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Inactivation of *NMB0419* encoding a Sel1-like repeat (SLR) protein in *Neisseria meningitidis* is associated with differential expression of genes belonging to the Fur regulon and reduced intra-epithelial replication

Running title:

Meningococcal *NMB0419* and the Fur regulon

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ABSTRACT

Neisseria meningitidis is a commensal microbe that colonizes the human nasopharynx but occasionally invades the bloodstream to cause life-threatening infection. *N. meningitidis* MC58 *NMB0419* encodes a Sel1-like repeat (SLR)-containing protein, previously implicated in invasion of epithelial cells. A gene-regulatory function was revealed in *Escherichia coli* expressing plasmid-borne *NMB0419* showing significantly increased epithelial adherence compared to wild type, due to increased expression of mannose-sensitive type 1 pili. While a meningococcal *NMB0419* mutant did not have altered epithelial adherence, in a transcriptome-wide comparison of wild type and an *NMB0419* mutant, a large proportion of genes differentially regulated in the mutant were involved in iron acquisition and metabolism. Fifty one and 38% of genes respectively up- and down-regulated in the *NMB0419* mutant had previously been identified as being induced and repressed by meningococcal Fur. An in vitro growth defect of the *NMB0419* mutant under iron-restriction was consistent with the down-regulation of *tbpAB* and *hmbR*, while an intra-epithelial replication defect was consistent with the down-regulation of *tonB*, *exbB* and *exbD*, based on a known phenotype of a meningococcal *tonB* mutant. Disruption of the N-terminal *NMB0419* signal peptide, predicted to export the protein beyond the cytoplasmic membrane, resulted in loss of functional traits in *N. meningitidis* and *E. coli*. Our study indicates that the expression of *NMB0419* is associated with transcriptional changes counterbalancing the regulatory function of Fur, offering a new perspective on regulatory mechanisms involved in meningococcal interaction with

epithelial cells, and suggests new insights into the roles of SLR-containing genes in other bacteria.

INTRODUCTION

Neisseria meningitidis is a human commensal bacterium that colonizes the nasopharyngeal mucosa of around 10% of healthy individuals, from where it occasionally invades the bloodstream to cause life-threatening infections such as septicaemia and meningitis (1). As well as colonizing the mucosal surface superficially, meningococci survive and replicate within epithelial cells, establishing a basis for invasion of deeper tissues to cause disease (2) and prolonged persistence on the nasopharyngeal mucosa as microbes exocytose to the surface again (3).

An important factor in meningococcal survival and replication in the human host is the ability of the microbe to assimilate iron in the face of avid sequestration by the host of all available iron in iron-binding proteins such as transferrin, lactoferrin and haemoglobin (4). In blood or other extracellular environments iron acquisition by the meningococcus is achieved through surface expression of receptors, such as TbpAB (transferrin binding proteins), LbpAB (lactoferrin binding proteins) and HmbR (haemoglobin binding protein) which interact with and mediate extraction of iron from their cognate host iron binding proteins. Within epithelial cells iron is mostly stored in ferritin, accessed by intracellular meningococci through ferritin degradation into low molecular weight iron complexes, which are then utilized *via* a Ton B-dependent receptor of unknown identity (5, 6). The expression of genes encoding all known iron receptors is regulated by the ferric uptake regulator (Fur) (7), a multi-functional protein also regulating the expression of genes involved in energy metabolism,

electron transfer, cytochrome metabolism, stress responses and production of secreted proteins related to the RTX family of cytotoxins. The Fur regulon has been studied in *N. meningitidis* using DNA microarrays (8, 9).

In relation to microbial iron uptake, Fur typically acts as a repressor of iron receptor genes to control the level of intracellular iron and to avoid cell damage mediated by reactive oxygen species. In an iron-restricted rather than a replete environment, as typically encountered by the meningococcus *in vivo*, the expression of these receptors is increased (4, 10). However, there is very little knowledge about microbial cellular components involved in the positive regulation of these iron receptors.

NMB0419 encodes a Sel1-like repeat (SLR) containing protein located within a genomic locus of eighteen open reading frames termed the division cell wall (*dcw*) cluster, involved in bacterial division and cell wall synthesis (11, 12). A functional promoter has been located immediately upstream of *NMB0419* (12). Sel1 (suppressor-enhancer of *lin*) was originally identified in *Caenorhabditis elegans* as a negative regulator of *lin-12*, encoding a membrane protein involved in the specification of cell fates during development (13). SLR proteins belong to the solenoid protein family and apparently play diverse biological roles as components of macromolecular complexes. SLR proteins have been described in the human pathogens *Helicobacter pylori*, *Legionella pneumophila* and *Pseudomonas aeruginosa* as important for virulence (reviewed in (14)), but remain largely uncharacterized in *Neisseria*.

In previous work, we established that *NMB0419* was involved – by an unknown mechanism – in meningococcal interaction with epithelial cells (15). In a study designed to investigate the involvement of the product of *NMB0419* in this phenotype, we initiated experiments with *Escherichia coli* expressing recombinant meningococcal NMB0419 (for which there is no *E. coli* homologue). We found, unexpectedly, that the result was up-regulation of genes involved in type 1 pilus (T1P) biosynthesis (an adhesin for which there is no homologue in *N. meningitidis*). Thus, NMB0419 was associated with transcriptional changes in *E. coli*, rather than acting as an adhesin. We have gone on to compare the transcriptomes of wild type (WT) and an *NMB0419* knock-out meningococcus, which revealed that the SLR-containing protein functions as a counterbalance to Fur in regulation of genes of the Fur regulon.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5 α (Invitrogen) and derivatives (Table 1) were grown on Luria-Bertani (LB) agar plates or in LB broth (Oxoid). *N. meningitidis* strain MC58 (B:15:P1.7,16b; ST-74) and derivatives (Table 1) were routinely grown on BHI (BD Biosciences) agar plates supplemented with 10% fetal bovine serum (FBS) (Invitrogen) or in BHI broth (BD Biosciences). In some experiments, *N. meningitidis* was cultured in RPMI1640 (Invitrogen) with or without 10% FBS supplementation. Bacteria grown on agar plates were incubated at 37°C in an atmosphere containing 5% CO₂ or in broth at 37°C with shaking at 200 rpm. Where appropriate, kanamycin (Km) or chloramphenicol was added to the growth medium to a final concentration of 50 and 20 μ g/ml, respectively.

General molecular biology techniques. Unless otherwise stated, standard recombinant DNA techniques, including DNA restriction digestion, agarose gel electrophoresis, DNA band recovery and DNA ligation, were carried out as described elsewhere (16). Chromosomal and plasmid DNA was prepared using the QIAprep Spin Miniprep Kit and QIAamp DNA mini kits (Qiagen), respectively.

Construction of pET based recombinant plasmids and generation of *E. coli* transformants. *NMB0419* was amplified by PCR from genomic DNA prepared from *N. meningitidis* strain MC58 using primers 0419PETF and 0419PETR (Table S1) and HotStar DNA polymerase (Qiagen) with Taq Extender PCR additive (Stratagene) according to the manufacturers' protocols, with the

addition of 5% DMSO (Sigma). The product was cloned into the NcoI and XhoI sites of the expression vector pET-29a (Novagen), generating the recombinant plasmid pET0419. *E. coli* DH5 α (Invitrogen) was transformed with pET0419 and pET, generating transformants DH5 α (pET0419) and DH5 α (pET), respectively. The coding sequence for the N-terminal signal peptide (identified using SignalP (17)) was removed from pET0419 using PCR primers SIGL0419F and SIGL0419R (Table S1) to create plasmid pET0419 Δ SIGL, in which the remaining coding sequence lies in frame with the start codon (verified by sequencing). The resulting *E. coli* transformant was named DH5 α (pET0419SIGL).

Construction of recombinant plasmids for genetic complementation of MC58 *NMB0419* mutants. Plasmid pMIDG0419 carrying *NMB0419* was created by insertion of an *NMB0419* amplicon prepared using primers pMIDG0419F and pMIDG0419R (Table S1) into BamHI and NheI sites of the broad host-range vector pMIDG201 (18), and the *aphA-3* gene was subsequently replaced at EcoRV sites with a chloramphenicol acetyltransferase (*cat*) gene amplified from plasmid pMC-Express (19) using primers EcoRVcatF and EcoRVcatR (Table S1). The resulting construct pMIDG0419 was transformed into *E. coli* S17.1 λ pir and into the MC58 *NMB0419* mutant through conjugation, generating MC58 (Δ *NMB0419*::Km) (pMIDG0419) selected using chloramphenicol (5 μ g/ml).

In addition, an in-frame deletion of the signal peptide coding sequence from *NMB0419* was made in pMIDG0419 by PCR amplification using primers SIGL0419F and SIGL0419R2, and re-circularization of the plasmid using T4

ligase, creating plasmid pMIDG0419 Δ SIGL, which was also conjugated into MC58 (Δ NMB0419::Km) to create MC58 (Δ NMB0419::Km) (pMIDG0419 Δ SIGL). The desired NMB0419 coding sequence was confirmed by sequencing.

The expression of *NMB0419* was detected in both complemented strains in log phase growth (in BHI) meningococci as determined by RT-PCR (Fig. S1), using primers NMB0419RTF and NMB0419RTR positioned downstream of the coding sequence for the N-terminal signal peptide domain (Table S1).

RT-PCR. *E. coli* RNA samples were prepared from DH5 α (pET0419) and DH5 α (pET) grown in LB broth (Oxoid) to OD₆₀₀ of 0.5 using the RNeasy kit (QIAGEN). RNA samples were treated with DNaseI (Invitrogen). As a negative control, RNA was incubated with RNaseA (Sigma) (1 μ g/ μ l) at 37°C for 20 min prior to reverse transcription. RT-PCR and verification of PCR products were performed as previously described (15). Primers are listed in Table S1.

Quantitative assessment of epithelial adherent and intracellular *E. coli*. Cells of the transformed human bronchial epithelial cell line 16HBE14 (20) were cultured at 37°C in 5% CO₂ in DMEM supplemented with 10% FBS and 2 mM L-glutamine (all from Invitrogen). Bacteria were suspended in culture media and added to confluent cell monolayers grown in 24-well tissue culture plates, at a multiplicity of infection (MOI) of 50 - 80. After incubation for 4 hours at 37°C, cell monolayers were washed vigorously three times with PBS and adherent bacteria were released from cells by treatment with 1% saponin (VWR) for 10 min at 37°C. Serial dilutions were plated out for CFU determination. Adherent bacterial CFU were expressed as percent of total growth. Inhibition of T1P-

mediated adherence was achieved by adding D-mannose (Sigma) 2% to the DMEM prior to inoculation of bacteria. The number of internalized *E. coli* was assessed using the gentamicin protection assay as previously described (15). In each experiment three independent biological replicates were performed.

Light microscopy. 16HBE14 cells were seeded (5×10^4 cells per well) onto glass cover slips (VWR) laid at the bottom of wells in 24-well tissue culture plates. Adherence assays were carried out as described above. After washing with PBS, samples were fixed on to the slides using 3.7% formaldehyde and stained with Giemsa (Sigma). Samples were visualized with an Olympus BH2-RFC light microscope and photographs taken at 100 x magnification.

Transmission Electron Microscopy (TEM). Copper palladium grids (300 mesh, TAAB Laboratories Equipment Ltd., UK) were gently floated on to 5 μ l of bacterial suspension for 5 min and air-dried. The bacteria were fixed on grids with 4% glutaraldehyde (Sigma) and stained with 1% uranyl acetate. Resulting samples were visualised using a FEI Technai G² transmission electron microscope.

Real time RT-PCR. RNA samples were prepared from MC58 (WT) and MC58 (Δ NMB0419::Km) (15) grown to an OD₆₀₀ of 0.75 using the FastRNA Pro Blue kit and FastPrep FP120 system (MP Biochemicals). Three independently prepared biological samples without detectable genomic DNA contamination as adjudged by Bioanalyzer (Agilent) analysis were used. RNA samples were reverse-transcribed to first-strand cDNA by using Superscript III and random primers (Invitrogen). Real-time PCR was performed using TaqMan technologies,

Universal PCR Master Mix, the StepOnePlus system (Applied Biosystems). Primer and reporter (FAM and NFQ labelled) sequences are listed in Table S1. Statistical analysis was carried out using the $2^{-\Delta\Delta CT}$ method (21) with 16S rRNA as the endogenous reference. Relative expression of *pilE* in MC58 ($\Delta NMB0419::Km$) was compared to that in MC58 (WT) and expressed as fold change.

Transcriptome analysis. *E. coli* microarray analysis was carried out using RNA samples prepared from DH5 α (pET0419) and DH5 α (pET) cultured in LB broth to OD₆₀₀ 0.5 and fluorescently labelled as described previously (22). Labelled RNA samples of three biological replicates and each with a pair of dye swaps for each strain were hybridized on ShEcoli arrays, which were obtained from the Institute of Food Research (Norwich, UK) and have been described previously (23). The hybridization and washing conditions were as previously described (22). Slides were scanned on a GenePix 4000B scanner (Axon) and data analysis performed in GenePix Pro6.0 and Genespring GX7.3 (Agilent) as previously described (22). Genes differentially expressed in DH5 α (pET0419) compared to DH5 α (pET) (Table S2) were selected using a t-test p-value with multiple testing using Benjamini and Hochberg False Discovery Rate (FDR) (24) at a cut-off of 0.05.

Accession numbers: The array design is available in ArrayExpress (Accession No. A-MEXP-1748; <http://www.ebi.ac.uk/arrayexpress>). Fully annotated microarray data have been deposited in ArrayExpress (accession number E-MEXP-2485).

Meningococcal RNA samples of three biological replicates was prepared for WT and mutant ($\Delta NMB0419::Km$) grown in supplemented RPMI1640 to an OD₆₀₀ of 0.5-0.7 using the FastRNA Pro Blue kit and FastPrep-24 (MP Biochemicals). All RNA samples were treated with DNase I (Invitrogen). RNA samples with a RIN > 9.5, as adjudged by Bioanalyzer analysis, were submitted to Beijing Genome Institute (www.bgi-international.com) for RNA sequencing analysis, which included removal of rRNA, RNA fragmentation, cDNA synthesis by random priming, size selection, PCR amplification, Illumina sequencing (HiSeq 2000), QC of raw data, alignment with MC58 genome, QC of alignment, calculating expression level (expressed as RPKM (25)) and expression level comparison for each pair (mutant vs WT) of biological replicates. We further filtered results of comparisons using FDR (described by Benjamini and Yekutieli(26)) < 0.01 and $-\log_2$ ratio < -0.5849 or > 0.5849 (fold change > 1.5). The genes that passed filter in all three sets of comparison was listed as up- and down-regulated in the *NMB0419* mutant (Table S3 and S4).

The raw data and processed data with description have been deposited in Gene Expression Omnibus under accession number GSE81572.

Iron utilization bioassay. *N. meningitidis* (10^7 CFU) were streaked out onto BHI agar plates containing 100 μ M of deferoxamine mesylate (Desferal) (Sigma) and supplemented with 10% of FBS, providing 15 μ M of bovine haemoglobin (5), an iron source that can be utilized by the meningococcus (27). Further tests were done by placing filter discs impregnated with 10 μ l iron supplements over the 10^7 CFU spread on the BHI agar plates containing Desferal

and FBS (as above). Iron supplements were haemoglobin (1.25 mM), iron (III) chloride (100 mM), apo-transferrin (40 mg/ml) and holo-transferrin (40 mg/ml). All agar plates were incubated at 37°C in 5% CO₂ for 17 hours.

Meningococcal intra-epithelial replication assay. Cells of the respiratory epithelial cell line COR-L23 (ATCC) were cultivated in RPMI1640 with 10% FBS and 1% non-essential amino acids (all from Invitrogen) and used in 24-well plates for assays. Gentamicin killing of extracellular meningococci was performed as previously described (15). After gentamicin killing, formulated media were added to designated wells to assess meningococcal intracellular replication. Formulated media were RPMI1640 and RPMI1640 with added Desferal (100 µM) or iron (III) chloride (25 µM). CFU were enumerated immediately (T0) and 4 hours (T4) after gentamicin killing. For each strain, CFU at T4 was divided by CFU at T0 to give the replication rate, which was further normalized to the value obtained for the MC58 (WT) for each media condition, and expressed as percent of WT replication.

PCR amplification of the *NMB0419* homologue from neisserial strains. Genomic DNA from *N. meningitidis* and *Neisseria lactamica* strains (grown on BHI agar) was prepared using a DNA mini kit (QIAGEN). PCR was performed using primers 0419PETF and 0419PETR (Table S1), HotStar DNA polymerase (Qiagen) and Taq Extender PCR additive (Stratagene) plus 5% DMSO (Sigma). PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN) and sequenced.

RESULTS

Expression of *NMB0419* in *E. coli* was associated with transcriptional changes, including increased expression of genes for type 1 pili (T1P) biogenesis. Expression of *NMB0419* was detected in *E. coli* DH5 α transformed with recombinant plasmid pET0419, but not in DH5 α (pET) alone (Fig. S2). DH5 α (pET0419) had an increased ability to adhere and invade to human epithelial cells, compared to DH5 α (pET) (adherent 14.3 % vs 2.1% with $p = 0.019$ and intracellular 0.004% vs 0.0007% with $p = 0.025$) (Fig. 1 A, B). Transcriptome analysis showed a set of genes being differentially regulated in DH5 α (pET0419) in comparison with DH5 α (pET) and amongst the top 15 up-regulated genes, eight were constituents of the *fim* operon encoding the type 1 pilus (T1P) (Table S2). RT-PCR confirmed up-regulation of *fimA* (encoding the major pilin) and *fimC* (encoding a minor pilin) in DH5 α (pET0419) (Fig. S3). Light microscopy showed a pattern of aggregative adherence (AA) (28) for strain DH5 α (pET0419) on epithelial cells (Fig. S4), consistent with T1P expression being a major contributor to AA (29). TEM showed numerous T1P-like structures (30) on the surface of DH5 α (pET0419) (Fig. S5), which were rarely seen on DH5 α (pET) (data not shown). The addition of D-mannose was shown to reduce DH5 α (pET0419) adherence to epithelial cells to 0.29% in comparison with the control (12.5%, without mannose) (Fig. 1C), confirming the role of T1P (31). The results showed that expression of *NMB0419* in *E. coli* was associated with increased expression in genes for T1P biogenesis, which resulted in increased adherence to and invasion of epithelial cells.

Type 4 pili (T4P) in the meningococcal NMB0419 mutant are expressed at WT level. Meningococci express T4P rather than T1P, the major pilin subunit being encoded by *pilE* (NMB0018) (11). We compared transcription of *pilE* in the NMB0419 mutant and WT MC58 using real time RT-PCR.

Expression of *pilE* was not significantly different between the strains ($p = 0.83$) with an expression ratio of 1.19 (mutant / WT) (Fig. S6). Electron microscopic inspection of WT and mutant cells revealed that T4P was expressed on the surface very similarly in each, in the form of single and bundled pili (Fig. S7).

Many genes differentially regulated in the NMB0419 mutant belong to the previously identified meningococcal Fur regulon. The transcriptional profiles of the NMB0419 mutant and its parental WT MC58 were compared using RNA-sequencing. Forty four and 47 genes (comprising 1.8% and 1.9% of the genome) respectively, were up- and down-regulated in the NMB0419 mutant compared to WT using a 1.5-fold change ($\log_2$ ratio of 0.5849) and FDR < 0.01 as the cut off in all three pairs of mutant / WT comparison. Most of the genes encoding products known to be important for iron uptake were down-regulated in the NMB0419 mutant. These were *tbpA* (NMB0461) and *tbpB* (NMB0460) encoding transferrin-binding proteins, *lbpA* (NMB1540) and *lbpB* (NMB1541) encoding lactoferrin-binding proteins, *hmbR* (NMB1668) encoding haemoglobin-binding protein, *frpB* (also *fetA*, NMB1988) encoding a siderophore receptor, and *exbB* (NMB1729), *exbD* (NMB1728) and *tonB* (NMB1730) involved in providing energy for these receptors. In contrast, *bfrA* (NMB1207) encoding the bacterial

iron storage protein bacterioferritin was up-regulated in the *NMB0419* mutant (Fig.2).

We compared our list of differentially-regulated genes with those previously described as being regulated by Fur in meningococci (9). Twenty-three out of 44 genes (52%) up-regulated in the *NMB0419* mutant were co-identified as being up-regulated (induced) by Fur (9). These included genes involved in energy metabolism and electron transfer, including 19 genes of the entire *nuo* operon (*NMB0241* – *NMB0259*) and 3 genes of the *pet* operon (*NMB2051* – *NMB2053*), as well as the above mentioned *bfrA* (Table S3). Eighteen of 47 genes (38%) down-regulated in the *NMB0419* mutant were co-identified as being down-regulated (repressed) by Fur (Fig. 3). These included the above-mentioned iron receptor genes; those encoding hypothetical iron receptors (such as *NMB0034* and *NMB0035 (efeO)*); and *frpA* and *frpC* encoding RTX-like secreted cytotoxins (Table S4). In addition to the co-identified genes, *NMB1989* – *NMB1991* (encoding a hypothetical iron ABC transporter system) were significantly down-regulated in the *NMB0419* mutant (Table S4).

The results indicate an apparent association between the genes differentially regulated in the *NMB0419* mutant and those previously identified as being part of the Fur regulon (9). Relevant experiments were carried out to ascertain whether the transcriptional changes in the *NMB0419* mutant would result in corresponding phenotypic changes.

The NMB0419 mutant is deficient in utilizing transferrin and haemoglobin under iron-restricted conditions and the signal peptide

domain is important for the function of NMB0419. FBS (providing 15 μ M of haemoglobin) containing additional Desferal (100 μ M) was able to support the growth of WT MC58 and MC58 (Δ NMB0419::Km) (pMIDG0419) but not the growth of the MC58 (Δ NMB0419::Km) and MC58 (Δ NMB0419::Km) (pMIDG0419 Δ SIGL) mutants (Fig. 4A). Further growth tests using the same agar plates with iron supplementation on filter discs showed that the *NMB0419* mutant was able to grow around a filter disc loaded with high concentrations of haemoglobin (1.25 mM) or iron (III) chloride (100 mM), but not with apo- or holo-transferrin (Fig. 4B). These results provided phenotypic validation that the level of down-regulation achieved of genes involved in iron uptake (*tbpAB* and *hmbR*) impaired the ability of the *NMB0419* mutant to utilize transferrin and haemoglobin for growth.

Functional characterization using MC58 (Δ NMB0419::Km) (pMIDG0419 Δ SIGL) indicated that NMB0419 is secreted and acts beyond the bacterial inner membrane (Fig. 4A). Exploiting the effect of NMB0419 on epithelial adherence of *E. coli* as a reporter system, strains bearing pET0419 Δ SIGL, a recombinant plasmid carrying *NMB0419* with an in-frame deletion of the signal peptide coding sequence, DH5 α (pET0419 Δ SIGL) and DH5 α (pET) (negative control) showed similar levels of adherence to 16HBE14 cells, at 3.0% and 2.3%, respectively, while DH5 α (pET0419) showed a significantly higher level of adherence (8.1%) ($p = 0.014$) (Fig. S8). These results show that the signal peptide is required for function both in *N. meningitidis* MC58 and *E. coli*.

Reduced intra-epithelial replication by the NMB0419 mutant.

Meningococci mutants in *tonB*, but not in *hmbR* or *fbpA*, replicate inside epithelial cells much more slowly than WT strains (5). We explored intra-epithelial meningococcal replication using the COR-L23 respiratory epithelial cell line, in which we have previously shown that the *NMB0419* mutant is less invasive than WT MC58. After an initial 24-hour infection period, gentamicin killing was performed to remove extracellular meningococci. The intracellular meningococci within COR-L23 respiratory cells were allowed to grow for another 4 hours in different (iron-replete and restricted medium), at which point the intracellular CFU count was assessed. With incubation in RPMI1640 media, the *NMB0419* mutant replicated less efficiently in COR-L23 cells than WT MC58 (to 45% of WT, $p = 0.0042$). When an iron-restricted growth condition was imposed by addition of Desferal to the external media, the replication efficiency of the *NMB0419* mutant was further reduced (to 25% of WT, $p = 0.0089$). However, when free iron (iron (III) chloride) was added to the growth media, the replication efficiency of the *NMB0419* mutant was comparable to that of WT ($p = 0.9$) (Fig. 5).

Homologues of NMB0419 with differing numbers of SLR domains are found throughout the genus Neisseria. By PCR and sequencing using *NMB0419*-specific primers, *NMB0419* was shown to be widespread in the genus *Neisseria* (Fig. 6). *NMB0419* homologues were found in every *N. meningitidis* strain examined ($n = 59$), including strains of serogroup A ($n = 3$), B ($n = 42$), C ($n = 10$) (Table 2), W, X, Y and Z (four and one each respectively, see Table S5) as well as in 28/28 *N. lactamica* (Table 2) and 2/2 *N. gonorrhoeae* strains (see Table

S5). The number of SLR domains encoded by different homologues varied strikingly, from 1 to 11 (Table 2). The *NMB0419* homologues from all 59 *N. meningitidis* strains encoded 1 – 8 SLR domains, whereas the *NMB0419* homologs from most (23/28) *N. lactamica* strains encoded 9 – 11 SLR domains. This variation mirrors the situation in *H. pylori* (reviewed in (14)) and *Haemophilus influenzae* (32). The functional consequences are unknown will be the subject of further study. It is intriguing to note that the highest numbers of SLR repeats (≥ 9) are in genes within commensal *N. lactamica* compared to the lower numbers within pathogenic *N. meningitidis*. In addition, we interrogated the information available at EMBL (<http://smart.embl-heidelberg.de>), which showed that higher numbers of SLR domains were common amongst SLR proteins encoded on the genomes of other commensal *Neisseria* spp. (*N. bacilliformis*, *N. cinerea*, *N. polysaccharea*, *N. sicca*, *N. subflava*) (Table S6).

DISCUSSION

In a previous study we have found the product of *NMB0419* of *N. meningitidis* MC58 to be involved in meningococcal interaction with epithelial cells (15), although it was not clear whether its functional role was as an adhesin, invasin or other component. In the present study, we started our investigation characterizing the effect of transformation of the non-adhesive and non-invasive *E. coli* strain DH5 α with the recombinant expression plasmid pET0419. DH5 α (pET0419) was significantly increased in its ability to adhere to epithelial cells, through up-regulation of genes involved in T1P biosynthesis and regulation. The adherence of DH5 α (pET0419) to the monolayer of epithelial cells formed a unique AA pattern on epithelial cells, which has been associated with the expression of T1P (29). The expression of T1P on the surface of DH5 α (pET0419) was further confirmed using electron microscopy. The importance of T1P in epithelial adherence by DH5 α (pET0419) was also confirmed using mannose competition assays. NMB0419 is predicted to contain an N-terminal signal peptide followed by four SLR domains. Using *E. coli* strain DH5 α (pET0419SIGL) with an in-frame deletion of the signal peptide coding sequence in pET0419, we showed that the signal peptide was necessary for NMB0419 to modulate epithelial adherence, indicating its functional location is beyond the cytoplasmic membrane. PSORTb 3.0.2 (www.psort.org/psortb/) and SignalP 4.1 (www.cbs.dtu.dk/services/SignalP/) predict that NMB0419 is exported beyond the cytoplasmic membrane and is extracellular, respectively. There is no prediction of a membrane location.

N. meningitidis strains express T4P rather than T1P, and in contrast to the situation described in *E. coli* with T1P, we could show no influence of *NMB0419* on pilus-mediated epithelial adherence. The meningococcal major pilin gene *pilE* was expressed at similar levels, as determined by qRT-PCR, in the WT and *NMB0419* mutant, and EM showed normal surface expression of T4P by the *NMB0419* mutant.

Genome wide RNA analysis demonstrated that, compared with WT, many genes encoding receptors involved in iron acquisition were down-regulated in the meningococcal *NMB0419* mutant. Since these genes are known to be regulated by Fur, we compared our list of differentially regulated genes with those previously identified as part of the meningococcal Fur regulon (9) and found a high degree of co-identification of genes down-regulated in the *NMB0419* mutant being repressed by Fur (38%), and genes up-regulated in the *NMB0419* mutant being induced by Fur (51%). The results suggest that the expression of *NMB0419* is associated with the up-regulation of a set of genes negatively regulated by Fur, and the down-regulation of a set of genes positively regulated by Fur. The inference is that the biological role of *NMB0419* expression is to counterbalance the regulatory function of Fur.

To determine whether there was a correlation between transcriptional changes and phenotype, we tested whether the down-regulation of iron receptor genes would affect the growth of the *NMB0419* mutant under iron-restricted conditions. The results showed that acquisition of haemoglobin and transferrin by the mutant was affected. Acknowledging that the initial meningococcal phenotype

of interest was epithelial intracellular multiplication, we went on to investigate whether the down-regulation of *tonB*, *exbB* and *exbD* would be enough to impair the *NMB0419* mutant's intracellular replication rate (mutations in meningococcal *tonB* lead to impaired intracellular replication, which is considered to result from blockade of iron uptake from degraded ferritin iron complexes that occurs via a TonB-dependent receptor of unknown identity (6)). In our experiments the *NMB0419* mutant replicated less efficiently within epithelial cells than the WT, and the effect was reversible by the addition of free iron, consistent with the *NMB0419* mutant lacking the ability to acquire transferrin degradation products.

Hooda et al. (33) have determined that the surface lipoprotein assembly modulator (Slam) protein (encoded by *NMB0313*) has an ancillary role in transporting lipoproteins, such as TbpB and LbpB, to the surface of *N. meningitidis*. *NMB0313* contains an N-terminal single tetratricopeptide repeat (TPR) domain which is similar in structure to Sel1-like domains. *NMB0313* is down-regulated in the *NMB0419* mutant together with other iron receptor genes (including *tbpB* and *lbpB*). The down-regulation of *NMB0313* in the *NMB0419* mutant is consistent with the proposition that the expression of *NMB0419* is not only associated with up-regulation of genes encoding iron receptors, but also their surface expression.

SLR proteins, like *NMB0419*, mediate their biological roles through their interaction with other proteins, forming protein complexes (14). While we have not determined the precise cellular location of *NMB0419*, as discussed above the most likely location is either periplasmic or extracellular. Where investigated

previously in Gram-negative bacteria, SLR proteins have been found to be periplasmic. For example, the SLR protein ExoR from *Sinorhizobium meliloti* has a similar domain structure to NMB0419 and has been demonstrated to interact in the bacterial periplasm with the ExoS/Chvltwo component system (TCS) (34). Our characterization of *NMB0419* in the meningococcus (using pMIDG0419 Δ SIGL) and *E. coli* (using pET0419 Δ SIGL) indicates a possible periplasmic functional location for NMB0419, similar to ExoR, which supports the possibility that NMB0419 is involved in sensing an environmental signal(s) in the periplasm, and exerts its regulatory role through interactions with a TCS(s) in the meningococcus and *E. coli*. If correct, it is likely that NMB0419 interacts with a different TCS in the meningococcus and *E. coli* resulting in the phenotypic changes in different biological processes, i.e. iron uptake and pilus biosynthesis, respectively. Four sets of TCS have been annotated in meningococcal genomes (11). Of these only the MisR/S (*NMB0595/NMB0594*) system has been extensively studied. However, MisR/S is only known to regulate *hmbR* but not other iron receptors (35), and microarray analysis did not show any evidence that the MisR/S and Fur regulons overlap (36, 37). In contrast, the MisR/S system in *N. gonorrhoea* has been reported to regulate *tbpB/A* through direct binding to the upstream region of the *tbpB* gene (38). The authors also determined that the DNA sequence in the cognate region upstream of the meningococcal *tbpB* gene is divergent from that found in *N. gonorrhoea*, consistent with previous findings in *N. meningitidis* (36, 37). Alternatively, NMB0419 could be extracellular or may have functions related to transport of lipoproteins involved in iron-regulation,

possibly similar to that described for the meningococcal outer membrane protein Slam (33). Our experimental data and the predictions that NMB0419 is exported beyond the cytoplasmic membrane make it unlikely that NMB0419 is acting as a transcriptional regulator and binding directly or indirectly to classical Fur motifs present in the promoters of Fur-regulated genes. Further investigation is required to identify the interaction partner(s) for NMB0419, the cellular location of these interactions and how these influence the expression of genes of the Fur regulon.

Fur is a well-studied global transcriptional repressor and activator for the genes involved not only in iron homeostasis but also in the stress response and other functions related to virulence and energy metabolism, enabling bacteria to survive in adverse environments (7, 39). Our finding that the expression of *NMB0419* was associated with transcriptional changes involving genes not only for iron uptake but also for virulence functions and energy metabolism, in a manner counterbalancing the regulatory function of Fur, is significant and novel. Factors associated with positive regulation of meningococcal iron receptors under iron-restricted conditions have not been described before, and may be important determinants for meningococcal survival in the host environment.

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Figure legends

Fig. 1. Increased epithelial adherence and internalization of *E. coli* due to the expression of *NMB0419* and type 1 pili. Adherent (A) and internalized (B) CFU of DH5 α (pET) and DH5 α (pET0419) recovered at 4 hour post infection of cultured 16HBE14 cells are expressed as percent of total bacteria. (C) Adherence of DH5 α (pET0419) and DH5 α (pET) were tested with (+) or without (-) mannose in the growth media. Error bars indicate standard error of means (SEM) of three biological replicates. Asterisks indicate CFU with a significant difference ($p < 0.025$) between the corresponding strains.

Fig. 2. Differential expression of genes encoding iron uptake and storage in the *NMB0419* mutant compared with WT MC58. The average ratio of three biological comparisons for each gene is expressed as Log₂ fold change. Error bars indicate SEM of three biological replicates.

Fig. 3. Venn diagram showing numbers of differentially regulated genes co-identified in the *NMB0419* mutant and in the previously reported Fur regulon (9).

Fig. 4. Growth of *N. meningitidis* on BHI agar containing Desferal and 10% FBS. (A) 10⁶ CFU from each meningococcal strain as indicated were streaked out on agar plates for overnight growth. (B) Growth of the *NMB0419* mutant around

discs impregnated with iron chloride or haemoglobin but no growth with apo-transferrin or holo-transferrin.

Fig. 5. Replication efficiency of intra-epithelial (COR-L23) meningococci under various media conditions. Intracellular meningococcal CFU taken at 4 hour time point after gentamicin killing was compared with CFU taken immediately after gentamicin killing to give a ratio. The ratio obtained for the *NMB0419* mutant (NMB0419) was further normalized to the ratio obtained for the MC58 (WT) under each condition expressed as percent of WT replication. The media conditions: “RPMI” – RPMI1640; “Desferal+” – addition of Desferal; “Iron+” – addition of iron chloride to RPMI. Asterisks indicate CFU with a significant difference ($p < 0.009$) between the WT and *NMB0419* mutant.

Fig. 6. Representative PCR products of *NMB0419* homologs amplified from genomic DNA of different *N. meningitidis* strains detected by gel electrophoresis. The corresponding number of SLR domains encoded by the strain specific *NMB0419* homolog (confirmed by sequencing) is indicated on top of each sample lane.

Table 1. Bacterial strains used in this study.

Strain name	Modification/plasmid	references
<i>E. coli</i>:		
DH5 α	WT	(16)
DH5 α (pET)	pET	This study
DH5 α (pET0419)	pET0419	This study
DH5 α (pET0419 Δ SIGL)	pET0419 Δ SIGL	This study
<i>N. meningitidis</i>:		
MC58	WT	(11)
MC58 (Δ NMB0419::Km)	pMIDG0419	(15)
MC58 (Δ NMB0419::Km) (pMIDG0419)	pMIDG0419	This study
MC58 (Δ NMB0419::Km) (pMIDG0419 Δ SIGL)	pMIDG0419 Δ SIGL	This study

Table 2. Number of SLRs encoded by *NMB0419* homologs from different *N. meningitidis* and *N. lactamica* strains.

Number of SLRs	Number of strains			
	NmA	NmB	NmC	<i>N. lactamica</i>
1	2	21	2	
2	1	10	2	1
4		10	6	2
7				1
8		1		1
9				4
10				7
11				12

NmA, NmB and NmC denote *N. meningitidis* serogroup A, B and C, respectively.

Table S1. Oligonucleotides used in cloning and PCR.

Name*	Sequence (from 5' to 3')	Application
0419PETF	AACATATGAAACAGACAGTCAAA TGG	Cloning of <i>NMB0419</i> into plasmid pET29a to generate pET0419
0419PETR	GTCTCGAGACCCGCTTCAGGC GTTGGTCATTG	
SIGL0419F	GGTGCATATGGATGACGTATCG GATTTTCG	Construction of in-frame deletion of signal peptide coding sequence to generate pET0419ΔSIGL
SIGL0419R	GTTTCATATGTATATCTCCTTCTT	
SIGL0419R2	GTTTCATATGTATATCTCCTTCTT AAAGTTAAAC	Paired with SIGL0419F to construct in- frame deletion of signal peptide coding sequence to generate pMIDG0419ΔSIGL
FIMAF	CCGTTTCAGTTAGGACAGGTTC	Detecting expression of <i>fimA</i> gene in <i>E.</i>
FIMAR	TCCGTTATTCAGGGTTGTTTC	<i>coli</i> by RT-PCR
FIMCF	GGCAAAAACAAGAGCAACTTG	Detecting expression of <i>fimC</i> gene in <i>E.</i>
FIMCR	CGAGCTGTAGCGTATTCTCAGT C	<i>coli</i> by RT-PCR
NMB0419F	ATGAAACAGACAGTCAAATGG	Detecting expression of <i>NMB0419</i> by
NMB0419R	ACCCGCCTTCAGGCGTTGGTCA TTG	RT-PCR in <i>E. coli</i> and <i>N. meningitidis</i>
NMB0419RTF	ATGAAACAGACAGTCAAATGG	Detecting expression of <i>NMB0419</i> with in-frame deletion of signal peptide
NMB0419RTR	ACCCGCCTTCAGGCGTTGGTCA TTG	
NMB0018PIL- EF	GCCATCGTCGGCATTGTTG	Forward primer, TaqMan PCR for <i>pilE</i>
NMB0018PIL- ER	TGCGCGGGCTGTGT	Reverse primer, TaqMan PCR for <i>pilE</i>
NMB0018PIL- EM1	CCTTCCTGCTTATCAAGACT	Reporter (FAM labelled), TaqMan PCR for <i>pilE</i>
NMrrnaA16S- B2F	GCTAATACCGCATACGTCTTGAG A	Forward primer, TaqMan PCR for 16S
NMrrnaA16S- B2R	TCGGCCGCTCGAATAGC	Reverse primer, TaqMan PCR for 16S
NMrrnaA16S- B2M2	AAGGTCCCCTGCTTTCT	Reporter (FAM labelled), TaqMan PCR for 16S
pMIDG0419F	ATACGGATCCAGAAGTAAAACCA TGAAACAGACAG	Cloning <i>NMB0419</i> into pMIDG201 to generate pMIDG0419
pMIDG0419R	ACGGGCTAGCGAGCTGTTCAAT AACCCGCCTTC	
EcoRVcatF	AAGATATCAGCTCTAACAAGCG GTCGGCA	Cloning the <i>cat</i> gene into pMIDG0419 to replace <i>aphA-3</i>
EcoRVcatR	AAGATCCCATGCATGCATAACAA GCGGT	

*xxxF and xxxR identify forward and reverse primers, respectively.

Table S2. List of genes up-regulated in *E. coli* DH5 α (pET0419) compared with DH5 α (pET) (selection cut off: 1.5 fold change and $P < 0.05$ with FDR).

Gene Name	Gene number	Fold change	p-value (FDR)	Gene product/function
<i>pheA</i>	b2599	1.6	2.11E-02	Chorismatemutase-P and prephenatedehydratase
<i>cysJ</i>	b2764	1.6	1.79E-02	Sulfite reductase (NADPH), flavoprotein beta subunit
<i>fimE</i>	b4313	1.5	3.74E-03	Recombinase involved in phase variation; regulator of <i>fimA</i>
<i>cspA</i>	b3556	1.8	3.34E-03	Cold shock protein 7.4, transcriptional activator of <i>hns</i>
<i>aceE</i>	b0114	1.9	2.87E-03	Pyruvate dehydrogenase (decarboxylase component)
<i>bglJ</i>	b4366	1.7	2.61E-03	2-component transcriptional regulator
<i>aceF</i>	b0115	1.9	1.49E-04	Pyruvate dehydrogenase (dihydrolipoyltransacetylase component)
<i>fimD</i>	b4317	10.9	1.31E-05	Outer membrane protein; export and assembly of type 1 fimbriae, interrupted
<i>b1017</i>	b1017	1.6	1.20E-05	Putative cytochrome
<i>fimC</i>	b4316	11.2	2.37E-06	Periplasmic chaperone, required for type 1 fimbriae
<i>fimG</i>	b4319	7.1	1.63E-06	Fimbrial morphology
<i>fimF</i>	b4318	7.2	1.68E-06	Fimbrial morphology
<i>fimA</i>	b4314	9.0	1.30E-06	Major type 1 subunit fimbrin (pilin)
<i>fimI</i>	b4315	10.2	4.35E-07	Fimbrial protein
<i>fimH</i>	b4320	7.1	2.63E-07	Minor fimbrial subunit, D-mannose specific adhesin

Table S3. List of genes up-regulated in the *NMB0419* mutant in comparison with WT MC58 filtered by $\log_2 > 0.5849$ and FDR < 0.01 .

Gene ID	Gene Name	fold change	log2 ratio	Fur induced (Delany et al., 2006)	Functional annotation
NMB0241	<i>nuoA</i>	2.2	1.1	Y	NADH dehydrogenase I subunit A
NMB0242	<i>nuoB</i>	2.5	1.3	Y	NADH dehydrogenase I subunit B
NMB0243	<i>nuoC</i>	2.3	1.2	Y	NADH dehydrogenase I subunit C
NMB0244	<i>nuoD</i>	2.4	1.3	Y	NADH dehydrogenase I subunit D
NMB0245	<i>nuoE</i>	2.5	1.3	Y	NADH dehydrogenase I subunit E
NMB0246	<i>nuoF</i>	2.3	1.2	Y	NADH dehydrogenase I subunit F
NMB0247		2.9	1.6		Hypothetical protein
NMB0248		2.1	1.1		Putative endonuclease
NMB0249	<i>nuoG</i>	2.3	1.2	Y	NADH dehydrogenase I subunit G
NMB0250	<i>nuoH</i>	2.3	1.2	Y	NADH dehydrogenase I subunit H
NMB0251	<i>nuoI</i>	2.4	1.3	Y	NADH dehydrogenase I subunit I
NMB0252		2.1	1.0		NADH-quinone oxidoreductase subunit B
NMB0253	<i>nuoJ</i>	2.0	1.0		NADH dehydrogenase I subunit J
NMB0254	<i>nuoK</i>	1.8	0.8	Y	NADH dehydrogenase subunit K
NMB0255		2.1	1.1		Cell filamentation protein Fic-like protein
NMB0256		1.9	0.9		Hypothetical protein
NMB0257	<i>nuoL</i>	1.9	0.9	Y	NADH dehydrogenase subunit L
NMB0258	<i>nuoM</i>	1.8	0.9		NADH dehydrogenase subunit M
NMB0259	<i>nuoN</i>	1.8	0.9	Y	NADH dehydrogenase subunit N
NMB0388		1.8	0.8		Sugar transporter
NMB0663	<i>nspA</i>	2.9	1.5	Y	NspA
NMB0717		2.3	1.2		Cytochrome
NMB0923		1.6	0.7		Cytochrome C
NMB0997	<i>dld</i>	1.6	0.7		D-lactate dehydrogenase
NMB1207	<i>bfrA</i>	1.6	0.7	Y	Bacterioferritin A
NMB1360	<i>pdxH</i>	1.7	0.8		Pyridoxamine 5'-phosphate oxidase
NMB1365		2.0	1.0		Hypothetical protein
NMB1370		1.9	0.9		Hypothetical protein
NMB1436	<i>lldE</i>	2.1	1.1	Y	L-lactate dehydrogenase complex protein
NMB1437	<i>lldG</i>	2.0	1.0	Y	L-lactate dehydrogenase complex protein
NMB1438	<i>lldF</i>	1.9	0.9	Y	L-lactate dehydrogenase complex protein
NMB1454		1.8	0.8		Ferredoxin, iron-sulfur protein, electron transfer
NMB1557		1.7	0.8		Hypothetical protein
NMB1590	<i>pgsA</i>	1.7	0.8		Hypothetical protein
NMB1613	<i>fumB</i>	2.2	1.2	Y	Fumarate hydratase
NMB1623	<i>pan1</i>	2.4	1.3	Y	Major anaerobically induced outer membrane protein
NMB1624		1.9	0.9		Hypothetical protein

NMB1677		2.1	1.1		Cytochrome C5
NMB1723	<i>fixP</i>	1.6	0.7	Y	Cytochrome c oxidase subunit III
NMB1723a		1.5	0.6		Cytochrome oxidase subunit 3
NMB1805		1.9	0.9	Y	Cytochrome C4
NMB1870	<i>petC</i>	1.6	0.7	Y	Cytochrome C1 precursor
NMB2052	<i>petB</i>	1.7	0.8		Cytochrome B
NMB2053	<i>petA</i>	2.0	1.0	Y	Ubiquinol-cytochrome c reductase iron-sulfur subunit

Table S4. List of genes down-regulated in the *NMB0419* mutant in comparison with WT MC58 filtered by $\log_2 < -0.5849$ and $FDR < 0.01$.

Gene ID	Gene Name	fold change	log2 ratio	Fur repressed (Delany et al., 2006)	Annotation
NMB0023	<i>pilA</i>	-1.6	-0.6		PilA type IV pilus assembly protein PilA
NMB0026		-2.8	-1.5		PilS cassette, cell projection
NMB0034		-2.6	-1.4	Y	High-affinity iron transporter
NMB0035	<i>efeO</i>	-1.7	-0.8	Y	Iron uptake system component
NMB0036		-1.8	-0.9		Transition metal ion binding
NMB0175	<i>zupT</i>	-6.2	-2.6	Y	Zinc transporter
NMB0201		-1.7	-0.8		Hypothetical protein
NMB0293		-3.3	-1.7		Iron complex outer membrane receptor protein
NMB0313		-1.8	-0.9		NADH-quinone oxidoreductase subunit D
NMB0378		-1.7	-0.8		Inorganic phosphate transporter, PiT family
NMB0460	<i>tbpB</i>	-2.6	-1.4	Y	Transferrin-binding protein B
NMB0461	<i>tbpA</i>	-1.9	-0.9	Y	Transferrin-binding protein A
NMB0503		-1.6	-0.7		Hypothetical protein
NMB0510		-1.7	-0.8		Hypothetical protein
NMB0584	<i>frpC</i>	-2.5	-1.3	Y	FrpC operon protein
NMB0585	<i>frpA</i>	-3.7	-1.9	Y	FrpA iron-regulated protein
NMB0744		-4.8	-2.3	Y	Hypothetical protein
NMB0747		-2.1	-1.0	Y	Bacterioferritin-associated ferredoxin
NMB0753		-1.9	-0.9		Hypothetical protein
NMB0855		-2.2	-1.1		intrinsic membrane component
NMB0856		-2.4	-1.3		Hypothetical protein
NMB0857		-2.1	-1.0		Hypothetical protein
NMB0858		-1.8	-0.9		Hypothetical protein
NMB0861		-2.0	-1.0	Y	Hypothetical protein
NMB0862		-2.3	-1.2		pyridoxamine 5'-phosphate oxidase
NMB0864		-5.4	-2.4		Hypothetical protein
NMB0865		-5.8	-2.5		NADH-quinone oxidoreductase subunit N
NMB0866		-5.3	-2.4		Intrinsic membrane component
NMB1368		-1.9	-0.9		ATP-dependent RNA helicase RhlE
NMB1377	<i>lldD</i>	-7.1	-2.8	Y	L-lactate dehydrogenase (cytochrome)
NMB1426		-1.8	-0.8		Hypothetical protein

NMB1458	<i>fumC</i>	-2.7	-1.4	Y	Fumarate hydratase
NMB1540	<i>lbpA</i>	-3.4	-1.8	Y	Lactoferrin-binding protein A
NMB1541	<i>lbpB</i>	-4.2	-2.1	Y	Lactoferrin binding protein B
NMB1668	<i>hmbR</i>	-1.7	-0.7		HmbR
NMB1728	<i>exbD</i>	-1.8	-0.9	Y	ExbD; biopolymer transport protein
NMB1729	<i>exbB</i>	-1.9	-1.0	Y	ExbB
NMB1730	<i>tonB</i>	-2.2	-1.1		TonB
NMB1762		-1.6	-0.7		Hemolysin activation/secretion protein
NMB1771		-1.9	-0.9		Hypothetical protein
NMB1794		-1.6	-0.7		Intrinsic membrane protein
NMB1796		-1.7	-0.8	Y	Fumarate reductase flavoprotein subunit
NMB1967		-2.9	-1.5		AraC family transcriptional regulator
NMB1988	<i>frpB (fetA)</i>	-6.7	-2.7	Y	FrpB iron-regulated outer membrane protein
NMB1989		-1.9	-0.9		Iron ABC transporter substrate-binding protein
NMB1990		-2.5	-1.3		Iron ABC transporter permease
NMB1991		-2.6	-1.4		Iron(III) ABC transporter permease

Table S5. Number of SLRs encoded by *NMB0419* homologs in different *Neisseria* strains.

Strain name	Meningococcal serogroup or neisserial species	Number of SLRs encoded by <i>NMB0419</i> homolog	Strain reference
NM001	B	1	Litt et al., 2004
NM002	B	2	Litt et al., 2004
NM004	B	1	Litt et al., 2004
NM008	B	1	Litt et al., 2004
NM009/NM010	B	1	Litt et al., 2004
NM021	B	2	Litt et al., 2004
NM036	C	4	Litt et al., 2004
NM037	B	2	Litt et al., 2004
NM050	B	2	Litt et al., 2004
NM058	B	1	Litt et al., 2004
NM066	B	4	Litt et al., 2004
NM067	C	4	Litt et al., 2004
NM069	B	4	Litt et al., 2004
NM081	C	4	Litt et al., 2004
NM088	C	4	Litt et al., 2004
NM092	B	1	Litt et al., 2004
NM100	C	4	Litt et al., 2004
NM106	B	1	Litt et al., 2004
NM107/NM108	B	1	Litt et al., 2004
NM117	B	2	Litt et al., 2004
NM119	B	4	Litt et al., 2004
NM131	B	1	Litt et al., 2004
NM145/NM146	C	2	Litt et al., 2004
NM154	C	1	Litt et al., 2004
NM156	B	4	Litt et al., 2004
NM167	B	1	Litt et al., 2004
NM184	B	2	Litt et al., 2004
NM186	B	2	Litt et al., 2004
NM188	B	4	Litt et al., 2004
NM191	B	4	Litt et al., 2004
NM200	B	1	Litt et al., 2004
SWZ107	B	1	Pizza et al., 2000
Z2491	A	1	Parkhill et al., 2000
528	B	1	Pizza et al., 2000
1000	B	1	Pizza et al., 2000
205900	A	1	Pizza et al., 2000
297-0	B	1	Pizza et al., 2000
860800(28)	Y	8	Pizza et al., 2000
90/18311(24)	C	2	Pizza et al., 2000

A22	W	1	Pizza et al., 2000
BZ133	B	1	Pizza et al., 2000
BZ147	B	1	Pizza et al., 2000
BZ169	B	4	Pizza et al., 2000
BZ198	B	1	Pizza et al., 2000
BZ232	B	2	Pizza et al., 2000
BZ83	B	4	Pizza et al., 2000
E26(27)	X	1	Pizza et al., 2000
E32	Z	1	Pizza et al., 2000
F6124	A	2	Pizza et al., 2000
FAM18	C	1	Bentley et al., 2007
H44/76	B	4	Pizza et al., 2000
L93/4286	C	4	Pizza et al., 2000
MC58	B	4	Tettelin et al., 2000
NG6/88	B	2	Pizza et al., 2000
NGE28	B	1	Pizza et al., 2000
NGF26	B	2	Pizza et al., 2000
NGH15	B	1	Pizza et al., 2000
NGH36(17)	B	8	Pizza et al., 2000
NGH38	B	1	Pizza et al., 2000
FA1090	<i>N. gonorrhoeae</i>	2	Pizza et al., 2000
SN4	<i>N. gonorrhoeae</i>	2	Pizza et al., 2000
L581	<i>N. lactamica</i>	11	Our collection
L583	<i>N. lactamica</i>	11	Our collection
L584	<i>N. lactamica</i>	10	Our collection
L585	<i>N. lactamica</i>	9	Our collection
L586	<i>N. lactamica</i>	8	Our collection
L590	<i>N. lactamica</i>	11	Our collection
L591	<i>N. lactamica</i>	11	Our collection
L593	<i>N. lactamica</i>	10	Our collection
L594	<i>N. lactamica</i>	10	Our collection
L596	<i>N. lactamica</i>	11	Our collection
L597	<i>N. lactamica</i>	11	Our collection
L600	<i>N. lactamica</i>	4	Our collection
L601	<i>N. lactamica</i>	4	Our collection
L603	<i>N. lactamica</i>	10	Our collection
L604	<i>N. lactamica</i>	10	Our collection
L607	<i>N. lactamica</i>	11	Our collection
L608	<i>N. lactamica</i>	9	Our collection
L613	<i>N. lactamica</i>	11	Our collection
L615	<i>N. lactamica</i>	11	Our collection
L624	<i>N. lactamica</i>	7	Our collection
L631	<i>N. lactamica</i>	11	Our collection
L628	<i>N. lactamica</i>	9	Our collection
L633	<i>N. lactamica</i>	10	Our collection

L640	<i>N. lactamica</i>	2	Our collection
L642	<i>N. lactamica</i>	11	Our collection
L643	<i>N. lactamica</i>	10	Our collection
L644	<i>N. lactamica</i>	11	Our collection
Y921009	<i>N. lactamica</i>	9	Our collection

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Table S6. SLR domain containing proteins encoded on the genomes of commensal *Neisseria* spp. (data source: <http://smart.embl-heidelberg.de>).

Strain name	Gene/Protein ID	Number of SLR domains	Protein length (amino acid)
<i>N. bacilliformis</i> ATCC BAA-1200	HMPREF9123_0587	11	474
	HMPREF9123_0795	6	328
	HMPREF9123_0878	3	144
	HMPREF9123_1788	12	527
	HMPREF9123_2053	8	354
	HMPREF9123_2720	4	227
<i>N. cinerea</i> ATCC 14685	NEICINOT_03126	9	467
<i>N. elongata subsp. glycolytica</i> ATCC 29315	NEIELOOT_00646	4	270
	NEIELOOT_01801	6	296
	NEIELOOT_01869	11	474
<i>N. flavescens</i> NRL30031/H210	NEIFLAOT_02136	10	468
<i>N. flavescens</i> SK114	NEIFL0001_0986	10	475
<i>N. mucosa</i> ATCC 25996	NEIMUCOT_04811	10	469
	NEIMUCOT_05081	6	263
	NEIMUCOT_05111	8	460
<i>N. mucosa</i> C102	HMPREF0604_00707	10	475
	HMPREF0604_01251	10	468
	HMPREF0604_01421	8	339
	HMPREF0604_01422	7	229
<i>N. polysaccharea</i> ATCC 43768	NEIPOLOT_01486	9	467
<i>N. shayegani</i> 871	HMPREF9371_0536	3	129
	HMPREF9371_0951	4	227
	HMPREF9371_0994	10	494
<i>N. sicca</i> ATCC 29256	NEISICOT_00897	6	286
	NEISICOT_01185	4	213
	NEISICOT_01186	2	74
	NEISICOT_02322	8	446
	NEISICOT_02910	11	470
	NEISICOT_03300	9	376
	NEISICOT_03302	9	384
<i>N. sicca</i> VK64	HMPREF1051_1458	8	446
	HMPREF1051_0449	4	197
	HMPREF1051_0450	3	96
	HMPREF1051_1764	6	286
	HMPREF1051_3116	4	199
	HMPREF1051_3119	7	305
	HMPREF1051_3118	6	267
	HMPREF1051_2711	10	454
<i>N. subflava</i> NJ9703	NEISUBOT_03120	10	420
	NEISUBOT_03121	4	146
	NEISUBOT_05066	10	475

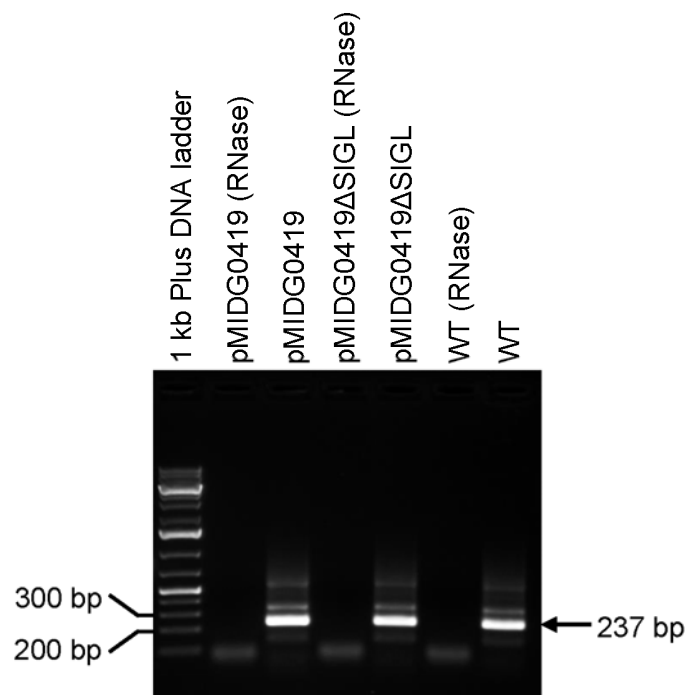


Fig. S1. Expression of *NMB0419* in *N. meningitidis* detected by RT-PCR and gel electrophoresis. Lanes contain products of RT-PCRs using RNA samples extracted from MC58 wild type strain (WT) and the *NMB0419* mutant carrying different recombinant plasmids as indicated. RNase indicates the RNA sample being RNaseA treated (negative control). Primers were NMB0419RTF and NMB0419RTR (product size, 237 bp). DNA ladder (1 kb plus) is also indicated.

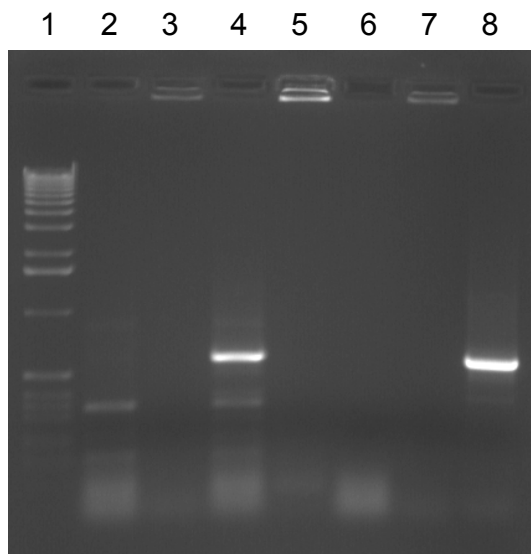


Fig. S2. Expression of *NMB0419* in *E. coli* detected by RT-PCR and gel electrophoresis. Lanes contain products of RT-PCRs with templates as follows: 1, 1 kb DNA ladder; 2, 4 and 6, RNA from DH5 α (pET), DH5 α (pET0419) and DH5 α , respectively; 3, 5 and 7, RNaseA-treated RNA (negative controls for samples in lanes 2, 4 and 6, respectively); 8, genomic DNA from MC58.

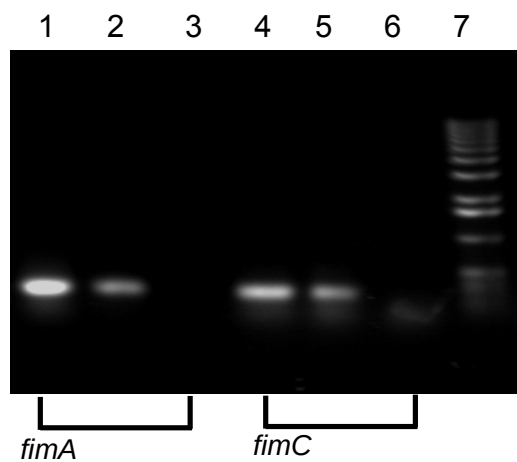


Fig. S3. *fimA* and *fimC* expression in *E. coli* detected by RT-PCR and gel electrophoresis. Lanes contain products of RT-PCRs with templates as follows: 1 and 4, RNA from *E. coli* DH5 α (pET0419); 2 and 5, RNA from DH5 α (pET); 3 and 6, RNaseA-treated RNA (negative controls representative of both RNA samples from DH5 α (pET0419) and DH5 α (pET)); 7, 1 kb DNA ladder.

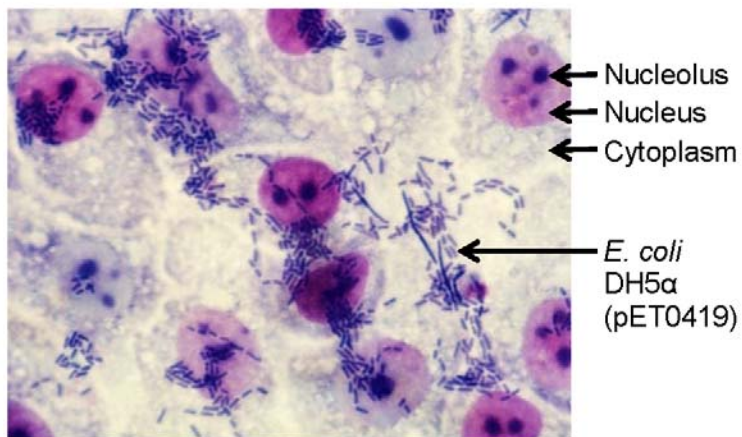


Fig. S4. Microscopic image (1000 X) of aggregative adherence of *E. coli* DH5α (pET0419) on 16HBE14 epithelial cells taken after 4 hour co-incubation and after Giemsa staining. Stacked-brick configuration of adherent *E. coli* (long arrow) and subcellular components (short arrows) are indicated.

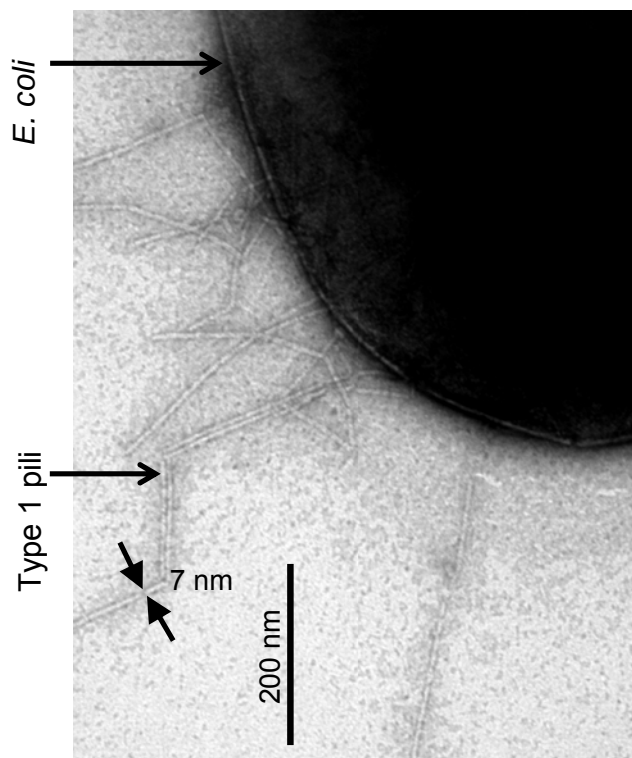


Fig. S5. T1P like structures on the surface of *E. coli* DH5α (pET0419) detected by transmission electron microscopy. Ruler: 200 nm.

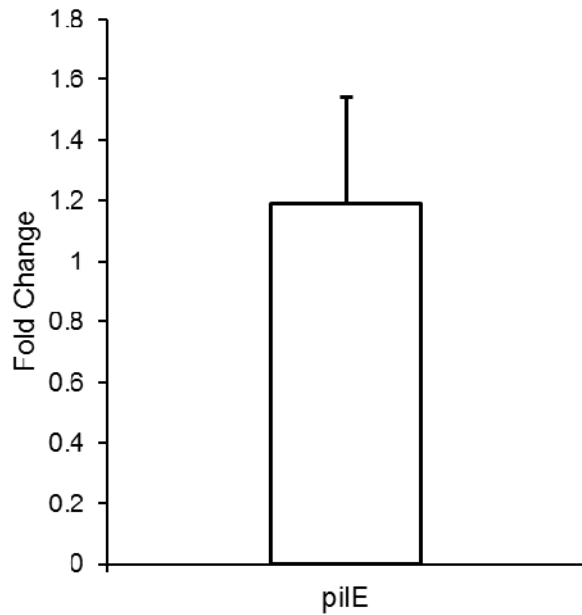
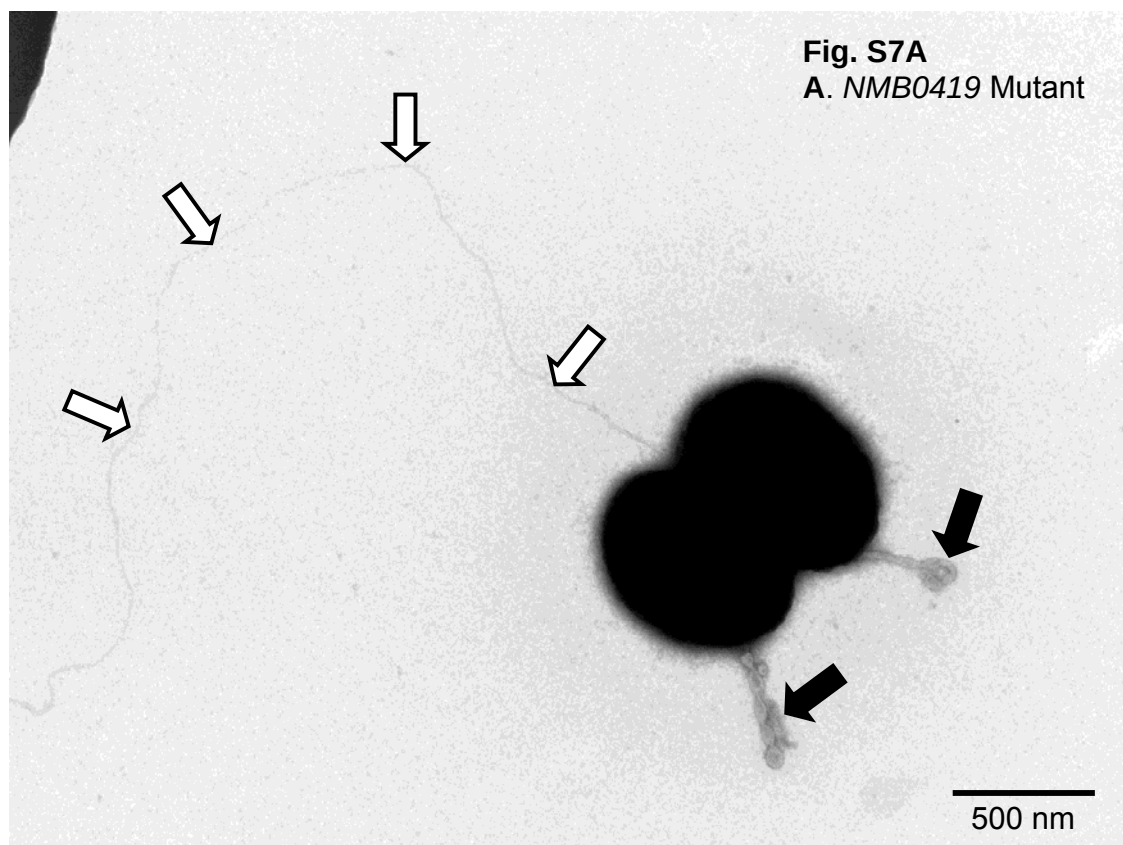


Fig. S6. Comparison of *pilE* (*NMB0018*) expression in the *NMB0419* mutant and WT *N. meningitidis* MC58 using real time RT-PCR. RNA extracted from the *NMB0419* mutant and WT MC58 was analysed using TaqMan technology for the expression level of *pilE*. Comparative results were expressed as fold changes (mutant / WT) with a p-value of 0.83. Error bars indicate SEM calculated from three pairs of biological samples.



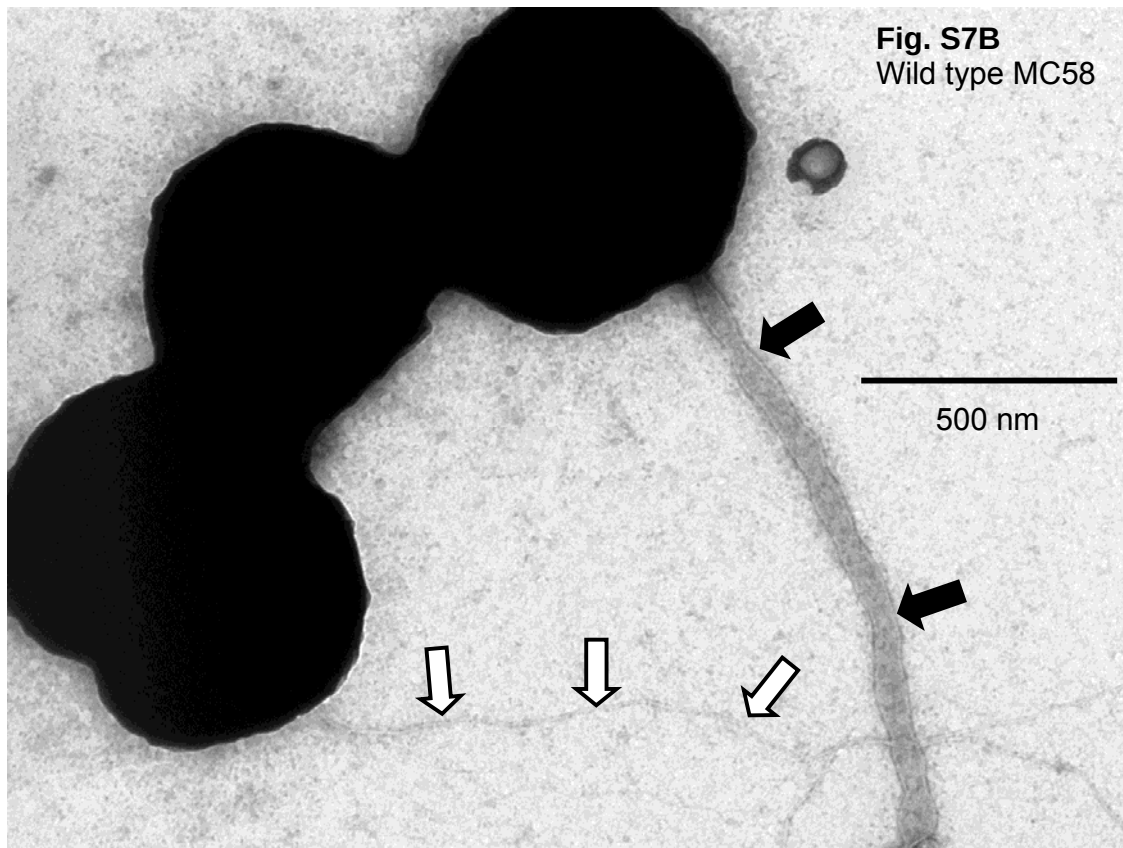


Fig S7. Expression of type 4 pili on the surface of the meningococci detected by transmission electron microscopy after uranyl acetate staining. Empty and solid arrows indicate a single and bundled type 4 pili, respectively, extending from meningococci. Ruler: 500 nm. **A.** *NMB0419* mutant. **B.** Wild type MC58.

Fig. S8.

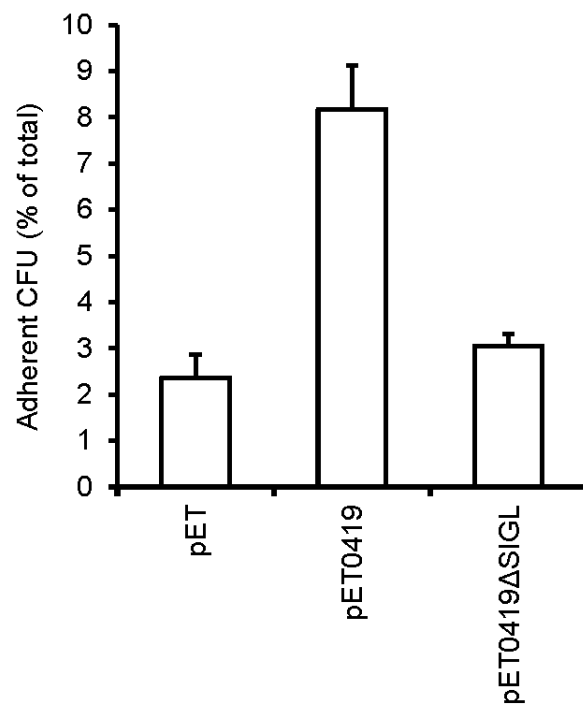


Fig. S8. The signal peptide is important for the function of NMB0419 in *E. coli*. Epithelial adherent CFU for DH5 α carrying plasmid pET, pET0419 or pET0419 Δ SIGL (with in-frame deletion of signal peptide coding sequence in *NMB0419*) are expressed as percent of total bacteria recovered at 4 hour post infection of 16HBE14 cells. Error bars indicate SEM of three biological replicates.