A recombinant commensal bacteria elicits heterologous antigen-specific immune responses during pharyngeal carriage

Supplementary Materials

Materials and Methods

Nucleic acid construct assembly

All nucleic acid constructs for transformation into *Neisseria lactamica* (Nlac) or genetically-modified Nlac (GM-Nlac) were maintained as plasmids in *E. coli* DH5α. All plasmids used in this study were derivatives of pUC19 and contain an ampicillin resistance gene that confers ampicillin resistance to successful transformants. Isothermal Assembly was used to generate plasmids from overlapping DNA fragments (Gibson Assembly Cloning Kit, New England Biolabs (NEB)), according to the manufacturer's instructions. A complete list of all plasmids used in this study is included in table S2. Routine PCR amplification was performed using high fidelity Q5 DNA Polymerase (NEB) and using extended primers (Sigma-Aldrich) on a Veriti thermal cycler (Thermo Fisher Scientific). A comprehensive list of primers used to generate various constructs is presented in table S3. Unless otherwise specified, all nucleic acid constructs for heterologous gene expression in GM-Nlac were targeted to 'Nlac heterologous construct insertion site number 1' (NHCIS1), an approximately 2 kilobase intergenic

chromosomal locus positioned between open reading frames: B2G52_06940 and B2G52_06945 (*49*).

Preparation of hypermethylated polymerase chain reaction.

Targeted mutagenesis of Nlac or GM-Nlac requires hypermethylated DNA, which is DNA in which all cytosine nucleotide residues are replaced with 5'-methylated cytosine nucleotide residues. Hypermethylated DNA can be generated in vitro using hypermethylated PCR. All hypermethylated PCR was performed using a mixture of deoxynucleotide triphosphates (dNTPs) containing 2'-deoxy-5-methylcytidine 5'-triphosphate (5-m-dCTP) (NEB) in place of 2'-deoxycytidine 5'-triphosphate (dCTP), to produce a methyl-dNTP mixture. The concentration of each dNTP in the methyl-dNTP mixture was 2.5 mM, with a total dNTP concentration of 10 mM. In a hypermethylated PCR reaction, the required volume of dNTP mixture is replaced by the same volume of methyl-dNTP mixture. The final concentration of dNTPs in a hypermethylated PCR reaction was therefore 0.8 mM. PCR using the methyl-dNTP mixture utilized Phusion high fidelity polymerase in high-GC-content buffer (GC buffer) (NEB) and thermal cycling conditions optimized for each reaction. Note that each primer pair amplified only the nucleic acid construct maintained within a given plasmid as hypermethylated DNA, and that the plasmid backbone including the origin of replication and ampicillin resistance gene was not amplified. Following purification of each hypermethylated PCR product (GeneJET PCR purification kit, Thermo Fisher Scientific), any plasmid template carried over was degraded by incubation for 1 hour at 37 ^{o}C in the presence of DpnI (NEB) in 1x CutSmart buffer (NEB). The

concentration of hypermethylated DNA was measured using a Nanodrop spectrophotometer (Shimadzu).

Transformation of *Neisseria lactamica* Y92-1009.

The recipient Nlac or GM-Nlac was cultured in tryptone soya broth supplemented with 0.2 % yeast extract (TSB) at 37 ºC, 5 % CO_2 with shaking (320 rpm) until the culture reached an optical density at 600nm (OD_{600nm}) = 0.3. The culture was then diluted x100 into fresh TSB and 10 µl aliquots of this suspension were spotted onto tryptone soya agar (TSA). Bacteria were incubated for 6 hours at 30 ºC, after which time a 10 µl aliquot of hypermethylated DNA suspension (50 nM) was spotted onto the barely visible colonies and allowed to dry in a microbiological safety cabinet. Plates were returned to the 30 ºC incubator and incubated for a further 9 hours. The colonies were collected from the plate surface and resuspended into 1 ml TSB. This suspension, putatively containing Nlac transformants, was diluted x5000 before 100 µl aliquots were spread across multiple (10-50) TSA plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Sigma-Aldrich) and incubated at 37 ºC, 5 % CO_2 overnight. GM-Nlac that was putatively transformed with a construct either introducing or deleting the *lacZ* coding sequence appeared as blue or white colonies, respectively. Alternatively, where the recipient Nlac or GM-Nlac strain was transformed with a construct containing the kanamycin resistance gene (*aphA3*), 100 µl aliquots of the suspension putatively containing Nlac transformants were spread onto TSA plates supplemented with kanamycin (50 µg ml^{-1}). Putative transformants were isolated and cultured overnight in TSB at 37 ºC, 5 % CO_2 with shaking (320 rpm). Glycerol stocks of each isolate were prepared and stored at -80 ºC

and the remainder of the culture was used to extract genomic DNA (GeneJET genomic DNA purification kit, Thermo Fisher Scientific), according to the manufacturer's instructions.

Verification of transformation and sequence fidelity.

Successful transformation of GM-Nlac isolates was verified by PCR of extracted genomic DNA. Primers were designed to anneal to chromosomal sequences that flank the specific site of insertion. Successful chromosomal integration of a construct resulted in amplification of a differently sized PCR product to the parental strain using the same primer pair. To verify the fidelity of coding sequences integrated into the NHCIS1 locus, primer pair HAEC1/2_FOR (5' – CTTCGTCGGTCTTTTTTTTGTTG – 3') and HAEC2_REV (5' – GAAACCCGGTGCGGAAAATG – 3') was used in multiple, low cycle number (18 cycles) PCRs to amplify the coding sequence from genomic DNA using Q5 high fidelity DNA Polymerase (NEB). The reactions were pooled, and the PCR products were purified (GeneJET PCR purification kit, Thermo Fisher Scientific) before being sequenced (Source Bioscience).

Inactivation of the NlaIII restriction endonuclease using a kanamycin resistance gene.

Plasmid pJL0005, containing a nucleic acid construct designed to disrupt the coding sequence of the endogenous *nlaIII* gene from Nlac strain Y92-1009 and to confer resistance to kanamycin upon transformation, was assembled from overlapping PCR fragments by isothermal assembly (Gibson Assembly Cloning Kit, NEB). The *nlaIII* gene codes for the type II restriction endonuclease NlaIII, which binds to a 4-nucleotide recognition sequence (5' – CATG – 3') and

cuts double-stranded DNA (*50*). The construct contains sequence identical to the coding sequence of the *nlaIII* gene, the reading frame of which is interrupted by the *aphA3* gene under transcriptional control of the endogenous Nlac *lst* gene promoter. Briefly, plasmid pJL0001, which contains a nucleic acid sequence designed to disrupt the coding sequence of *nlaIII* with two genes: a copy of the green fluorescent protein-derivative CLOVER and the kanamycin resistance gene, *aphA3*, was used as template for PCR using primer pairs: (i) *ΔnlaIII:aphA3*VECTOR_FOR and *ΔnlaIII:aphA3*VECTOR_REV, and (ii) *ΔnlaIII:aphA3*INSERT_FOR and *ΔnlaIII:aphA3*INSERT_REV, to yield PCR products (i) *ΔnlaIII:aphA3*VECTOR and (ii) *ΔnlaIII:aphA3*INSERT. The PCR products *ΔnlaIII:aphA3*VECTOR and *ΔnlaIII:aphA3*INSERT were mixed at a stoichiometry of 1:3 along with 1x Gibson Assembly master mix (total volume = 20 µl) and incubated at 50 ^{o}C for 1 hour. Circularized plasmids in the assembly mixture were transformed into highly competent *E. coli* DH5α (NEB) according to the manufacturer's instructions. Transformed *E. coli* were selected for on Luria Bertani (LB) agar supplemented with 50 µg ml^{-1} kanamycin (Sigma-Aldrich) and 100 µg ml^{-1} ampicillin (Sigma-Aldrich). Plasmid pJL005 was isolated from an overnight culture of transformed *E. coli* (GeneJET plasmid miniprep kit, Thermo Fisher Scientific) and was used as template in hypermethylated PCR using Phusion DNA polymerase in high-GC content buffer (GC buffer) (NEB) to produce hypermethylated PCR product: *ΔnlaIII:aphA3* (hm*ΔnlaIII:aphA3*). Following addition of 1 x CutSmart buffer (NEB) and restriction endonuclease DpnI (NEB) to hm*ΔnlaIII:aphA3* for 1 hour at 37 ^{o}C, to digest the template plasmid, hm*ΔnlaIII:aphA3* was used to transform wild-type (WT) Nlac strain Y92-1009. Successful transformants were selected for on plates containing tryptone soya agar (TSA) supplemented with 0.2% yeast extract (Sigma-Aldrich) and kanamycin (50 µg ml^{-1}) (Sigma-

Aldrich). Transformed, kanamycin-resistant, *ΔnlaIII* GM-Nlac (*ΔnlaIII*) was isolated and stored as frozen glycerol stocks at -80 ºC. Deletion of the *nlaIII* gene was confirmed by amplification of the *nlaIII* chromosomal locus using primer pair: *nlaIII*LOCUS_FOR and *nlaIII*LOCUS_REV.

Deletion of the endogenous Nlac β-galactosidase gene (*lacZ*).

Plasmid pJL0006, containing a nucleic acid construct designed to completely remove the coding sequence of the endogenous *lacZ* gene from Nlac strain Y92-1009 upon transformation, was assembled from overlapping PCR fragments by isothermal assembly (Gibson Assembly Cloning Kit, NEB). Briefly, PCR products: (i) 5PRIMEEND*ΔlacZ* and (ii) 3PRIMEEND*ΔlacZ* were amplified from genomic DNA (gDNA) extracted from WT Nlac Y92-1009 (GeneJET genomic DNA purification kit, Thermo Fisher Scientific), using primer pairs: (i) 5PRIMEEND*ΔlacZ*_FOR and 5PRIMEEND*ΔlacZ*_REV, and (ii) 3PRIMEEND*ΔlacZ*_FOR and 3PRIMEEND*ΔlacZ*_REV. A third PCR product: *ΔlacZ*VECTOR, was amplified from circular pUC19 using primer pair: *ΔlacZ*VECTOR_FOR and *ΔlacZ*VECTOR_REV. PCR fragments were mixed at a stoichiometry of 3:3:1 (5PRIMEEND*ΔlacZ*:3PRIMEEND*ΔlacZ*:*ΔlacZ*VECTOR) along with 1x Gibson Assembly master mix (total volume = 20 µl) and incubated at 50 ºC for 1 hour. Circularized plasmids in the assembly mixture were transformed into highly competent *E. coli* DH5α (NEB) according to the manufacturer's instructions. Transformed *E. coli* were selected for on LB agar supplemented with 100 µg ml^{-1} ampicillin (Sigma-Aldrich). Plasmid pJL006 was isolated from an overnight culture of transformed *E. coli* (GeneJET plasmid miniprep kit, Thermo Fisher Scientific) and was used as template in hypermethylated PCR using Phusion DNA polymerase in GC buffer to produce hypermethylated PCR product: *ΔlacZ* (hm*ΔlacZ*). Following addition of 1 x CutSmart

buffer (NEB) and restriction endonuclease DpnI (NEB) to hm*ΔlacZ* for 1 hour at 37 ºC, to digest the template plasmid, hm*ΔlacZ* was used to transform WT Nlac strain Y92-1009. Successful transformants were screened for on LB agar supplemented with X-gal (40 μg ml^{-1}) (Sigma-Aldrich) and were identifiable by growth as white-colored colonies. Untransformed, WT Nlac instead grow as otherwise-identical but blue-colored colonies due to the activity of β-galactosidase. Transformed, *ΔlacZ* GM-Nlac (JRL0001) was isolated and stored as frozen glycerol stocks at -80 ºC. Deletion of the *lacZ* gene was confirmed by amplification of the *lacZ* chromosomal locus using primer pair: *lacZ*LOCUS_FOR and *lacZ*LOCUS_REV.

Assessing the effect of the meningococcal *porA* upstream activation sequence on gene expression in GM-Nlac.

Plasmid pJL0007 contains a nucleic acid construct designed to target a copy of the endogenous, Nlac β-galactosidase gene, under transcriptional control of the Nlac *porB* promoter, to the intergenic chromosomal locus: Neisseria heterologous construct insertion site number 1 (NHCIS1). Circular pJL0007 was used as template for PCR amplification using primer pair: NHCIS1-*lacZ*VECTOR_FOR and NHCIS1-*lacZ*VECTOR_REV, yielding PCR fragment NHCIS1-*lacZ*VECTOR. Plasmid pJL0005 was used as template for PCR amplification of the endogenous Nlac *lst* gene promoter, using primer pair: *lst*promoter_FOR and *lst*promoter_REV, yielding PCR fragment *lst*promoter. PCR fragments *lst*promoter and NHCIS1-*lacZ*VECTOR were mixed at a stoichiometry of 3:1 along with 1x Gibson Assembly master mix (total volume = 20 μl) and incubated at 50 ºC for 1 hour. Circularized plasmids in the assembly mixture were transformed into highly competent *E. coli* DH5α (NEB) according to the manufacturer's

instructions. Transformed *E. coli* were selected for on LB agar supplemented with 100 μg ml^{-1} ampicillin (Sigma-Aldrich). The resultant plasmid, pJL0009, contains a nucleic acid construct designed to target a copy of the endogenous, Nlac β-galactosidase gene, under transcriptional control of the Nlac *lst* promoter, to NHCIS1. Genomic DNA extracted from WT Nmen strain MC58 (GeneJET genomic DNA purification kit, Thermo Fisher Scientific) was used as template for amplification of the 400 base pair nucleotide sequence immediately upstream of the *porA* promoter sequence, using primer pair: *porA*upstream_FOR and *porA*upstream_REV, to yield PCR fragment *porA*UAS. Plasmid pJL0009 was used as template for PCR amplification using primer pair: NHCIS1-lst400-*lacZ*_FOR and NHCIS1-lst400-*lacZ*_REV, yielding PCR fragment PCR-pJL0010. PCR fragments *porA*UAS and PCR-pJL0010 were mixed at a stoichiometry of 3:1 along with 1x Gibson Assembly master mix in a total volume of 20 μl and incubated at 50 ^{o}C for 1 hour. Circularized plasmids in the assembly mixture were transformed into highly competent *E. coli* DH5α (NEB) according to the manufacturer's instructions. Transformed *E. coli* were selected for on LB agar supplemented with 100 μg ml^{-1} ampicillin (Sigma-Aldrich). The resultant isolated plasmid, pJL0010, contains a nucleic acid construct designed to target a copy of the endogenous, Nlac β-galactosidase gene, under transcriptional control of the Nlac *lst* promoter, flanked immediately upstream (5') by 400 bp of the meningococcal *porA* UAS, to NHCIS1 (fig. S1). Plasmid pJL0010 was subsequently used as the template for multiple PCR amplifications, each one designed to shorten the length of the *porA* UAS to a defined number of base pairs. Primers: (i) NHCIS1-lst250-*lacZ*_FOR, (ii) NHCIS1-lst200-*lacZ*_FOR, (iii) NHCIS1-lst150-*lacZ*_FOR, (iv) NHCIS1-lst100-*lacZ*_FOR and (v) NHCIS1-lst50-*lacZ*_FOR, were each used in separate PCR reactions with a common reverse primer: NHCIS1-*lst*XX-*lacZ*_REV to produce the PCR fragments: (i) PCR-pJL0011, (ii) PCR-pJL0012, (iii) PCR-

pJL0013, (iv) PCR-pJL0014 and (v) PCR-pJL0015. Aliquots of DNA solutions containing PCR fragments PCR-pJL0011 to PCR-pJL0015 (1 µg total DNA) were supplemented with 1x CutSmart buffer (NEB) and restriction endonuclease SalI (NEB) in a total volume of 50 µl and incubated at 37 ºC for 1 hour. Following cleanup of the digested PCR (GeneJET PCR purification kit, Thermo Fisher Scientific), 1 µl of each SalI-digested PCR fragment was supplemented with 1x DNA ligase buffer (Thermo Fisher Scientific) and T4 DNA Ligase (Thermo Fisher Scientific). The SalI half-sites at either end of the digested PCR fragments ligated together, to yield circular plasmids, which were then transformed into highly competent *E. coli* DH5α (NEB) according to the manufacturer's instructions. Transformed *E. coli* were selected for on LB agar supplemented with 100 µg ml^{-1} ampicillin (Sigma-Aldrich). Plasmids (i) pJL0011, (ii) pJL0012, (iii) pJL0013, (iv) pJL0014 and (v) pJL0015 were isolated from overnight cultures of transformed *E. coli* (GeneJET plasmid miniprep kit, Thermo Fisher Scientific) and were used, along with plasmids (vi) pJL0009 and (vii) pJL0010, as templates in hypermethylated PCR using primer pair: 5PRIMEENDNHCIS1_FOR and 3PRIMEENDNHCIS1_REV and Phusion DNA polymerase in 'GC buffer' (NEB). These reactions yielded the hypermethylated PCR products: (i) hmNHCIS1-*lst*(250)-*lacZ*, (ii) hmNHCIS1-*lst*(200)-*lacZ,* (iii) hmNHCIS1-*lst*(150)-*lacZ*, (iv) hmNHCIS1-*lst*(100)-*lacZ*, (v) hmNHCIS1-*lst*(50)-*lacZ*, (vi) hmNHCIS1-*lst*-*lacZ* and (vii) hmNHCIS1-*lst*(400)-*lacZ*. Following addition of 1 x CutSmart buffer (NEB) and restriction endonuclease DpnI (NEB) to each hypermethylated PCR product for 1 hour at 37 ºC, to digest the template plasmid, each hypermethylated PCR product was used to transform strain JRL0001 (*ΔlacZ*). Successful transformants were screened for on LB agar supplemented with X-gal (40 µg ml^{-1}) (Sigma-Aldrich) and were identifiable by growth as blue-colored colonies. Transformed GM-Nlac

strains: (i) JRL1002, (ii) JRL1003, (iii) JRL1004, (iv) JRL1005, (v) JRL1006, (vi) JRL1007 and (vii) JRL1001 were isolated and stored as frozen glycerol stocks at -80 ^{o}C. Insertion of the gene expression constructs into the chromosome of each strain was confirmed by amplification of the NHCIS1 locus using primer pair: NHCIS1LOCUS_FOR and NHCIS1LOCUS_REV.

Assay of β-galactosidase specific activity.

GM-Nlac strains JRL1001 through JRL1007 were inoculated into TSB and grown to mid-log phase at an optical density (OD) $_{600nm}$ = 0.4. Bacteria were pelleted by centrifugation, washed once in PBS then resuspended in 0.5 ml bacterial lysis buffer (BLB, 0.05 M Tris HCl (pH 8.0) + 10 % (v/v) glycerol + 0.1 % Triton X-100), supplemented with protease inhibitor cocktail (Sigma-Aldrich). Bacterial suspensions were chilled on ice prior to lysis via sonication (3 x 15 sec bursts). Immediately following sonication, each sample was supplemented with phenylmethylsulfonyl fluoride (PMSF), to a final concentration of 1 mM. Sonicated samples were centrifuged at 17,000 *g* for 10 minutes at 4 ^{o}C and the supernatants collected. Triplicate assays of β-galactosidase (β-gal) activity in each supernatant were made using the β-galactosidase Assay Kit (Life Technologies), according to the manufacturer's instructions. The specific activity of β-gal was calculated by normalizing the nmol of o-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed over a given period (in minutes) by the protein concentration of each lysate (in mg). Protein concentration in each sample was measured using the DC Protein Assay reagent (BioRad), in comparison to a bovine serum albumin (BSA) standard curve.

Integrating the *nadA* coding sequence or a negative control sequence into NHCIS1.

Plasmid pJL0012 contains a nucleic acid construct designed to target a copy of the endogenous, Nlac β-galactosidase gene, under transcriptional control of an optimally-UAS-enhanced (200 bp), endogenous Nlac *lst* promoter, to the intergenic chromosomal locus: NHCIS1 (fig. S1). Circular pJL0012 was used as template for PCR amplification using primer pair: NHCIS1-lst200(X)-*lacZ*_FOR and NHCIS1-lst200(X)-*lacZ*_REV, yielding PCR fragment PCR-pJL0016. A gBLOCK gene fragment (Integrated DNA Technologies) was synthesized, coding for a synthetic, non-phase variable gene promoter followed by a non-coding linker sequence (5' – ATCTATTATATAAC – 3'), sandwiched between XhoI (5') and NotI (3') restriction sites (gBLOCKpJL0016). gBLOCKpJL0016 contained sequences at either end that overlapped with the ends of PCR-pJL0016, making it compatible with isothermal assembly for incorporation into a complete plasmid. The synthetic promoter was a hybrid of the meningococcal *porA* and *porB* promoters from Nmen strain MC58, insofar as it was largely the same sequence as the *porA* gene promoter, but in which the homopolymeric guanosine nucleotide tract that separates the -35 and -10 boxes of the RNA polymerase binding site in the WT *porA* promoter is replaced with the same 17 bp sequence present in the *porB* promoter. PCR-pJL0016 and gBLOCKpJL0016 were mixed at a stoichiometry of 1:5 along with 1x Gibson Assembly master mix (total volume = 20 µl) and incubated at 50 ^{o}C for 1 hour. Circularized plasmids in the assembly mixture were transformed into highly competent *E. coli* DH5α (NEB) according to the manufacturer's instructions. Transformed *E. coli* were selected for on LB agar supplemented with 100 µg ml^{-1} ampicillin (Sigma-Aldrich). Plasmid pJL016 was isolated from an overnight culture of transformed *E. coli* (GeneJET plasmid miniprep kit, Thermo Fisher Scientific). Plasmid pJL0016 was used as template for PCR amplification using primer pair: NHCIS1-*nadA*-*lacZ*FOR and NHCIS1-*nadA*-

*lacZ*REV, yielding PCR fragment PCR-pJL0017. A gBLOCK gene fragment (Integrated DNA Technologies) was synthesized, coding for the *nadA* coding sequence under transcriptional control of the optimally-UAS-enhanced, hybrid *porA*/*porB* promoter, flanked at either end by an XhoI (5') or NotI (3') restriction site, and with sequence overlapping the ends of PCR-pJL0017 (gBLOCKpJL0017). PCR-pJL0017 and gBLOCKpJL0017 were mixed at a stoichiometry of 1:5 along with 1x Gibson Assembly master mix (total volume = 20 µl) and incubated at 50 ^{o}C for 1 hour. Circularized plasmids in the assembly mixture were transformed into highly competent *E. coli* DH5α (NEB) according to the manufacturer's instructions. Transformed *E. coli* were selected for on LB agar supplemented with 100 µg ml^{-1} ampicillin (Sigma-Aldrich). Plasmid pJL017 was isolated from an overnight culture of transformed *E. coli* (GeneJET plasmid miniprep kit, Thermo Fisher Scientific). Plasmids (i) pJL0016 and (ii) pJL0017 were used as templates in hypermethylated PCR using primer pair: 5PRIMEENDNHCIS1_FOR and 3PRIMEENDNHCIS1_REV and Phusion DNA polymerase in 'GC buffer' (NEB). These reactions yielded the hypermethylated PCR products: (i) hmNHCIS1-(Y)-*lacZ* and (ii) hmNHCIS1-*nadA*-*lacZ*. Following addition of 1 x CutSmart buffer (NEB) and restriction endonuclease DpnI (NEB) to each hypermethylated PCR product for 1 hour at 37 ^{o}C, to digest the template plasmid, each hypermethylated PCR product was used to transform strain JRL0001 (*ΔlacZ*). Successful transformants were screened for on LB agar supplemented with X-gal (40 µg ml^{-1}) (Sigma-Aldrich) and were identifiable by growth as blue-colored colonies. Transformed GM-Nlac strains: (i) 4YB2 and (ii)4NB1 were isolated and stored as frozen glycerol stocks at -80 ^{o}C. Insertion of the gene expression constructs into the chromosome of each strain was confirmed by amplification of the NHCIS1 locus using primer pair: NHCIS1LOCUS_FOR and NHCIS1LOCUS_REV.

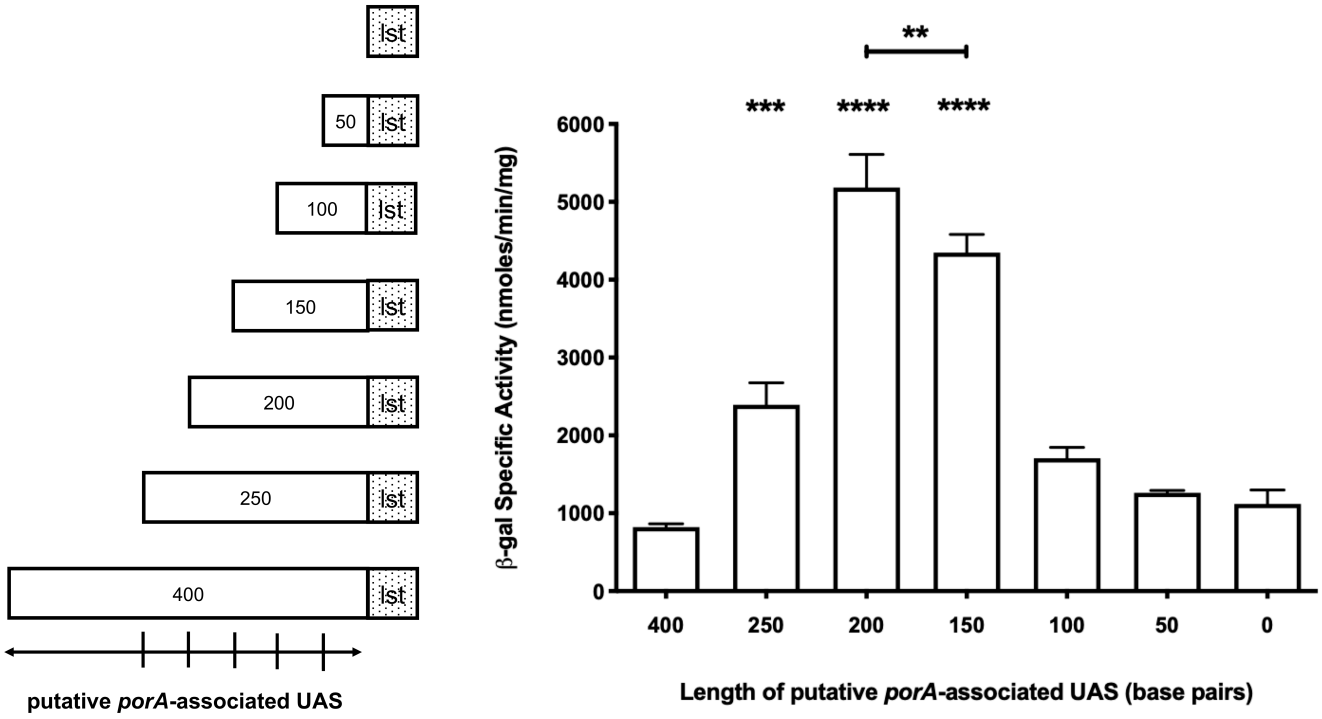


Fig. S1. The nucleotide sequence upstream of the meningococcal *porA* gene is an upstream activation sequence (UAS) that enhances gene expression. Specific Activity of β-galactosidase measured in lysates of GM-Nlac strains: JRL1001 through JRL1007 (RHS). Each strain contains a chromosomally-integrated construct in the NHCIS1 locus, wherein *lacZ* expression is driven by either the endogenous *lst* gene promoter alone (0 bp, strain JRL1001), or the endogenous *lst* promoter preceded (5') by increasing lengths of the putative *porA*-associated UAS. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ $^{****}p \leq 0.0001$ as calculated by a one-way ANOVA with Tukey's multiple comparisons test, differences from 0 base pairs are shown, except where indicated. Bars represent mean ± SD (n = 3 replicates per group).

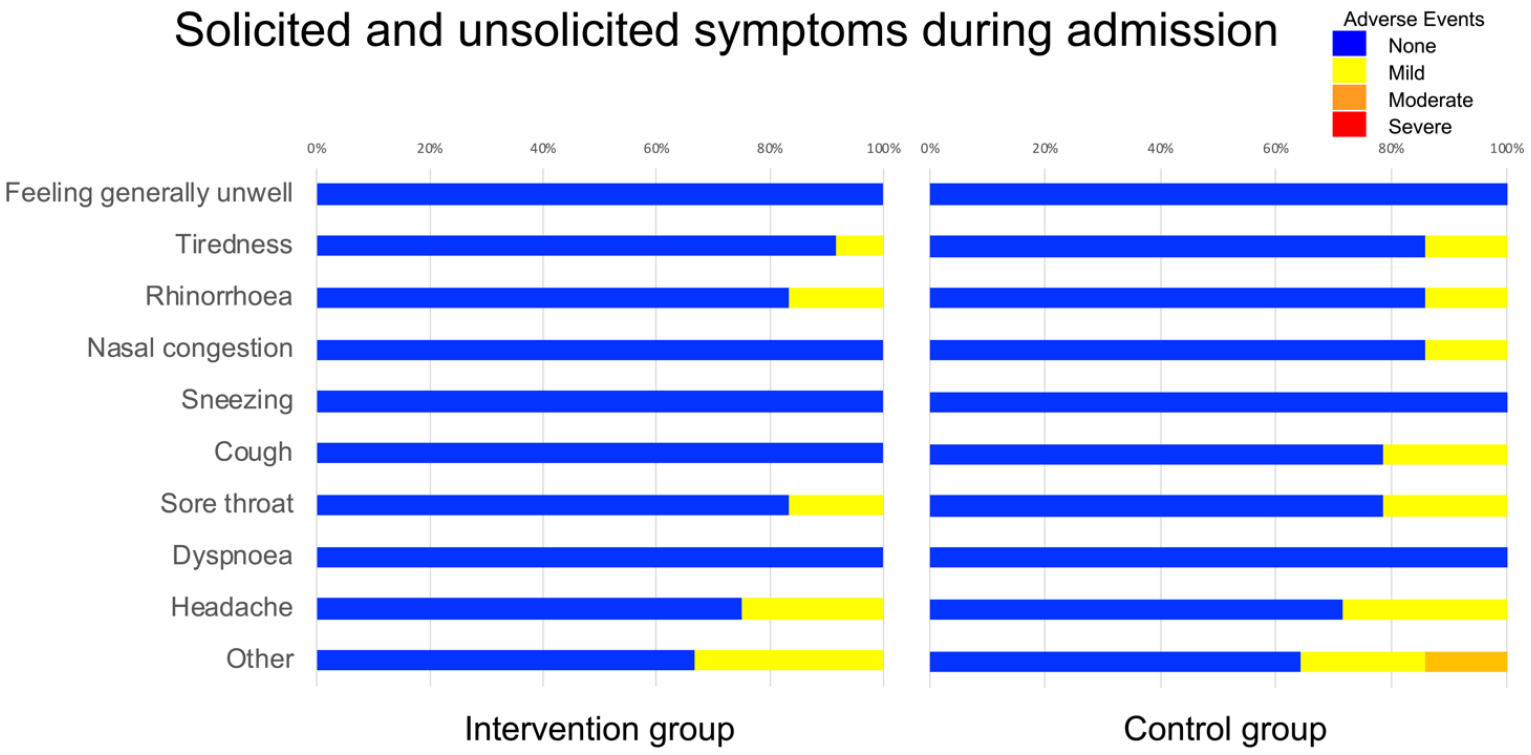


Fig. S2. Adverse events reported during the admission period of the controlled human infection model experiment. The percentage of challenge participants who reported solicited or any other symptoms during admission is shown, comparing the intervention and control groups. The indicated severity is the maximal severity of each symptom reported by individual participants at any point during the 4.5-day admission period. All symptoms self-resolved or resolved with simple analgesia.

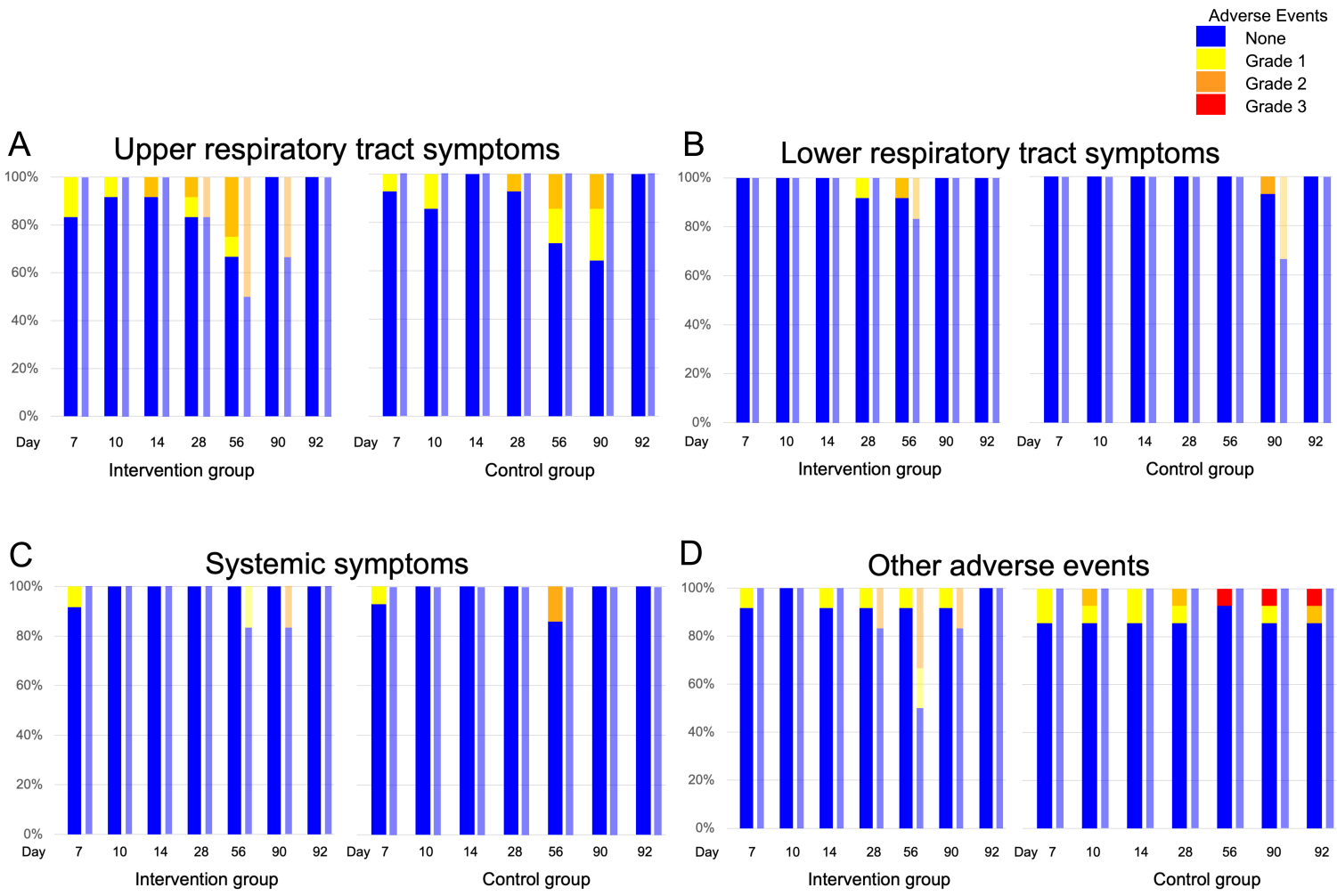


Fig. S3. Adverse events reported during the follow-up period of the controlled human infection model experiment. The percentage of participants in each group who, during the follow-up period of the study (post-residential to pre-clearance) reported **(A)** upper respiratory tract symptoms (including coryza, sore throat, ear pain and epistaxis), **(B)** lower respiratory tract symptoms (including cough and exacerbation of asthma), **(C)** systemic symptoms (malaise, feeling generally unwell, aching, lymphadenopathy) or **(D)** other adverse events. The indicated severity is the maximal severity of each symptom reported by individual participants at any point during the follow-up period. Challenge volunteers are shown in the thicker columns and contact volunteers in the thinner columns for each visit day. Symptoms continuing over more than one visit are shown in both.

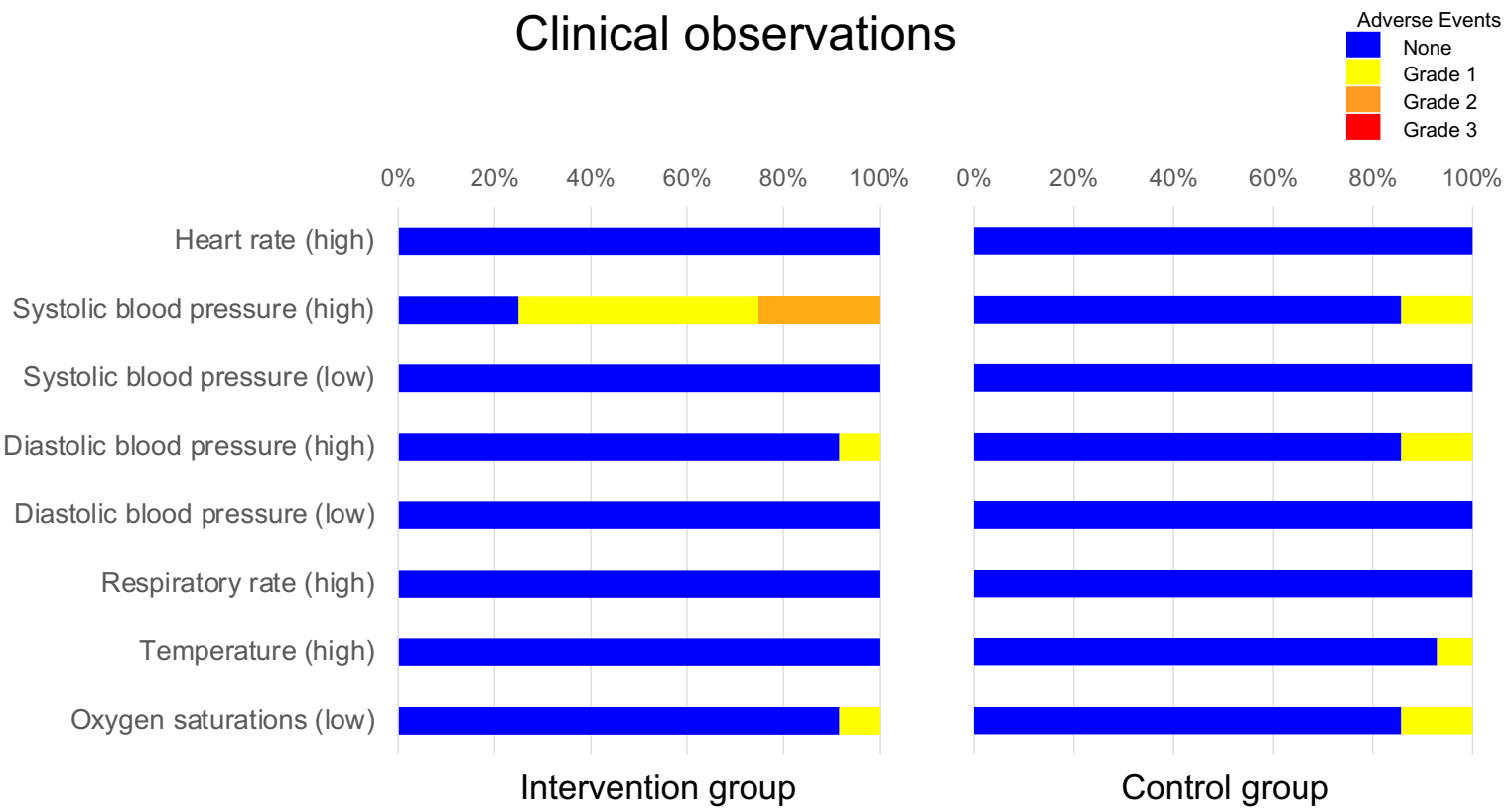
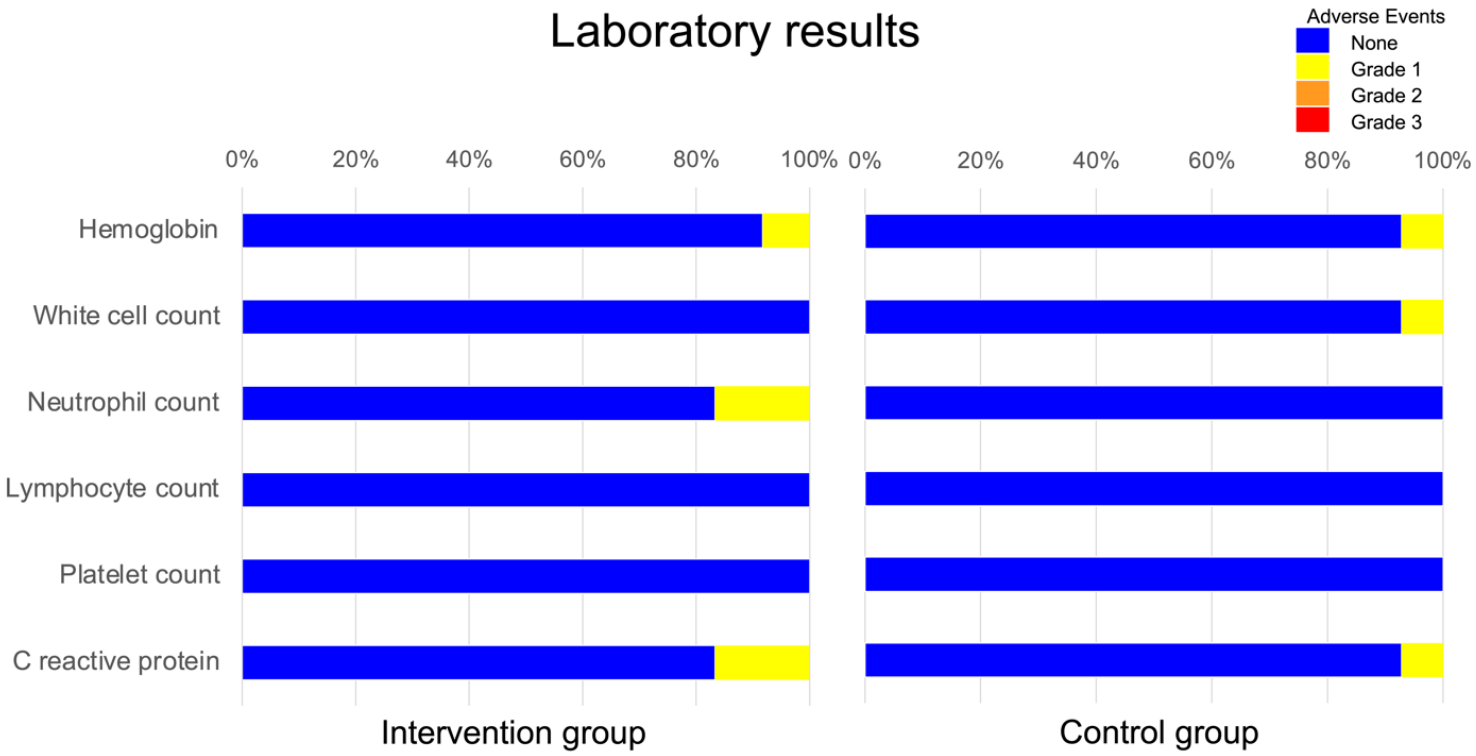


Fig. S4. Clinically observed adverse events during the admission and follow-up periods of the controlled human infection model experiment. The percentage of challenge participants in each group with abnormal clinical observations during the admission period or at follow up visits, graded according to pre-determined adverse event ranges. The maximal severity of each observation recorded for individual participants is shown, with comparison between the intervention and control groups. Three individuals in the intervention group had Grade 2 systolic hypertension during the admission period, two of these reported substantial stress unrelated to the study at that time. The participants with Grade 1 pyrexia and low saturations were all otherwise clinically well at the time, and all self-resolved.

A



B

Parameter	Units	None	Grade 1	Grade 2	Grade 3
Hemoglobin (M)	g/L	126 - 170	115 - 125	100-114	< 100
Hemoglobin (F)	g/L	114 - 150	105 - 113	90-104	< 90
White cell count	x 10^9/L	3.5 - 11.5	11.6 – 15 or 2.5 – 3.4	15.1-20 or 1.5 – 2.49	> 20 or < 1.5
Neutrophils	x 10^9/L	1.5 - 7.5	1.0 - 1.49	0.50 - 0.99	<0.5
Lymphocytes	x 10^9/L	1.0 - 4.0	0.75 - 0.99	0.50 - 0.74	<0.5
Platelets	x 10^9/L	136 - 450	125 - 135	100 - 124	<100
C reactive protein	mg/L	0 - 9	10 - 19	20 - 50	>50

Fig. S5. Laboratory parameter adverse events during the admission and follow-up periods of the controlled human infection model experiment. (**A**) The percentage of challenge participants in each group with abnormal laboratory results during the admission period or at follow-up visits, graded according to pre-determined adverse event ranges. (**B**) The maximal severity of each laboratory parameter is shown, with comparison between the intervention and control groups. Two participants in the intervention group had a rise in C-reactive protein (CRP) on Day 14. One of these participants was clinically well but had run a marathon on both Day 12 and 13. The other had concurrent upper respiratory tract symptoms with Grade 1 neutropenia, which resolved by the next follow up visit. One participant in the intervention group had a Grade 1 neutropenia on Day 7 associated with mild upper respiratory tract symptoms. All other laboratory parameter adverse events were in clinically well participants. One participant in the control group had a CRP within the Grade 1 range throughout the study including prior to inoculation. This was related to an underlying condition rather than the study. Other than CRP in this participant, all laboratory parameters spontaneously returned to the normal range.

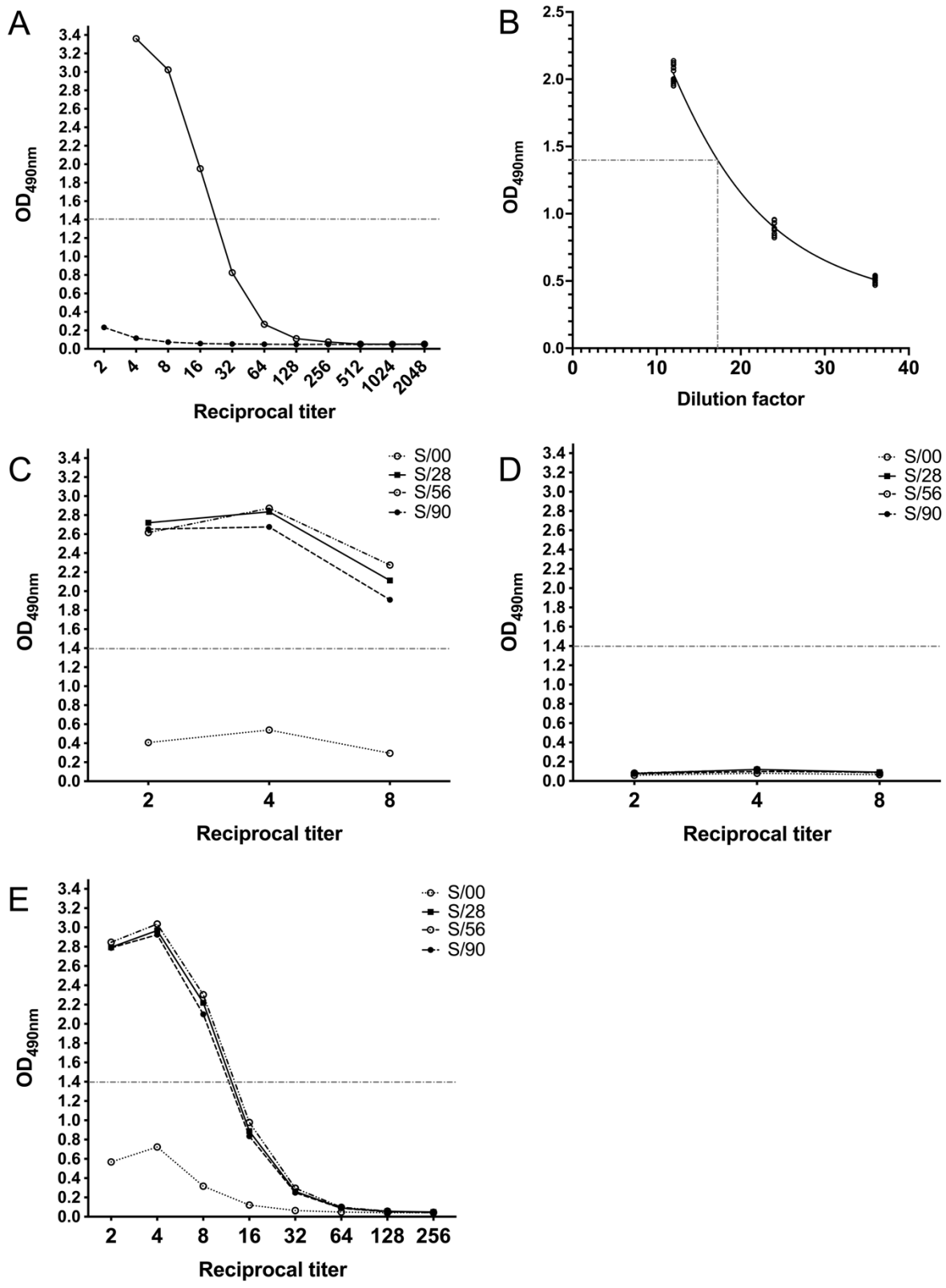

A
B
C
D
E
OD490nm
Reciprocal titer
Dilution factor
S/00
S/28
S/56
S/90

Fig. S6. Determination of endpoint titer for anti-sNadA IgG in human serum samples. (**A**) Serum samples NA8746 (●) and NA9136 (○) were extracted from whole blood taken from the same human male before and 28 days after vaccination with Bexsero, respectively. Sera were serially diluted two-fold in PBS across the range shown. Duplicate aliquots of each serum dilution were incubated in sNadA-coated wells of an ELISA plate, and captured anti-sNadA IgG was detected as an increase in OD_{490nm} using biotinylated, anti-human IgG monoclonal antibody, streptavidin-HRP and OPD substrate. Points represent mean of duplicate measurements. An OD_{490nm} = 1.4 (indicated) represents a positive signal in this ELISA and sits on the linear portion of this representative reference curve. (**B**) Serum NA9136 was diluted 1:12, 1:24 and 1:36 (n = 10) and the anti-sNadA IgG titer of each serial dilution was measured in duplicate using the anti-sNadA ELISA. Each point represents the mean of duplicate measurements. Data were fitted to a four-parameter logistic (4PL) nonlinear regression. The reciprocal titer of serum NA9136 corresponding to a measurement of OD_{490nm} = 1.4 was interpolated from the reference curve (= 17.245). (C and D) Serum from Participant 15 (**C**) and Participant 5 (**D**) was isolated from whole blood taken prior to inoculation with GM-Nlac (S/00) and at 28, 56 and 90 days post-inoculation. Sera were diluted two-fold in PBS across the range of concentrations shown. Duplicate aliquots of each serum dilution were incubated in sNadA-coated wells of an ELISA plate, and captured anti-sNadA IgG was detected. (**E**) Where a signal of $OD_{490nm} \geq 1.4$ was detected, all serum samples from that participant were diluted two-fold in PBS across the broader range of dilutions shown**.** Duplicate aliquots of each serum dilution were incubated in sNadA-coated wells of an ELISA plate, and captured anti-sNadA IgG was detected . The reciprocal anti-sNadA IgG endpoint titer of each serum sample was determined as the reciprocal of the last dilution tested that generated an $OD_{490nm} \geq 1.4$. Reciprocal anti-sNadA IgG titers of participant

15's serum samples are therefore: (i) S/00 = < 2 (undetectable), (ii) S/28 = 8, (iii) S/56 = 8 and (iv) S/90 = 8.

Participant ♯	Control (4YB2)				
	0	28	56	90	Max fold change
1	16	16	16	16	**1**
2	<2	<2	<2	<2	**1**
3	<2	<2	<2	<2	**1**
4	8	8	8	8	**1**
5	<2	<2	<2	<2	**1**
6	<2	<2	<2	<2	**1**
7	<2	<2	<2	<2	**1**
8	<2	<2	-	<2	**1**
9	<2	<2	<2	<2	**1**
10	<2	<2	<2	<2	**1**
11	<2	<2	<2	<2	**1**

Participant ♯	Intervention (4NB1)				
	0	28	56	90	Max fold change
15	<2	8	8	8	**8**
16	<2	<2	<2	<2	**1**
17	<2	<2	4	4	**4**
18	<2	<2	<2	<2	**1**
19	<2	8	8	8	**8**
20	<2	<2	<2	<2	**1**
21	4	8	4	4	**2**
22	<2	<2	<2	<2	**1**
23	<2	4	<2	<2	**4**
24	<2	<2	<2	<2	**1**

Fig. S7. Colonization with NadA-expressing GM-Nlac elicits seroconversion against NadA. Sera were assayed for anti-sNadA IgG using an endpoint ELISA. The reciprocal endpoint titer of each serum was considered to be the reciprocal titer of the least dilute serum sample tested that generated an optical density $(OD)_{490nm} \geq 1.4$. A reciprocal endpoint titer of anti-sNadA IgG < 2 was considered to have a value of 1 for the purposes of calculating fold change, which was always in comparison to sera from Day 0. Participants in whose serum there was a detectable reciprocal endpoint titer of anti-sNadA IgG (≥ 2-fold increase) at one or more time points are filled in gray. Serum samples that were not analyzed because the participant did not attend are denoted as -.

Participant ♯	Control (4YB2)			
	0	28	56	90
1	10.7	14.8	15.2	21.9
2	<2	<2	<2	<2
3	5.1	7.4	8.2	7.4
4	**<2**	**<2**	**<2**	**9.7**
5	<2	2.7	2.5	2.6
6	2.8	6.8	5.1	2.6
7	2.6	<2	<2	<2
8	3.5	10.6	-	5.8
9	<2	<2	2.5	<2
10	<2	<2	3.4	<2
11	3.7	7.3	4.7	5

Participant ♯	Intervention (4NB1)			
	0	28	56	90
15	10.1	24.2	29.2	25.9
16	<2	<2	2.9	<2
17	9.2	10.2	11.7	9.3
18	10.1	8.9	11.6	10.4
19	<2	6.5	13.8	15.6
20	<2	<2	4.7	<2
21	<2	<2	6.5	7
22	3.2	5.3	6.9	7.2
23	<2	<2	<2	<2
24	2.6	8.4	32	31.1

Fig. S8. Serum bactericidal antibody titers in response to colonization by GM-Nlac. Sera were evaluated for serum bactericidal antibody activity versus Nmen strain 5/99 using human complement. Interpolated reciprocal SBA titers, representing the interpolated dilution factor of each serum sample at which meningococcal cells were killed by 50% as compared to controls, for each participant at each time point are shown. Participants whose serum contained a non-protective SBA titer (< 4) at Day 0 but in whom there was a protective SBA titer (≥ 4) at Day 90 are filled in gray. SBA titers in serum derived from participants that acquired meningococcal carriage are shown in bold. In concomitant assays, reference serum NA9136 was calculated to have a mean reciprocal SBA titer of 82.09 ± 29.14 against Nmen strain 5/99 (n = 46). Serum samples that were not analyzed because the participant did not attend are denoted as -.

Participant #	Day 00			Day 28		
	α	β	[IgA] (µg/ml)	α	β	[IgA] (µg/ml)
1			–			318.8
2		✓	107.7			135.4
3		✓	167.7		✓	165.0
4		✓	–		✓	256.2
5	✓		246.8			234.3
6	✓	✓	559.6		✓	178.2
7		✓	193.7	✓	✓	74.2
8			324.8			130.6
9		✓	263.4	✓		228.8
10			245.2			240.5
11			183.7			206.9
12			343.5			138.5
13	✓		267.4	✓	✓	124.4
14			206.2			166.9
15	✓	✓	517.4	✓	✓	–
16			170.4			151.3
17			90.3		✓	262.6
18			98.6			241.6
19	✓	✓	368.7	✓	✓	143.9
20			201.3			193.5
21		✓	123.7			165.5
22	✓		209.2	✓		151.8
23			–			–
24	✓	✓	234.9	✓	✓	174.2
25			373.7			N/A
26			307.6			N/A

Fig. S9. Results of salivary IgA ELISAs. Saliva samples isolated at Day 0 and Day 28 from all participants were diluted 3x and assayed for anti-sNadA IgA in single wells coated with either coating buffer alone, or coating buffer plus sNadA. Wells in which the resulting OD_{490nm} equaled or exceeded the threshold value of Mean + 3SD of appropriately-coated wells (sNadA-coated wells (α): 0.07; uncoated wells (β): 0.068) are denoted with a tick symbol. Saliva samples considered to be positive for anti-sNadA IgA (containing a reciprocal titer of anti-

sNadA IgA > 3 in the absence of signal in uncoated wells) are shown in gray. The total IgA concentration (in μg ml^{-1}) of each saliva sample was interpolated by reference to a standard curve generated using human IgA isolated from colostrum. Saliva samples wherein there was insufficient remaining volume to perform the total IgA measurement are denoted as -.

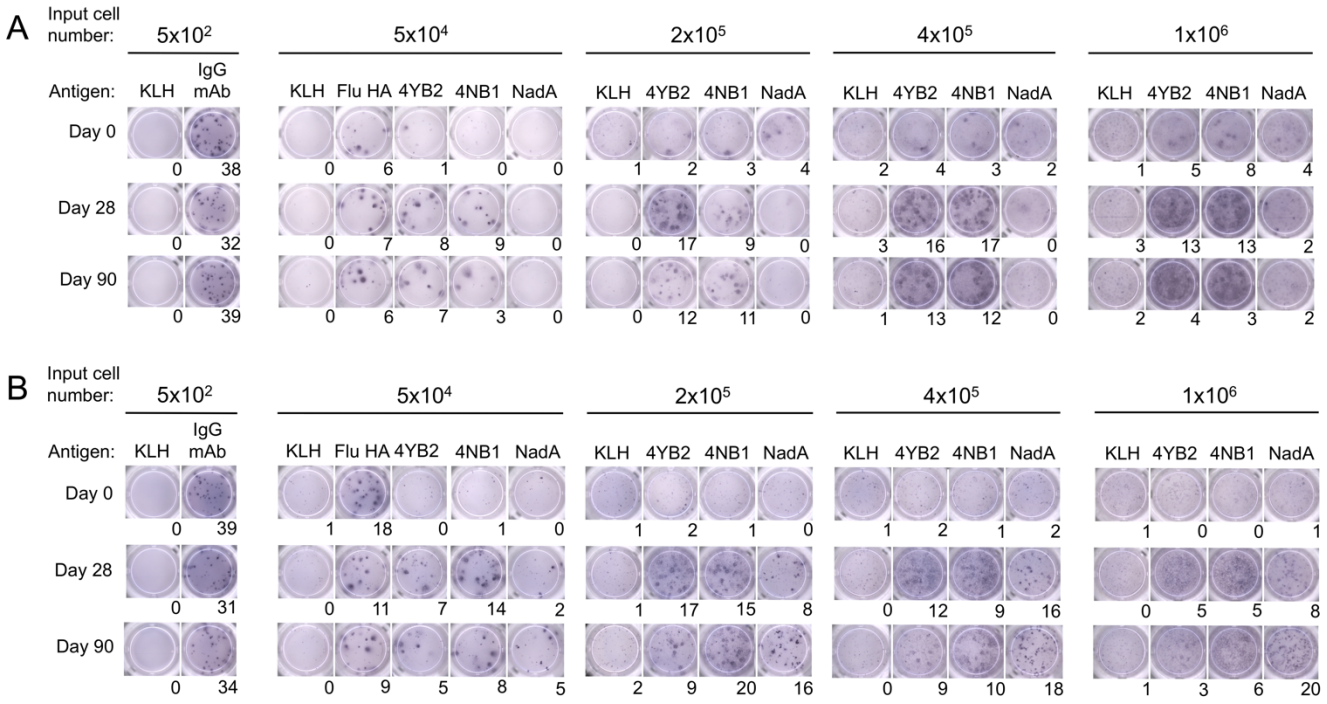


Fig. S10. Representative outputs from IgG-secreting memory B cell ELISpot assay on participants colonized with GM-Nlac. (A and B) PBMC isolated at Day 0, Day 28 and Day 90 from Participants 3 (**A**) and 19 (**B**) were polyclonally stimulated for 5-days, allowing memory B cells to differentiate into antibody-secreting cells. Cells were then added at the concentrations shown to duplicate wells containing membranes coated with the following antigens: keyhole limpet hemocyanin (KLH), rat anti-human IgG monoclonal antibody (mAb) clone M1310G05 (IgG mAb), influenza hemagluttinin (FluHA), deoxycholate-extracted outer membrane vesicle preparation (dOMV) derived from strain 4YB2 (4YB2), dOMV derived from strain 4NB1 (4NB1) or the soluble domain of NadA. One representative well for each condition is shown. Antigen-specific antibody-secreting cells (or IgG-secreting cells captured by the anti-IgG mAb) were lysed in hypotonic buffer and human IgG was detected. The number of spot-forming units (SFU) per membrane, as counted by the AID ELISpot reader, is shown underneath each image.

Table S1: Bacterial strains used in this study.

Species	Strain	Designation	Genotype	Derived:	Reference
Neisseria lactamica	Y92-1009	ND: P1.ND,ND: F4-8: ST-3493	WT		[20]
	ΔnlaIII		Y92-1009 *ΔnlaIII:aphA3*	Y92-1009	This work
	JRL0001		Y92-1009 *ΔlacZ*	Y92-1009	This work
	JRL1001		Y92-1009 *ΔlacZ* NHCIS1::[*lst*400]*lacZ*	JRL0001	This work
	JRL1002		Y92-1009 *ΔlacZ* NHCIS1::[*lst*250]*lacZ*	JRL0001	This work
	JRL1003		Y92-1009 *ΔlacZ* NHCIS1::[*lst*200]*lacZ*	JRL0001	This work
	JRL1004		Y92-1009 *ΔlacZ* NHCIS1::[*lst*150]*lacZ*	JRL0001	This work
	JRL1005		Y92-1009 *ΔlacZ* NHCIS1::[*lst*100]*lacZ*	JRL0001	This work
	JRL1006		Y92-1009 *ΔlacZ* NHCIS1::[*lst*50]*lacZ*	JRL0001	This work
	JRL1007		Y92-1009 *ΔlacZ* NHCIS1::[*lst*]*lacZ*	JRL0001	This work
	4YB2	GMO (Control)	Y92-1009 *ΔlacZ* NHCIS1::(X)-*lacZ*	JRL0001	This work
	4NB1	GMO (Intervention)	Y92-1009 *ΔlacZ* NHCIS1::*nadA-lacZ*	JRL0001	This work
Neisseria meningitidis	MC58	B: P1.7,16-2: F1-5: ST-74	WT		[51]
	ΔsiaD		MC58 *ΔsiaD:aphA3*	MC58	This work
	ΔnadA		MC58 *ΔnadA:aphA3*	MC58	This work
	N54.1	P1.21,16: F3-7: ST-8510	WT		[17]

Table S2: Primers used in this study.

Name	Sequence (5' □ 3')	Product	Template
*ΔnlaIII*VECTOR_FOR	GGGCAAATTGGTCGATCTAGAGACCTGCAGGCATGCAAGCTTG	*ΔnlaIII*VECTOR	pUC19
*ΔnlaIII*VECTOR_REV	CATTCTGCTTGTAAGGCGACTCTAGAGGATCCCCGGG		
*nlaIII*LOCUS_FOR	CGGGTGATTAGCTCAGTTGG	*nlaIII*LOCUS	gDNA (Y92-1009)
*nlaIII*LOCUS_REV	GGTTTTCATTGTGCCGATAACGG		
*ΔnlaIII:aphA3*INSERT_FOR	GAATGGAGTTTAAAGGAAATCATATGGCCAAAATGCGCATTAGTCCG	*ΔnlaIII:aphA3*INSERT	pJL0001
*ΔnlaIII:aphA3*INSERT_REV	CAATTTCAGACGGCATAGATCTAGAATAATTCATCCAACAGGATATAATATTTG		
*ΔnlaIII:aphA3*VECTOR_FOR	GATGAATTATTCTAGATCTATGCCGTCTGAAATTGCGGCCGCGCGGCCAAATATACATCGGAC	*ΔnlaIII:aphA3*VECTOR	pJL0001
*ΔnlaIII:aphA3*VECTOR_REV	GCGCATTTTGGCCATATGATTTCCTTTAAACTCCATTC		
5PRIMEEND*ΔlacZ*_FOR	CAGACAGCATATCGGGCGATG	5PRIMEEND*ΔlacZ*	gDNA (Y92-1009)
5PRIMEEND*ΔlacZ*_REV	CAATTTCAGACGGCATAGATCCTCCTAATTTGAAACATCGCTC		
3PRIMEEND*ΔlacZ*_FOR	ATCTATGCCGTCTGAAATTGGGGATATATGCTAACCGCAG	3PRIMEEND*ΔlacZ*	gDNA (Y92-1009)
3PRIMEEND*ΔlacZ*_REV	GTCTCTAGACATACATCCGCTCATCGC		
*ΔlacZ*VECTOR_FOR	GAGCGGATGTATGTCTAGAGACCTGCAGGCATGCAAG	*ΔlacZ*VECTOR	pUC19
*ΔlacZ*VECTOR_REV	CGCCCGATATGCTGTCTGGACTCTAGAGGATCCCCGG		
*lacZ*LOCUS_FOR	GGGTACAGTCAATCGGTTTCTTTG	*lacZ*LOCUS	gDNA (Y92-1009)
*lacZ*LOCUS_REV	GAAAGGGGGCGTGTGTTC		
5PRIMEENDNHCIS1_FOR	CTCTAGAGTCCTGATACCGAGCTTTTCCCATG	5PRIMEENDNHCIS1	gDNA (Y92-1009)
5PRIMEENDNHCIS1_REV	AAAACAAACTTGTCGACTTCAGACGGCGTTGCACAGTTTTACTCCATG		
3PRIMEENDNHCIS1_FOR	GTTTTTAGATGCCGTCTGAATGCTGAAGTAGAAAACCAGC	3PRIMEENDNHCIS1	gDNA (Y92-1009)
3PRIMEENDNHCIS1_REV	GTCTCTAGACTGAAAGAAGCTATCACCTTCATAAATAAG		
NHCIS1VECTOR_FOR	GCTTCTTTCAGTCTAGAGACCTGCAGGCATGCAAG	pUC19NHCIS1	pUC19
NHCIS1VECTOR_REV	GGGAAAAGCTCGGTATCAGGACTCTAGAGGATCCCCGG		
*porB*promoter_FOR	CAACGCCGTCTGAAGTCGACAGTTTGTTTTTTCGGGCGGG	*porB* promoter	gDNA (Y92-1009)
*porB*promoter_REV	ATTCGCTAATAACATATGATTCCTTTTTTGGTTAAGAAATTTAAGCG		
Nlac*lacZ*_FOR	AACCAAAAAAGGAATCATATGTTATTAGCGAATTATTATCAAGATCC	*lacZ*	gDNA (Y92-1009)
Nlac*lacZ*_REV	GATACCAATCTTTGCAGAAAGCTTATAACCGGATACTTATATCGAAATTG		
*porB*terminator_FOR	GTATCCGGTTATAAGCTTTCTGCAAAGATTGGTATC	*porB* terminator	gDNA

*porB*terminator_REV	CTACTTCAGCATTCAGACGGCATCTAAAAACAG		(Y92-1009)
NHCIS1LOCUS_FOR	CAAAGGTAATCAGGTAACGGCTCAT	NHCIS1LOCUS	gDNA (Y92-1009)
NHCIS1LOCUS_REV	CAACAGGGTAAATTCCGGAGGTC		
*lst*promoter_FOR	GCAACGCCGTCTGAAGTCGACTCGGCAACTGTCGGAATATCTG	*lst* promoter	pJL0005
*lst*promoter_REV	ATTCGCTAATAACATATGTGTATTCCTTTAAACTCC		
NHCIS1-*lacZ*VECTOR_FOR	GGAGTTTAAAGGAATACACATATGTTATTAGCGAATTATTATC	NHCIS1-*lacZ*VECTOR	pJL0007
NHCIS1-*lacZ*VECTOR_REV	CAGTTGCCGAGTCGACTTCAGACGGCGTTGCACAGTTTTACTCCATG		
NHCIS1-lst400-*lacZ*_FOR	GATATTTGTTCTGAAAATCGGCAACTGTCGGAATATCTGC	PCR-pJL0010	pJL0009
NHCIS1-lst400-*lacZ*_REV	GAAATCAAGCCGAATGTCGACTTCAGACGGCGTTG		
porAupstream_FOR	CAACGCCGTCTGAAGTCGACATTCGGCTTGATTTCGATACACCC	*porA* UAS	gDNA (MC58)
porAupstream_REV	GATATTCCGACAGTTGCCGATTTTCAGAACAAATATCTGATAAATGCCGCAAC		
NHCIS1-lst250-*lacZ*_FOR	CATCGTACGTCGACGAGCTAAGGCGAGGCAACGCC	PCR-pJL0011	pJL0010
NHCIS1-lst200-*lacZ*_FOR	CATCGTACGTCGACGTGCCGCGTGTGTTTTTTTATGGCG	PCR-pJL0012	pJL0010
NHCIS1-lst150-*lacZ*_FOR	CATCGTACGTCGACGGCAGCAGCGCATCGGC	PCR-pJL0013	pJL0010
NHCIS1-lst100-*lacZ*_FOR	CATCGTACGTCGACAAACACAACGTTTTTGAAAAAATAAGCTATTG	PCR-pJL0014	pJL0010
NHCIS1-lst50-*lacZ*_FOR	CATCGTACGTCGACTCATTTTTAAAATAAAGGTTGCGGCATTTATC	PCR-pJL0015	pJL0010
NHCIS1-lst200(X)-*lacZ*FOR	ATCTATTATATAACGCGGCCGCATATTCGGCAACTGTCGGAATATCTG	PCR-pJL0016	pJL0012
NHCIS1-lst200(X)-*lacZ*REV	CGCCCGAAAAACCATTTTTCAGAACAAATATCTGATAAATG		
NHCIS1-*nadA*-*lacZ*FOR	AACTACGAATGGTAAGCGGCCGCATATTCGGCAACTG	PCR-pJL0017	pJL0016
NHCIS1-*nadA*-*lacZ*REV	GTGTTTCATGCTCATCTCGAGTTCCTTTTGTAAATTTG		
5PRIMEEND*ΔnadA*_FOR	GTGCCACCTTCTAGACCGACAAAAAGGCCGTCTGAAC	5PRIMEEND*ΔnadA*	gDNA (MC58)
5PRIMEEND*ΔnadA*_REV	GCCGAATATGACGTCGGCGTTGGTGGTTTCATCC		
3PRIMEEND*ΔnadA*_FOR	GAATTATTCTAGGCGGCCGCGGGAGAAAATATAACGACATTTGC	3PRIMEEND*ΔnadA*	gDNA (MC58)
3PRIMEEND*ΔnadA*_REV	GCCTTTTGCTCTAGACGGCCGGATAGAAAATAAAAAC		
*ΔnadAaphA3*_FOR	GAAACCACCAACGCCGACGTCATATTCGGCAACTGTCG	*ΔnadAaphA3*	pJL0005
*ΔnadAaphA3*_REV	GTTATATTTTCTCCCGCGGCCGCCTAGAATAATTC		
MC58*ΔnadA*VECTOR_FOR	TTTTCTATCCGGCCGTCTAGAGCAAAAGGCCAGC	*ΔnadA:aphA3*VECTOR	pJL0005
MC58*ΔnadA*VECTOR_REV	CGGCCTTTTTGTCGGTCTAGAAGGTGGCACTTTTC		
5PRIMEEND*ΔsiaD*_FOR	GAAAAGTGCCACCTTCTAGACAGAGGATTGGCTATTACATATAG	5PRIMEEND*ΔsiaD*	gDNA (MC58)
5PRIMEEND*ΔsiaD*_REV	CAGTTGCCGAATATGACGTCAAGTATATTAGGGGCTCAATTAG		

3PRIMEEND*ΔsiaD*_FOR	GAATTATTCTAGGCGGCCGCAACAAATCCTAAAGGAATTATAGGC	3PRIMEEND*ΔsiaD*	gDNA (MC58)
3PRIMEEND*ΔsiaD*_REV	GCTGGCCTTTTGCTCTAGAATATAAAGCGCGTAAGGCTATAG		
*ΔsiaDaphA3*_FOR	GAGCCCCTAATATACTTGACGTCATATTCGGCAACTGTCGGAATATC	*ΔsiaDaphA3*	pJL0005
*ΔsiaDaphA3*_REV	TAATTCCTTTAGGATTTGTTGCGGCCGCCTAGAATAATTC		
trunc.pUC19*ΔsiaD*_FOR	TAGCCTTACGCGCTTTATATTCTAGAGCAAAAGGCCAGCAAAAG	*ΔsiaD:aphA3*VECTOR	pJL0005
trunc.pUC19*ΔsiaD*_REV	ATGTAATAGCCAATCCTCTGTCTAGAAGGTGGCACTTTTCGG		
Band1FOR	NNNNCCTACGGGNGGCWGCAG	Band 1	gDNA (GM-Nlac)
Band1REV	GACTACHVGGGTATCTAATCC		
Band3dFOR	CACTCGGGGCGTATGTTCAATTTG	Band 3	gDNA (GM-Nlac)
Band3dREV	GCACATTGATTTGTTTCGTAAAAGCGATTTC		

Table S3: Plasmids used in this study.

Plasmid	Construct	Derivative of:	Reference
pUC19	pMB1 amp^R		[52]
pJL0001	*ΔnlaIII*::CLOVER(2)-*aphA3*	pUC19	This work
pJL0005	*ΔnlaIII*::*aphA3*	pJL0001	This work
pJL0006	*ΔlacZ:*DUS	pUC19	This work
pJL0007	NHCIS1::[porB]*lacZ*	pUC19	This work
pJL0009	NHCIS1::[lst]*lacZ*	pJL0007	This work
pJL0010	NHCIS1::[lst400]*lacZ*	pJL0009	This work
pJL0011	NHCIS1::[lst250]*lacZ*	pJL0010	This work
pJL0012	NHCIS1::[lst200]*lacZ*	pJL0010	This work
pJL0013	NHCIS1::[lst150]*lacZ*	pJL0010	This work
pJL0014	NHCIS1::[lst100]*lacZ*	pJL0010	This work
pJL0015	NHCIS1::[lst50]*lacZ*	pJL0010	This work
pJL0016	NHCIS1::[porA/porB200](X)-[lst]*lacZ*	pJL0012	This work
pJL0017	NHCIS1::[porA/porB200]*nadA*-[lst]*lacZ*	pJL0016	This work
pJL0018	*ΔsiaD::aphA3*	pSC101	This work
pJL0019	*ΔnadA::aphA3*	pSC101	This work