGCGGATGCAAAATCAAAAATCAT
GACATTGGAAACACAGTATCTGCTGTCTGAAAATGGTAAATTGGATATTCAAACAAATGCTGTTTTCC
ACGAGCATTTGCCATTCCTGCCCCGTTTCGGTTTGCGCTTTTTCTTAGACGAACAGAAAACACCTTTT
ACCTACTTAGGTTATGGGGCGGGTGAAAGCTATATTGATAAACATCAGGCGACCAAACTTGGTATTTA
TTCGACAACGGCAGGGGAAAATCATGTTGGCTATCTCAAGCCTCAAGAGAACGGCAGCCATTATGGAT

GCTTTTATGTTCAAAACGATATGATCCGGGTGGAGTCGGGGCAGCCGTTTAGTTTCAACCTGTCCCCT
TATACGCAAGAAGAACTGACGCAGAAAAAACATTCTTATGAATTGGTTTGTTCCGGGTATGATGTATT
GTGTATCGACTATAAGATGAGCGGTATCGGTTCGAATTCTTGCGGGCCTAATTTGAAGCCGCAATATC
GTTTGATTGAAAATAACATCAATTTCGATATAAGTATCCGGTTATAA

Translated amino acid sequence - β-D-galactosidase:

MLLANYYQDPEITRINALPHHSYFIPFDKKDKVDQFSRENSSFFTSLNGMWQFAYYPSMQDLPESPDE
IAFTKQINVPSNWQNHGFDAHQYTNINYPFPFDPPFVPLENPCGVYQKQVNLKKNINKRYLLVLEGVD
SCSYIYVNHQFVGYGSISHSTNEFDITDYLHDGENTLTVFVLKWCAGSYLEDQDKFRMSGIFRDVYLL
EREHHYLQDLNIRTVLSEDLSLGQICLDLNFAGDAGDVGVSLFDTDGQIVQAGSAITTDKQRMQIRLD
NIPLTKSRLWNAENPALYTLVLNTKEEIITQKIGFRKVEVKNGVLLLNNQPIKFKGVNRHDSDPKTGY
AISVAQAVTDLSLMKKHNINAIRTAHYPNSPWFCELCDKYGFYVISESDIESHGAAFQAISHPEPSIF
LNVENPNEEPRIRQQTIDNFCYFAREPLYRAALLERTKANIERDKNRSSILIWSLGNESGYGENFEYC
AKWVKERDPDRLVHYESSIYQHSAYQNNTGHLDLYSEMYSDTEAIDAYFADHSQTKKPFLLCEYSHAM
GNSNGDMEDYFQTFNKYSGCCGGFIWEWCDHAQYITPTKLGYGGDFGEKIHDGNFCVDGLVSPERVPH
SNLLEVKNVNRPVRANLRGEQIELYNYFDFTNLKDILCVKYEWVKNGQITGTGTLAVDCEPHHSQILP
IQLPKEREGLLWLNLYYCASRQTDLLPAGHHFGFDQIILSKEYTPAIGSDKDDCPPLEITETVRQIVV
RNNRYYFEFNKLTGIIDEIKVNGKAFIHKPLAWNIWRAPTDNDRLIRSQWQNAGYDQMYSKVYDICAH
RQGNGVVVSVKSALVADAKSKIMTLETQYLLSENGKLDIQTNAVFHEHLPFLPRFGLRFFLDEQKTPF
TYLGYGAGESYIDKHQATKLGIYSTTAGENHVGYLKPQENGSHYGCFYVQNDMIRVESGQPFSFNLSP
YTQEELTQKKHSYELVCSGYDVLCIDYKMSGIGSNSCGPNLKPQYRLIENNINFDISIRL*

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.

***porB* terminator nucleotide sequence (5’→3’):**

TCTGCAAAGATTGGTATCAACAAAAAGCCTGTCGTCAGACAGGCTTTTTTTCTGTTTTCTGTTTTTAG
AT

Alternate *fhbp*$_{x.yz}$ genes present in transformative constructs 2, 3 and 4; nucleotides coding for amino acid residues different to those in the respective wild type proteins are shown as lower case lettering, with those amino acids also denoted in bold text:

***fhbp*$_{1.13}$_(H248L) nucleotide sequence (5’→3’):**

ATGACTAGGTCCAAACCCGTCAATCGTACGGCATTTTGCTGTCTATCTCTTACCGCCGCCCTGATATT
GACTGCGTGCAGCTCAGGAGGGGGCGGTGTAGCAGCTGATATTGGAGCCGGGCTAGCGGACGCACTTA
CAGCTCCGCTGGATCATAAGGACAAAGGCTTGCAATCCCTAACACTTGATCAGTCGGTTCGAAAGAAC
GAAAAACTGAAGTTGGCCGCGCAAGGCGCTGAGAAAACCTATGGTAATGGAGACAGCCTAAACACGGG
GAAGCTTAAAAATGATAAGGTGTCACGGTTCGACTTTATCCGCCAGATAGAAGTTGATGGCAAACTGA
TTACCTTGGAGTCTGGTGAATTCCAAGTGTATAAGCAGTCCCACTCCGCGTTGACCGCATTGCAAACG
GAACAGGTTCAAGACTCCGAGGATTCTGGCAAAATGGTGGCCAAGCGGCAGTTTCGCATTGGTGATAT
CGCGGGCGAACATACCTCCTTCGACAAACTTCCGAAAGGAGGCTCCGCCACGTATCGGGGTACCGCTT
TTGGCTCTGATGACGCAGGCGGGAAGCTGACGTACACAATTGATTTCGCTGCCAAACAAGGTCACGGA
AAGATCGAGCATCTAAAATCCCCCGAACTGAACGTTGAACTGGCGACCGCCTATATTAAGCCGGACGA

AAAACGCCACGCGGTGATCTCTGGCTCCGTTCTGTATAATCAGGATGAGAAGGGGTCTTACTCCCTGG
GCATTTTTGGCGGCCAAGCACAGGAAGTGGCTGGTTCAGCCGAGGTCGAAACCGCCAATGGCATTCAT
ttgATTGGGCTGGCCGCCAAACAATAG

Translated amino acid sequence – FHbp1.13 (H248L):

MTRSKPVNRTAFCCLSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKN
EKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGKLITLESGEFQVYKQSHSALTALQT
EQVQDSEDSGKMVAKRQFRIGDIAGEHTSFDKLPKGGSATYRGTAFGSDDAGGKLTYTIDFAAKQGHG
KIEHLKSPELNVELATAYIKPDEKRHAVISGSVLYNQDEKGSYSLGIFGGQAQEVAGSAEVETANGIH
LIGLAAKQ*

*fhbp2.19*_(L130R G133D) nucleotide sequence (5'→3'):

ATGACACGTAGCAAGCCGGTGAACCGAACCGCGTTCTGTTGCTTGTCCCTAACTGCCGCTCTTATCCT
GACAGCCTGCTCGTCTGGTGGAGGGGGCGTCGCGGCAGACATAGGTGCTGGATTGGCCGATGCGCTAA
CGGCACCCCTTGACCACAAAGATAAGTCTCTGCAGAGCTTGACGCTAGACCAATCAGTACGCAAAAAT
GAGAAGCTTAAACTGGCTGCCCAGGGGGCAGAAAAGACTTACGGCAACGGTGATTCGTTGAATACCGG
AAAACTAAAGAACGACAAAGTTTCTAGGTTTGATTTCATTCGGCAAATCGAGGTTGATGGGCAGCTTA
TAACCCTGGAATCCGGCGAGTTTCAAATTTATAAACAGGATCATTCAGCAGTTGTTGCGCTACAAATC
GAGAAAATTAATAACCCGGACAAGATCGATTCCCTGATTAACCAGCGGTCCTTCcggGTGTCTgatCT
GGGTGGGGAACACACAGCCTTCAATCAGCTGCCCTCTGGTAAGGCTGAGTACCATGGAAAAGCCTTTT
CCTCCGACGATGCGGGTGGCAAGCTTACCTATACGATCGATTTTGCCGCTAAGCAAGGACACGGTAAA
ATCGAACATTTGAAGACCCCGGAACAAAACGTCGAGCTGGCATCTGCGGAATTGAAAGCCGATGAGAA
GTCTCATGCAGTTATTTTGGGCGACACGCGGTACGGCGGCGAGGAAAAGGGTACCTATCATCTGGCGC
TGTTCGGCGATCGCGCGCAGGAGATCGCCGGCTCTGCTACGGTGAAGATTCGGGAAAAAGTTCACGAG
ATTGGCATCGCCGGTAAACAATGA

Translated amino acid sequence – FHbp2.19 (L130R G133D):

MTRSKPVNRTAFCCLSLTAALILTACSSGGGGVAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKN
EKLKLAAQGAEKTYGNGDSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQIYKQDHSAVVALQI
EKINNPDKIDSLINQRSF**R**VS**D**LGGEHTAFNQLPSGKAEYHGKAFSSDDAGGKLTYTIDFAAKQGHGK
IEHLKTPEQNVELASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVKIREKVHE
IGIAGKQ*

*fhbp3.45*_(E254A) nucleotide sequence (5'→3'):

ATGACCCGCTCTAAGCCGGTAAACCGGACAGCTTTCTGTTGCCTTTCACTGACGGCCGCGTTGATTCT
AACCGCATGCTCCTCGGGCTCCGGCTCCGGGGGCGGTGGAGTTGCTGCCGACATCGGGACCGGCCTTG
CAGATGCTCTGACTGCCCCCTTGGACCACAAAGATAAGGGTCTAAAATCTCTTACTCTGGAAGATAGC
ATTTCCCAAAATGGCACGTTGACCCTATCCGCACAGGGTGCCGAAAAGACGTTTAAAGTTGGGGATAA
GGATAATTCCCTTAATACAGGCAAACTGAAGAACGACAAAATCTCGCGCTTTGATTTCGTTCAGAAAA
TTGAGGTTGATGGTCAGACCATCACCCTAGCCTCAGGAGAGTTCCAGATCTACAAGCAAGACCACTCC
GCTGTGGTGGCCCTTCAGATTGAAAAGATCAACAATCCCGATAAAATTGACTCTTTGATCAATCAACG
GTCTTTTCTGGTTTCCGGTTTGGGCGGAGAGCATACTGCGTTTAACCAACTACCGTCCGGCAAAGCGG
AATATCACGGGAAGGCATTCTCTTCAGATGACGCCGGCGGAAAATTGACATACACGATTGACTTCGCA

GCGAAACAGGGGCATGGCAAGATTGAGCACCTGAAAACGCCGGAGCAAAATGTTGAACTGGCTTCCGC
CGAGCTGAAGGCGGACGAAAAATCCCACGCTGTCATCCTGGGCGATACCCGCTATGGTTCCGAAGAGA
AAGGCACGTATCACCTGGCCTTGTTTGGCGACCGGGCCCAAGAAATTGCAGGTTCTGCGACCGTGAAA
ATTCGGGAGAAGGTTCATgccATCGGAATTGCCGGCAAACAGTAA

Translated amino acid sequence – FHbp3.45 (E254A):

MTRSKPVNRTAFCCLSLTAALILTACSSGSGSGGGGVAADIGTGLADALTAPLDHKDKGLKSLTLEDS
ISQNGTLTLSAQGAEKTFKVGDKDNSLNTGKLKNDKISRFDFVQKIEVDGQTITLASGEFQIYKQDHS
AVVALQIEKINNPDKIDSLINQRSFLVSGLGGEHTAFNQLPSGKAEYHGKAFSSDDAGGKLTYTIDFA
AKQGHGKIEHLKTPEQNVELASAELKADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVK
IREKVH**A**IGIAGKQ*

There is only one functional gene in construct 5, coding for *Neisseria* Adhesin A (NadA), a type V autotransporter from *N. meningitidis* strain MC58. The *nadA* gene is codon-optimised to maximise expression in *N. lactamica* and is transcriptionally controlled by the *porA*-associated-UAS-enhanced, *porA/porB* hybrid promoter. The *nadA* gene is flanked on either end by sequence homologous to two separate, 1000bp regions of the gene coding for the endogenous *lacZ* gene of *N. lactamica*, to target construct 5 into the screening marker of the previous transformative cassettes, i.e. insertionally inactivating the *lacZ* gene introduced along with the *fhbp*$_{x.yz}$ gene(s). A schematic of the features included in construct 5 is shown in Figure 7.

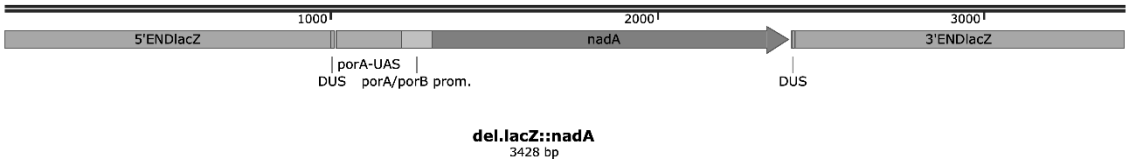


Figure 7: To-scale schematic of transformative construct 5, used to knock a functional, *N. lactamica* codon-optimised *nadA* gene into the *lacZ* coding sequence integrated into NHCIS1 by one of the four previous transformative constructs. Each FHbp-expressing GM-*N. lactamica* strain was transformed with the same version of construct 5: strain 4FA313_(H248L) was transformed to yield strain 4FAN313_H; strain 4FB313_(H248L) was transformed to yield strain 4FBN313_H; strain 4FC313 was transformed to yield 4FCN313; and strain 4FD313_(E254A) was transformed to yield 4FDN313_E. Each of the strains expressing mutant FHbp variants and NadA became the background for subsequent transformation with 3'END*lacZ*:Synth.*lacZ*:3'ENDNHCIS1.

The ∆*lacZ::nadA* construct in its entirety (3,428 bp) consists of:

1. Sequence homologous to a 999bp region proximal to the 5'END of the coding sequence of the *N. lactamica lacZ* gene.

   5'END*lacZ* nucleotide sequence (5'→3'):

GCGAATTATTATCAAGATCCTGAAATCACGAGAATCAATGCGTTGCCGCACCATAGCTATTTTATCCC
TTTTGATAAAAAAGATAAGGTAGATCAGTTTTCTAGGGAAAATTCTTCGTTTTTTACATCATTAAATG
GAATGTGGCAATTCGCATATTATCCGAGTATGCAGGATTTGCCTGAAAGTCCGGACGAAATCGCTTTT
ACGAAACAAATCAATGTGCCTTCAAATTGGCAGAATCACGGGTTTGATGCCCATCAATATACTAATAT
TAATTATCCTTTCCCTTTCGATCCGCCTTTTGTTCCTTTAGAGAACCCTTGCGGGGTATATCAAAAGC
AGGTCAATCTGAAAAAGAATATAAATAAGCGGTATTTATTAGTCCTTGAAGGGGTTGATTCCTGTTCT
TATATATATGTAAACCATCAATTTGTAGGATATGGTTCTATCAGCCACAGTACCAATGAATTTGATAT
TACCGATTATCTTCACGATGGTGAAAACACCCTTACCGTATTTGTCCTGAAATGGTGTGCCGGAAGCT
ACTTGGAAGATCAGGATAAATTTAGAATGTCGGGAATTTTCCGAGATGTATATTTATTGGAAAGGGAG
CATCACTATTTGCAAGACTTGAATATTCGAACCGTGCTTTCTGAAGATTTATCATTGGGGCAGATTTG
TCTGGATTTAAATTTTGCGGGGGATGCGGGGGATGTCGGAGTGTCATTGTTTGATACGGACGGGCAAA
TTGTTCAAGCAGGCAGCGCAATCACGACAGATAAACAACGGATGCAAATCCGCCTTGATAATATTCCT
TTAACCAAATCCCGACTCTGGAATGCGGAAAACCCTGCCCTTTATACCTTGGTATTAAACACAAAAGA
AGAAATCATTACCCAGAAGATCGGATTCCGCAAGGTAGAGGTCAAAAACGGGGTATTGCTGCTTAATA
ATCAGCCTATTAAATTCAAAGGGGTAAACCGGCACGATAGCGATCCC

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 *N. lactamica* 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-UAS’). 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 Item 15, Figure 3).

   ***porA*-associated UAS nucleotide sequence (5’→3’):**

GTGCCGCGTGTGTTTTTTTATGGCGTTTTAAAAAGCCGAGACTGCATCCGGGCAGCAGCGCATCGGCT
CGCACGAGGTCTGCGCTTGAATTGTGTTGTAGAAACACAACGTTTTTGAAAAAATAAGCTATTGTTTT
ATATCAAAATATAATCATTTTTAAAATAAAGGTTGCGGCATTTATCAGATATTTGTTCTGAAAA

4. A synthetic, hybrid promoter (labelled as ‘porA/porB prom.’) consisting primarily of sequence derived from the *porA* gene of wild type *N. meningitidis*, but wherein the homopolymeric guanosine nucleotide tract that separates the -10 and -35 boxes of that promoter has been replaced by the 17bp, non-phase variable sequence that separates the -10 and -35 boxes of the *N. lactamica porB* promoter (lower case lettering, below).

   ***porA/porB* hybrid promoter nucleotide sequence (5’→3’):**

ATGGTTtttcgggcgggaacattTATAATTGAAGACGTATCGGGTGTTTGCCCGATGTTTTTAGGTTT
TTATCAAATTTACAAAAGGAACTCGAG

5. An *N. lactamica* codon-optimised, synthetic version of the *nadA* gene from *N. meningitidis* strain MC58.

   ***nadA* nucleotide sequence (5’→3’):**

ATGAGCATGAAACACTTTCCGTCGAAAGTACTGACAACCGCAATTTTAGCTACATTTTGTAGCGGCGC
CTTGGCAGCCACCAGCGACGATGACGTAAAAAAAGCCGCCACTGTCGCTATCGTGGCGGCCTACAACA
ACGGCCAGGAAATCAACGGTTTTAAAGCCGGTGAAACCATCTATGACATCGGCGAAGACGGTACTATC
ACCCAAAAAGATGCTACGGCGGCAGACGTTGAAGCAGACGATTTCAAAGGCTTGGGCCTGAAAAAAGT
GGTCACCAACTTGACCAAgACCGTGAACGAAAATAAACAgAACGTCGATGCCAAAGTAAAAGCAGCGG
AAAGCGAAATTGAAAAGTTGACCACGAAATTAGCAGAtACCGACGCGGCTCTGGCCGATACCGACGCT
GCGCTGGACGAgACCACCAATGCatTGAATAAACTGGGTGAAAATATCACCACgTTCGCCGAAGAAAC
GAAAACCAACATCGTGAAAATTGACGAgAAATTGGAaGCGGTGGCCGAcACCGTCGATAAACACGCGG
AAGCGTTCAATGACATCGCCGATAGCTTGGACGAAACcAACACAAAGGCAGATGAAGCGGTTAAGACG
GCCAATGAAGCAAAgCAAACTGCCGAAGAgACAAAACAgAACGTGGACGCCAAAGTCAAAGCgGCCGA
aACaGCGGCcGGCAAAGCtGAAGCAGCCGCAGGCACAGCgAACACAGCCGCGGAtAAAGCAGAAGCAG
TAGCGGCCAAAGTCACCGAcATCAAAGCCGATATCGCGACTAACAAAGCGGATATCGCCAAAAACTCA
GCACGTATCGACTCTTTGGACAAAAACGTAGCGAACTTACGTAAAGAAACCCGCCAGGGTTTGGCTGA
ACAAGCGGCTTTGTCTGGATTGTTCCAACCCTATAACGTCGGTCGCTTTAACGTCACGGCAGCCGTTG
GCGGGTATAAAAGCGAATCGGCTGTCGCTATAGGCACCGGTTTCCGCTTCACTGAGAACTTTGCCGCG
AAAGCCGGCGTTGCCGTAGGCACCTCCTCCGGCTCATCCGCCGCTTATCACGTTGGCGTCAACTACGA
ATGGTAA

Translated amino acid sequence – Nad*A*:

MSMKHFPSKVLTTAILATFCSGALAATSDDDVKKAATVAIVAAYNNGQEINGFKAGETIYDIGEDGTI
TQKDATAADVEADDFKGLGLKKVVTNLTKTVNENKQNVDAKVKAAESEIEKLTTKLADTDAALADTDA
ALDETTNALNKLGENITTFAEETKTNIVKIDEKLEAVADTVDKHAEAFNDIADSLDETNTKADEAVKT
ANEAKQTAEETKQNVDAKVKAAETAAGKAEAAAGTANTAADKAEAVAAKVTDIKADIATNKADIAKNS
ARIDSLDKNVANLRKETRQGLAEQAALSGLFQPYNVGRFNVTAAVGGYKSESAVAIGTGFRFTENFAA
KAGVAVGTSSGSSAAYHVGVNYEW*

6. Sequence homologous to a 1000bp region proximal to the 3’END of the coding sequence of the *N. lactamica lacZ* gene.

3’END*lacZ* nucleotide sequence (5’→3’):

CCGATTCAGCTTCCCAAAGAACGGGAAGGATTGCTATGGTTGAACCTATATTACTGCGCTTCCCGGCA
AACTGATTTATTGCCGGCAGGACATCATTTCGGTTTCGATCAAATTATTTTGAGTAAGGAATACACTC
CTGCCATAGGATCGGATAAAGATGATTGCCCGCCGTTGGAAATAACCGAAACAGTGAGACAAATCGTT
GTTCGGAACAATCGATATTATTTTGAATTTAACAAGCTCACGGGGATAATTGACGAAATTAAAGTCAA
TGGCAAGGCTTTCATCCATAAACCATTGGCTTGGAATATTTGGCGCGCGCCGACGGATAATGACCGCC
TGATTCGTTCCCAATGGCAAAATGCCGGTTATGATCAAATGTATTCCAAGGTTTACGATATTTGCGCG
CATCGGCAAGGAAACGGTGTTGTCGTGTCTGTGAAAAGTGCATTGGTTGCGGATGCAAAATCAAAAAT
CATGACATTGGAAACACAGTATCTGCTGTCTGAAAATGGTAAATTGGATATTCAAACAAATGCTGTTT
TCCACGAGCATTTGCCATTCCTGCCCCGTTTCGGTTTGCGCTTTTTCTTAGACGAACAGAAAACACCT
TTTACCTACTTAGGTTATGGGGCGGGTGAAAGCTATATTGATAAACATCAGGCGACCAAACTTGGTAT
TTATTCGACAACGGCAGGGGAAAATCATGTTGGCTATCTCAAGCCTCAAGAGAACGGCAGCCATTATG
GATGCTTTTATGTTCAAAACGATATGATCCGGGTGGAGTCGGGGCAGCCGTTTAGTTTCAACCTGTCC
CCTTATACGCAAGAAGAACTGACGCAGAAAAAACATTCTTATGAATTGGTTTGTTCCGGGTATGATGT
ATTGTGTATCGACTATAAGATGAGCGGTATCGGTTCGAATTCTTGCGGGCCTAATTTGAAGCCGCAAT
ATCGTTTGATTGAAAATAACATCAATTTCGATATAAGTATCCGGTTAT

There is only one functional gene in construct 6, a rationally-designed, synthetic *lacZ* gene (Synth.*lacZ*) coding for β-D-galactosidase, under transcriptional control of the endogenous *N. lactamica lst* gene promoter. The Synth.*lacZ* gene is coded to be as different as possible to the 5'END*lacZ* and 3'END*lacZ* regions that remain in the chromosome of FHbp variant- and NadA-expressing GM-*N. lactamica* strains at the nucleotide sequence level, whilst maintaining the wild type amino acid sequence of β-D-galactosidase. This disrupts the length of regions of nucleotide homology between the chromosomal remnants of the endogenous, *N. lactamica lacZ* in NHCIS1 and the sequence of Synth.*lacZ* in the transformative construct, reducing the likelihood of off-target integration of the construct. Indeed, the region of Synth.*lacZ* that corresponds to the 5'END*lacZ* region is only 64.36% similar to the sequence of the endogenous gene, whilst the region of Synth.*lacZ* that corresponds to the 3'END*lacZ* region is only 63.87% similar. The Synth.*lacZ* gene is flanked at its 5' end by the 3'END*lacZ* sequence, and at its 3' end by the 3'ENDNHCIS1 sequence. The result of successfully transforming FHbp variant- and NadA-expressing GM-*N. lactamica* strains with this construct is essentially to integrate a functional copy of the *lacZ* gene back into the GM-*N. lactamica* chromosome at the 3' end of the modified NHCIS1 locus. A schematic of the features included in construct 6 is shown in Figure 8.

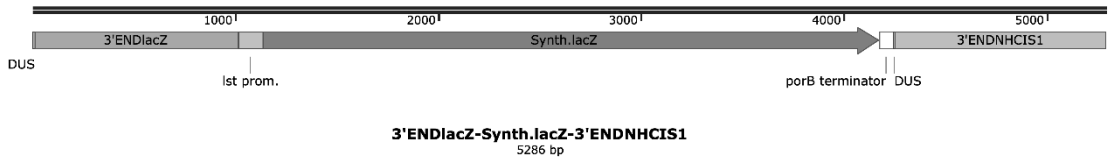


Figure 8: To-scale schematic of transformative construct 6, used to knock a functional, rationally-designed *lacZ* gene into the junction between the 1000bp remnant of the endogenous *N. lactamica lacZ* gene and the 3'END of the NHCIS1 chromosomal locus in GM-*N. lactamica* strains transformed to express one mutated FHbp variant and NadA. Each FHbp variant- and NadA-expressing GM-*N. lactamica* strain was transformed with the same version of construct 6: strain 4FAN313_H was transformed to yield strain 4NFA313_H; strain 4FBN313_H was transformed to yield strain 4NFB313_H; strain 4FCN313 was transformed to yield 4NFC313; and strain 4FDN313_E was transformed to yield 4NFD313_E.

The 3'END*lacZ:*Synth.*lacZ*:3'ENDNHCIS1 construct in its entirety (5,286 bp) consists of:

1. Sequence homologous to a 1000bp region proximal to the 3'END of the coding sequence of the *N. lactamica lacZ* gene.

   3'END*lacZ* nucleotide sequence (5'→3'):

CCGATTCAGCTTCCCAAAGAACGGGAAGGATTGCTATGGTTGAACCTATATTACTGCGCTTCCCGGCA
AACTGATTTATTGCCGGCAGGACATCATTTCGGTTTCGATCAAATTATTTTGAGTAAGGAATACACTC
CTGCCATAGGATCGGATAAAGATGATTGCCCGCCGTTGGAAATAACCGAAACAGTGAGACAAATCGTT
GTTCGGAACAATCGATATTATTTTGAATTTAACAAGCTCACGGGGATAATTGACGAAATTAAAGTCAA
TGGCAAGGCTTTCATCCATAAACCATTGGCTTGGAATATTTGGCGCGCGCCGACGGATAATGACCGCC
TGATTCGTTCCCAATGGCAAAATGCCGGTTATGATCAAATGTATTCCAAGGTTTACGATATTTGCGCG
CATCGGCAAGGAAACGGTGTTGTCGTGTCTGTGAAAAGTGCATTGGTTGCGGATGCAAAATCAAAAAT
CATGACATTGGAAACACAGTATCTGCTGTCTGAAAATGGTAAATTGGATATTCAAACAAATGCTGTTT
TCCACGAGCATTTGCCATTCCTGCCCCGTTTCGGTTTGCGCTTTTTCTTAGACGAACAGAAAACACCT
TTTACCTACTTAGGTTATGGGGCGGGTGAAAGCTATATTGATAAACATCAGGCGACCAAACTTGGTAT
TTATTCGACAACGGCAGGGGAAAATCATGTTGGCTATCTCAAGCCTCAAGAGAACGGCAGCCATTATG
GATGCTTTTATGTTCAAAACGATATGATCCGGGTGGAGTCGGGGCAGCCGTTTAGTTTCAACCTGTCC
CCTTATACGCAAGAAGAACTGACGCAGAAAAAACATTCTTATGAATTGGTTTGTTCCGGGTATGATGT
ATTGTGTATCGACTATAAGATGAGCGGTATCGGTTCGAATTCTTGCGGGCCTAATTTGAAGCCGCAAT
ATCGTTTGATTGAAAATAACATCAATTTCGATATAAGTATCCGGTTAT

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

   ***lst* promoter nucleotide sequence (5'→3'):**

ATATCGGCAACTGTCGGAATATCTGCTAAAATTCCGCATTTTCCGCACCGGGTTTCCGCACCGGGACA
CTCGGGGCGTATGTTCAATTTGTCGGAATGGAGTTTAAAGGAATACACAT

3. A rationally-designed, synthetic copy of the *lacZ* gene (Synth.*lacZ*), coding for the cytoplasmic enzyme β-D-galactosidase. Nucleotide substitutions designed to reduce the length of tracts of homology between Synth.*lacZ* and the two 1000bp remnants of the endogenous *N. lactamica lacZ* gene, whilst maintaining the fidelity of the β-D-galactosidase amino acid sequence are shown in lower case lettering.

   Synth.*lacZ* nucleotide sequence (5'→3'):

ATGCTGTTGGCAAACTACTACCAAGACCCGGAAATTACCCGCATCAACGCACTCCCGCAtCATTCCTA
CTTCATCCCATTCGACAAgAAAGACAAAGTTGACCAATTTTCGCGCGAgAAcTCCAGCTTCTTCACAT
CCCTGAACGGCATGTGGCAgTTCGCtTACTACCCTTCTATGCAAGACcTGCCGGAATCTCCCGAtGAg
ATtGCGTTCACTAAACAgATaAACGTACCGAGCAACTGGCAaAACCATGGCTTcGACGCGCAcCAgTA
CACCAACATcAACTACCCGTTtCCATTtGACCCtCCgTTCGTACCCCTGGAAAATCCGTGTGGTGTCT
ACCAgAAACAaGTTAACCTGAAgAAAAACATCAACAAACGTTAccTcCTGGTtCTGGAgGGcGTgGAC
agcTGCagcTACATCTAcGTgAAtCACCAGTTcGTtGGTTACGGCAGCATTTCCCATtcCACgAACGA
GTTcGAcATCACTGAcTACTTGCATGACGGcGAgAATACgCTCACtGTCTTCGTAtTGAAGTGGTGCG
CgGGCtcCTAtCTGGAgGAcCAAGACAAgTTCCGCATGTCCGGTATCTTtCGGGACGTcTACTTGcTc
GAgCGCGAACAcCATTACCTGCAGGATCTCAACATCCGCACtGTcCTGAGCGAgGACCTcagcCTcGG
CCAaATcTGCCTTGAcCTGAACTTCGCCGGTGAcGCCGGCGACGTgGGTGTcagTCTcTTCGACACCG
AtGGCCAGATCGTgCAgGCCGGTTCCGCCATtACTACgGACAAgCAgCGTATGCAgATTCGtCTGGAc
AAcATcCCgTTGACTAAAAGtCGTttgTGGAAcGCCGAgAAtCCGGCATTGTAcACgcTtGTTCTGAA
TACCAAAGAgGAgATTATcACgCAAAAAATtGGCTTtCGtAAAGTTGAAGTGAAgAATGGCGTGcTGT
TGCTGAAcAACCAaCCGATCAAgTTtAAGGGtGTTAAtCGCCATGActcCGACCCTAAAACGGGGTAC
GCTATTTCCGTCGCCCAAGCCGTCACGGACCTGTCACTGATGAAAAAACACAATATCAACGCGATTCG

CACTGCACATTATCCGAATTCCCCCTGGTTCTGCGAACTGTGTGACAAATATGGGTTTTACGTGATCA
GTGAAAGCGACATTGAATCACACGGTGCAGCCTTCCAGGCTATCTCCCATCCGGAACCGTCAATTTTC
CTTAACGTGGAAAACCCCAACGAAGAACCGCGGATCCGCCAACAAACAATCGACAACTTTTGCTACTT
CGCTCGTGAACCGTTGTATCGTGCGGCACTGCTGGAACGTACCAAAGCCAACATTGAACGTGACAAAA
ACCGCTCTTCCATTTTGATTTGGTCTTTGGGCAACGAGAGCGGCTACGGCGAAAACTTCGAATACTGC
GCAAAATGGGTTAAAGAACGCGATCCTGATCGTTTGGTCCACTACGAATCAAGCATCTATCAGCATAG
CGCATACCAAAATAACACCGGTCATTTGGATCTATACAGTGAAATGTACTCCGATACGGAAGCCATTG
ATGCCTACTTTGCAGACCACAGCCAGACCAAAAAACCGTTCCTGCTATGTGAATACAGCCACGCCATG
GGCAATTCCAACGGTGACATGGAAGATTACTTTCAAACCTTTAACAAATACTCCGGCTGTTGCGGCGG
TTTCATCTGGGAATGGTGTGACCACGCACAATATATCACCCCGACGAAATTGGGCTACGGTGGCGACT
TTGGAGAGAAAATCCATGATGGCAATTTCTGTGTCGATGGGTTGGTTAGCCCTGAACGCGTACCCCAC
TCGAATCTGTTGGAGGTTAAGAACGTTAACCGCCCGGTCCGCGCTAACCTGAGGGGTGAACAAATAGA
ATTGTACAACTACTTCGATTTCACCAACTTAAAAGACATCTTGTGCGTAAAATACGAATGGGTCAAAA
ATGGTCAAATTACTGGCACCGGTACACTGGCGGTCGACTGCGAACCCCACCACTCCCAGATTTTGCCT
ATcCAaCTGCCGAAGGAGCGTGAgGGTcTcTTgTGGcTtAAtCTGTACTATTGtGCCagcCGTCAGAC
cGACCTGCTCCCTGCgGGCCACCACTTtGGCTTtGACCAgATcATCCTGTCAAAAGAGTAtACCCCCG
CGATTGGCAGCGACAAgGACGAcTGtCCaCCtCTGGAGATCACtGAgACCGTCCGCCAGATTGTGGTc
CGTAAtAACCGTTACTACTTCGAgTTCAAtAAATTGACTGGtATTATCGATGAGATcAAgGTGAACGG
tAAAGCCTTTATTcCAcAAACCGCTCGCCTGGAAcATCTGGCGtGCCCCCACCGAcAAcGAtCGTTTGA
TcCGCTCACAGTGGCAgAACGCGGGCTACGAcCAgATGTACTCTAAAGTcTAtGACATCTGtGCACAC
CGCCAgGGCAAtGGcGTCGTTGTCTCGGTAAAGTCGGCGCTCGTCGCAGACGCCAAATCGAAgATtAT
GACGCTGGAgACCCAATACTTGCTCagcGAgAACGGCAAACTGGACATCCAgACCAACGCaGTGTTTC
AtGAACAcCTCCCGTTtTTaCCACGCTTTGGCCTcCGtTTCTTtCTGGATGAgCAAAAgACCCCGTTC
ACTTAtCTGGGCTAcGGCGCCGGCGAgTCTTACATCGACAAgCAcCAAGCCACgAAATTGGGCATcTA
cTCCACCACCGCCGGCGAgAACCATGTcGGtTAccTgAAACCGCAGGAAAATGGTTCCCAcTACGGcT
GTTTcTAcGTGCAgAAtGAcATGATtCGCGTAGAAAGCGGCCAACCcTTCTCCTTTAAttTaagcCCG
TACACCCAgGAAGAgTTGACCCAAAAgAAaCACTCCTACGAgCTCGTCTGcagcGGATAcGACGTCct
cTGCATTGAtTAcAAAATGTCTGGcATTGGCTCCAACAGCTGTGGCCCCAACcTGAAACCTCAgTACC
GCCTCATCGAgAACAAtATtAAcTTtGAcATTTCCATTCGCCTCTAG

Translated amino acid sequence – (Synth.)β-D-galactosidase:

MLLANYYQDPEITRINALPHHSYFIPFDKKDKVDQFSRENSSFFTSLNGMWQFAYYPSMQDLPESPDE
IAFTKQINVPSNWQNHGFDAHQYTNINYPFPFDPPFVPLENPCGVYQKQVNLKKNINKRYLLVLEGVD
SCSYIYVNHQFVGYGSISHSTNEFDITDYLHDGENTLTVFVLKWCAGSYLEDQDKFRMSGIFRDVYLL
EREHHYLQDLNIRTVLSEDLSLGQICLDLNFAGDAGDVGVSLFDTDGQIVQAGSAITTDKQRMQIRLD
NIPLTKSRLWNAENPALYTLVLNTKEEIITQKIGFRKVEVKNGVLLLNNQPIKFKGVNRHDSDPKTGY
AISVAQAVTDLSLMKKHNINAIRTAHYPNSPWFCELCDKYGFYVISESDIESHGAAFQAISHPEPSIF
LNVENPNEEPRIRQQTIDNFCYFAREPLYRAALLERTKANIERDKNRSSILIWSLGNESGYGENFEYC
AKWVKERDPDRLVHYESSIYQHSAYQNNTGHLDLYSEMYSDTEAIDAYFADHSQTKKPFLLCEYSHAM
GNSNGDMEDYFQTFNKYSGCCGGFIWEWCDHAQYITPTKLGYGGDFGEKIHDGNFCVDGLVSPERVPH
SNLLEVKNVNRPVRANLRGEQIELYNYFDFTNLKDILCVKYEWVKNGQITGTGTLAVDCEPHHSQILP
IQLPKEREGLLWLNLYYCASRQTDLLPAGHHFGFDQIILSKEYTPAIGSDKDDCPPLEITETVRQIVV
RNNRYYFEFNKLTGIIDEIKVNGKAFIHKPLAWNIWRAPTDNDRLIRSQWQNAGYDQMYSKVYDICAH
RQGNGVVVSVKSALVADAKSKIMTLETQYLLSENGKLDIQTNAVFHEHLPFLPRFGLRFFLDEQKTPF
TYLGYGAGESYIDKHQATKLGIYSTTAGENHVGYLKPQENGSHYGCFYVQNDMIRVESGQPFSFNLSP
YTQEELTQKKHSYELVCSGYDVLCIDYKMSGIGSNSCGPNLKPQYRLIENNINFDISIRL*

4. A transcriptional terminator (labelled as 'porB terminator') derived from the sequence immediately downstream of the *porB* gene from wild type *N. meningitidis* strain MC58.

   ***porB* terminator nucleotide sequence (5'→3'):**

TCTGCAAAGATTGGTATCAACAAAAAGCCTGTCGTCAGACAGGCTTTTTTTCTGTTTTCTGTTTTTAG
AT

5. 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 *N. lactamica* and internalised by the bacteria as part of natural competence.
6. Sequence homologous to the 3'END of the NHCIS1 locus of *N. lactamica* strain Y92-1009.

   3'ENDNHCIS1 nucleotide sequence (5'→3'):

TGCTGAAGTAGAAAACCAGCAAGAAGGTAAAAAGAAAGAAGCAGTTTTTTGGATTTTAGATGTTACCG
CAATTGGTTTCCTTTCCTAAAATTTGTTTAAATTATTTGCAATATTAATATAAACTGGATATTAATGA
TGAGGATTCAAAAAGGCATACTGAATATAATTTGTACAAAATATTTGCAGTATTTAAAAATGTTGGTT
CGTATATGAAAAGTTAAAAATGCCAAAATGTACAGTTGCTAAACTGTAAAACTGCTAAAGCAACAAAA
CATAAAAAGGAATGCAGGGATGCGATCACTACATCTTTTTATTCCGTAAGCATTTATGACTTTACGGT
CAACTGCTACTCTATGTTTCCAGCTTTTCAGCTCCCTATTTTCGAATATTGGACGAGGCATTTTCATC
AGTGTCGTAATGCCGACCGAAACCCTCACAAACCATATTGGTTCTTGTGGCAGCAACACCTATCCGTT
TGTTCAAGCGGCCACAAGAGTAACATGATTGGCTGGTGGCATTGGCTTTAATCTCTTCGATATGAACT
CCATTTTTAGCTGCACCTTCTTTCAGCGTATGCAACAGTGGGGATGGGGCAACTATCTTCTGGTGGAG
TTTGTTGACTTCAATCTGTATGCACTGTAGGTTGAGCAGATTCAGTTTTTTGATACAGATTATCCGGC
TTTGTTCGGCAATTCTGTTGGCGAACGTATAGTAGAGCTGTCGTCTTGCCGCTTTCAATTTGCGGTAG
GTATTTTACCATTCCATGCGTAGCCGCTCGGTACGTTTGAGCCAAATGTTATCTTCGCCATTTGTACC
AATTTGTTTTTACATTAGGCTGTGTTTTAGTAATCTATTGATTTCAATTATTTGCAAGGGAAAAGACA
ATTATTTTCCGGTTAGGAATAAACCTATCCTATTGAATATATTGAAGCCAAGTACGCTTATCAACACT
ATATTAAAACACAGCCTTTTTTAATATAGTAGACACAATCTTTCCTTATTTATGAAGGTGATAGCTTC
TTTCAG

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 which 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 stepwise insertion of three gene expression cassettes into an intergenic chromosomal locus (NHCIS1) (for overview, see Figure 9). The first gene expression cassette contained the coding sequence of one of four different variants of FHbp, each specifically mutated to disrupt the interaction of the gene product with human complement Factor H (see Figure 12). The expression of each *fhbp* gene is under transcriptional control of a synthetic, hybrid promoter, which is designed to allow for constitutive expression of

the gene product to a high level (see Item 24, Figure 6). To provide a means of identifying putative transformants, each *fhbp* gene was flanked at its 3' end by a copy of the *N. lactamica lacZ* gene, which meant putative transformants grew as blue colonies on growth medium containing X-Gal. Into this copy of the *lacZ* gene, a second nucleic acid construct was inserted, coding for an *N. lactamica* codon-optimised version of the *nadA* gene, under transcriptional control of the same promoter as the *fhbp* gene (see Item 24, Figure 7). Finally, β-D-galactosidase activity was restored to the strains by integration of a rationally-designed, synthetic copy of the *lacZ* gene (Synth.*lacZ*) (see Item 24, Figure 8).

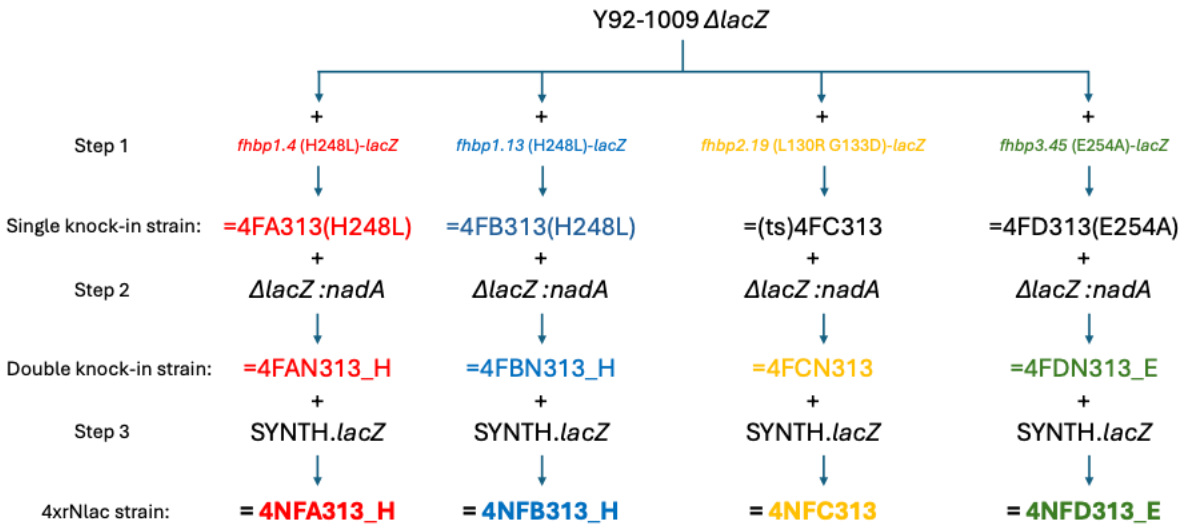


Figure 9: Schematic representation of stepwise transformations to yield 4xrNlac strains.

1. Heterologous antigen expression

Using flow cytometry based assays and antigen-specific immunological reagents, each GMO has been shown to express both (i) FHbp and (ii) NadA on its surface:

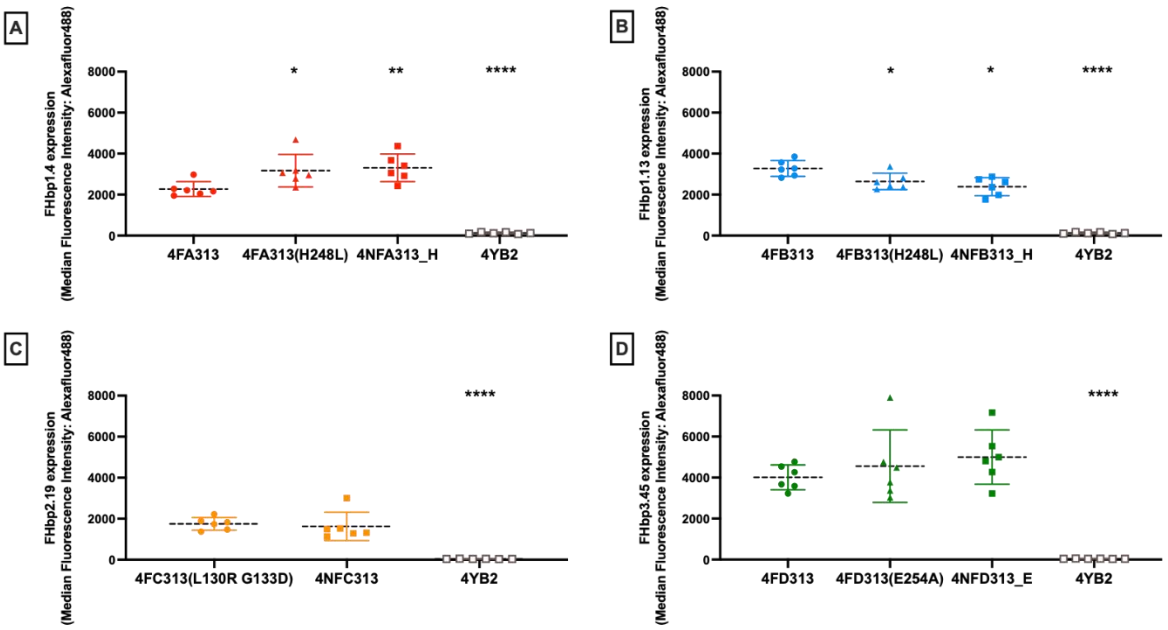


Figure 10: Surface expression of FHbp epitopes recognised by JAR monoclonal antibodies on 4xrNlac strains. GM-*N.lactamica* strains engineered to express: (i) the wild type (WT) versions of FHbp: 1.4 (Panel **A**, red: 4FA313), 1.13 (Panel **B**, blue: 4FB313) or 3.45 (Panel **D**, green: 4FD313) alone; (ii) mutagenised versions of each FHbp variant: FHbp 1.4(H248L) (Panel **A**: 4FA313(H248L)), FHbp 1.13(H248L) (Panel **B:** 4FB313(H248L)) or FHbp 3.45(E254A) (Panel **D**: 4FD313(E254A)) alone; (iii) a mutant version of FHbp 2.19 (Panel **C**, gold: 4FC313 L130R G133D), wherein amino acid residue substitutions L130R and G133D have been made to the N terminal domain to increase its thermostability, alone; (iv) the same mutagenised versions of the FHbp variants described in (ii) and (iii), *in addition to* the meningococcal autotransporter adhesin, NadA (Panel **A**: 4NFA313_H, Panel **B**: 4NFB313_H, Panel **C**: 4NFC313 and Panel **D**: 4NFD313_E); or the genetically-modified but wild type-equivalent strain, 4YB2 (grey), were grown to log phase and prepared for flow cytometry. Approximately one hundred million (10^8) colony-forming units of each strain were incubated with either mouse-derived, anti-FHbp variant 1 monoclonal antibody (JAR4) at a dilution of 1:500 (Panels **A** and **B**), or mouse-derived, anti-FHbp variant 2/3 monoclonal antibody (JAR11) at a dilution of 1:1000 (Panels **C** and **D**) in 5% FBS-dH_2O for 30 minutes at RT. After washing, samples were labelled by incubation with a 1:250 dilution of polyclonal, goat-derived, anti-mouse IgG conjugated to Alexafluor488 in 5% FBS-dH_2O for 30 minutes at RT in the dark (**ALL** Panels). After washing, all samples were fixed by incubation in 3.75% formaldehyde, 1% methanol for 15 minutes at RT in the dark, followed by resuspension into 5% FBS-dH_2O. The fluorescence intensity of each sample was measured using a BD FACSAria flow cytometer, and the Median Fluorescence Intensity of gated 'singlet' events for each population are reported. *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$ Repeated Measures 1-way ANOVA with Dunnett's test vs. panel-specific MFI of GM-*N. lactamica* expressing WT FHbp variants as Control. Dotted bars represent Mean ± standard deviation (SD) (n=6).

Note that a direct comparison of Median Fluorescence Intensity (MFI) of the 4xrNlac strains between panels labelled with the same JAR mAb (i.e. Panel A vs. Panel B; or Panel C vs. Panel D) is not reliably quantitative, insofar as it is plausible that the

epitope recognised by the antibody is constrained differently in different FHbp variants, and the mAb may have different binding kinetics to this epitope in each of these contexts. As such, these data cannot be reliably interpreted as showing, for example, that the level of FHbp 2.19 (L130R G133D) expression on the surface of GM-*N. lactamica* is lower than the level of FHbp 3.45 (E254A) expression. Instead, these data show that there is either only ever a modest (variant 1) or non-measurable (variant 2/3) effect of either: (i) performing site-directed mutagenesis on the *fhbp* gene, or (ii) transforming the strain to co-express NadA, on the expression levels of FHbp. Importantly, all the 4xrNlac strains express an epitope bound by an anti-FHbp variant mAb on their surface, producing populations of labelled bacteria with a Median Fluorescence Intensity much larger than that of the genetically modified, but wild type with respect to gene content mutant, 4YB2.

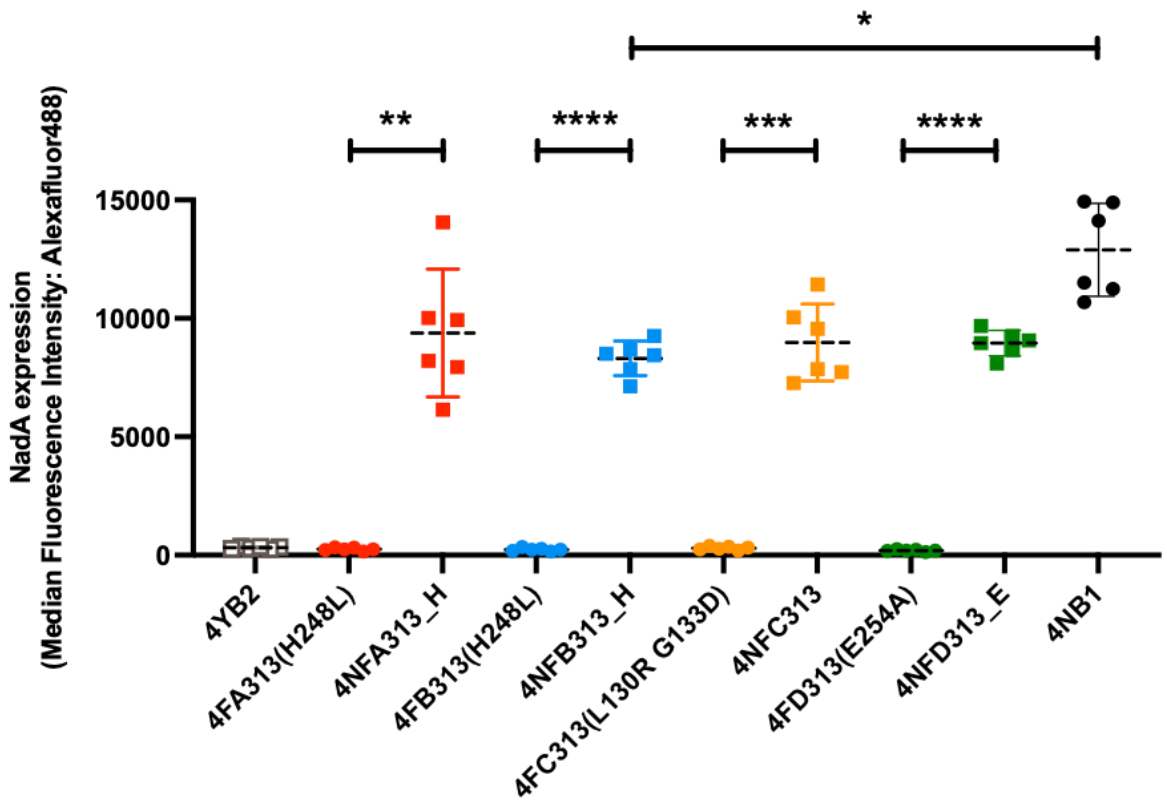


Figure 11: Surface expression of NadA epitopes recognised by sNadA-affinity purified polyclonal antibodies on 4xrNlac strains. GM-*N. lactamica* strains engineered to express: (i) mutagenised versions of FHbp: 1.4(H248L) (4FA313(H248L), ●), 1.13(H248L) (4FB313(H248L), ●), FHbp 2.19(L130R G133D) (4FC313(L130R G133D), ●) or 3.45(E254A) (4FD313(E254A), ●) alone; (ii) the same as described in (i) *in addition to* the meningococcal autotransporter adhesin NadA: (4NFA313_H, ■, 4NFB313_H, ■, 4NFC313 ■, 4NFD313, ■); (iii) NadA alone (4NB1, ●); or (iv) neither of these antigens (4YB2, □), were grown to log phase and prepared for flow cytometry. Approximately one hundred million (10^8) colony-forming units of

each strain were incubated with rabbit-derived, sNadA-affinity purified, anti-NadA polyclonal antibody ([PE-JRL-3]) at a dilution of 1:250 in 5% FBS-dH_2O for 30 minutes at RT. After washing, samples were labelled by incubation with a 1:100 dilution of polyclonal, goat-derived, anti-rabbit IgG conjugated to Alexafluor488 in 5% FBS-dH_2O for 30 minutes at RT in the dark. After washing, all samples were fixed by incubation in 3.75% formaldehyde, 1% methanol for 15 minutes at RT in the dark, followed by resuspension into 5% FBS-dH_2O. The fluorescence intensity of each sample was measured using a BD FACSAria flow cytometer, and the Median Fluorescence Intensity of gated 'singlet' events for each population are reported. NB: only selected comparisons are shown: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ Repeated Measures 1-way ANOVA with Tukey's multiple comparisons test. Dotted bars represent Mean ± standard deviation (SD) (n=6).

Note that, unlike the measurement of surface FHbp expression, where our reagents might interact differently with different protein variants, all GM-*N. lactamica* strains in this experimental series express the same antigen and have been labelled with the same polyclonal mixture of antibodies. This infers that signal intensity (i.e. MFI) in this series of experiments is reliably quantitative between strains. As compared to strain 4NB1, which expresses only NadA from its NHCIS1 locus, all the 4xrNlac strains have lower MFIs, which suggests they each have fewer molecules of NadA on their surfaces than does 4NB1. However, only one strain (4NFB313_H) produced a population with MFIs significantly lower than that produced by the population of 4NB1, and there were no significant differences between the MFIs of any of the double knock-in strains. It is therefore plausible that co-expression of FHbp and NadA comes at the expense of the highest levels of NadA surface expression.

2. Heterologous antigen function

2.1 Abrogation of resistance to complement-mediated killing and resistance of the GMOs to uptake of horizontally acquired (capsule biosynthesis) genes

The major difference between *N. lactamica* and *N. meningitidis*, and the primary virulence determinant of the latter, is the ability of the meningococcus to synthesise and deposit upon its surface a capsular polysaccharide. The main role of this capsule is to offer the meningococcus significant protection against killing by normal human serum, enabling the bacterium to survive within the bloodstream of naïve or unvaccinated individuals. The consequence of this is the rapid onset sepsis syndrome defined as invasive meningococcal disease (IMD), which can be fatal in as little as four hours following infection.

Another significant, but supplementary meningococcal adaptation that enhances its survival in the bloodstream (and potentially within tissues that can become pervaded with serum/plasma) is the expression of the outer membrane protein, Factor H binding protein (FHbp). FHbp is not a true 'virulence factor' of *N. meningitidis*, insofar as expression of the protein is not a prerequisite for pathogenesis and is dispensable during infection, however the established function of FHbp during an episode of IMD

is to bind human complement Factor H (hFH). This has the effect of inhibiting the activation of the complement cascade via the alternative pathway at the bacterial surface, which in turn reduces the intensity of complement-mediated killing of the bacterium in the bloodstream [47]. No other functions for FHbp beyond this immunomodulatory role have been satisfactorily demonstrated, meaning that heterologous expression of this antigen in *N. lactamica* is not expected to result in changes to the trophic requirements of the bacterium, nor to the interactions it makes with host cells or other bacterial species resident in the nasopharynx. Combined with the fact that *N. lactamica* is unable to synthesise and deposit a polysaccharide capsule on its surface and remains acutely sensitive to complement-mediated killing (Figure 13), and in addition is seemingly resistant to taking up capsule biosynthesis genes from its environment (see Figure 14), we posit that there will be no significant changes to the niche, lifestyle or tropism of genetically modified, FHbp-expressing *N. lactamica*, and therefore that any risks to colonised human participants posed by heterologous expression of FHbp in *N. lactamica* are negligible. As a further precaution however, and considering lots of published evidence that FHbp can be specifically mutated to disrupt its ability to bind human complement Factor H with no detriment to its immunogenicity, the decision was made to abrogate the hfH-binding ability of *N. lactamica*-expressed FHbp, insofar as the effect of expression of these FHbp derivatives on the surface of the GMOs resulted in these bacteria binding no more human complement Factor H than does a 'genetically-modified but wild type-equivalent with respect to gene content' strain of *N. lactamica*, 4YB2 (Figure 12).

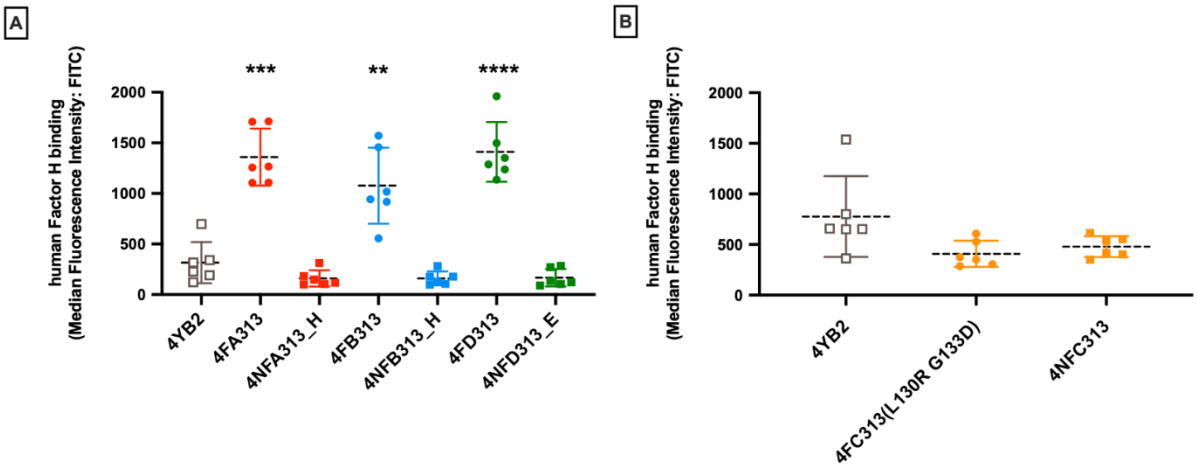


Figure 12: Binding of human Factor H to GM-*N. lactamica* strains expressing (modified) FHbp variants (and NadA, where applicable). GM-*N. lactamica* strains engineered to express one of: (i) the wild type version of FHbp: 1.4 (4FA313, ●), 1.13 (4FB313, ●) or 3.45 (4FD313, ●); (ii) mutagenised versions of FHbp: 1.4(H248L) (4NFA313, ■), 1.13(H248L) (4NFB313, ■) or 3.45(E254A) (4NFD313, ■); or (iii) a mutant version of FHbp 2.19 (4FC313(L130R G133D), ● and 4NFC313, ■), wherein amino acid residue substitutions L130R and G133D have been introduced to the N terminal domain; or the genetically-modified but wild type-equivalent strain, 4YB2 (□), were grown to log phase and prepared for flow cytometry. Approximately one hundred million (10^8) colony-forming units of each strain were incubated with human Factor H

(hfH) at either 20 μg ml^{-1} (Panel **A**) or 40 μg ml^{-1} (Panel **B**) in 5% FBS-dH_2O for 30 minutes at RT. After washing, hfH bound to the bacterial surface was labelled by incubation with a 1:25 dilution of anti-human Factor H monoclonal antibody OX-24 directly conjugated to FITC [OX-24-FITC] in 5% FBS-dH_2O for 30 minutes at RT in the dark. After washing, all samples were fixed by incubation in 3.75% formaldehyde, 1% methanol for 15 minutes at RT in the dark, followed by resuspension into 5% FBS-dH_2O. The fluorescence intensity of each sample was measured using a BD FACSAria flow cytometer, and the Median Fluorescence Intensity of gated 'singlet' events for each population are reported. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$ Repeated Measures 1-way ANOVA with Dunnett's test vs. panel-specific 4YB2 measurements as Control. Dotted bars represent Mean ± standard deviation (SD) (n=6).

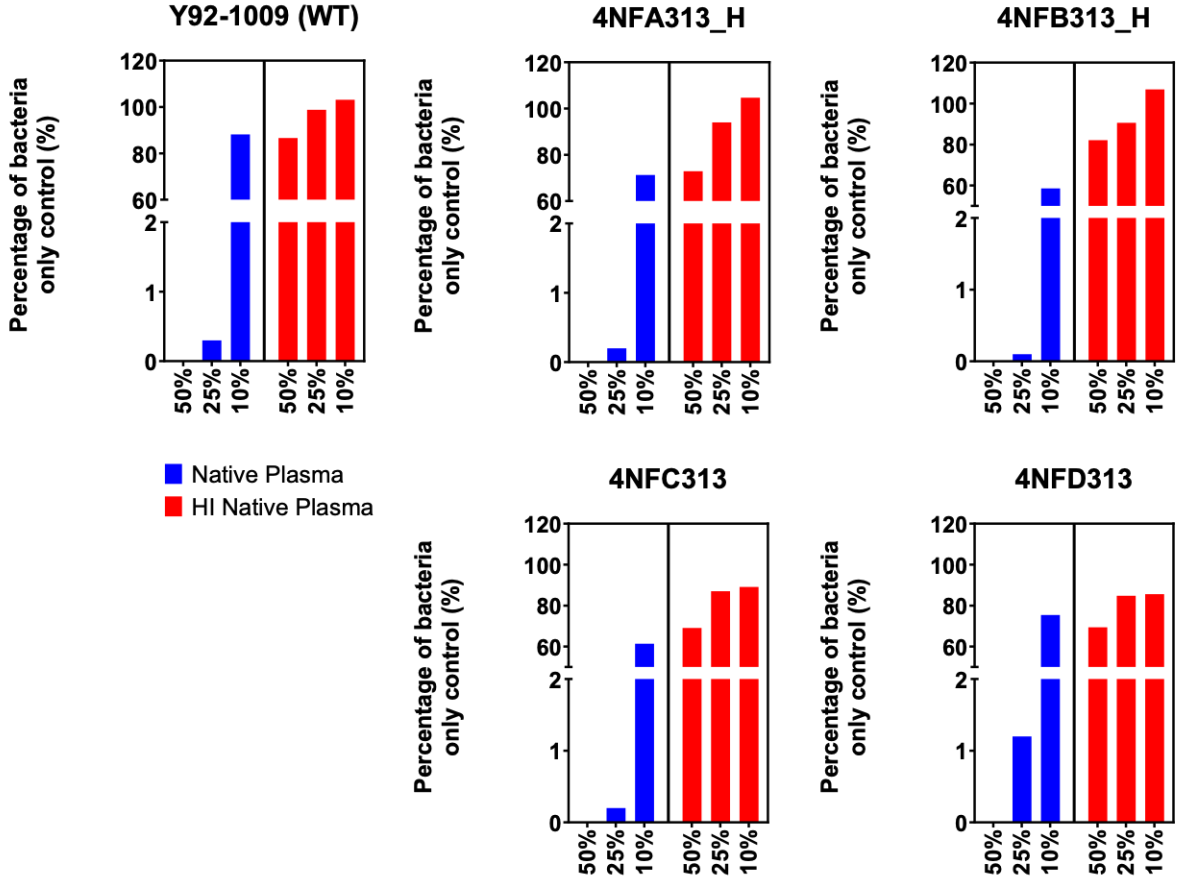


Figure 13: The 4xrNlac GMO strains remain acutely susceptible to complement-mediated killing. Wild type *N. lactamica* strain Y92-1009, or the genetically modified derivatives thereof that express both Neisseria Adhesin A (NadA) and one of four different variants of specifically mutagenized Factor H-binding protein (FHbp): FHbp1.4(H248L) (4NFA313_H), FHbp1.13(H248L) (4NFB313_H), FHbp2.19 (L130R G133D) (4NFC313) or FHbp3.45(E254A) (4NFD313_E), were grown to mid-log phase. Aliquots of each bacterial suspension containing 6 x 10^5 CFU were resuspended into either Tryptone Soya Broth + 0.2% yeast extract (TSB) alone, or TSB supplemented to a final concentration of 50%, 25% or 10% (v/v) with either Native Human Plasma (blue bars), or heat-inactivated plasma from the same, random consenting donor (red bars). Bacteria were incubated at 37 ^oC, 5% CO_2 for 1 hour, after which the number of viable CFU were enumerated by serial dilution and plating. Data are presented as the number of CFU growing on plates after overnight

incubation, as a percentage of the number of CFU growing on plates inoculated from the TSB only control condition (n=1).

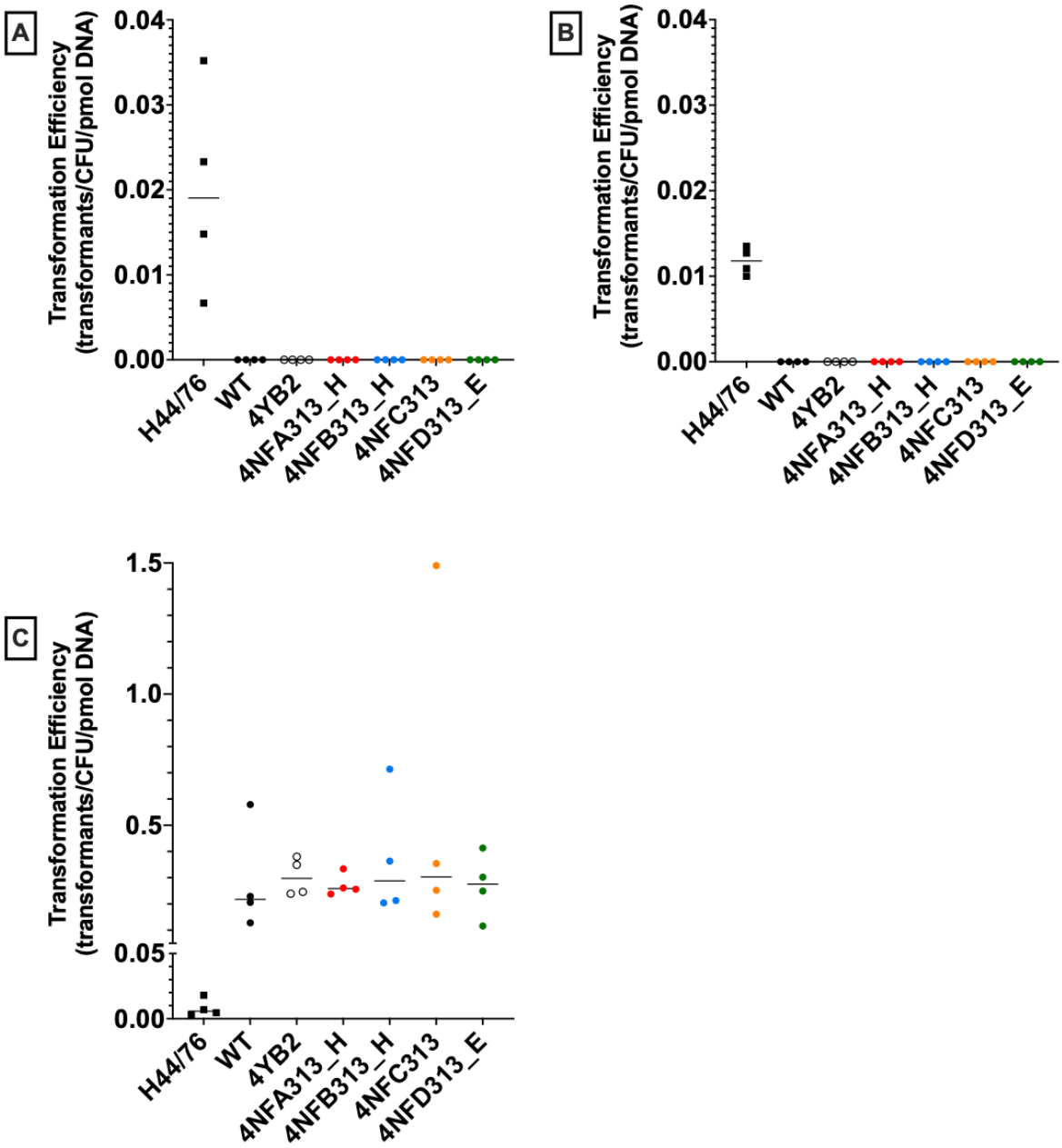


Figure 14: *N. lactamica* derivatives are naturally competent and can be transformed, but are refractory to horizontal gene acquisition from *N. meningitidis* even under optimised laboratory conditions. The wild type *N. lactamica* strain, Y92-1009 (WT, ●), or genetically-modified derivatives thereof, engineered to either: (i) remain wild type in regard to gene content (4YB2, **O**) or (ii) to express both the meningococcal autotransporter adhesin, NadA and one of four mutagenised versions of meningococcal FHbp: FHbp1.4(H248L) (4NFA313, ●); FHbp1.13(H248L) (4NFB313, ●); FHbp2.19(L130R G133D) (4NFC313, ●); or 3.45(E254A) (4NFD313, ●); or the wild type *N. meningitidis* strain H44/76 (■), were grown to log phase, normalised to OD_{600nm} = 0.3 and then incubated for 3 h in the presence of 150ng (0.0001 pmol)

of chromosomal DNA purified from one of: (i) *N. meningitidis* strain MC58 ∆*siaD:aphA3* (**A**), (ii) *N. meningitidis* strain MC58 ∆*nadA:aphA3* (**B**) or (iii) GM-*N. lactamica* strain Y92-1009 ∆*nlaIII:aphA3* (**C**). Aliquots of each bacterial suspension were diluted as appropriate and plated on CBA supplemented with 50 μg/ml kanamycin. Each suspension was also serially-diluted tenfold and plated to enumerate the total number of CFU/ml. Plates were incubated overnight at 37 °C, 5% CO_2. The number of kanamycin-resistant colonies on selective plates was adjusted for volume and dilution to give the number of kanamycin-resistant transformants/ml. Data are presented as the number of transformants per CFU per pmol of gDNA. Bars denote Median (n=4).

Note: consistent with the evidence that *N. lactamica* serves as a repository of genetic information for uptake into the meningococcus [48], transformation of representative *N. meningitidis* strain, H44/76 with gDNA derived from the *N. lactamica* mutant strain, Y92-1009 ∆*nlaIII:aphA3*, yielded kanamycin-resistant colonies at a low, but non-zero frequency (Figure 14C). Due to the absence of the *nlaIII* gene from H44/76, these transformants represent untargeted, *de novo* integration of the kanamycin resistance gene into the meningococcal chromosome. The same is true for H44/76 transformants yielded following exposure to MC58 ∆*nadA*:*aphA3* gDNA, since H44/76 is a *nadA*$^-$ meningococcal strain. In contrast, 2 different sources of meningococcal gDNA, each containing a kanamycin resistance gene at a different locus (one of which, *siaD*:*aphA3*, was integrated into the capsule biosynthesis operon), was unable to generate a single kanamycin-resistant mutant of *N. lactamica* (Figure 14A and Figure 14B). From the above experiments, we estimate the transformation frequency for any *N. lactamica* Y92-1009 derivative with either of these sources of meningococcal gDNA is less than 1 in 2.1 x 10^9 CFU.

Importantly, when being transformed with gDNA derived from an *N. lactamica* Y92-1009 derivative (i.e. ∆*nlaIII:aphA3*), which is appropriately methylated so as to bypass the restriction endonuclease activity that protects the integrity of the *N. lactamica* genome, there was no significant difference between the transformation efficiencies (TE) of the wild type (WT) commensal, or any of its GM-*N. lactamica* derivatives (Figure 14C). Whilst the wild type strain used in this experiment has never been transformed in the laboratory, both the 4YB2 strain and the 4xrNlac strains have undergone either 2 or 4 separate transformation events, respectively, during their derivation. The fact that the number of transformants yielded does not increase with increasing numbers of historical transformation events is encouraging. It means that transformability is not a self-selecting phenotype and that we are not inadvertently selecting for *N. lactamica* strains that are inherently more transformable. This is important because it means that transformed *N. lactamica* strains carried as part of a CHIME are only as likely as a wild type strain to horizontally acquire nucleic acids from their environment, and whilst this almost certainly occurs at a non-zero frequency, the frequency is empirically very low.

2.2 In vitro culture and bacterial binding to and internalisation by a eukaryotic cell line

The relationship between the growth of the 4xrNlac strains in liquid culture, as measured by culture turbidity at an optical density of 600nm (OD_{600nm}) and the corresponding bacterial viability, measured in CFU ml^{-1} after serial dilution and plating, was examined.

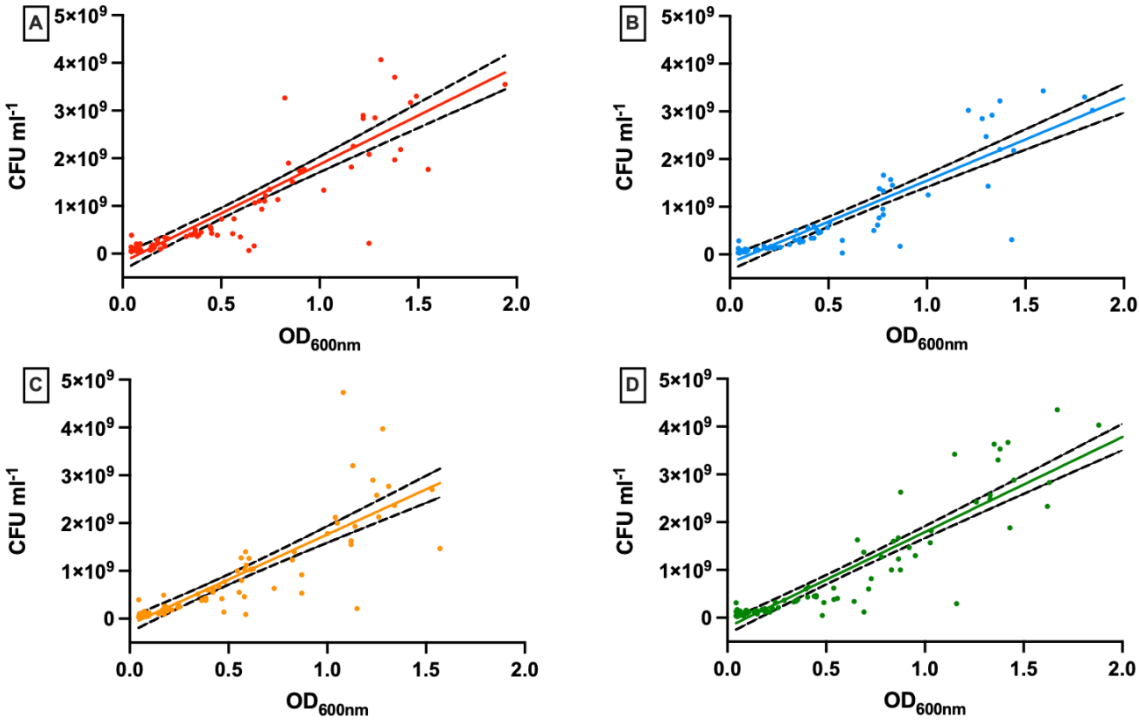


Figure 15: Culture turbidity at OD_{600nm} and viability of the 4xrNlac strains positively correlate. Genetically modified derivatives of *N. lactamica* strain Y92-1009, each engineered to express both NadA and one of four different, specifically mutagenised variants of FHbp: 4NFA313_H (**A**, expressing FHbp1.4(H248L), red), 4NFB313_H (**B**, expressing FHbp1.13(H248L), blue), 4NFC313 (**C**, expressing FHbp2.19(L130R G133D), gold) or 4NFD313_E (**D**, expressing FHbp3.45(E254A), green), were inoculated into tryptone soya broth supplemented with 0.2% yeast extract (TSB) and cultured at 37 °C, 5% CO_2 with shaking at 360 rpm. At regular intervals across multiple cultures (n=9), the OD_{600nm} of each culture was measured, and an aliquot of the same material was serially diluted 10-fold before being plated to enumerate the number of CFU ml^{-1}. For each plot, the data were fitted to a simple linear regression (solid, coloured line) and the 95% confidence intervals are shown (dotted black lines).

The R squared values of the lines fitted to each simple linear regression in Figure 15 are as follows: 4NFA313_H = 0.7737, 4NFB313_H = 0.8140, 4NFC313 = 0.7137 and 4NFD313_E = 0.8462. These data show that each of the 4xrNlac strains can be cultured in TSB and that the number of viable bacteria per millilitre of culture can be reliably estimated during log phase (i.e. $0.25 \leq OD_{600nm} > 1.0$), which will facilitate manipulation of all four cultures to produce the challenge agent at the desired ratio of 1:1:1:1.

Note that unlike the other 4xrNlac strains, 4NFC313 did not produce cultures with OD_{600nm} more than 1.6 arbitrary units (Figure 15C). This is due to the propensity of this strain to autoaggregate during stationary phase culture in TSB, where the bacteria form into dense spherical pellicles that drop out of suspension in the absence of culture agitation. Autoaggregation is not uncommon in commensal bacteria, with an autoaggregative phenotype in *N. lactamica* attributable to the phase-variable expression of the type V autotransporter protein, AutA [49]. Whilst it is not clear whether expression of AutA is responsible for the observed phenotype in strain 4NFC313 (there are likely other unidentified causes), it is worth noting that a small proportion of cultures using this strain do not autoaggregate (data not shown), which infers the involvement of a stochastic process like phase variation in triggering this phenotype. By retracing the lineage of strain 4NFC313 (see Figure 9) and performing repeated liquid cultures on its progenitors, it was observed that neither strain 4FCN313, nor strain (ts)4FC313 exhibited an autoaggregative phenotype (data not shown). This infers that the observed autoaggregation likely results from an off-target, potentially stochastic change in the genome of 4NFC313 that occurred in one of the culture steps surrounding the insertion of the Synth.*lacZ* gene.

In our previous application to perform a Deliberate Release of GM-*N. lactamica*, we demonstrated that the NadA-expressing GM-*N. lactamica* strain, 4NB1 bound to HEp-2 cells (which are a HeLa cell derivative) in significantly larger numbers per cell than did either wild type *N. lactamica* or the genetically modified but wild type-equivalent strain, 4YB2. These data were generated using the gentamicin protection assay, which was repeated for the 4xrNlac strains to assess their interactions with a representative cell line (Figure 16).

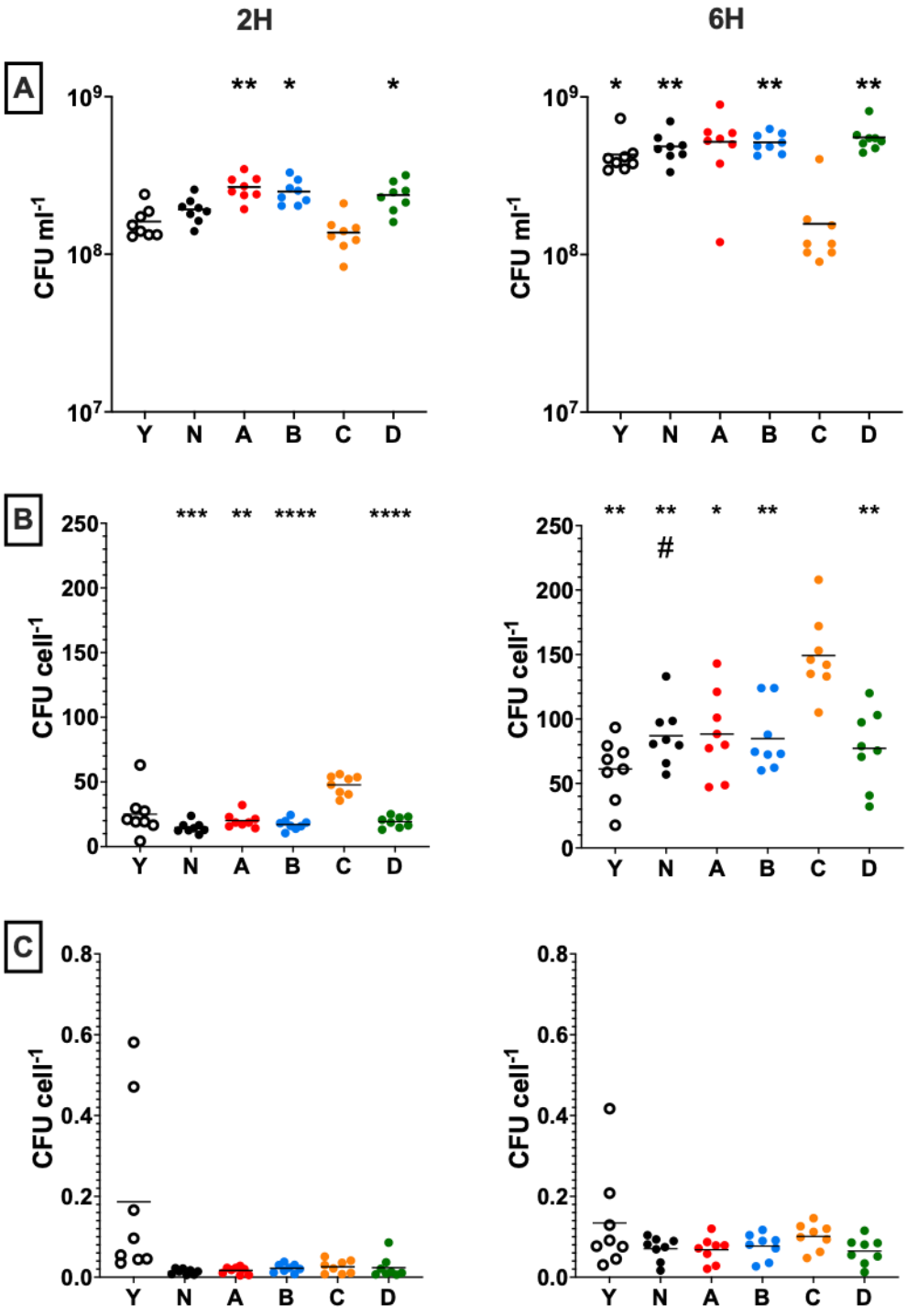


Figure 16: GM-*N. lactamica* binds to and is internalised within the HEp-2 cell line. GM-*N. lactamica* strains engineered to express: (i) the NadA adhesin alone (4NB1, 'N',●), (ii) the NadA adhesin and one of four mutagenised versions of FHbp: FHbp1.4(H248L) (4NFA313, 'A', ●); FHbp1.13(H248L) (4NFB313, 'B', ●); FHbp2.19(L130R G133D) (4NFC313, 'C', ●); or 3.45(E254A) (4NFD313, 'D', ●); or (iii) the genetically-modified but wild type-equivalent strain, 4YB2 ('Y',**O**), were grown to log phase, washed, resuspended into Dulbecco's Modified Eagle Medium supplemented with 10% HI-FBS (hereafter, DMEM), then used to infect duplicate wells of HEp-2 (HeLa) cells at an initial multiplicity of infection (MOI) of 100 (t=0). Infected cells were incubated at 37 °C, 5% CO_2. Thirty minutes prior to each timepoint, supernatants were aspirated from each well, and aliquots of each supernatant were serially diluted 10-fold and plated to

enumerate bacteria (in CFU ml^{-1}) (**A**). The infected HEp-2 (HeLa) cells were washed 5 times with excess, sterile PBS, then for each duplicate set of wells one well was supplemented with fresh DMEM alone, whilst the other was supplemented with DMEM plus 100 $\mu g\ ml^{-1}$ gentamicin and 10 $\mu g\ ml^{-1}$ penicillin G. Cells were returned to the incubator at 37 ^oC, 5% CO_2 for 30 minutes, after which all wells were washed a further 5 times with excess PBS before addition of 250 μl of a solution of 2% (w/v) saponin in PBS. Cells were incubated at 37 ^oC, 5% CO_2 for 15 minutes to allow saponisation, after which each well was supplemented with 750µl of PBS and robustly agitated to disrupt the HEp-2 (HeLa) cell monolayer and release intracellular bacteria. Aliquots of non-antibiotic-treated, saponised lysates (**B**) and antibiotic-treated, saponised lysates (**C**) were 10-fold serially diluted in PBS and plated to enumerate bacteria (in CFU ml^{-1}), which was normalised to the mean number of HEp-2 cells in each well (resulting in CFU $cell^{-1}$). The values shown in (**B**) are assumed to represent bacteria both adherent to and internalised within the HEp-2 (HeLa) cells at each timepoint, whilst the values shown in (**C**) are assumed to represent only intracellular bacteria. The viability counts shown in (**A**) are those derived from supernatants in wells that were not subsequently treated with antibiotics. $*p \leq 0.05$,$**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$ Repeated Measures 1-way ANOVA with Tukey's multiple comparisons test vs. 'C'; $^{\#}p \leq 0.05$ Repeated Measures 1-way ANOVA with Tukey's multiple comparisons test vs. 'Y'. Bars denote Mean (n=8).

Note that, as in our previous disclosure, the binding of strain 4NB1 to HEp-2 cells was significantly higher than the binding of strain 4YB2 to the same cells (Figure 16B, 6H). Except for strain 4NFC313, which bound to cells in significantly higher numbers than any other GM-*N. lactamica* strain, even at the earlier timepoint (Figure 16B, 2H), the binding of the 4xrNlac strains to HEp-2 cells was not significantly different to either 4NB1 or 4YB2 (Figure 16B, 6H). These results can plausibly be explained by considering the measurements of NadA surface expression (Figure 11), where it was posited that co-expression of FHbp with NadA might come at the cost of the highest levels of expression of NadA. Although it is unknown whether the number of NadA molecules expressed on the surface of bacteria modulates their ability to bind cells, or whether the observed increase in cell binding attributable to NadA expression is simply binary (i.e. ON or OFF), these data are consistent with the former.

The autoaggregative phenotype of strain 4NFC313 (discussed above) is evident in these data. Measurements of CFU per ml in the cell line supernatants (Figure 16A) are impacted by this phenotype, insofar as the number of 4NFC313 CFU ml^{-1} are significantly lower than other GM-*N. lactamica* strains at both 2H and 6H, whilst no other GM-*N. lactamica* strain has a different viability from any other GM-*N. lactamica* strain (excluding 4NFC313) at either time point. This is consistent with the aggregation of 4NFC313 disrupting the assumed relationship that one bacterial cell gives rise to one CFU following culture on agar medium. The precipitation of the autoaggregative phenotype as early as 2H is likely due to the difference in composition between the TSB bacterial growth medium and the cell line-sustaining DMEM. Figure 15 shows that autoaggregation of this strain does not occur in TSB until stationary phase, which occurs after approximately 5-6 hours of culture (data not shown).

Importantly, heterologous expression of both NadA and FHbp by GM-*N. lactamica* has no significant impact on the propensity of the bacteria to become internalised by HEp-2

cells (Figure 16C), insofar as the strains remain non-invasive even after 6H of infection. Previous work has demonstrated that heterologous antigen expression can increase the internalisation of *N. lactamica* by these cells, insofar as a GM-*N. lactamica* strain, 4OA2, which expresses the meningococcal antigen OpcA, was internalised in significantly larger numbers than the wild type (or strain 4NB1) after 6 hours of infection (data not shown).

Although the relevance of binding to and becoming internalised by a HeLa cell derivative is questionable in relation to how these bacteria will behave during colonisation of the human nasopharyngeal epithelium, these data are reassuring insofar as they indicate expression of the heterologous antigens, especially FHbp, do not result in a significant increase in the number of cells becoming internalised by a cell to which they bind (i.e. there is no indication that expressing these proteins will fundamentally change the lifestyle of the 4xrNlac strains from that lived by the wild type commensal during infection).

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 (see Item 24). As such, the cassette is stably maintained as an inheritable unit in the bacterial genome. Note that the gene expression cassettes do not contain sequences capable of directing extrachromosomal replication of the sequence.

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

In our previous application to conduct a Deliberate Release of genetically modified *N. lactamica,* we disclosed data showing that serial *in vitro* passage of the NadA-expressing (4NB1) and 'empty vector' (4YB2) GMOs on solid media resulted in genetic changes consistent with the natural *Neisseria* process of phase variation and limited chromosomal rearrangements (see Item 11). These changes were subsequently shown to be more extensive than nasopharyngeal carriage of these strains for the same length of time, inferring that survival within its niche has a stabilising effect on the bacterial genome [21]. Importantly, we demonstrated that after 28 days of serial passage, the coding sequence of the chromosomally-integrated, NHCIS1-targeted *nadA* and *lacZ* genes retained 100% identity to the sequence present in the gene expression constructs, and that the level of expression of the NadA protein was not significantly changed.

For this application, we have dispensed with serial *in vitro* passage of the GMOs, on the grounds that: (i) our challenge agent will be made using bacteria that have been cultured *in vitro* only a maximum of three times since the isolation of the strains and the subsequent verification of their heterologous nucleic acid sequences, (ii) our

gene expression cassettes have been targeted to the same chromosomal locus as in the 4NB1 and 4YB2 strains (NHCIS1), which we have shown not to be a recombination hotspot in our previous work, and (iii) our previous data draws the relevance of this analysis into question, insofar as the selective pressures and evolutionary bottlenecks that the strains will encounter during their use in our experiment differ so radically between *in vitro* culture and *in vivo* carriage [21]. Specifically, this means that without performing a release of these strains into humans, we cannot know precisely what effects a period of carriage will have on the genomic stability of these bacteria. For the purposes of clarity however, it is important to note that we anticipate the vast majority of changes to the genome to reduce a mutated bacterium's relative level of fitness for its niche, with the most likely outcome being loss of the mutated derivative from carriage.

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.

Figure 10 (Item 25) shows the levels of expression of each mutagenized FHbp variant on the surface of the 4xrNlac strains, as determined by flow cytometric analysis using one of two monoclonal antibodies (mAbs): JAR4 or JAR11, which each bind to an epitope conserved amongst FHbp variant group 1, or variant group 2/3, respectively.

Figure 11 (Item 25) shows the levels of expression of NadA on the surface of: (i) the 4xrNlac strains, (ii) GM-*N. lactamica* strains expressing only mutagenized versions of each FHbp variant, (iii) a GM-*N. lactamica* strain expressing only NadA (4NB1), which was used in a previous CHIME, and (iv) a GM-*N. lactamica* strain that has been genetically modified to express the *lacZ* gene from NHCIS1, but which does not contain a coding sequence under transcriptional control of the *porA*-associated UAS-enhanced, hybrid *porA/porB* gene promoter (4YB2), and which was also used in a previous CHIME; as determined by flow cytometric analysis using an affinity-purified polyclonal antibody mixture ([PE-JRL-3]), raised in rabbits against the soluble domain of the NadA protein (sNadA).

The Specific Activity of β-galactosidase measured in a GM-*N. lactamica* 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). Note that although the Specific Activity of β-galactosidase was not measured in each of the four 4xrNlac strains under consideration herein, the *lacZ* and Synth.*lacZ* genes coded for in these organisms have sufficient activity to allow the discrimination of blue/white colonies on X-gal-containing medium.

29. The activity of the gene product.

Figure 12 (Item 25) shows that through specific mutation of key amino acid residues in the FHbp variants, we have abrogated the ability of the protein to bind human complement Factor H (hFH). Mutant strains expressing wild type versions of each of the four FHbp variants bound significantly more hFH than did the genetically-modified but wild type-equivalent strain, 4YB2 (which has been used in a previous CHIME). In contrast, the 4xrNlac strains were shown to bind only as much hFH as strain 4YB2, despite our flow cytometric analysis demonstrating surface expression of FHbp epitopes on the surface of each strain (Figure 10).

The NadA protein is an epithelial cell-binding adhesin. Although the receptor for NadA has yet to be elucidated, we have shown that recombinant expression of the *nadA* gene in *N. lactamica* significantly increases the propensity of the bacteria to bind to the HEp-2 cell line (Figure 16B, 6H and [50]). Our data suggests that co-expression of FHbp and NadA in the same strain might come at the cost of the highest levels of expression of NadA (Figure 11), which might be the reason why there was no significant difference between the numbers of 4YB2 bacteria and 4xrNlac bacteria that adhered to HEp-2 cells (Figure 16B). Importantly, expression of NadA and FHbp had no effect on the propensity of the 4xrNlac bacteria to become internalised by the HEp-2 cell line during a prolonged *in vitro* infection period (Figure 16C).

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 participants, at the appropriate time points, throat swabs and nasal washes will be performed. The contents of each throat swab will be eluted into PBS, which in parallel to nasal wash will be used to culture viable *Neisseria* species on X-gal-supplemented GC agar plates. Note that only a limited subset of *Neisseria* species have been demonstrated to form colonies on this media after overnight incubation, specifically the pathogenic *Neisseria* (i.e. *Neisseria meningitidis* and *Neisseria gonorrhoeae*)*, Neisseria lactamica* and *Neisseria bacilliformis*. Putative *Neisseria lactamica* colonies will be identified on the basis of colony morphology and colouration in the first instance, being the only *Neisseria* species from the above subset to grow as blue colonies on X-gal-containing medium. Note that the identity of *Neisseria lactamica* can be confirmed through biochemical testing, using an API-NH kit from bioMérieaux if necessary. Confirmation of the recovery of genetically modified organisms can be achieved through amplification of the NHCIS1 locus in any given colony using PCR. Amplification of the NHCIS1 locus from genomic DNA derived from wild type *Neisseria lactamica* will produce a 2.2 kb product but the same reaction will produce an approximately 10 kb product in any one of the 4xrNlac strains.

The composition of the resident 4xrNlac population can be determined through qPCR, for which we have developed a bespoke assay of 4 primer/probe sets, each specific for one of the four different *fhbp* coding sequences.

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 GMOs and this will be their first release, in two experimental medicine studies involving controlled infection of healthy adult volunteers (Item 34).

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

The GM-*N. lactamica* strains will be administered intranasally to healthy adults enrolled as challenge participants in two experimental medicine studies (Item 34). We anticipate that this will result in nasopharyngeal colonisation with one or more of the GMOs in the majority of challenge participants, which may persist for the duration of the study but will be cleared prior to the end of study participation (Item 34). We do not expect the GM-*N. lactamica* strains to have any increased pathogenicity (Item 25) in comparison to the wild type organism, which is non-pathogenic in immunocompetent individuals, with only very few reported cases of association with disease in immunocompromised individuals (Item 12b). In order to mitigate any potential risk to human health, participants will be enrolled according to strict eligibility criteria (Item 66) with infection control procedures in place to minimise the risk of onward transmission, particularly to immunocompromised individuals (Item 77). The risk assessment and mitigation strategies as relate to human health are discussed in detail in part A4.

N. lactamica is a human restricted commensal bacterium, therefore 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,

These 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 4xrNlac GMO strains to be any more reactogenic than either wild type *N. lactamica*, or the two GM-*N. lactamica* strains used in our previous Deliberate Release into human participants. Considering one of the two GMOs used in a previous CHIME expressed NadA to high levels (strain 4NB1), and that there were no safety concerns associated with infection using this organism in the previous study; it is logical that the most likely potential source of any change to

the reactogenicity profile of the 4xrNlac GMOs is their expression of an FHbp variant. Herein, we have presented evidence that: (i) the heterologous gene product, FHbp can no longer bind human complement Factor H (Item 25, Figure 12), (ii) expression of heterologous FHbp has no significant effect on the internalisation of the GMOs into a HeLa cell line derivative (Item 25, Figure 16C), and (iii) the 4xrNlac strains remain refractory to transformation with exogenous, non-*N. lactamica*-derived nucleic acids (Item 25, Figure 14). It is also important to note that FHbp in the anti-serogroup B meningococcal vaccines is well tolerated whilst being immunogenic, so it is therefore unlikely that the FHbp proteins have superantigenic qualities that might adversely affect their reactogenicity. Collectively, we posit these data mean the lifestyle and infection dynamics of these GMOs will not significantly differ from those of wild type *N. lactamica* or GM-*N. lactamica* strains 4YB2 and 4NB1, which are all asymptomatic colonisers of the human nasopharynx during both natural (WT only) and experimentally-induced infection.

Note that in colonised individuals it is highly likely the GMOs will 'bleb' to produce outer membrane vesicles (OMV), which contain *N. lactamica*-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 *N. lactamica*-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 [51]. 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,

In our previous application to conduct a Deliberate Release of genetically modified *N. lactamica,* we disclosed data showing that neither the NadA-expressing nor wild type-equivalent GMOs (4NB1 and 4YB2, respectively) showed any evidence of increased pathogenicity compared to wild type *N. lactamica* 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 and genetically modified *N. lactamica* strains 4NB1 and 4YB2, were injected i/p at either a low (1.7×10^5 CFU) or a high (approx. 2.5×10^7 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 contrasts with i/p challenge with serogroup B *N. meningitidis* where 1/5 mice survived following a challenge with 2×10^6 CFU. There were no survivors following a dose of 2×10^7 CFU [52].

For this application we have dispensed with this model on the grounds of relevance; specifically, the nebulous quality of 'pathogenicity' following i/p injection into mice does not intuitively correlate with what is expected to occur following intranasal

administration into the bacteria's natural host. Because strains 4NB1 and 4YB2 have both been used in a previous CHIME with no reported adverse events attributable to the infection, and given the disclosures summarised in Item 33a, we do not anticipate any increases to either the pathogenicity, or the potential for pathogenicity, in these strains.

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* increases the propensity of the bacteria to bind to human epithelium. Although our previous CHIME was not powered to detect an effect of NadA expression on the rate of colonisation following challenge, it is worthwhile to note that there was a 100% colonisation rate in participants who were challenged with 10^5 CFU of strain 4NB1, whilst the colonisation rate in those challenged with 10^5 CFU of the control strain, 4YB2 was 78.5% [50]. Although both colonisation fractions are experimentally useful in terms of the workability of the challenge model, a larger sample size is required to test whether the observed difference is statistically significant.

If we consider our other data: Item 25, Figure 16B shows that expression of NadA by *N. lactamica* strain 4NB1 results in a significant increase in the number of bacteria binding to the HEp-2 cell line as compared to the control strain, 4YB2. The same figure also shows that the numbers of 4xrNlac bacteria binding to HEp-2 cells is not significantly different to the control. In addition, Figure 11 suggests that co-expression of FHbp and NadA on the surface of GM-*N. lactamica* may come at the cost of the highest levels of NadA expression, and that the 4xrNlac strains likely have fewer NadA molecules on their surface than does 4NB1. Taken together, a legitimate question is therefore whether the 4xrNlac strains will colonise more like the 4NB1 or 4YB2 strain when used in a CHIME. However, even in the worst case scenario, where the level of NadA expression measured in the 4xrNlac strains confers no cell-binding advantages, we would anticipate the 4xrNlac strains to colonise at least as efficiently as 4YB2.

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,

Not applicable

ii. communicability,

Not applicable

iii. infective dose,

Not applicable

iv. host range and possibility of alteration,

Not applicable

v. possibility of survival outside of human host,

Not applicable

vi. presence of vectors or means of dissemination,

Not applicable

vii. biological stability,

Not applicable

viii. antibiotic-resistance patterns,

Not applicable

ix. allergenicity

Not applicable

x. availability of appropriate therapies

Not applicable

e. Other product hazards

Not applicable

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.

The four GM *N. lactamica* strains will be administered intra-nasally as a multi-strain challenge agent (designated 4xrNlac) to healthy adult participants in two experimental medicine studies; an initial pilot study followed by a randomised controlled experimental medicine study. These studies will utilise our model of controlled human infection (CHI) using *Neisseria lactamica* to study host-pathogen interactions; specifically, to establish whether there is a role for experimental colonisation of the human nasopharynx with 4xrNlac in generating a 'protected from colonisation' phenotype upon rechallenge with the same multi-strain challenge agent. This outcome is important in the context of the observations made in previous *N. lactamica*-based CHI studies, wherein colonisation by *N. lactamica* was shown to

displace carriage of co-carried meningococcal strains in a serogroup-independent manner, and to prevent acquisition of the meningococcus for the duration of the period of *N. lactamica* carriage [17]. Whilst the basis of this phenomenon has yet to be fully elucidated, we have subsequently shown that anti-Neisserial adaptive immunity plays at least a partial role in regulating the overall bacterial burden in the nasopharynx [53] whilst others have demonstrated definitively that the anti-serogroup B, sub-capsular polysaccharide, protein-based meningococcal vaccine 4CMenB (Bexsero) does not confer protection from meningococcal colonisation [54, 55] (NB: The effect of Trumenba on nasopharyngeal colonisation with *N. meningitidis* is still under investigation). Additional outcome measures will include the generation of immunological responses against FHbp, NadA and *N. meningitidis*.

The initial pilot study will follow a clinical protocol entitled "**Safety, colonisation and immunogenicity following nasal inoculation with genetically modified Neisseria lactamica expressing Factor H binding protein and Neisseria Adhesin A – a pilot controlled human infection study"**, with a short title of **"The GM-Nlac study – Pilot".** In this study, ten healthy adult participants will be nasally inoculated with 4xrNlac and followed up with regular outpatient visits to review safety, colonisation and immunogenicity over 30 days. Oral antibiotics will be administered at day 28 to clear colonisation (Item 73.3). The aims of this study will be to establish that nasopharyngeal colonisation can be induced safely in an adequate fraction of participants, and successfully cleared with oral antibiotic treatment, with equivalence to that seen following inoculation with wild-type *N. lactamica*, and the GM-*N. lactamica* strains 4YB2 and 4NB1 in our previous studies [50, 53]. The co-primary objectives for this study will be:

1. To establish the safety of nasal inoculation of healthy adult participants with four strains of genetically modified *Neisseria lactamica* expressing FHbp and NadA
2. To demonstrate successful induction of nasopharyngeal colonisation following nasal inoculation with genetically modified *Neisseria lactamica* expressing FHbp and NadA

Following successful completion of this pilot study, a double-blind randomised placebo-controlled experimental medicine study will be conducted according to a clinical protocol entitled "**Safety, colonisation and immunogenicity following nasal inoculation with genetically modified *Neisseria lactamica* expressing Factor H binding protein and Neisseria Adhesin A – a Randomised Controlled experimental medicine study**", short title "**The GM-Nlac study – Main**". In this study, 62 healthy adult participants will be randomised 1:1 to inoculation with 4xrNlac (Intervention arm) or sham control (Control arm). Participants will attend outpatient follow up visits to assess safety, colonisation and immunogenicity until day 56 post inoculation at which point they will receive oral antibiotics to clear colonisation (Item 73.3). After a washout period of at least 7 days, participants will return for a rechallenge phase, where all participants in both arms will receive an intranasal

inoculation with 4xrNlac. Safety, colonisation and immunogenicity will be monitored at outpatient follow up visits over a further 56 days, with administration of oral antibiotics to all challenged participants at day 56 post rechallenge (Item 73.3).

The primary outcome measure of this study will be the proportion of Intervention arm participants who, after becoming colonised with one or more 4xrNlac strains after Inoculation 1, become colonised with any 4xrNlac strain after Inoculation 2; *as compared to* the proportion of Control arm participants who, after receiving placebo at Inoculation 1, become colonised with any 4xrNlac strain after Inoculation 2.

The primary objective for this study will be to establish whether experimentally induced nasopharyngeal colonisation with genetically modified *Neisseria lactamica* expressing FHbp and NadA is protective against repeat colonisation following rechallenge with the same strains.

Secondary objectives will include analysis of the kinetics and density of colonisation with the genetically modified *N. lactamica* strains, and immunological parameters potentially associated with colonisation such as serum bactericidal antibody, FHbp variant specific or NadA specific B and T cell responses and polarity.

For both studies, participants who receive an intranasal inoculum of 4xrNlac or control (PBS, “Main” study only) will be enrolled as “challenge participants”. To assess for any onward transmission of the GM strains, individuals who share a bedroom with challenge participants will be enrolled as “contact participants”.

The future use of the GMOs may be (1) to enable investigation of mucosal immunity against meningococcal antigens during nasal carriage under controlled conditions (2) to test vaccines containing NadA and FHbp for their ability to protect against colonisation by strains expressing the immunologically cognate antigens, (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) and (4) (if a ‘protected against colonisation with 4xrNlac GM-*N. lactamica*’ is detected upon rechallenge) to deplete the reservoir of infection of *Neisseria meningitidis*.

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

The GM-Nlac – Pilot study is expected to commence on the 1st of February 2026 (pending all necessary approvals) and will run for approximately 3 months with an expected completion date of approximately 30th April 2026. The study will have an enrolment target of 10 adult challenge participants. All challenge participants will receive one intranasal inoculum of 4xrNlac, following which we anticipate that 60-100% of challenge participants will become colonised with one or more GM-*N. lactamica* strains. All challenge participants will have follow-up visits up to 30 days

post challenge, and will receive a single dose of oral ciprofloxacin to clear colonisation prior to study completion (Item 12e and Part A4).

The first challenge participant will be inoculated individually and will have a safety review including bloods and safety data by day 6-8 post inoculation. Providing there are no safety concerns, further challenge participants will be inoculated individually or in pairs, with safety reviews at day 6-8 post inoculation, up to a total of 5 participants. The remaining challenge participants will be inoculated in groups of a maximum of five with safety reviews at day 6-8 post inoculation. The decision to inoculate the next group of challenge participants will be taken by the investigators, with discussion with an external data and safety monitoring board if there are any safety concerns.

The GM-Nlac – Main study is expected to commence on the 1st November 2026 (pending all necessary approvals) and will run until study completion (anticipated to be 31st May 2031). All challenge participants will have follow-up visits up to 56 days following each inoculation, with oral antibiotics administered to clear colonisation prior to the end of each challenge phase (Item 73.3). The study will have an enrolment maximum of 62 adult challenge participants.

Up to approximately 31 challenge participants in the Intervention arm will receive two doses of intranasal 4xrNlac, one at the start of each challenge phase. Up to approximately 31 challenge participants in the Control arm will receive one dose of intranasal 4xrNlac at the beginning of the second challenge phase. All challenge participants will have follow-up visits up to 56 days following each inoculation, and will receive oral antibiotics to clear colonisation prior to the end of each challenge phase (Item 73.3). Challenge participants will be inoculated in groups of a maximum of ten with safety reviews at day 6-8 post inoculation. The decision to inoculate the next group of challenge participants will be taken by the investigators, with discussion with an external data and safety monitoring board if there are any safety concerns.

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 conducted at the Southampton NIHR Clinical Research Facility (NIHR-CRF) 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 study staff will be given study-specific training.

37. The size of the site.

The release will take place in the Southampton NIHR-CRF, at University Hospital Southampton, Tremona Rd, Southampton, **SO16 6YD**. The size of the room in which the release will occur is approximately 15m^2.

Relevant facilities at the Southampton NIHR-CRF include 13 consulting rooms (2 configured for infectious participants); a containment level 3 laboratory for microbiological work, two environmental laboratories with "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 (https://www.southamptoncrf.nihr.ac.uk/our-facilities). 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 inoculum will be administered as nose drops (500 µL into each nostril) with the participant lying supine with neck extended. The inoculation procedure will be carried out in one of the containment level 2 environmental chambers within the NIHR-CRF to assure the inoculum will be administered to the participant only, without posing any risk of infection to other people or the environment. Staff will wear disposable gloves, apron and a surgical mask during the inoculation procedure. After inoculation the participant will lie supine for 5 minutes. After the challenge the room will be cleaned following hospital guidelines [56] (Item 84).

39. The quantity of organisms to be released.

Challenge participants will receive an intranasal inoculum containing approximately 100,000 (10^5) CFU of each of the four GM strains for a total of 4 x 10^5 CFU in 1 mL in phosphate buffered saline (PBS). The dose of inoculum will be prepared according to a dilution calculation based on the batch viability of inoculum vials. It is anticipated that there will be some variability in the actual dose administered, which will be monitored by viable count of the residual inoculum following each inoculation. Any individual inoculum found to be >5 or >10 times the intended total dose will be reported as an adverse event, or adverse event of special interest, respectively, and will trigger reassessment of the batch viability and recalculation of the batch dilution calculation if required.

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.

All procedures to investigate the carriage of the GMOs by the participant (throat swabs, nasal washes, nasal swabs) will be undertaken in the containment level 2 environmental chambers within the NIHR-CRF. Staff will wear appropriate personal protective equipment during the inoculation procedure (Item 38). Before and after the inoculation they will wash their hands. After the inoculation the room will be cleaned following hospital guidelines [56] (Item 84).

42. The post-release treatment of the site.

Following the inoculation procedure the environmental chamber will be cleaned according to hospital guidelines [56] (Item 84).

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

While participants are present in the NIHR-CRF, all facilities and waste potentially contaminated with the GMOs will be cleaned or inactivated and disposed of according to hospital infection control guidelines [56] (Item 84) and site SOPs for the disposal of GM contaminated waste (Item 82).

Previous studies have not demonstrated transmission of *N. lactamica* from colonised adults to bedroom sharers [50], or from mothers to their babies [57] and so the risk of onward transmission is considered to be low (Item 54, Part A4). Risk assessment and mitigation strategies to minimise the potential for onward transmission are discussed in full in part A4. Bedroom sharers of challenge participants will be enrolled as contact participants to assess for onward transmission. Challenge and contact participants will be enrolled according to specific eligibility criteria (Item 66) including the exclusion of those with regular occupational or household contact with children under 5 years, who are known to more commonly become colonised with wild type *N. lactamica.* Throughout the study, challenge and contact participants will follow standard infection control procedures to minimise onward transmission. These include regular hand washing, avoidance of high transmission risk activities with anyone other than with their declared and consented bedroom sharer, and limitation of contact with potentially vulnerable individuals (Item 77). All challenge participants will receive oral antibiotics at the end of each challenge phase to clear nasopharyngeal colonisation with the GM-*N. lactamica* strains (Item 73.3). Contact participants will provide a throat swab sample to look for transmission of the GM-*N. lactamica* strains at the end of their study involvement, and if they develop any respiratory or systemic symptoms potentially suggestive of infection during the study. Any contact participants found to be colonised will receive oral antibiotics to clear colonisation. All challenge participants and any contact participants who have received antibiotics to clear colonisation of the GM strains will return for confirmation of clearance 1-4 days after completion of antibiotics (Item 73.3).

In the unexpected event that the GMO causes disease in challenge or contact participants, then treatment with standard antibiotics (e.g. ceftriaxone) will be instituted. In the extremely unlikely 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 and FHBp antigens and has been shown to prevent meningococcal disease at the population level [58]. 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*, and remains acutely sensitive to killing by normal human plasma (Item 25, Figure 13).

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 release of these GMOs.

A previous CHIME using GM-*N. lactamica* that expressed either NadA (strain 4NB1), or contained a gene expression cassette without a coding sequence (i.e. a wild type-equivalent, but genetically modified control) (strain 4YB2) has been conducted at the University of Southampton (DEFRA REF: 17/R50/01). In this work, we showed that:

(i) colonisation of participants with the NadA-expressing commensal was safe
(ii) a subset of participants colonised by the NadA-expressing commensal produced NadA-specific IgG-secreting plasma B cells (B_{PLAS}) and experienced an at-least 2-fold increase in serological anti-NadA IgG titres
(iii) all participants colonised by the NadA-expressing commensal experienced an increase in the proportion of circulating NadA-specific IgG-memory B cells (B_{MEM})
(iv) a subset of participants colonised by the NadA-expressing commensal seroconverted to become protected against IMD caused by a NadA-overexpressing strain of *N. meningitidis*, 5/99, consistent with the universally acknowledged correlate of protection against IMD (i.e. a strain-specific, serum bactericidal antibody (SBA) reciprocal titre greater than or equal to 4)
(v) there was no detectable transmission of either the NadA-expressing or wild type-equivalent (i.e. control) GMO from colonised participants to bedroom-sharing close contacts at any point over a 90-day carriage period [50].

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

The release will take place in the environmental chamber within Southampton NIHR Clinical Research Facility, at University Hospital Southampton, Tremona Rd, Southampton, **SO16 6YD**.

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. Challenge participants are likely to encounter other humans. Therefore we will monitor transmission to contact participants (and provide eradication treatment if colonised) and require both challenge and contact participants to abide by infection control rules to limit transmission to any other individuals (Item 77).

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 which limits the impact of the release to modulation of the nasopharyngeal microbiome. The competitive relationship observed between *N. lactamica* and *N. meningitidis* [17], 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 (data not shown) 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*.

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 the human nasopharynx, most commonly in pre-school aged children. Healthy adult volunteers, screened and enrolled as

challenge participants in the proposed clinical studies will be primary recipients of the GM strains. We anticipate that this will induce nasopharyngeal colonisation with at least one of the GM-*N. lactamica* strains for up to 56 days following each 4xrNlac challenge. This colonisation will be cleared with antibiotic therapy at the end of each challenge phase (Item 73.3).

During these periods of colonisation, the challenge participants will be living within their normal community and so it is possible that there could be onward transmission to close contacts, although the risk of onward transmission is considered to be low (Item 54, Part A4). Bedroom sharers will be enrolled as contact participants to assess for transmission and all participants will follow strict infection control guidelines (Item 77) to minimise the risk of transmission to any other contacts. Mitigation strategies to minimise the risk of transmission are discussed further in Item 77, and assessments of the risk of spread and impact on human health are discussed further in part A4.

53. Any known planned developments or changes in land use in the region which 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.

Note that the wild type organism we have genetically modified, *Neisseria lactamica* strain Y92-1009, is a human-adapted, commensal, non-pathogenic bacterium. The GM derivatives of this organism described herein are all non-attenuated, and are expected to survive and multiply within their biological niche following colonisation.

Transmission of *N. lactamica* is believed to occur person-to-person, hypothesised to occur through mechanisms similar to those studied for the meningococcus, i.e. exchange of respiratory and throat secretions (saliva) or during close and/or lengthy contact. 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 [59-61]. Therefore, following inoculation of challenge participants, there is a chance that their close contacts (i.e. contact participants) may also become carriers of one or more strains of the GMO. However, it is very important to note that we have never observed *N. lactamica* transmission during any of our studies. Arguably the most potent example of this is the observation that *N. lactamica*-infected mothers did not detectably transmit the organism to their offspring during the postnatal period [57]. In addition to this, a previous study of the genetic diversity of *N. lactamica* isolates within households suggested that infants were most likely to

acquire *N. lactamica* from other children, rather than adults, within their household [62]. One possible explanation is that adult humans are effectively a dead-end for *N. lactamica* transmission chains.

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. 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, Table 3).

Interactions with the environment

57. The predicted habitat of the organism.

The natural habitat of *N. lactamica* is humans. Challenge participants enrolled to the proposed clinical studies will be the primary recipients of the GMOs.

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 and/or a single copy of the appropriate *fhbp* gene will be released to the extracellular milieu of the nasopharynx. The DNA containing one or both genes 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 species and the balance of DNA repair systems within each organism, which will determine the recombinogenicity of a given species. The gene expression cassettes contain no means to initiate their own replication, which means that to be maintained there is an absolute requirement for integration of the gene into the recipient's DNA (be this chromosomal or plasmid-based).

To facilitate engineering of the 4xrNlac strains, both genes have been closely linked to at least one Neisserial DNA uptake sequence (DUS), which significantly increases the likelihood of naked DNA from the GMOs 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* (NB: DUS vary slightly in sequence between *Neisseria* species, with uptake mechanisms biased in favour of their specific DUS subtype, or 'dialect' [33]). Indeed, there is evidence of frequent, interspecific genetic exchange amongst the *Neisseriaceae* [63]. The canonical DUS of *N. lactamica* is also that favoured by the potentially pathogenic *Neisseria* species, *N. meningitidis* and *N. gonorrhoeae*. As a result, DNA released from the GMOs will be preferentially targeted and bound by the DNA uptake machinery of these pathobionts, should they co-habit the same individual's nasopharynx. Whilst the risk of this will be minimised in our Pilot colonisation study, wherein we will screen for pre-existing meningococcal carriage prior to challenge and exclude meningococcal carriers, throughout both studies there remains the slim probability that *N. meningitidis* will be naturally acquired during the period of *N. lactamica* colonisation. Realistically, there is no practical way to eliminate this risk.

Accepting that dissemination of our *N. lactamica* codon-optimised version of these genes is therefore possible, it becomes important to consider the impact this will have on the recipient organisms:

For those organisms unable to express the genes, 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 these genes, serving as a potential route of indirect transmission to other (*Neisseria*) species in the future. Importantly however, there is no reason why this version of the *nadA* or *fhbp* gene is any more likely to be disseminated than naturally occurring alleles in currently-circulating strains of *N. meningitidis.* The fact that NadA and FHbp remain meningococcal-specific outer membrane proteins suggests that these genes are *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 can express the *nadA* and/or *fhbp* genes, then the situation becomes 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* from the *porA/porB* hybrid*,* non-phase variable promoter 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 for meningococcal carriage strains to downregulate NadA expression following the establishment of colonisation [4], it is reasonable to assume that NadA expression constitutes a survival liability to the meningococcus beyond the initial phases of the colonisation process. Therefore, unlike acquiring a natural *nadA* allele from the meningococcus, complete with a phase variable promoter that can be downregulated or switched OFF, acquisition of our engineered version of *nadA* would mean the recipient bacterium constitutively produces NadA, which could ultimately act as a target for immune-mediated killing of the bacteria. The only conceivable ways 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).

Strains that horizontally acquire the GMO-derived *fhbp* genes will have the same issue regarding constitutive protein expression as a survival liability, as explored above. In addition, the human complement Factor H-binding ability of the gene products have all been abrogated, meaning there will be no conceivable selective advantage to maintain the expression of these genes over the longer term.

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 *N. lactamica* genome. This incompatibility arises because many other bacteria lack DNA methylases capable of protecting their DNA against the restriction endonuclease activities of *N. lactamica* (NB: the *N. lactamica* genome codes for 2 restriction endonucleases with 4 bp recognition and cleavage sequences, NlaIII and NlaIV). As a result, most heterotypic DNA taken up by *N. lactamica* is degraded before recombination can take place. The GMOs are similarly refractory to transformation with heterotypic DNA (see Item 25, Figure 14).

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

The 4xrNlac strains do 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. This is also true of each FHbp variant, which have all been specifically mutated to abrogate the binding of human complement Factor H. Indeed, the 4xrNlac strains have been shown to be no more competent than wild type bacteria in assimilating homotypic DNA (Item 25, Figure 14C), indicating that a 'transformable' phenotype is not self-selecting.

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 *N. meningitidis* chromosomal DNA). *N. lactamica*-colonised individuals are likely to be protected from (re-)acquisition of *N. meningitidis* by an as-yet undefined mechanism, although it is not possible to eliminate the risk of *N. lactamica* and *N. meningitidis* co-carriage in the same participant. However, as evidenced in Item 25, Figure 14, the 4xrNlac strains remain refractory to horizontal gene transfer from the meningococcus.

As discussed in Item 59a, persistent expression of these antigens most likely represents a survival liability, insofar as they eventually become targeted by adaptive immune responses. The likely outcomes of this negative selection pressure are: (i) the loss of the gene from the genome, (ii) mutation of the coding sequence to disrupt immunogenic epitopes or (iii) mutation of the sequences governing regulation of gene expression.

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 genomic integrity of the 4xrNlac GMOs, with respect to the lack of uptake of markers from *N. meningitidis* chromosomal DNA is demonstrated in Item 25, Figure 14. We interpret this as meaning that the likelihood of the GMOs assimilating genetic material from other organisms in the nasopharynx is negligible.

With respect to the stability of the introduced genetic material over time, as discussed in Item 33b, we have previously demonstrated that the NHCIS1 locus is not a recombination hotspot and that heterologous genes in this locus are unchanged following 28 days of serial, *in vitro* passage on bacterial growth medium. Because: (i) growth within its biological niche has a stabilising effect on the *N. lactamica* genome compared to *in vitro* culture, and (ii) our challenge agent will be comprised of bacteria that will have been cultured a maximum of three times prior to administration into humans, we have dispensed with a similar analysis of the stability of the 4xrNlac genomes following serial *in vitro* culture. Whilst analysis of the genomic stability of 4xrNlac isolates taken from participants is one of the study objectives, by its nature this can only be performed post-challenge.

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.

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

Neisseria lactamica carriage is restricted to humans. It is carried exclusively in the nasopharynx and is believed to transmit 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%, $P < 0.05$) [59]. 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%) [61]. 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 [64].

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 [65]. However, other studies have suggested that such household

transmission may occur chiefly from infants and children rather than from adults, as discussed in Item 54 [57, 62].

From these data we conclude that household members, in particular bedroom-sharers of the challenge 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 [64], 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 (see Item 77).

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.

The only natural reservoir of *N. lactamica* is the human nasopharynx, so excessive population increase of the organisms in the environment would imply that there has been transmission of the 4xrNlac strains beyond the index cases (i.e. *N. lactamica*-colonised challenge participants). It is important to note that we have never observed transmission of wild type or GM *N. lactamica* from colonised human adults in any of our studies. However, because there will always be the risk that the unattenuated GMOs *could* transmit within the community following release, a foreseeable consequence might be circulation of the 4xrNlac strains in the local paediatric population. Whilst all the data disclosed herein indicates this is not a cause for concern, and that the GMOs would most likely circulate like a wild type strain, we will exclude participants with household or regular occupational contact with children under the age of five years.

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* in the short-term, specifically in terms of the ability of the GMO to colonise the nasopharynx in individuals not currently colonised with a strain of *N. lactamica*, or in a situation where an individual has been exposed to a wild type organism and one or more of the 4xrNlac strains simultaneously. However, over the longer term constitutive expression of NadA may prove a survival liability (see Item 59a).

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

The target recipients in the proposed clinical studies will be healthy human adults. Challenge and contact participants will undergo a screening assessment by a study doctor, including a physical examination, vital signs and screening investigations such as blood and urine tests (challenge participants) and an ECG (challenge and contact participants). Eligibility will be assessed according to strict inclusion and exclusion criteria to ensure that participants will not be at any increased risk of disease or harm from colonisation with the GM-*N. lactamica* strains or any other study procedures such as antibiotic administration, and that the potential risk of onward transmission to other humans, particularly to immunocompromised individuals, is minimised. The eligibility criteria as relate to the potential for onward transmission and participant safety are detailed below, further assessment of the risks and mitigation strategies are outlined in part A4.

Inclusion criteria:

- Healthy adults aged 18 to 45 years (challenge participants) or 55 years (contact participants) inclusive on the day of enrolment
- Able and willing (in the investigator's opinion) to comply with all study requirements
- Able to correctly answer all questions in the pre-consent questionnaire
- Provide written informed consent to participate in the study
- Provide written agreement to abide by infection control guidelines during the study period (Item 77)
- Provide written consent to allow the study team to discuss the participant's medical history with the General Practitioner (challenge participants only)
- For females of child-bearing potential, willingness to practice continuous effective contraception during the study and a negative pregnancy test on the days of screening (all participants) and inoculation (challenge participants only)
- Agreement to take antibiotic eradication therapy according to the study protocol

Exclusion criteria:

- Individuals who have a current infection at the time of inoculation (challenge participants only)
- Use of systemic antibiotics within the period 30 days prior to the initial challenge (challenge participants only)
- Any confirmed or suspected immunosuppressive or immune-deficient state, specifically terminal complement component deficiencies, eculizumab use, known HIV infection, malignancy, asplenia, recurrent severe infections and chronic (more than 14 days) immunosuppressant medication within the past 6 months (topical steroids are allowed)
- Use of immunoglobulins or blood products within 3 months prior to enrolment

- Any abnormal finding on clinical examination or screening investigations, assessed by the investigator to be clinically significant. In the event of abnormal test results, confirmatory repeat tests may be requested.
- Pregnancy, lactation or intention to become pregnant during the study
- Any other significant disease, disorder, or finding which may significantly increase the risk to the participant, affect their participation in the study or impair interpretation of the study data, for example recent surgery to the nasopharynx
- History of allergy or intolerance to any component of the inoculum (challenge participants only)
- Contraindications to the use of ciprofloxacin, specifically hypersensitivity to quinolones, a history of tendon disorders related to quinolone use, epilepsy, a personal or family history of aneurysm or congenital heart valve disease, and prolonged QT interval
- Contraindications to the use of ceftriaxone, specifically known hypersensitivity to cephalosporins or severe penicillin allergies (e.g. anaphylaxis or Stevens Johnson Syndrome)
- Household, close social or regular occupational contact with persons known to be immunosuppressed, specifically HIV infection with a CD4 count <200 cells/mm3; asplenia; any malignancy, recurrent, severe infections and chronic (more than 14 days) immunosuppressant medication within the past 6 months (topical steroids are allowed)
- Household or regular occupational contact with children under 5 years or an older child with a tendency to co-sleep with the participant

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 immune responses to the 4xrNlac strains following intranasal exposure and subsequent colonisation will be measured following both primary challenge and re-challenge. It is anticipated that following primary challenge one or more strains of the GMOs that comprise 4xrNlac will colonise the nose and throat of susceptible challenge participants, but at this stage it is unknown what characterises susceptibility to or protection from colonisation in humans. Note that in a previous CHIME, two GM-*N. lactamica* strains established colonisation in either 78.5 % (Control arm) or 100 % (Intervention arm) of challenge participants following primary challenge with 10^5 CFU [50] so it is possible that some participants will be challenged with 4xrNlac but will not become colonised by the constituent GMOs. The pre-existing immune parameters of these non-colonised participants are of interest in this study, as they may represent one possible ‘fingerprint of protection’, which is defined as the combination of immunological responses that underwrite a ‘protected from GM-*N. lactamica* colonisation’ phenotype. It is these possible ‘fingerprints of protection’ that the study attempts to evoke through primary challenge and then test through its re-challenge component. In challenge participants who become colonised by one or more strains

of GMO following primary challenge, it is anticipated that the unattenuated organisms will insert themselves into the microbiome of the upper respiratory tract, from where the normal microbial surveillance mechanisms of the host will detect, process and respond to them (a process presumed to involve passage through M cells, followed by internalisation by phagocytes/dendritic cells, the presentation of GMO-derived antigens in conjunction with major histocompatibility complexes, with downstream T cell activation and subsequent effector cell function). Once *in situ*, the colonising GMO(s) will stimulate immune responses in the mucosal compartment, the complexity and intensity of which will vary between participants and be modulated by each participant's unique combination of vaccination status and history of asymptomatic infection by Neisseriaceae. Note that it is extremely likely that all challenge participants will have had previous asymptomatic exposure to circulating strains of *Neisseria*, which infers their adaptive immune responses to the GMO will be 'recall responses' that were primed by other strains of bacteria with whom GM-*N. lactamica* shares structural similarities. Immune responses to GMO-derived antigens will include both innate and adaptive responses, with the latter further broken down into cell-mediated and serological responses. The dynamics of these processes will vary, but longitudinal upper respiratory tract sampling will enable changes in immune parameters in response to GM-*N. lactamica* colonisation to be tracked.

After the initial period of carriage, the GMO(s) will be eradicated using an appropriate sterilising antibiotic, and then time will be allowed for the antibiotic to wash out of the participant and for the adjusted microbiome of the treated participants to stabilise. In those participants who were previously colonised with the GMO(s), it is hypothesized that the various immunological responses to carriage will have generated a 'protected from GM-*N. lactamica* colonisation' phenotype, which will prevent the same individuals becoming colonised by the GMOs following rechallenge. It is possible that the immunological underpinnings of this phenotype differ from participant to participant, and that more than one immunological 'fingerprint of protection' is evident in our cohort.

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.

The only established interaction with non-target organisms in the context of controlled human infection with *N. lactamica* is the apparent exclusion of resident *N. meningitidis* from the nasopharynx of *N. lactamica*-colonised individuals in a serogroup-independent manner [17]. The precise mechanism that underpins this inverse association is still unknown, but plausibly manifests 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, co-cultivation of these bacteria in a mixed-species biofilm [66], corroborates our own, unpublished findings.

Whilst it is anticipated that colonisation with the 4xrNlac strains will reduce the likelihood of meningococcal reacquisition, co-carriage of these species is still possible. Fortunately, our selective culture medium also supports the growth of *N. meningitidis*, and our staff are trained to identify *N. meningitidis* colonies. If colonies characteristic of meningococcal colonisation are identified on plates, then a battery of confirmatory diagnostic tests are available. Whilst no direct action will be taken upon discovery of meningococcal co-carriage in a given participant, because the presence of the related organism will confound downstream serological data from this individual, there is provision in our protocols to recruit another participant and protect the power of our study.

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 GMOs will be further transformed by exogenous nucleic acid that changes the biology of the GMOs, and (b) that the GMOs donate nucleic acid that modifies the biology of other commensals or pathobionts carried by humans infected by the GMOs.

Regarding (a), we have shown in Item 25, Figure 14 that the 4xrNlac strains remain refractory to uptake of meningococcal genomic DNA under optimised laboratory conditions, suggesting that the risk of this occurring is negligible. However, the *N. lactamica* chromosome does contain regions of 'acquired' DNA, identified as such on the basis of a GC nucleotide content different from the chromosomal average [67]. 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* [19];[13]. It is also important to note that the capacity of *N. lactamica* to become transformed with exogenous nucleic acid is not self-selecting and self-enriching, insofar as the 4xrNlac strains, which have each undergone 4 deliberate, *in vitro* transformations, are no more likely to become transformed by homotypic DNA than is a wild type strain (Item 25, Figure 14C). It is impossible to predict exactly which nucleic acids will be encountered and taken up by the 4xrNlac strains during carriage, but the robust restriction modification systems of the species, as evidenced both by our experimental data and the relative paucity of historical transformations, suggests that the risk of radically shifting the evolutionary trajectory of the organisms is only as likely to occur as it would in a wild type *N. lactamica* strain.

Regarding (b), there is evidence of frequent, interspecific genetic exchange from *Neisseria lactamica* into *Neisseria meningitidis* [22];[23];[24];[25], with genetic material from the former being readily incorporated into the latter and the subsequent generation of hybrid genotypes. This process is driven by the abundance of Neisserial DNA Uptake Sequences (DUS), which are hypothesised to act as a conservative, rather than diversifying evolutionary force and bias natural competence in favour of 'self' DNA, effectively promoting 'safe sex' between conspecifics [20]. Whilst certain genetic differences between these species have implications for clinically relevant phenotypes such as relative sensitivities to penicillin [24], there is nothing about the 4xrNlac strains to suggest these bacteria would facilitate this transfer any more than would a wild type *N. lactamica* strain. Indeed, as discussed in Item 59a, should the escape of alleles coding for the heterologous meningococcal antigens into other bacterial species occur, their incorporation would likely serve only as a detriment. Constitutive antigen expression would represent a metabolic burden that negatively impacts the transformed bacterium's relative fitness for its niche, and could potentially act as an additional target for strong, sterilising immunological responses.

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 controlled human infection with *N. lactamica* is the apparent exclusion of resident *N. meningitidis* from the nasopharynx of *N. lactamica*-colonised individuals in a serogroup-independent manner [17]. *N. lactamica* strains are not anticipated to survive for long outside of their adapted niche.

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 previously been released in a clinical study, but they have been extensively handled in the laboratories at University of Southampton (under Health & Safety Executive notification GM57/14.3) and at UK Health Security Agency Porton Down. We have substantial experience in monitoring nasopharyngeal

colonisation, transmission and safety in controlled human infection studies with the wild-type parent strain, and with the GM strains 4NB1 and 4YB2. Colonisation and clearance of challenge participants, any onward transmission and any unexpected effects will be monitored using local and study-specific processes and standard operating procedures as detailed below.

1. Colonisation of challenge participants

Challenge participants are those individuals enrolled to the clinical studies who receive an intranasal inoculum of 4xrNlac or control (PBS). We anticipate that most challenge participants who are inoculated with 4xrNlac will develop nasopharyngeal colonisation with at least one of the GM strains. Colonisation may be spontaneously cleared or may continue for the duration of the challenge phase, i.e. from nasal inoculation until antibiotic eradication therapy is administered. This will be a period of 28 days for the pilot study, and 1-2 periods of 56 days for the Main study.

Throughout these periods, nasopharyngeal colonisation will be monitored at regular follow up visits (see Item 76) by culture of nasopharyngeal samples (throat swabs and nasal washes) on selective media containing X-gal (see Item 30 for more details). 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). All challenge participants will be screened for pre-existing *Neisseria* colonisation at approximately 5 days prior to inoculation, and excluded if they are already colonised with *N. lactamica*. Therefore any positive culture of *N. lactamica* at any time point following inoculation will be assumed to be one or more of the GM strains for the purposes of the study conduct. Colonisation density will be estimated by colony count.

Presence and relative abundance of each of the four GM strains will be verified by strain-specific qPCR.

2. Onward transmission of GM *N. lactamica* strains

Transmission is possible to close human contacts via respiratory droplets, with the highest risk of transmission to individuals sharing a bedroom with colonised participants (see Item 62).

In our previous GM-*N. lactamica* CHIME using strains 4NB1 and 4YB2, we monitored all challenge participants at every follow up visit for environmental shedding of the GM strains by attempting culture from a face mask worn for 1 hour, and from a 300 L/min air sampler conducted in an environmental chamber. No GM-*N. lactamica* colonies were detected from any mask or air sample, despite abundant nasopharyngeal colonisation being detectable from culture of nasopharyngeal samples. Additionally, bedroom sharers of challenge participants were enrolled to

monitor for onward transmission with regular throat swab samples taken throughout the study. All bedroom sharer throat swab samples were negative throughout the study, i.e. no onward transmission was detected even to those individuals sharing a bedroom with participants colonised with the GM-*N. lactamica* strains.

Therefore, we anticipate that the risk of environmental shedding and onward transmission is low. In the proposed studies, any individual sharing a bedroom with a challenge participant will be screened and consented *a priori* as a contact participant. Contact participants will be screened for transmission of the GMOs by a throat swab taken at the end of the study, at an additional visit if symptoms suggestive of respiratory or systemic infection occur, or if they, or their corresponding challenge participant, withdraw prior to the end of the study. Any contact participants found to be colonised will be given antibiotic therapy to clear colonisation.

Although unlikely, we recognise that onward transmission to other human contacts is possible. In order to minimise the risk of transmission, both challenge and contact participants will be required to abide by infection control rules (Item 77), including abstinence from intimate contact or bedroom sharing with any individual other than those consented and enrolled as their bedroom-sharer. These rules will form part of a pre-consent questionnaire, and the written informed consent form which will be signed by all participants prior to enrolment. Potential challenge and contact participants will not be enrolled to the study if they have any household, regular occupational or close social contact with individuals known to be immunocompromised, or pre-school children, during the study period.

It is not feasible to monitor any further onwards transmission through the wider community. However, we do not expect the GMO to propagate widely relative to wild type strains of *N. lactamica*.

3. Antibiotic clearance of GMOs

The GM strains have confirmed sensitivity to ciprofloxacin, rifampicin and ceftriaxone (Item 12e, Table 3). In our previous wild type and GM-*N. lactamica* controlled human infection studies we have demonstrated that a single 500mg dose of oral ciprofloxacin is 100% effective in clearing nasopharyngeal colonisation within 24-48 hours [50].

After assessment of the risks and benefits of each potential antibiotic therapy, a single dose of oral ciprofloxacin is considered to be the safest and most effective method to reliably clear colonisation with GM-*N. lactamica*. This risk assessment is detailed in Part A4.

Therefore, all challenge participants will receive a single dose of oral ciprofloxacin to clear potential nasopharyngeal colonisation of the GM strains at day 28 in the pilot study, and at the end of each challenge phase (day 56 following each inoculation, both control and intervention groups) in the Main study, or at the end of their

participation in the study if they withdraw prior to these timepoints. Contact participants will receive ciprofloxacin at the end of their involvement in the study if they are found to be colonised with the GM-*N. lactamica* strains. Alternative effective antibiotics may be used if clinically indicated, e.g. rifampicin in the event of pregnancy, or ceftriaxone if parenteral treatment were indicated. A throat swab will be taken 1-4 days following completion of antibiotic treatment to confirm clearance.

4. **Unexpected effects / disease**

As discussed throughout this application, we do not expect the GM strains 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. In our previous controlled human infection studies using wild-type *N. lactamica* and GM strains 4NB1 and 4YB2 there were no symptoms attributed to nasopharyngeal colonisation with any strain. However, study participants will be monitored at regular study visits (Item 76) including assessment of vital signs, biochemical and haematological markers and the development of any symptoms or occurrence of any adverse events. If any symptoms develop between study visits then participants will be encouraged to inform the study team via a 24 hour telephone number, and additional visits will be arranged if appropriate. Additional investigations such as additional blood tests, blood cultures and respiratory viral PCR screens will be arranged as required, following review by a study doctor. In the event of significant symptoms developing which are felt to be potentially attributable to GM-*N. lactamica* disease, then treatment with appropriate oral or parenteral antibiotics will be commenced.

An independent Data Safety Monitoring Board (DSMB) will be established prior to the start of the study to provide oversight of safety and study conduct. The DSMB will meet at agreed intervals during the studies, in the event of any safety concerns arising, or if the Chief Investigator feels independent advice or review is required. The DSMB will 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 potentially attributable to any of the GM strains). The outcome of each DSMB review, including recommendations regarding study continuation, will be communicated directly to the study investigators and documentation of all reviews will be kept in the trial master file.

As *N. lactamica* is a human restricted commensal it will not transmit or have any other effect outside of human hosts.

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.

Clinical samples (throat swabs and nasal swabs) will be taken from participants to assess for nasopharyngeal colonisation with the GMOs at pre-defined time points as per the study protocols (see Item 76). These samples will be analysed by culture in

the first instance, using standard microbiological techniques and biochemical tests (API, bioMérieux) if required. Putative *Neisseria* colonies*,* identified on the basis of colony morphology and growth on selective (GC) agar, will be discriminated as *N. lactamica* in the first instance by metabolism of X-gal (generation of a blue breakdown product), see Item 7 for further details.

All challenge participants will be screened for *N. lactamica* colonisation at a pre-challenge visit (approximately 5 days prior to inoculation) and excluded if positive. Therefore, any putative *N. lactamica* colonies from clinical samples taken at any time point beyond inoculation will be assumed to be GM strain(s) for the purpose of study conduct.

The presence and relative abundance of the four GM strains will subsequently be verified using bespoke qPCR assays. Total genomic DNA will be extracted from the remaining volume of each sample eluate, and the number of genome copies of each of the four GM-*N. lactamica* strains will be enumerated. These assays specifically amplify unique sections of each rationally-designed *fhbp* gene, coded within the modified NHCIS1 locus and will therefore detect the presence of the GMOs with 100% specificity.

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

Given the proposed methodology for determining the *N. lactamica* colonisation status of participants, specifically the culture of viable bacteria on GC selective agar medium supplemented with X-gal, during the study it would only be possible to look for the transfer of donated genetic material to a very narrow range of other nasopharyngeal bacteria (i.e. those capable of growth on the selective agar). Even then, given that expression of both NadA and FHbp are effectively ‘markerless’, having no notable effect on colony morphology, it would realistically only be possible to detect if the Synth.*lacZ* gene, coding for β-galactosidase, is transferred to these other bacteria. Transfer of Synth.*lacZ* into the small subset of species capable of growth under these conditions would be characterised by the metabolism of X-gal and the formation of a blue-coloured colony, which would have a morphology likely different to that of GM-*N. lactamica* and consistent with that of a wild type strain of the recipient bacterium. Although both the *nadA* and *fhbp* genes are closely linked to the Synth.*lacZ* gene in the NHCIS1 locus of the 4xrNlac strains, and it is possible all three genes could be transferred together in a single unit, it is equally feasible that only one or both heterologous antigen genes, divorced from the Synth.*lacZ* gene, could transform a non-target bacterium.

Given all the data disclosed herein, including the attenuation of human Factor H binding for each FHbp variant (Item 25, Figure 12), and the anticipation that heterologous allele escape into other non-target species is more likely to serve as an evolutionary disadvantage or survival liability over the longer term, we do not intend to actively look for evidence of gene transfer into the small subset of nasopharyngeal

commensals that grow on our selective media. We posit this is reasonable given the above, and the fact that such surveillance, whilst labour and resource-intensive, would not capture horizontal genetic exchange into what are presumably the vast majority of nasopharynx-colonising bacteria (specifically, any species that does not grow to form colonies on the selective medium). Any such surveillance would be further confounded by the growth of any bacterial colonies that naturally express β-galactosidase: firstly, a β-galactosidase-expressing bacterium may also become transformed with our Synth.*lacZ* gene, but undergo no identifiable change in colony colouration (i.e. they grow as blue-coloured colonies anyway); and secondly, because each participant has a unique microbiome that changes over time, it would be impossible to determine whether the presence of a blue-coloured colony in one sample that was not detected in previous samples was because of horizontal genetic exchange with our GMOs, or the result of having acquired a new, naturally-circulating, β-galactosidase-expressing bacterium. Therefore, were we to include investigational triggers for these events in our clinical protocols, we would likely invest a lot of time and resource into trying to track an extremely rare and ultimately inconsequential event.

Although, as stated, we are not planning to proactively monitor the small subset of bacterial species that grow on our selective medium for evidence of horizontal genetic exchange from our GMOs, *if one wanted to detect such events* in isolated, putative transformants, then it would be possible to do so using one of many bespoke PCRs, which either amplify the artificial, rationally-designed nucleic acid sequences of the heterologous antigen genes, or take advantage of the unnaturally close proximity of specific nucleotide sequences such as the *lacZ* coding sequence and the endogenous, *N. lactamica lst* gene promoter. A culture-independent technique that could be employed to detect horizontal genetic exchange at a population level is metagenomic sequencing from upper respiratory tract samples, followed by bioinformatic identification of the nucleotide sequences that flank our known, target sequences.

76. Duration and frequency of the monitoring.

Challenge participants (those who receive an intranasal inoculum of 4xrNlac, or control for Main study only) will be monitored for nasopharyngeal colonisation with the GM strains, and for any symptoms or safety concerns, for the duration of their involvement in the study, at scheduled follow up visits as detailed in the study specific protocols. They will also be reviewed at additional visits if required, for example if any symptoms suggestive of respiratory tract or systemic infection occur in between planned visits.

For the pilot study, follow up visits will be planned for days 4, 7, 14 and 28 post inoculation. Antibiotic therapy will be given at day 28 to clear colonisation.

In the Main study, follow up visits will be planned for 1, 2, 4 and 8 weeks following each of the two inoculations. Antibiotic therapy will be given at the 8 week (day 56) visit.

Contact participants (bedroom-sharers of challenge participants) will be monitored for transmission of any of the GM strains with a throat swab taken at the day 28 (Pilot study) or rechallenge day 56 (Main study) visit of their corresponding challenge participant. If they develop any symptoms suggestive of possible respiratory tract or systemic infection during the course of the study then they will be invited to attend an additional visit at which point a throat swab will be taken. Any contact participant found to be colonised with a GM-*N. lactamica* strain will receive antibiotic therapy to clear colonisation at the end of their participation in the study.

The Pilot study is expected to commence on the 1st of February 2026 (pending all necessary approvals) and will run for approximately 3 months with an expected completion date of 30th April 2026. The Main study is expected to commence on the 1st November 2026 (pending all necessary approvals) and will run until study completion (anticipated to be 31st May 2031).

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.

As detailed in Items 62 and 73, *N. lactamica* colonisation is restricted to humans, with the greatest risk of transmission being to those who share a bedroom with colonised individuals. In our previous GM *N. lactamica* study we did not detect shedding from colonised participants, nor transmission to their bedroom sharers so we anticipate that the risk of onward transmission is low. However, to minimise any potential transmission from participants to other contacts and the wider community, with particular consideration of potentially more vulnerable individuals, the following control measures will be used:

1. **Eligibility criteria**

Challenge and contact participants will be enrolled according to specific inclusion and exclusion criteria which will be assessed at an initial screening visit by a study doctor. Those criteria pertaining to onwards transmission and participant safety are detailed in Item 66. Those relevant to minimising onward transmission include:

Inclusion criteria:

- Able to correctly answer all questions in the pre-consent questionnaire
- Provide written agreement to abide by infection control guidelines during the study period
- Agreement to take antibiotic eradication therapy according to the study protocol

Exclusion criteria:

- Occupational, household or intimate contact with immunosuppressed persons, specifically HIV infection with a CD4 count <200 cells/mm^3; asplenia; any malignancy, recurrent, severe infections and chronic (more than 14 days) immunosuppressant medication within the past 6 months (topical steroids are allowed)
- Occupational or household contact with children under 5 years or an older child with a tendency to co-sleep with the participant
- Pregnancy, lactation or intention to become pregnant during the study

2. Infection control rules

Both challenge and contact participants will be required to adhere to specific infection control rules for the duration of their involvement in the study. These will be explained in a written participant information sheet which participants will receive prior to screening and they will be verbally explained by a study doctor at the screening appointment. Understanding of these rules will be confirmed as part of a pre-consent questionnaire, and agreement to abide by these rules will be included in the written informed consent form. These rules are as follows:

- Participants must wash their hands before leaving their home and following any contact with respiratory secretions e.g. sneezing / blowing nose
- Participants must be contactable by mobile phone, which has the study emergency phone number programmed in, and contact the clinical study team if they have any symptoms suggestive of respiratory or systemic infection
- Participants must be able to be return to the NIHR-CRF within 24 hours
- Participants must avoid attending heavily crowded environments such as nightclubs for the two weeks following inoculation of the challenge participant
- Participants must avoid close contact with individuals known to be potentially vulnerable for the duration of their involvement in the study. Close contact is defined as:
    - Face to face contact < 2m for >15 minutes
    - Staying overnight in the same accommodation
- Potentially vulnerable individuals include
    - Immunocompromised individuals
    - Children < 5 years
- Participants must avoid activities associated with a high risk of transmission with any individuals other than their declared and consented bedroom-sharer. High risk transmission activities include:
    - Bedroom sharing
    - Intimate/sexual contact
    - Contact that may involve transfer of respiratory secretions e.g. kissing
    - Sharing cutlery or drinking vessels

3. Clinical procedures

The inoculation procedure and any clinical sampling to investigate the carriage of the GMOs by the participant (throat swabs, nasal washes, nasal swabs) will be undertaken in the containment level 2 environmental chambers within the NIHR-CRF. Study team members will wear appropriate personal protective equipment (disposable apron, gloves and surgical mask).

4. **Laboratory safety**

GM-*N. lactamica* is routinely handled in University of Southampton laboratories under Health & Safety Executive notification GM57/14.3. The GM-*N. lactamica* stock vials, inoculum and all participant samples potentially contaminated with the GM strains will be processed according to study specific SOPs within category 2 microbiological safety cabinets by trained personnel wearing appropriate personal protective equipment (disposable gloves and laboratory coat).

All waste potentially contaminated with the GMOs will be inactivated and disposed of according to local and site SOPs for the disposal of GMO contaminated waste (Items 82 and 84).

5. **Informing other stakeholders**

Participants' GPs will be notified about their inclusion in these studies. 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 paused or stopped as per protocol, and this information will be passed on immediately to UKHSA and the participant's GP. Hampshire and Isle of Wight Health Protection Team South East will be informed when the clinical studies commence.

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

The front entrance to the Southampton NIHR Clinical Research Facility is manned at a reception desk during working hours. All other entrances, and this entrance out of hours, are locked with entry permitted by keycard only. Southampton NIHR Clinical Research Facility is located within University Hospital Southampton and is regularly monitored by onsite security services who are able to attend quickly in the event of any security concerns.

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

The infection control procedures instituted at 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/respiratory 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 maximum of a few clinical waste bags 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.

All waste potentially contaminated with the GMOs will be treated according to site standard operating procedures (SOPs) for disposing of GM contaminated waste. Briefly: materials used during the preparation of the inoculum will be decontaminated overnight in efficacious biocide (e.g. an at-least 2 % (w/v) Virkon solution or an at-least 5 % Biocleanse solution). All potentially GM-contaminated materials will be disposed of into a clinical waste bag or sharps bin which will be placed into an autoclave box. All waste will then 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.

Emergency response plans

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

N. lactamica colonisation is restricted to the human nasopharynx. Onward spread of the GM *N. lactamica* strains is possible to close contacts of colonised participants. The highest risk of transmission is to bedroom sharers (Item 62) and so any individuals sharing a bedroom with a challenge participant will be enrolled as a contact participant to monitor transmission, with antibiotic clearance prior to study completion, as detailed in Item 73.3. Onward transmission from challenge or contact participants to other contacts, or into the wider community, is possible, but will be minimised by the eligibility criteria and infection control procedures detailed in Item 77.

It is not feasible to routinely monitor further onwards transmission through the wider community. However we do not expect the GMO to propagate widely relative to wild type strains of *N. lactamica*.

Unexpected spread within the community might be detected if microbiology laboratories reported an unusually high number of *N. lactamica* isolates detected in throat swabs taken for other clinical indications. Hampshire and Isle of Wight Health Protection Team South-East will be informed when the clinical studies commence and so any reported increase in colonisation could be linked to our studies and any available isolates could be confirmed to be the GM strains using the strain specific PCR (Item 7). 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 [68], or alternatively to vaccinate with the NadA and FHbp containing vaccine Bexsero, which has been shown to protect against the occurrence of invasive meningococcal disease [58].

The GM *N. lactamica* strains are not expected to cause any disease. In the extremely unlikely event that *N. lactamica* was identified as a possible cause of disease in an individual not enrolled in the study, the index case could be effectively treated with ceftriaxone, which the GM strains are acutely susceptible to (Item 12e), and contacts could have prophylaxis with ciprofloxacin. Bexsero vaccine would be available if public health authorities deemed it necessary to protect larger populations.

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

The inoculation procedure and any clinical sampling to investigate the carriage of the GMOs by the participant (throat swabs, nasal washes, nasal swabs) will be undertaken in an environmental chamber within the NIHR-CRF. The chamber will be cleaned according to hospital guidelines [56] (Item 84), specifically the glass windows and inside walls of the hood will be cleaned with 70 % (v/v) alcohol solution (e.g. ethanol or industrial methylated spirits) after each participant visit, and the environmental suite will be cleaned with actichlor at the end of each day of participant visits.

Laboratory or clinical area spills will be decontaminated according to site standard operating procedures (SOPs) for disposing of GM-contaminated waste. The spillage will be covered with an appropriate liquid biocide (e.g. an at-least 2 % (w/v) Virkon solution or an at-least 5 % Biocleanse solution) with a minimum of 10 mins of contact time. The area will then be cleaned again with 70% alcohol solution (e.g. ethanol or industrial methylated spirits). All potentially contaminated materials will be disposed of as GM waste (Item 82).

Clinical carriage can be eradicated with a single dose of 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 *N. lactamica* exclusively colonises humans, containing onward transmission between individuals will 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 [68] or alternatively (if there is disease caused by the GMO) to vaccinate with the NadA and FHbp containing vaccine Bexsero which has been shown to protect against the occurrence of invasive meningococcal disease [58]. 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 [68]. Bexsero vaccine 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
- United Kingdom Health Security Agency

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Application for consent to release a GMO

Part A2: data or results from any previous releases of the GMO

Give information on data or results from any previous releases of this GMO by you either inside or outside the European Community [especially the results of monitoring and the effectiveness of any risk management procedures].

Not applicable, this is the first application for deliberate release of this GMO.

Part A3: Details of previous applications for release

Give details of any previous applications to release the GMO made to the Secretary of State under the 2002 Regulations or to another Member State under the Deliberate Release Directive 2001/18/EC.

Not applicable, this is the first application for deliberate release of this GMO.

Part A4: Risk assessment and a statement on risk evaluation

Risk Assessment: environmental impact of the release of the GMOs

We propose the deliberate release of four genetically modified strains of *N. lactamica* (4NFA313_H, 4NFB313_H, 4NFC313, 4NFD313_E) in the multi-strain challenge agent 4xrNlac, in the two clinical studies described in Item 34. Potential hazards associated with this deliberate release have been evaluated in the following risk assessments with consideration of the likelihood of occurrence and the potential severity of any consequences. These assessments have been conducted considering pre-existing scientific knowledge and expertise, extensive pre-clinical testing of the strains and proposed safety, infection control, monitoring and emergency response procedures and processes.

Risk assessment: factors affecting dissemination

N. lactamica carriage is restricted to the human nasopharynx with peak carriage in pre-school aged children [14, 15]. As detailed in Item 62, extrapolating from the much larger knowledge base around *N. meningitidis* transmission, it is likely that *N. lactamica* can be transmitted from person-to-person through close contact, with the highest risk of transmission being between household contacts, and particularly bedroom sharers [59, 61, 64]. While less data is available regarding *N. lactamica* transmission, there are some data to support the potential for transmission within a household [65], although transmission from children and infants may be of more importance than from adults [62]. We have not previously demonstrated transmission of *N. lactamica* from experimentally colonised individuals to either bedroom sharers [50] or from colonised mothers to their babies [57]. Despite extensive assessment, we have not previously demonstrated environmental shedding of *N. lactamica* from experimentally colonised participants [50].

Onward transmission of the GMOs to close contacts, particularly bedroom sharers, is therefore a potential, although likely low, risk. The risk of transmission to other contacts and to the wider community is lower. If onward transmission were to occur, the likely consequence of this for those individuals would be asymptomatic carriage of one or more GM-*N. lactamica* strains which may continue for several months. As the GM-*N. lactamica* strains are not expected to cause any symptoms or disease, the risk of harm to these individuals would be negligible. These risks and the mitigation strategies which will be in place are summarised in Table 4.

Potential adverse effect	Likelihood	Consequence	Mitigation strategies	Overall Risk
Transmission to bedroom sharers	Moderate	Negligible	• Bedroom sharers screened, consented and enrolled as "contact participants" with assessment for transmission at the end of the study, or if any potentially relevant symptoms occur during the study (Item 73.2) • Any GM-*N. lactamica* colonised contact participants will receive antibiotics to clear colonisation (Item 73.3)	Negligible
Transmission to other close contacts	Low	Negligible	• Eligibility criteria – challenge and contact participants with household or occupational contact with immunocompromised individuals or children < 5 years will not be enrolled (Item 66) • Infection control rules to minimise potential spread to other contacts – both challenge and contact participants (Item 77) • Antibiotic clearance of colonisation at the end of the challenge period – all challenge participants and any colonised contact participants (Item 73.3)	Negligible
Transmission to the wider community	Very low	Negligible		Negligible
Transmission to study staff	Very low	Negligible	• Standard operating procedures and staff training to minimise any potential for transmission (Items 38, 41, 43, 77) ○ Clinical procedures ○ Laboratory procedures ○ Cleaning of clinical areas ○ Disposing of GMO contaminated waste	Negligible
Transmission within the CRF / hospital	Very low	Negligible		Negligible

Table 4: Dissemination of the GM-*N. lactamica* strains – risk assessment and mitigation strategies

Risk assessment: human health impact

Wild type *N. lactamica* is a harmless commensal organism, commonly carried in the nasopharynx of healthy pre-school children. Although there are a few case reports of *N. lactamica* involvement in disease (see Table 4 in [39]), these are universally secondary complications arising from immunosuppression or trauma, and are exceptionally rare amongst these groups (Item 12d). Table 5 summarises the assessment of the risk of experimental colonisation with GM-*N. lactamica* to immunocompromised individuals, assuming no increase in pathogenicity.

The GM-*N. lactamica* strains are not expected to have any increased pathogenicity or risk to human health in comparison to the wild-type organism, chiefly due to the lack of capsule expression, which is the primary virulence factor for *N. meningitidis.* Assessment of the risk of the genetic modification having otherwise increased the transmissibility or pathogenic potential of GM-*N. lactamica* is summarised in Table 6.

The GM strains have confirmed sensitivity to ciprofloxacin, rifampicin and ceftriaxone (Item 12e, Table 3). Challenge participants will receive oral antibiotics to clear colonisation with the GM-*N. lactamica* strains at the end of the study (The GM-Nlac study – Pilot) and at the end of each challenge phase (The GM-Nlac study – Main). Any contact participants who are found to be colonised will also receive antibiotic clearance (Item 73.3).

In our prior studies, a single dose of oral ciprofloxacin has been used to clear *N.* lactamica colonisation with 100% of participants cleared within 48 hours of administration, including the GM-*N. lactamica* strains 4NB1 and 4YB2 [50]. The recent MHRA warning regarding ciprofloxacin use is noted, with the recommendation that fluroquinolones should only be prescribed where other commonly recommended antibiotics are inappropriate, due to the risk of long-lasting adverse drug reactions including tendonitis [69]. However, the risk of complications following a **single dose** of ciprofloxacin as meningococcal prophylaxis is considered to be extremely small and hence is still recommended in UKHSA guidance [68]. Other potential antibiotic agents were considered such as rifampicin, which would require a longer course and interferes with the oral contraceptive pill, and parenteral ceftriaxone, which would involve the risks associated with venous access. A single dose of oral ciprofloxacin is therefore felt to be the safest and most effective method to clear GM-*N. lactamica* colonisation. To mitigate the very low risk associated with this, participants will be screened for any contra-indication to ciprofloxacin [70] such as hypersensitivity to quinolones, a history of tendon disorders related to quinolone use, epilepsy, a personal or family history of aneurysm or congenital heart valve disease, and prolonged QT interval, with an ECG check at screening (Item 66).

Safety data will be collected at each follow up visit, including vital signs, laboratory parameters, and the occurrence of any symptoms or adverse events. Both challenge and contact participants will have a 24-hour telephone number to contact the study team in case any symptoms develop between visits, with additional visits and

investigations as appropriate. Symptoms and adverse events will be assessed for their likelihood of being related to colonisation with the GMOs. In the event of symptoms occurring which are considered to be potentially attributable to GM-*N. lactamica* disease, participants will be treated with oral or parenteral antibiotics as appropriate to the clinical situation. The treatment options, risks and mitigation strategies for antibiotic treatment are discussed further in **Risk assessment: emergency response**, below.

An independent Data Safety Monitoring Board (DSMB) will be established prior to the start of the study to provide oversight of safety and study conduct. The DSMB will meet at agreed intervals during the course of the studies, in the event of any safety concerns arising, or if the Chief Investigator feels independent advice or review is required.

Potential hazard	Likelihood	Consequence	Mitigation strategies	Overall Risk
N. lactamica disease in immunocompromised individuals	Extremely low • Very few case studies of *N. lactamica* disease despite high levels of carriage in children globally	Moderate	Challenge and contact participants will **not** be enrolled if they meet any exclusion criterion (Item 66) including (in summary): • Confirmed or suspected immunocompromise • Clinically significant abnormal finding on screening examination or investigations • Household or regular occupational contact with people known to be immunocompromised or children < 5 years • Pregnancy Challenge and contact participants will agree to abide by infection control rules throughout the study (Item 77), including (in summary): • Avoidance of heavily crowded environments (e.g. nightclubs) in the two weeks following challenge • Limitation of contact with any individuals known to be immunocompromised or vulnerable • Avoidance of high-risk transmission activities with anyone other than their declared and consented bedroom-sharer Availability of effective antibiotics (Item 12e)	Extremely low

Table 5: Risk assessment of *N. lactamica* disease in immunocompromised individuals, assuming no increase in pathogenicity or transmission over wild-type *N. lactamica*

Characteristic	Considerations	Pre-clinical testing	Risk of increasing pathogenicity
Expression of NadA	NadA not necessary nor sufficient for virulence in *N. meningitidis* No increase in pathogenicity demonstrated in previous CHIME with GM-*N. lactamica* expressing NadA	NadA expression demonstrated (Item 25, Figure 11)	Negligible
Expression of modified FHbp	FHbp not necessary nor sufficient for virulence in *N. meningitidis* Potential immunomodulatory role of FHbp due to binding of human complement Factor H (hFH) (Item 25) FHbp modified to disrupt ability to bind hFH	FHbp expression demonstrated (Item 25, Figure 10) Lack of hFH binding activity demonstrated (Item 25, Figure 12)	Negligible
Interactions with human cells	Expression of NadA associated with increase binding to HEp-2 cells	No increase in the internalisation of the GMOs by HEp-2 cells (Item 25, Figure 16)	Negligible
Genetic stability	*N. lactamica* known to be highly resistant to horizontal gene transfer (Item 10) Main risk would be uptake of *N. meningitidis* capsule biosynthesis genes	GMOs refractory to horizontal gene acquisition from *N. meningitidis* (Item 25, Figure 14)	Negligible
Susceptibility to killing by human serum		Susceptibility to complement-mediated killing demonstrated (Item 25, Figure 13)	None
Susceptibility to antibiotics		All four GM-*N. lactamica* strains acutely susceptible to front-line antibiotics (Item 12e, Table 3)	None

Table 6: Risk assessment of genetic modification increasing the pathogenicity of *N. lactamica*

Risk assessment: environmental impact

The human nasopharynx is the only natural environmental niche of *N. lactamica*; it does not survive elsewhere. The release of these GM-*N. lactamica* strains will therefore have no environmental impact.

Risk assessment: monitoring the GMO

The GMO will be monitored by clinical assessment of, and samples taken from challenge and contact participants enrolled to the clinical studies. Throughout their involvement in the study, participants will have access to a 24-hour telephone number to contact the study team if they develop any symptoms or have any concerns. Participants will attend the NIHR-CRF for scheduled follow up visits plus additional visits if required (Item 76). At each visit participants will have a clinical review including vital signs and physical examination if required. Clinical samples including blood tests, throat and nose swabs and nasal washes will be taken.

The risk of onward transmission from these participants to study team members, other individuals within the CRF and within the hospital have been considered in the section **Risk assessment: factors involved in dissemination**.

Additional risks associated with monitoring are therefore the risks of clinical assessment and sampling, which are low to moderate frequency but of negligible to low severity. Specifically, these are:

- Venesection – localised discomfort, bruising and/or vaso-vagal symptoms. If participants are known to be liable to vaso-vagal responses to venesection then blood tests will be performed with the participant supine to mitigate this risk.
- Respiratory sampling – localised discomfort which is transient and self-limiting, and rarely transient epistaxis.

Information about the clinical procedures and these risks will be included in the written participant information sheets. All visits will occur within the NIHR-CRF, where all clinical rooms have emergency call bells. Trained clinical staff, including ALS trained study doctors, will be present within the NIHR-CRF and able to attend the participant in the case of any concerns during a study visit. The NIHR-CRF is situated within University Hospital Southampton NHS Foundation Trust with resuscitation teams and the emergency department available if required. SOPs are in place for the management and transfer of unwell study participants.

Risk assessment: emergency response

In the unlikely event of symptoms considered to be potentially attributable to GM-*N. lactamica* disease in participants, they would be treated with oral or parenteral antibiotics as appropriate to the clinical situation. The GM strains have confirmed sensitivity to ciprofloxacin, rifampicin and ceftriaxone (Item 12e, Table 3). Each of these antibiotics are in regular clinical use and have known adverse reactions of varying likelihood and severity [70-72]. However, as the likelihood of these antibiotics being required is very low (as a treatment course rather than a single dose in the case of ciprofloxacin), the overall risk of adverse events related to antibiotic treatment is considered to be extremely low. Mitigation strategies will include the exclusion of challenge and contact participants with contraindications to the use of ciprofloxacin (as detailed in **Risk assessment: human health impact**), and those with known hypersensitivity to cephalosporins or with severe penicillin allergies (e.g. anaphylaxis or Stevens Johnson Syndrome). The choice of antibiotic, if required, would be made with consideration of the individual case risk / benefit profile.

If there is onward spread of the GMO detected in the community, it is unlikely that any action would be required as we do not expect the GM strains to cause any symptoms or disease. However in the extremely unlikely event that disease did occur, then the index case could be effectively treated with routinely used antibiotics as above, and 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 vaccination with the NadA and FHbp containing vaccine Bexsero which has been shown to protect against the occurrence of invasive meningococcal disease [58]. The likelihood of Bexsero being required, and therefore the risk of an adverse reaction [73] occurring as a result, is negligible.

Part A5: Assessment of commercial or confidentiality of information contained in this application.

Identify clearly any information that is considered to be commercially confidential. A clear justification for keeping information confidential must be given.

Not applicable

Part A6: Statement on whether detailed information on the description of the GMO and the purpose of release has been published

Make a clear statement on whether a detailed description of the GMO and the purpose of the release have been published, and the bibliographic reference for any information so published.
This is intended to assist with the protection of the applicant's intellectual property rights, which may be affected by the prior publication of certain detailed information, e.g. by its inclusion on the public register.

A detailed description of the GMO and the purpose of this release have not been published. The technology used to derive the GMOs are proprietary to the University of Southampton, comprising International Publication Number: WO 2017/103593 A1.