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Transcriptional Profiling of *Neisseria meningitidis* Interacting with Human Epithelial Cells in a Long-Term *In Vitro* Colonization Model

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Neisseria meningitidis **is a commensal of humans that can colonize the nasopharyngeal epithelium for weeks to months and occasionally invades to cause life-threatening septicemia and meningitis. Comparatively little is known about meningococcal gene expression during colonization beyond those first few hours. In this study, the transcriptome of adherent serogroup B *N. meningitidis* strain MC58 was determined at intervals during prolonged cocultivation with confluent monolayers of the human respiratory epithelial cell line 16HBE14. At different time points up to 21 days, 7 to 14% of the meningococcal genome was found to be differentially regulated. The transcriptome of adherent meningococci obtained after 4 h of coculture was markedly different from that obtained after prolonged cocultivation (24 h, 96 h, and 21 days). Genes persistently upregulated during prolonged cocultivation included three genes (*hfq*, *misR*/*phoP*, and *lrp*) encoding global regulatory proteins. Many genes encoding known adhesins involved in epithelial adherence were upregulated, including those of a novel locus (spanning *NMB0342* to *NMB0348* [*NMB0342-NMB0348*]) encoding epithelial cell-adhesive function. Sixteen genes (including *porA*, *porB*, *rmpM*, and *fbpA*) encoding proteins previously identified by their immunoreactivity to sera from individuals colonized long term with serogroup B meningococci were also upregulated during prolonged cocultivation, indicating that our system models growth conditions *in vivo* during the commensal state. Surface-expressed proteins downregulated in the nasopharynx (and thus less subject to selection pressure) but upregulated in the bloodstream (and thus vulnerable to antibody-mediated bactericidal activity) should be interesting candidate vaccine antigens, and in this study, three new proteins fulfilling these criteria have been identified: NMB0497, NMB0866, and NMB1882.**

Neisseria meningitidis is a common colonist of the human nasopharyngeal epithelium, persisting in this location as a commensal organism for weeks or months (1, 2). From here, in a process that is poorly understood (but which apparently reflects human genetic predisposition, environmental factors, and the particular meningococcal strain present), it may rarely invade deeper tissues and the bloodstream to cause septicemia and meningitis (3–6). The dichotomous phenotypes of colonization and invasion are widely considered to be established early following the arrival of meningococci in the nasopharynx, with most invasive infections being thought to occur soon after this point. Late invasion is apparently rare (7). The interaction of meningococci with the nasopharyngeal epithelium has been a major driving force shaping the bacterial gene pool (8). While attention has understandably been focused largely on organisms isolated from the bloodstream, transcriptional changes promoting this phenotype cannot influence the gene pool, as these organisms are doomed, either through host bactericidal activity terminating infection or (all too often) through fatal infection removing organisms (and the host) from the pool. It is the bacterial transcriptome in organisms on the epithelial surface that not only determines the dynamics of the colonization process but also must be pivotal in the process of invasion. Transcriptomal studies focused on this phase of infection reveal changes during colonization that may increase or reduce the likelihood of invasion and identify critical bacterial determinants with vaccine potential. Published work in this area has been confined to studies which can identify only early events in the colonization process, having monitored bacterial/epithelial cell cocultivation for no longer than 6 h (9, 10). Here we report cocultivation of meningococci with human respiratory tract epithelial cells extended to a period of 21 days. The meningococcal transcriptome over this period was found to diverge markedly from the pattern found at 4 h, providing new insights into gene expression of potential relevance to invasive disease.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *N. meningitidis* MC58 (serogroup B:15:P1.7.16b; ST-74) and its isogenic mutant strains (see below) were used. Meningococcal strains were routinely propagated at 37°C with 5% CO_2 on GC agar (Difco) supplemented with 1% Vitox (Oxoid) (sGC) or at 37°C in brain heart infusion (BHI) broth (BD Biosciences) supplemented with 1% Vitox (sBHI broth).

Culturing of human epithelial cells. The 16HBE14 bronchial epithelial cell line is derived from primary human bronchial epithelial cells transformed by the simian virus 40 (SV40) large T antigen (11). COR-L23 (catalogue no. 92031929) cells are derived from human lung carcinoma cells with epithelial morphology and were obtained from the European Collection of Cell Culture (Health Protection Agency, Porton Down, United Kingdom). The Chang epithelial cell line (ATCC CCL20.2) was obtained (in 2001) from the American Type Culture Collection (ATCC). The lung carcinoma cell line A549 (ATCC CCL-185) was obtained from

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the ATCC. The human tonsillar epithelial (HTE) cell line (transformed using human papillomavirus E6/E7) was a kind gift from Michael Apicella of the University of Iowa. All cell lines were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (HIFBS) (all reagents from Invitrogen) with a humidified incubator at 37°C with 5% CO_2.

Cocultivation model and control samples. Epithelial cells grown to >90% confluence in either wells of 24-well plates (Cellstar; Greiner) or T75 flasks (BD Falcon; VWR) with permanently hydrophilic surfaces were used for determining meningococcal viable counts or preparing total RNA from adherent meningococci. For scanning electron microscopy (as well as for light microscopy using Giemsa staining), epithelial cells were grown on glass coverslips (13-mm diameter; British Drug House [BDH]) in 24-well plates. *N. meningitidis* cells grown on sGC plates were suspended in DMEM for initial inoculation at a multiplicity of infection of 30. The infected or noninfected (control) epithelial monolayers were incubated as specified above. Intermediate washes with phosphate-buffered saline (PBS) and the addition of fresh culture medium were carried out at 16-h intervals followed by 8-h intervals. The morphological integrity of epithelial monolayers was routinely monitored with an inverted microscope (Olympus). Representative monolayers were stained with trypan blue (Invitrogen) and viewed under the microscope. Images were photographed by using a C-35AD-4 (Olympus) camera (see Fig. S1 in the supplemental material).

RNA isolated from *N. meningitidis* cells cultured in DMEM for 3 h (DMEM3h) was used as a control sample. Bacteria grown on sGC plates were resuspended in supplemented DMEM to an optical density at 600 nm (OD_{600}) of 0.2 in tissue culture flasks (T25) and incubated identically to the epithelial cell cocultivation preparations for 3 h.

Epithelial cell adherence and gentamicin protection assays. At the desired cocultivation time points, infected monolayers were washed with PBS (three times with 1 ml per well), followed by incubation with 1% saponin (Sigma) for 10 min at 37°C (for the epithelial cell adherence assay). Bacteria as well as debris of human cells were separated by pipetting and plated onto sGC agar plates for CFU determinations. For the gentamicin protection assay, gentamicin (Sigma) was added to each sample at 150 μg/ml with supplemented DMEM, and the sample was incubated for 1 h at 37°C under 5% CO_2. The resulting samples were further washed with PBS (3× 1 ml), and intracellular bacteria were released by using saponin and plated for enumeration.

Scanning electron microscopy. At desired cocultivation time points, infected monolayers were washed as described above and incubated overnight at 4°C in 3% glutaraldehyde (Sigma), followed by a 30-min incubation with 1% osmium tetroxide (OsO_4) (Sigma). Ethanol-dehydrated samples were processed by using a critical-point drying apparatus (Emitech K850) according to the manufacturer's instructions and subsequently mounted onto scanning electron microscopy stubs (JEOL) for further coating with palladium-gold using a Mini Sputter Coater (Polaron SC7620). The resulting samples were photographed by using a JEOL JSM-6390 scanning electron microscope.

RNA extraction. Infected monolayers at 4 h, 24 h, 96 h, and 21 days in T75 flasks were washed with PBS and incubated with 0.5% trypsin containing 0.2% EDTA (Sigma) for 5 min at 37°C, followed by the addition of ice-cold PBS and pipetting (to separate cells and bacteria). The resulting mixture was centrifuged at 300 × *g* for 3 min in a prechilled centrifuge (Jouan) to pellet epithelial cells. The resulting supernatant or DMEM3h control samples were mixed with RNAprotect (Qiagen), incubated for 5 min at ambient temperature, and centrifuged for 10 min at 3,000 × *g*. The pellets were subjected to RNA extraction by using the FastPrep FP120 system (MP Biochemicals) according to the manufacturer's instructions. The RNA samples were further treated with DNase (Invitrogen), purified by using an RNeasy kit (Qiagen), and analyzed by using PCR and a Bioanalyzer (Agilent) to monitor RNA quality and contamination of human RNA and bacterial genomic DNA (gDNA). RNA samples selected from each biological group were tested for contamination of genomic DNA by using primers for the meningococcal gene *NMB0152* (see Table S1 in the supplemental material), the HotStar PCR kit (Qiagen), and a DNA Engine thermal cycler (Bio-Rad) for 40 cycles, according to the manufacturer's recommendations. One-fifth of each PCR mixture was examined by agarose gel electrophoresis and ethidium bromide staining and visualized on a UV transilluminator. No PCR product of the expected molecular weight (that of positive-control MC58 genomic DNA as the template) was detected (data not shown). There was no human RNA or genomic DNA contamination, as measured by Bioanalyzer analysis, i.e., no distinct peaks observed at a high molecular weight (see Fig. S2 in the supplemental material).

Microarray hybridization and analysis. Labeling of RNA (1 μg) and genomic DNA (0.1 μg) obtained from MC58 was performed as previously described (12). Briefly, RNA samples were labeled by using random primers, a Superscript III kit (Invitrogen), and Cy5 (GE Healthcare), with an initial sample denaturation step at 72°C and a subsequent elongation step at 42°C for >2 h. DNA samples were labeled by using random primers, a large-fragment DNA polymerase I kit (Invitrogen), and Cy3 (GE Healthcare), with an initial sample denaturation step at 95°C and a subsequent elongation step at 37°C. The pan-*Neisseria meningitidis* array NMGv2.2.0 was designed by the Bacterial Microarray Group at St. George's University of London (BμG@S) (http://bugs.sgul.ac.uk/) and manufactured on the Agilent SurePrint 8×15 K platform (Agilent), with each gene being represented by three different oligonucleotides (40- to 60-mers). Each array slide was cohybridized with one of the Cy5-labeled RNA samples and Cy3-labeled gDNA (universal reference) by using the Agilent hybridization kit and microarray hybridization oven with rotating hybridization chambers. The resulting slides were washed by using an Oligo aCGH & ChIP-on-Chip wash buffer kit (Agilent) and scanned by using a high-resolution microarray scanner (Agilent). Data were extracted and processed from each scanned image by using Agilent Feature Extraction v10.7.3.1 (Agilent) and further analyzed by using GeneSpring software (Agilent).

Normalization of microarray data and comparison of transcriptomes between time points, e.g., 4 h versus 3 h with DMEM treatment, were performed as previously described (12). Briefly, the signal intensities resulting from hybridization of Cy5-labeled cDNA derived from meningococcal RNA were normalized to the signal intensities from Cy3-labeled genomic DNA on each spot (per-spot normalization). The normalized spot values of technical replicates for each gene were averaged and normalized to the 50th percentile (median) values of each array with locally weighted scatterplot smoothing (LOWESS) applied for smoothing using 20% of the data (per-gene/per-array normalization). To identify genes differentially expressed between DMEM3h (control) and other biological groups (4 h, 24 h, 96 h, and 21 days), the normalized intensities for each gene derived from averaged biological replicates within a group were compared by using analysis of variance (ANOVA). Cutoff values of a 1.5-fold change ($P < 0.01$, corrected for multiple testing using Benjamini-Hochberg correction) were used. Correlation of gene expression profiles was calculated as previously described (13). Principal-component analysis (PCA) was performed by using all genes that passed quality control testing and default settings in GeneSpring. Gene clustering was done with GeneSpring using a hierarchical algorithm, and transcriptome similarity was measured by Euclidean distance.

DAVID (14) was used to identify differentially regulated gene groups according to their associated functional annotations. Benjamini multiple correction ($P < 0.01$) was used to select the most significantly enriched functional groups.

Quantitative real-time reverse transcription-PCR (RT-PCR). RNA samples from three fresh biological replicates were prepared as described above and were reverse transcribed to first-strand cDNA by using Superscript III and random primers (Invitrogen). Real-time PCR was performed as described previously (12), using TaqMan technologies, Universal PCR Master Mix, and the StepOnePlus system (Applied Biosystems). The primers were designed and manufactured by using Assay-by-Design

(Applied Biosystems) and are listed in Table S1 in the supplemental material. The reporter dye and quencher were 6-carboxyfluorescein (FAM) and NFQ, respectively. We used 10 ng total RNA per reaction. The reference gene was 16S rRNA. Statistical analysis was based on methods reported previously by Livak and Schmittgen (15), and detailed statistical formulations are described in Applied Biosystems user bulletin no. 2 (P/N4303859).

Construction and characterization of meningococcal mutants. Genes in the locus spanning *NMB0342* to *NMB0348* (*NMB0342–NMB0348* locus) share identical sequences with homologous genes in *N. meningitidis* strain 8013 (16, 17). Strain MC58 was transformed with genomic DNA from the corresponding isogenic mutants of strain 8013 (kindly provided by Vladimir Pelicic of the Imperial College London) made through transpositional mutagenesis (18). A backcross transformation strategy previously used in meningococcal studies (19) was used to ensure that the phenotypic changes in each mutant strain were due to the mutation of the targeted gene rather than the incorporation of unintended mutations at other chromosomal loci. It must be conceded that, although very unlikely, it is not impossible that despite this backcross approach, undetectable recombination may still have occurred in other loci. Briefly, DNA from first-generation (kanamycin-resistant) mutants was used to retransform the kanamycin-sensitive parental strain (MC58) to generate second-generation mutants. The resulting transformants, grown on sGC plates containing kanamycin (50 μg/ml), were selected for PCR amplification using gene-specific primers (see Table S1 in the supplemental material) to confirm insertion of a Kan^r cassette in the targeted genes *NMB0342*, *NMB0344*, *NMB0345*, *NMB0347*, and *NMB0348* (see Fig. S3 in the supplemental material). The PCR products were sequenced by using the BigDye reaction kit (Applied Biosystems) and an ABI3730XL sequencer (Applied Biosystems).

For bacterial growth curves, *N. meningitidis* cells (MC58 and its isogenic mutants) were grown for 16 h on sGC plates, harvested, and diluted in sBHI broth or DMEM supplemented with 10% HIFBS to give an initial OD_{600} of 0.2. The resulting bacterial suspension was incubated at 37°C with gentle shaking, and OD_{600} readings were taken intermittently.

Expression of targeted or adjacent genes was studied by using RT-PCR. RT was performed by using RNA extracted from each mutant grown on sGC plates, the SuperScript III kit (Invitrogen), and reverse primers (see Table S1 in the supplemental material). The resulting cDNA was used as the template for PCR using the HotStar PCR kit (Qiagen). The primers used for RT-PCR are listed in Table S1 in the supplemental material.

Statistical significance was calculated for the differences between adherent CFU obtained from wild-type strain MC58 and those obtained from each of its isogenic mutant strains by using the two-tailed Student *t* test.

Nucleotide sequence accession numbers. The array design is available at BμG@Sbase (accession no. A-BUGS-45 [http://bugs.sgul.ac.uk/]) and also at ArrayExpress (accession no. A-BUGS-45 [http://www.ebi.ac.uk/microarray-as/ae/]). Fully annotated microarray data have been deposited in the BμG@Sbase database (accession no. E-BUGS-141) and also in the ArrayExpress database (accession no. E-BUGS-141), which is in compliance with minimum information about a microarray experiment (MIAME) protocols.

RESULTS

Establishing and characterizing a meningococcal epithelial colonization model. We sought to establish a meningococcal/human epithelial cell cocultivation model using immortalized epithelial cells that would support meningococcal serogroup B strain MC58 adherence for a long period and at a sufficient bacterial density to yield enough meningococcal RNA (ca. 1 μg/sample) for transcriptome analysis. Five cell lines were tested in a system of confluent culture in flat-bottomed flasks, with twice-daily washing and replenishment of culture medium (DMEM) (see Fig. S4 in the supplemental material). At 28 h, there were significant numbers of meningococci adherent to 16HBE14 and COR-L23 cells but no detectable meningococci on Chang, A549, or transformed human tonsillar epithelial (HTE) cells (data not shown for the latter two lines). 16HBE14, the SV40-immortalized bronchial epithelial cell line used by Grifantini et al. (10) for their transcriptomal study, was the only cell line supporting meningococcal adherence at 48 h cocultivation and beyond, with the CFU per monolayer being comparable at 28, 48, and 96 h but with a further increase at 21 days (see Fig. S4 in the supplemental material). During cocultivation, epithelial cells were examined daily for gross signs of cell detachment from the plastic surface, and cell viability was examined periodically by trypan blue staining (see Fig. S1 in the supplemental material). Giemsa staining revealed that by 48 h, the majority of COR-L23 cells had lost their nucleoli, with no meningococci to be seen on the cell surface (Fig. 1A). In contrast, 16HBE14 cells retained distinct nucleoli within nuclei, and numerous adherent meningococci were present (Fig. 1B). The 16HBE14 cell line was the only cell line that could survive an extended period of cocultivation with *N. meningitidis* strain MC58 under our experimental conditions and therefore was selected for further study.

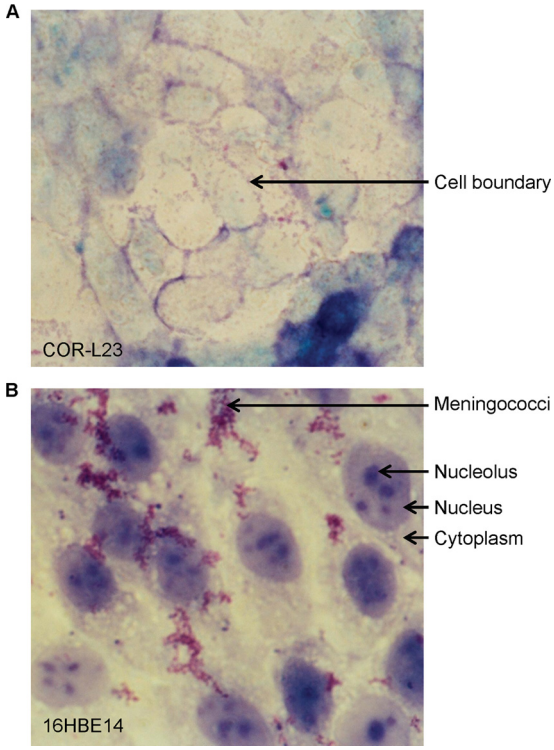

FIG 1 Viability of epithelial cells cocultivated with *N. meningitidis* MC58. Epithelial cell monolayers cocultivated with bacteria for 48 h were washed and stained with Giemsa. (A) COR-L23 cells at a ×400 magnification. The arrow indicates an example of a cell boundary. (B) 16HBE14 cells at a ×1,000 magnification. Arrows indicate examples of cytoplasm, nucleolus, nucleus, and a group of meningococci.

Meningococcus-16HBE14 cell interactions were characterized during extended periods of cocultivation by scanning electron microscopy. After 10 h, meningococci were present as microcolonies, and the surface of the epithelial cells was marked by short microvillus-like membrane protrusions (20, 21) (Fig. 2A). After 48 h, elongated filopodium-like protrusions were also observed

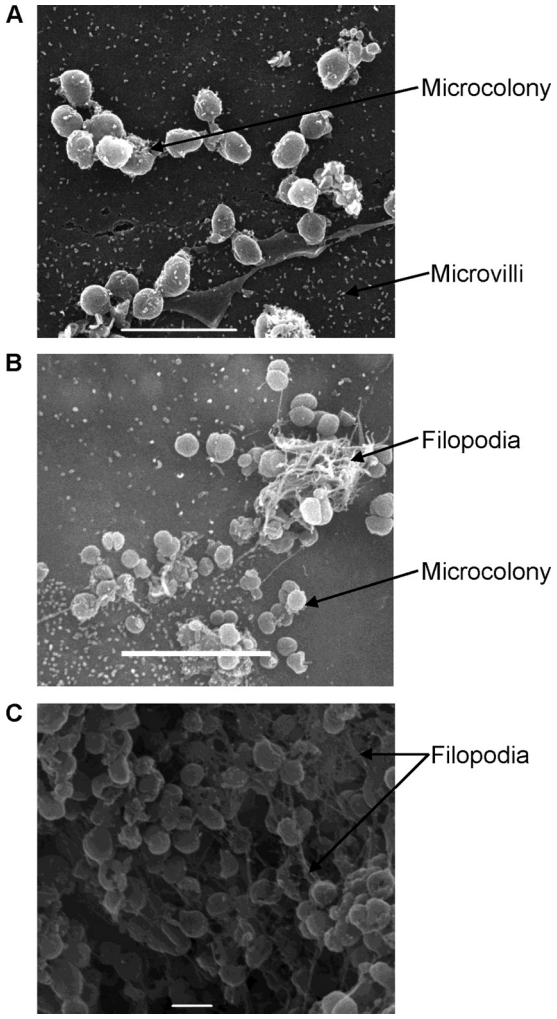

FIG 2 Scanning electron microscopy images of 16HBE14 cells cocultivated with *N. meningitidis* MC58. Images were taken at 10 h (A), 48 h (B), and 21 days (C) of cocultivation, with white bars indicating 5, 5, and 1 μm, respectively.

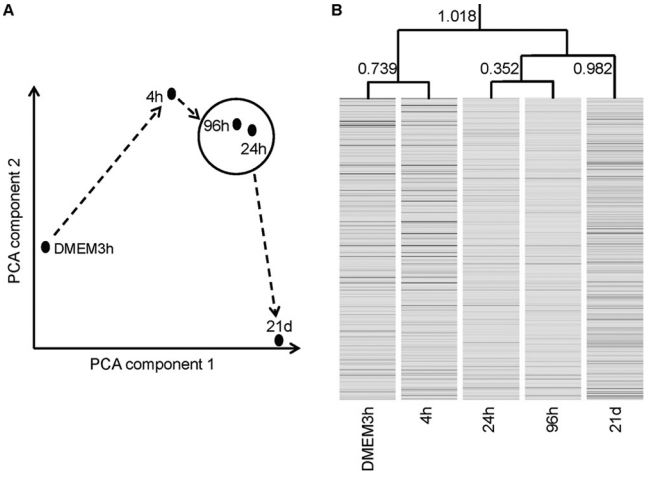

FIG 3 Comparison of meningococcal transcriptomes obtained at different cocultivation time points. (A) PCA. Solid dots in the plot represent the relative positions of samples at different cocultivation time points. Dashed arrows represent the passage of time. (B) Gene clustering with similarity between time points measured by Euclidean distance, as indicated.

(20, 21), entangled with the meningococcal microcolonies (Fig. 2B), as previously described (22). After 21 days, meningococci were found to be extensively entangled with filopodium-like membrane protrusions, with increased numbers of straight, fine, pilus-like filaments connecting bacteria (Fig. 2C). Comparison with images obtained at earlier time points (10 h and 48 h) indicates that the number of meningococci at 21 days was increased, which correlates with the increased number of adherent CFU at 21 days of cocultivation (see Fig. S4 in the supplemental material).

Reliability and comparability of transcriptomes. RNA samples prepared from adherent meningococci at each of four cocultivation time points (4 h, 24 h, 96 h, and 21 days) were reverse transcribed to cDNA, labeled, and hybridized to pan-*N. meningitidis* DNA arrays. Four biological replicates were made at each time point except the last, at which time there were 3 replicates. RNA samples from meningococci grown to mid-exponential phase (3 h in DMEM) were processed similarly and hybridized to the meningococcal DNA array to generate a reference transcriptome for later comparisons (termed the DMEM3h transcriptome). Transcriptomes at each time point were compared. Replicates for each time point were closely correlated, ranging from 83% to 99% (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). Correlations between time points ranged between 62% and 99% (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). The correlation between DMEM3h (control) and other samples ranged between 68 and 85%. The highest correlations were found, as expected, between biological replicates; the next highest correlation was found between transcriptomes at 24 h and 96 h, which was in turn higher than those between these time points and 4 h, with the 21-day transcriptomes being the least similar to those at earlier time points. This is interpreted as there being substantial transcriptional changes occurring early, followed by a period of relative transcriptional consistency before late changes supervene, plausibly reflecting a biologically relevant model of epithelial colonization. Transcriptomes from individual biological replicates were also compared by using principal-component analysis (PCA). PCA grouped biological replicates into three main clusters, and within each cluster, transciptomes for each time point group were closely related (see Fig. S5 in the supplemental material), which was in agreement with the correlation analysis (see the data at http://bugs.sgul.ac.uk/E-BUGS-141).

Overall transcriptome comparison between cocultivation time points: the transcriptome at 4 h was markedly different from those at prolonged coincubation times. Meningococcal transcriptomes recovered at each time point were consolidated and analyzed by using PCA. Transcriptomes at all meningococcal/epithelial cocultivation time points were clearly separated from the DMEM3h transcriptome with respect to both PCA components 1 and 2, representing 37% and 28.5% variances, respectively (Fig. 3A). Transcriptomes determined at the four cocultivation time points were shifted mainly along PCA component 2, with those at 4 h and 21 days being positioned at the top and bottom of the scale and those at 24 h and 96 h being clustered together at an intermediate position (Fig. 3A).

The four consolidated transcriptomes derived from cocultures were compared individually with the DMEM3h transcriptome (4

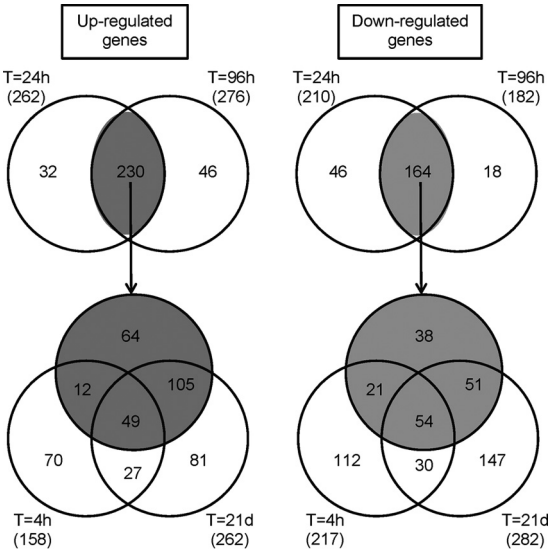

FIG 4 Venn diagrams showing numbers of differentially regulated genes coidentified at different cocultivation time points. The shaded circles in three-way Venn diagrams represent 230 up- and 164 downregulated genes coidentified at 24 h and 96 h (in two-way Venn diagrams). The total numbers of differentially regulated genes for each time point are indicated in parentheses.

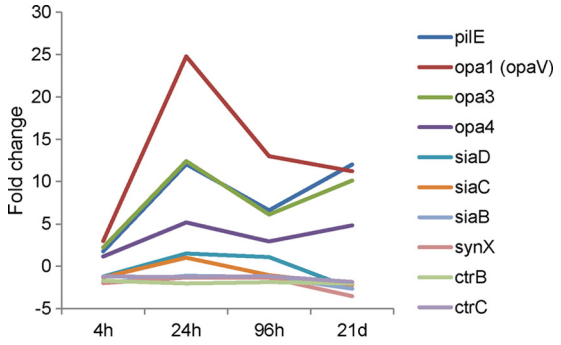

FIG 5 Transcriptional profiles of selected genes. Levels of meningococcal gene expression at different cocultivation time points (as indicated on the *x* axis) and for DMEM at 3 h were compared and are expressed as fold changes.

pairwise comparisons, e.g., 4 h versus DMEM3h), seeking significant differences in individual gene expression. A total of 819 genes that showed a >1.5-fold change with *P* values of <0.01 (after correcting for multiple comparisons) for at least 1 of the 4 pairwise comparisons were identified as being differentially expressed at various time points (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). (From here on, the terms up- and downregulated refer to these differences.) Using this selected set of genes and a clustering algorithm, we further established the Euclidean distance relation between transcriptomes recovered at different time points (Fig. 3B). Transcriptomes at 24 h and 96 h were most similar, with a distance of 0.352, while that at 4 h was closest to the reference at 3 h in DMEM (distance = 0.739). The transcriptome at 21 days was not similar to that at any other time point but was closer to the combined group at 24 h and 96 h (distance = 0.928) than anything else.

A total of 160, 262, 267, and 264 genes were upregulated, and 222, 210, 182, and 288 genes were downregulated, at 4 h, 24 h, 96 h, and 21 days, respectively; between 7% and 14% of 2,062 genes on our array platform were differentially regulated at any time point (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). A total of 230 genes were upregulated, and 164 genes were downregulated, at both 24 h and 96 h (Fig. 4). A total of 49 and 54 genes were up- and downregulated, respectively, at all time points from 4 h to 21 days compared to transcription after 3 h of incubation in DMEM (see the data at http://bugs.sgul.ac.uk/E-BUGS-141).

Differentially expressed genes were grouped functionally into Clusters of Orthologous Groups (COGs), defined by the U.S. National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) (see Fig. S6 in the supplemental material). Genes belonging to the groups "transcription," "translation," "posttranslational modification," and "cell wall/membrane biogenesis," mostly downregulated at 4 h, had different patterns of transcription at later times (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). There were 105 and 51 genes up- and downregulated, respectively, during prolonged (24 h, 96 h, and 21 days) coincubation (Fig. 4). Among the differentially expressed genes, three were identified as transcriptional regulators, *hfq* (*NMB0748*), *phoP* (also termed *misR*) (*NMB0595*), and *lrp* (*NMB1650*), all upregulated at later time points (24 h, 96 h, and 21 days), compared to a lower level of expression at 4 h (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). This suggests that there is substantial, genome-wide resetting of transcriptional activity in meningococci during a prolonged interaction with epithelial cells.

Up- and downregulated genes at different time points were further analyzed by using DAVID (14), a program for gene enrichment analysis according to associated functional annotations. Significant gene enrichment ($P < 0.01$ with Benjamini correction) for 5 functional groups was found in all upregulated gene lists (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). All 5 functional groups were associated with bacterial surface structures at all time points. A further two were enriched in surface structures only at 21 days (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). Among downregulated genes at 21 days, 11 functional groups were identified as being significantly enriched, with most being related to DNA modification (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). Here we focus on those meningococcal genes which encode proteins involved directly in epithelial interaction or encode outer membrane proteins with vaccine potential.

Genes involved in epithelial adhesion and invasion. Two groups of adhesins, type IV pili and opacity proteins, are considered to be crucial for adherence of meningococci to human epithelial cells (23). The *pilE* (*NMB0018*) gene, which encodes the major pilin subunit, together with 7 *pilS* genes (*NMB0019*, *NMB0020*, and *NMB0022* to *NMB0026*), was upregulated at all time points (Fig. 5) (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). Upregulation of *pilE* was marginal (1.7-fold) at 4 h but much higher at later time points (6.6- to 12-fold) (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). Other genes involved in pilus biogenesis (24, 25) were mostly not differentially regulated. Of the 25 genes encoding products potentially involved in pilus biogenesis and function, *pilP* (*NMB1811*), *pilV* (*NMB0547*), and *pilN* (*NMB1809*) were downregulated, and *pilT* (*NMB0768*) was upregulated, at various time points (see the data at http://bugs.sgul.ac.uk/E-BUGS-141).

opa1 (*opaV*) (*NMB0042*), *opa3* (*NMB1465*), and *opa4* (*NMB1636*), encoding opacity proteins previously shown to be

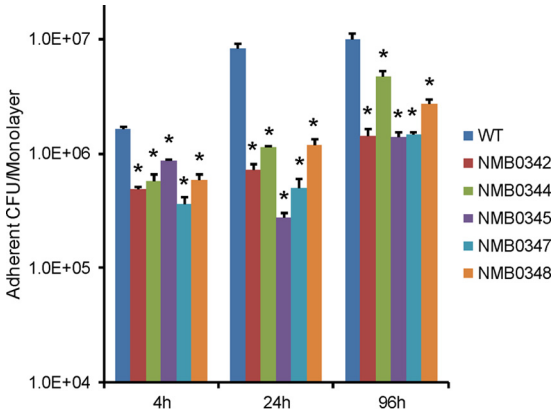

FIG 6 Epithelial cell adherence assay of meningococcal mutants. Monolayers of 16HBE14 cells were incubated with wild-type (WT) *N. meningitidis* MC58 and its isogenic mutants (indicated as NMB0342, NMB0344, NMB0345, NMB0347, and NMB0348, for the genes being disrupted) for 4 h, 24 h, and 96 h. Numbers of adherent meningococcal CFU were obtained from three biological replicates. Error bars indicate standard errors of the means. Asterisks indicate CFU with a significant difference ($P < 0.02$) between the corresponding mutant and the wild type.

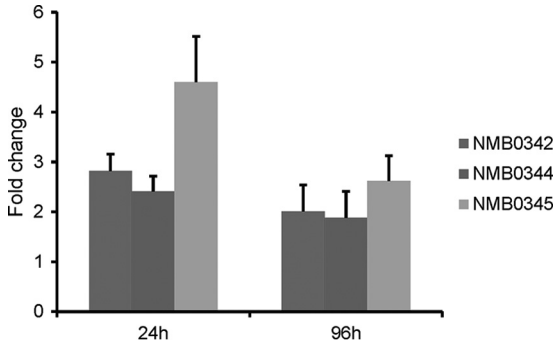

FIG 7 Comparison of gene expression using real-time RT-PCR. Independent biological RNA samples were prepared from MC58 cells harvested after 24 h and 96 h of cocultivation with 16HBE14 epithelial cells and compared to RNA samples from MC58 cells incubated for 3 h in DMEM alone (DMEM3h). The results of comparisons are expressed as fold changes. Error bars indicate standard errors of the means calculated from three biological replicates.

involved not only in adhesion but also in invasion of epithelial cells (26), were upregulated at all time points (Fig. 5) (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). Genes encoding other known adhesins (23), *opcA* (*NMB1053*), *nhhA* (*NMB0992*), *app* (*NMB1985*), *nadA* (*NMB1994*), *hrpA1* (*NMB0497*), and *hrpA2* (*NMB1779*), were not differentially expressed. *mspA* (*NMB1998*) was downregulated at all time points (see the data at http://bugs.sgul.ac.uk/E-BUGS-141).

NMB1965, encoding the L-glutamate transporter GltT, involved in epithelial cell invasion and intracellular survival (27, 28), was upregulated at 96 h (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). *NMB1963*, encoding a putative periplasmic transporter functioning on the same pathway and likely to be involved in epithelial cell adhesion/invasion (27), was upregulated at 24 h and 96 h (see the data at http://bugs.sgul.ac.uk/E-BUGS-141).

NMB0964, encoding the TonB-dependent zinc receptor ZnuD, involved in epithelial adhesion and invasion (29), was upregulated at 4 h and 96 h (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). *NMB1882*, also encoding a TonB-dependent receptor, was upregulated at 96 h but downregulated at 21 days (see the data at http://bugs.sgul.ac.uk/E-BUGS-141).

The majority of genes involved in meningococcal capsular polysaccharide biosynthesis and transport (*NMB0067* to *NMB0070*, *NMB0072*, and *NMB0073*), believed to negatively influence adherence and invasion of human epithelial cells (30), were downregulated at various time points (Fig. 5) (see the data at http://bugs.sgul.ac.uk/E-BUGS-141).

Expression of different lipooligosaccharide (LOS) phenotypes influences not only meningococcal survival in human blood (31) but also interactions with epithelial surfaces (32, 33). Genes involved in LOS biosynthesis, *lgtE* (*NMB1926*), *lgtA* (*NMB1927*), *lgt* (*NMB1072*), *lpxK* (*NMB0672*), and *lpxA* (*NMB0178*), were downregulated at various time points (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). Genes involved in LOS transport (34) were not differentially expressed, except for *lptB* (*NMB0356*), which was marginally upregulated at 24 h and 96 h (see the data at http://bugs.sgul.ac.uk/E-BUGS-141).

The products of *NMB0342* and *NMB0345* have been implicated in meningococcal epithelial cell adhesion, survival in human blood, and virulence (35, 36). *NMB0345* is recognized by sera obtained from individuals colonized by meningococci (2). These genes and other adjacent genes form the cluster *NMB0342–NMB0348*, all upregulated during prolonged coincubation with 16HBE14 epithelial cells, especially at 24 h and 96 h (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). We have chosen to characterize this locus further.

The *NMB0342–NMB0348* locus. Mutants were constructed in strain MC58 through insertion of a kanamycin-resistant cassette into the open reading frame of *NMB0342* (encoding intracellular septation protein A), *NMB0344* (encoding a BolA/YrbA family protein), *NMB0345* (encoding a predicted cell binding factor), *NMB0347* [encoding D-tyrosyl-tRNA(Tyr) deacylase], and *NMB0348* (encoding tRNA-dihydrouridine synthase A). Semiquantitative RT-PCR was performed to investigate the effect of each mutation on the expression of downstream genes; no polar effects were demonstrated (see Fig. S7 in the supplemental material).

There was no difference in growth between any of the mutants and wild-type strain MC58 in the DMEM used for cocultivation of meningococci with epithelial cells (see Fig. S8 in the supplemental material). The resulting mutants, as well as wild-type strain MC58, were tested for their ability to adhere to 16HBE14 cell layers at 4 h, 24 h, and 96 h. In comparison with MC58, each mutant strain demonstrated significantly ($P < 0.02$) reduced adherence, with the differences increasing at 24 h and 96 h (Fig. 6).

Transcription of *NMB0342*, *NMB0344*, and *NMB0345* was investigated by using quantitative real-time RT-PCR. Independent biological samples were prepared from meningococci (MC58) harvested after 24 h and 96 h of cocultivation with 16HBE14 epithelial cell layers and compared to samples from bacteria incubated for 3 h in DMEM alone. All three genes were upregulated, between 1.7- to 4.5-fold at both time points (Fig. 7).

Genes encoding surface antigens with vaccine potential. Sixteen genes encoding proteins identified by PSORTb (37) to be located in the bacterial outer membrane (and hence to have vaccine potential) were found to be differentially regulated (up- or downregulated) in the present study (Table 1). They included six genes which were previously investigated as meningococcal vaccine candidates. These

TABLE 1 Differentially expressed genes encoding outer membrane proteins[a]

Gene identification	Gene name	FC at each cocultivation time point			
		4 h	24 h	96 h	21 days
NMB0345			4.1	3.0	
NMB0382	*rmpM*	−2.7	4.8	2.6	2.7
NMB0460	*tbpB*	−1.6	−1.8	−1.7	−2.8
NMB0497		−2.1	−2.6	−2.8	−3.1
NMB0866		−2.7	−2.5	−1.9	−2.3
NMB1405					−2.4
NMB1429	*porA*	−2.8	5.0	4.2	2.3
NMB1541	*lbpB*	−1.5	−1.8	−1.7	−2.3
NMB1567			3.1	2.8	1.7
NMB1768		1.7	1.6		1.7
NMB1882				1.5	−1.7
NMB1969	*ausP*	−1.7	−1.7	−1.7	−1.7
NMB1988	*fetA*	−1.6			
NMB1998	*mspA*	−1.8	−1.6	−1.5	−1.7
NMB2039	*porB*	−4.0	3.7	2.4	4.2
NMB2050			−2.0	−2.2	

[a] Predicted by using the PSORTb algorithm. Only values with a *P* value of <0.01 are shown. *NMB0345*, *NMB0382*, *NMB1429*, and *NMB2039* were also identified previously by Williams et al. (2) (Table 2).

TABLE 2 Differentially expressed genes encoding immunoreactive proteins[a]

Gene identification	Gene name	FC at each cocultivation time point			
		4 h	24 h	96 h	21 days
NMB0018	*pilE*	1.8	12.1	6.6	12.0
NMB0128	*rplA*	−2.2	4.0	3.6	1.7
NMB0143	*rplD*	−1.6	10.0	7.3	1.7
NMB0345			4.1	3.0	
NMB0382	*rmpM*	−2.7	4.8	2.6	2.7
NMB0546	*adhA*	−2.6		1.8	
NMB0554	*dnaK*				2.5
NMB0604			2.1	2.2	
NMB0634	*fbpA*		3.5	2.5	
NMB1313	*tig*		2.1	1.6	1.6
NMB1429	*porA*	−2.8	5.0	4.2	2.3
NMB1533				1.8	
NMB1710	*gdhA*		3.5	2.9	1.5
NMB1972	*groEL*		1.9	1.5	2.0
NMB2039	*porB*	−4.0	3.7	2.4	4.2
NMB2159	*gapA-2*		2.0	2.8	

[a] Immunoreactive proteins were previously identified by using sera from individuals colonized with serogroup B meningococci (2). Only values with a *P* value of <0.01 are shown.

genes are *NMB1541* (encoding the lactoferrin binding protein LbpB) and *NMB0460* (encoding the transferring binding protein TbpB) (38–40), which were downregulated at all time points, and genes encoding PorA (NMB1429), PorB (NMB2039), and RmpM (NMB0382), recently shown to be present as protein complexes (41), which shared a different expression pattern: downregulated at 4 h and upregulated at later time points. *NMB1882*, identified as a candidate vaccine antigen on the strength of being upregulated during incubation in nonbactericidal human blood (13), was upregulated upon coincubation with 16HBE14 cells by 96 h but downregulated at 21 days.

Two further proteins of unknown function listed in Table 1, NMB0866 and NMB0497, were downregulated at all time points upon coincubation with 16HBE14 cells but were found previously by Hedman et al. (13) to be upregulated after 20 and 240 min of incubation in nonbactericidal human blood. Three more genes, *NMB1405*, *NMB1988*, and *NMB2050*, also downregulated at various time points in the present study, were also downregulated in blood (13).

NMB0345, *NMB1567*, and *NMB1768*, all encoding proteins of unknown function, were upregulated at various time points during prolonged cocultivation with 16HBE14 cells, while *NMB1969* and *NMB1998*, encoding autotransporters (13, 42, 43), were downregulated at all time points.

Grifantini et al. (10) carried out a short time course study otherwise comparable to ours, coincubating MC58 with 16HBE14 cells for up to 3 h. They identified over 50 genes that were highly upregulated at at least one time point in this period and investigated 9 of these genes by fluorescence-activated cell sorter (FACS) analysis. Two of the nine genes, *NMB0787* and *NMB0995*, were also upregulated in the present study, in each case not at 4 h but at 24 and 96 h and still at 21 days.

Williams et al. (2) identified 43 vaccine candidate genes through immunoreactivity of their products with sera obtained from individuals colonized by serogroup B meningococci. Sixteen of these genes were found to be upregulated in the present study (Table 2), with 15 of 16 being upregulated at all three later time points (24 h, 96 h, and 21 days), including *porA*, *porB*, *rmpM*, and *groEL*, previously found to also be highly immunoreactive to sera from immunized mice (41) or from convalescent meningococcal patients (44) in later studies. PorA is one component of the investigational serogroup B meningococcal vaccine 4CMenB (Bexsero) (45). However, genes encoding the three other major antigenic components, factor H binding protein (NMB1870), neisserial heparin binding antigen (NMB2132), and the adhesin NadA (NMB1994), were not found to be differentially expressed in our study.

DISCUSSION

Published transcriptomic studies of meningococci adherent to epithelial cells have until now been confined to short periods of cocultivation (up to 6 h) (9, 10). However, it is well known that meningococci colonize the human nasopharynx for weeks to months (1, 2), and therefore, such short-term adherence studies cannot throw light on the importance of meningococcal gene products that act later in the process of host-pathogen interactive biology. Here we have investigated differences over time in the transcriptome of serogroup B meningococci adherent to cultured respiratory epithelial cells in a prolonged-colonization model. Principal-component analysis indicates that transcriptomes obtained at 24 h, 96 h, and 21 days are quite different from that seen at 4 h (Fig. 3A). Transcriptomes at 24 h and 96 h appeared to be very similar to each other but substantially different from that at 21 days (Fig. 3B), comparisons emphasized in Venn diagrams of differentially expressed genes at the three different time points (Fig. 4). Since it is generally considered that where invasive disease occurs, meningococci invade soon after they arrive in the nasopharynx, the transcriptome at relatively early cocultivation time points may be more representative of the biological state of meningococci poised for invasion than that seen after prolonged cocultivation. In contrast, sustained upregulation upon prolonged

cocultivation may identify a meningococcal gene set relevant to the commensal state.

Comparing our results with those of previously reported studies of short-term cocultivation, 107 of the 347 meningococcal genes identified by Grifantini et al. (10) as being differentially regulated over 3 h of contact with 16HBE14 cell monolayers were also found in our differential gene lists (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). Of 55 genes that Grifantini et al. (10) found to be substantially upregulated at some point in the 180 min of their experiment, 18 were similarly expressed in our study. Seven were differentially expressed in the opposite direction, while the remaining 30 did not show significant differential expression at our time points (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). Differences in methodology, and in particular in the microarrays used, growth conditions for control samples, and the statistical algorithm employed, require caution in the interpretation of such comparisons. Agreement between studies should be seen more as hypothesis strengthening rather than proving a causative relationship between specific gene expression and biology. However, it is interesting to note that three genes involved in sulfur acquisition and metabolism (*cysD*, *cysJ*, and *cysN*), identified as being upregulated in the study by Grifantini et al. (10) and hypothesized to encode products involved in the epithelial infection process, were also upregulated in our study.

Grifantini et al. (10) used FACS analysis to detect meningococcal proteins on the bacterial surface, comparing adhering and nonadhering bacteria. *NMB0787* and *NMB0995*, encoding surface-expressed proteins upregulated in adherent organisms, were also upregulated in our study.

In a second reported short-term cocultivation study, Dietrich et al. (9) found 67 meningococcal genes upregulated during a 6-h cocultivation with HEp-2 cell monolayers. Twenty-one of these genes were also found to be upregulated in our gene list (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). However, differences in experimental settings, especially with different cell lines being used, likely account for the disparities between the present study and that of Dietrich et al. (9).

The modest correlation of our results with the results of those two studies should be seen in the context of the fact that only 6 genes identified as being substantially upregulated by Grifantini et al. (10) were also identified in the list of differentially expressed genes in the study by Dietrich et al. (9): *NMB0517*, *NMB0787*, *NMB0881*, *NMB0994*, *NMB0995*, and *NMB1017*. It is noteworthy that two of these six genes (*NMB0787* and *NMB0995*) in the set identified by Grifantini et al. (10) to be surface exposed in epithelial cell-adherent meningococci were highly upregulated in our study after cocultivation with 16HBE14 monolayers for >24 h (fold change [FC], 2.4 to 7.4) although not differentially expressed at 4 h. Focusing on *NMB0995*, Kuwae et al. (46), in agreement with our results, reported upregulation following infection of a human pharyngeal epithelial cell line only after 6 h, a protein not detected on the meningococcal surface at the early adhesion stage (2 h). The gene, termed *nafA* (for neisserial antiaggregation factor A) by those authors, encodes a surface-expressed protein which interacts with components of the meningococcal type IV pilus to prevent excess autoaggregation and the formation of microcolonies on epithelial surfaces during colonization. It is highly conserved in sequence between meningococcal strains, a *nafA* mutant is less virulent in mice following intraperitoneal challenge, and purified protein elicits bactericidal antibodies in mice (10, 46); these factors all establish its vaccine candidacy. Extending such studies, we have shown previously that *nafA* is upregulated in meningococci cocultivated with nonbactericidal fresh human blood (13).

In a third study, a serogroup B meningococcal carriage strain (alpha-710) or MC58 was cocultivated with FuDa epithelial cells for 6 h, and their resulting transcriptomes were compared (47). Eight of the 22 genes described previously by Joseph et al. (47) as being the most highly differentially regulated in MC58 were also differentially regulated in the present study. Seven of the eight genes were similarly upregulated (*NMB0879*, *NMB0995*, and *NMB1368*) or downregulated (*NMB0483*, *NMB1398*, *NMB1541*, and *NMB1753*) (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). It is noteworthy that *NMB0995* was identified in all studies (9, 10, 47) to be upregulated in MC58 in response to cocultivation with epithelial cells.

Considering meningococcal gene transcription during prolonged cocultivation, it is of note that the 16 differentially regulated genes encoding proteins previously identified as being immunoreactive to sera from individuals colonized by meningococci (2) were all upregulated for this period (Table 2), supporting the proposition that our system models growth conditions *in vivo* during the commensal state. The transcriptional regulators Hfq, PhoP (MisR), and Lrp were upregulated during prolonged (>24 h) cocultivation, establishing their importance in modulating genome-wide patterns of gene expression during established colonization. Hfq binds small RNAs and is widely involved in gene regulation: *hfq* mutants are attenuated for epithelial cell adherence (48). Employing proteomic approaches, two studies using meningococcal *hfq* mutants have identified a number of deregulated proteins (49, 50). Genes encoding some deregulated proteins were also found in our differentially regulated gene lists. These genes included *pilE*, *opa*, *fbpA* (*NMB0634*), *metH* (*NMB0944*), *glyA* (*NMB1055*), *gdhA* (*NMB1710*), *groEL* (*NMB1972*), *NMB0946*, *NMB1590*, and *NMB1796* (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). All were upregulated during prolonged coincubation (24 h, 96 h, and 21 days) but not at 4 h, as was *hfq* (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). The meningococcal two-component system PhoP/Q regulates a broad range of different genes (51, 52), and a *phoP* mutant is also attenuated for epithelial cell adherence (53). Meningococcal PhoP-regulated genes have been identified in serogroup B ($n = 117$) (52) and serogroup C ($n = 281$) (51). Of these genes, 23 reported by Tzeng et al. (52) and 45 reported by Newcombe et al. (51) were also found to be differentially regulated during prolonged (>24 h) cocultivation (see the data at http://bugs.sgul.ac.uk/E-BUGS-141). Neisserial Lrp is of unknown function, but in other Gram-negative bacteria, it regulates genes involved in various metabolic pathways, including pilus biogenesis (54). Our sustained cocultivation model should assist in delineating the role of these regulators in long-term epithelial cell colonization by meningococci.

In Fig. S6 in the supplemental material, the most enriched category of differentially expressed genes was translation, with most genes being initially downregulated at 4 h but being upregulated during prolonged (>24 h) coincubation with epithelial cells. A similar trend of differential regulation of genes was also observed for those genes categorized in the posttranslational modification, protein turnover, and chaperone categories. This suggests that there is increased protein synthesis activity during prolonged cocultivation. The opposite was observed for meningococci growing

in human blood (13), with genes involved in protein synthesis being increasingly downregulated at later cocultivation time points. Thus, there appears to be a similarity in protein synthesis activity in meningococci growing in blood and early (4 h), but not prolonged, cocultivation with epithelial cells.

Using another method of functional grouping of differentially expressed genes (DAVID), the most enriched functional categories were all related to meningococcal surface structures. The most upregulated (FC, 10 to 37) 10 genes at 21 days of cocultivation were those encoding pili (*pilE* and 5 *pilS*), opacity proteins (*NMB0442* and *NMB1465*), and two hypothetical proteins (*NMB0046* and *NMB1116*). Their upregulation was not as high at 4 h (FC, 1.65 to 5.1). It has been reported that, compared to 4 h of attachment to epithelial cells, pili are less exposed at the meningococcal surface at 9 h, allowing tight attachment through other adhesins, particularly opacity proteins (55). In contrast, we found upregulation of pilus genes at 21 days, and this correlated with high numbers of adherent meningococci (Fig. 2C; see also Fig. S4 in the supplemental material). It is likely that meningococci constantly colonize new areas of the epithelial monolayer as an adaptation to their environment. It may partly explain why meningococci are able to persist on the nasopharyngeal epithelia of an individual for weeks or months.

We were concerned to demonstrate that 16HBE14 cells in culture remained viable after prolonged coincubation (96 h and 21 days) with meningococci. At 48 h, light microscopic inspection of Giemsa-stained 16HBE14 and COR-L23 cells cocultured with meningococci revealed intact cells associated with many meningococci in the case of the former but dead/dying cells with very few meningococci in the case of the latter (Fig. 1; see also Fig. S4 in the supplemental material). Dead epithelial cells do not associate with meningococci to any substantial extent, a reflection of the fact that association is not nonspecific but receptor mediated (23). We interpret the even higher numbers of meningococci associated with 16HBE14 cells at the later time points to indicate the epithelial cells' continuing viability. A second line of evidence comes from the results of the gentamicin protection assay (see Fig. S4 in the supplemental material). Epithelial cells must be intact and metabolically active to afford protection for intracellular meningococci from externally applied gentamicin.

The five mutants of the *NMB0342–NMB0348* locus all showed significant reductions in epithelial cell adherence. As there was no polar effect of any of the gene disruptions studied, the defect in epithelial cell adherence for each mutant was attributable to disruption of the targeted gene. The biological functions of these genes are unknown, except that *NMB0345* is homologous to *cj0596* from *Campylobacter jejuni*, encoding a protein known to be important for epithelial cell adherence, colonization of mouse intestinal epithelium, and biofilm formation (56). Further work will be required to ascertain whether specific genes in the *NMB0342–NMB0348* locus have a role in outer membrane protein assembly, as was suggested for *cj0596* (57), and in promoting epithelial-bacterial cell interactions and/or colonization.

We have proposed previously (13) that meningococcal surface-expressed proteins encoded by genes that are relatively downregulated in the nasopharynx but upregulated in the bloodstream should be comparatively invariant in sequence and attractive candidate vaccine antigens. Three genes found to be upregulated upon coincubation with human blood (*NMB0497*, with a FC of 1.7; *NMB0866*, with a FC of 1.2; and *NMB1882*, with a FC of 2.3) (13) fulfilled this criterion. *NMB0497* and *NMB0866* were both downregulated at all time points in the present study, while *NMB1882* was downregulated at 21 days.

NMB0497, NMB0866, and NMB1882 contain 1, 6, and 12 peptide loops, respectively, predicted to project beyond the meningococcal outer membrane by PRED-TMBB (58), and each loop shows a high degree of sequence conservation between homologues from different meningococcal strains available at the KEGG database (http://www.genome.jp/kegg), compared by using ClustalW. If these proteins are surface located and abundant, they may prove to be useful components to broaden the coverage of existing vaccine formulations, such as the investigational MenB vaccine Bexsero (59), to prevent invasive infections caused by serogroup B strains of *N. meningitidis*.

Modeling of prolonged meningococcal colonization by epithelial cell cocultivation provides insight into gene expression during this important, but experimentally relatively inaccessible, phase of human infection. Comparison of transcriptomes obtained at early (4 h) and late (24 h, 96 h, and 21 days) cocultivation time points suggests that the regulators PhoP/Q, Hfq, and Lrp have a role in differentially regulating the meningococcal gene repertoire, enabling adaptation at different stages of colonization. Functional grouping showed that transcriptional changes in genes involved in protein synthesis at the early cocultivation time point (4 h) resembled those of meningococci growing in blood (13). This indicates that at an early stage of colonization, bacteria are metabolically adapted for growth in blood. The higher levels of expression of adhesin genes at later time points suggest their importance for prolonged nasopharyngeal epithelial cell colonization.

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