Meningococcal biofilm growth on an abiotic surface – a model for epithelial colonization?

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Neisseria meningitidis colonizes the human nasopharynx asymptotically, often for prolonged periods, but occasionally invades from this site to cause life-threatening infection. In the nasopharynx aggregated organisms are closely attached to the epithelial surface, in a state in which the expression of components of the bacterial envelope differs significantly from that found in organisms multiplying exponentially in liquid phase culture or in the blood. We and others have hypothesized that here they are in the biofilm state, and to explore this we have investigated biofilm formation by the serogroup B strain MC58 on an abiotic surface, in a sorbarod system. Transcriptional changes were analysed, focusing on alteration in gene expression relevant to polysaccharide capsulation, lipooligosaccharide and outer-membrane protein synthesis – all phenotypes of importance in epithelial colonization. We report downregulation of genes controlling capsulation and the production of core oligosaccharide, and upregulation of genes encoding a range of outer-membrane components, reflecting phenotypic changes that have been established to occur in the colonizing state. A limited comparison with organisms recovered from an extended period of co-cultivation with epithelial cells suggests that this model system may better mirror natural colonization than do short-term meningococcal/epithelial cell co-cultivation systems. Modelling prolonged meningococcal colonization with a sorbarod system offers insight into gene expression during this important, but experimentally relatively inaccessible, phase of human infection.

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INTRODUCTION

Neisseria meningitidis is an obligate human commensal known to cause meningitis and septicaemia in children and adolescents, with significant mortality and morbidity. Many healthy individuals may be colonized during their lifetime, with approximately 10% of the population carrying meningococci asymptotically in their nasopharynx at any one time (Caugant *et al.*, 1994; Maiden & Stuart, 2002). *N. meningitidis* and *Neisseria lactamica* isolates can colonize the nasopharynx of the same individual for prolonged periods, lasting up to 22 months (Ala'Aldeen *et al.*, 2000; Bennett *et al.*, 2005). The likelihood of colonization varies significantly between individuals, and very few develop invasive disease. The carriage of *N. meningitidis* and other commensal neisseriae is thought to lead to the induction of specific immunity

against meningococcal disease (Gold *et al.*, 1978; Goldschneider *et al.*, 1969). Understanding the biology of colonization and possible progression to invasion of the nasopharyngeal mucosa, from where bacteria may spread to the bloodstream and the meninges, causing sepsis and meningitis, is of great importance but there is a lack of tractable experimental models to study this early phase of infection. The most attractive of these, in which nasopharyngeal tissue explants collected at surgery are colonized experimentally with meningococci (Exley *et al.*, 2009), yield numbers of organisms too small for full-scale transcriptomal studies.

Micro-organisms which share the same ecological niche as *N. meningitidis* form biofilms – matrix-enclosed populations of bacteria adherent to each other and/or to surfaces (Costerton *et al.*, 1995) – as adjudged by electron microscopy of biopsy samples of chronically infected adenoidal mucosa from paediatric patients (Coticchia *et al.*, 2007). The biofilm state is believed to confer a number of advantages on the bacteria, including increased resistance to antimicrobial agents and host immune responses (Costerton *et al.*, 1987; Davies, 2003). Biofilm formation has been proposed as a mechanism which

Abbreviations: LOS, lipooligosaccharide; OMV, outer-membrane vesicle.

The array design and fully annotated microarray data for this study are available in BμG@Sbase (accession no. E-BUGS-75; <http://bugs.sgul.ac.uk/E-BUGS-75>) and ArrayExpress (accession no. E-BUGS-75).

Two supplementary tables, listing the genes up- and downregulated in *N. meningitidis* MC58 wild-type and its capsule-negative mutant, are available with the online version of this paper.

enables bacteria to achieve long-term mucosal colonization of the host (Luke *et al.*, 2007). Biofilms form through a sequence of ordered events: attachment, maturation, maintenance and dissolution. The time taken to form a biofilm varies between bacteria; a structure interpreted to be a meningococcal biofilm has been reported to form as early as 12 h after application of organisms to glass coverslips (Yi *et al.*, 2004).

An *in vivo* model for the study of biofilm formation has yet to be developed but various *in vitro* abiotic surface models have been studied. These can be categorized as 'static' (for example incubation of micro-organisms on plastic surfaces); or 'flow', where a constant supply of fresh nutrients and hydrodynamic shear forces are applied. So-called biofilms formed by meningococci have been investigated in two studies (Lappann *et al.*, 2006; Yi *et al.*, 2004); both focused on important bacterial determinants of host-microbial interaction. Extrapolating from their finding that capsulate strains (in contrast to a capsule null mutant) did not apparently form an appreciable biofilm in their flow model, Lappann *et al.* (2006) suggested that polysaccharide capsule inhibits development of the biofilm state. Yi *et al.* (2004) suggested that meningococci isolated from the carriage state are better able to form biofilms than invasive isolates, ascribing this to different levels of capsule expression. Lipooligosaccharide (LOS) biosynthesis has also been investigated in the context of meningococcal biofilm formation, with reduction in full-length LOS shown to increase the quantity of biofilm formed (Yi *et al.*, 2004).

Here we report the transcriptional profile of *N. meningitidis* MC58 wild-type and its capsule-deficient mutant growing on an abiotic surface, providing insight into the biofilm state in which they persist, and into the extent to which this model reflects the interactive biology of *N. meningitidis* and the host epithelial surface.

METHODS

Bacterial strains and growth conditions. *N. meningitidis* MC58 (serogroup B:15:P1.7.16b; ST-74) and its isogenic capsule-negative strain (cap⁻) (Virji *et al.*, 1995) were used. Neisserial strains were routinely propagated at 37 °C with 5% CO₂ on GC agar (Difco) supplemented with 1% Vitox (Oxoid), or at 37 °C in Mueller-Hinton (MH) broth (Oxoid) supplemented with 1% Vitox (sMH).

Microtitre plate assay. The microtitre plate assay was performed by modification of the published method (O'Toole *et al.*, 1999). This assay is based on the ability of bacteria to form biofilms on polystyrene (Cellstar). A 16 h plate of *N. meningitidis* was harvested into sMH, adjusted to an OD₆₀₀ of 0.05 and inoculated into polystyrene 96-well plates (Cellstar). The plates were incubated statically for 24 h and then rinsed with distilled water, baked for 1 h at 60 °C and stained with crystal violet (Pro-Lab Diagnostics). The crystal violet was solubilized by adding ethanol/acetone (80:20, v/v) and the biofilms were quantified by measuring A₆₀₀.

Sorbarod model and RNA extraction. The sorbarod experimental system has been used to establish continuous culture biofilms of

Streptococcus pneumoniae, *Staphylococcus aureus* and *Pseudomonas* spp. (Hodgson *et al.*, 1995; Waite *et al.*, 2001). Meningococcal sorbarod biofilms were prepared by modification of the published method (Hodgson *et al.*, 1995). Cellulose sorbarods (Ilacon, Tonbridge, Kent, UK) consist of cylindrical paper sleeves (10 mm in diameter and 20 mm in length) encasing compacted concertinas of cellulose fibres. The packed cellulose filling provides a large surface area for bacterial adhesion whilst the extensive inter-fibre spaces avert system blockage. The sterilized sorbarod was housed in an enclosed glass unit (Fig. 1), manufactured to our design by Terri Westlake (Artistic and Scientific Glassblowing, Kidlington, Oxfordshire, UK) for use in a class I safety cabinet within a biosafety level 3 containment facility. A 16 h plate culture of *N. meningitidis* MC58 was harvested into sMH and the concentration adjusted to 10⁸ c.f.u. ml⁻¹. The sorbarod, pre-wetted with 1 ml sMH, was inoculated with 1 ml of this suspension, following which sMH was delivered continuously at a rate of 0.1 ml min⁻¹ by means of an eight-channel 205S peristaltic pump (Watson Marlow). Temperature control was achieved by means of the water jacket, attached to a 37 °C recirculating water bath. At designated times biofilm effluent was collected and the viable bacterial count determined. Following initial loss of loosely attached cells into eluted medium, steady-state conditions (approx. 10⁸ c.f.u. per sorbarod and 10⁷ c.f.u. per ml) were achieved where the adherent biomass and the rate of cell release from the membrane remained constant. Steady state was attained after 24 h. After 48 h, individual sorbarods were harvested into 3 ml RNA protect (Qiagen) and the fibres disaggregated by vortexing. RNA was extracted from the bacteria recovered, and from 5 ml of the inoculation culture, using a FastRNA Pro Blue kit (Q BioGene/MP Biochemicals). Samples were further treated with DNase I (Invitrogen) and cleaned with an RNeasy

Fig. 1. Schematic representation of the glass sorbarod apparatus. The unit is clamped upright in a class I microbiological safety cabinet (Envair) and fresh medium is delivered from above. Eluted spent medium and dispersed cells are collected in a reservoir for investigation or safe disposal.

kit (Qiagen). Experiments were repeated five times on different days (biological replicates).

Light microscopy studies of meningococcal biofilm on epithelial cells and sorbarod. Confluent monolayers of 16HBE14, a SV40-transformed human bronchial epithelial cell line (Gruenert *et al.*, 1988) were used as previously (Grifantini *et al.*, 2002) for investigation of bacterial–host cell interaction. Cells were seeded at a density of 2×10^5 per well on to glass slides placed in individual wells of a 24-format microtitre plate, and incubated without agitation in DMEM supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (Life Technologies) at 37 °C, in an atmosphere of air supplemented with 5% CO₂. Suspensions of plate-grown bacteria in fresh medium were added to confluent epithelial cells (confluence checked microscopically) at an m.o.i. of 100:1, and the plates were incubated under the same conditions. The cells were washed and refreshed with culture medium at 6, 18 and 24 h post-infection. The slides were fixed in 4% formaldehyde, stained with Giemsa solution and photographed with an Olympus BH2-RFCA.

Light microscopy studies of the sorbarod biofilm were carried out in order to demonstrate the presence of biofilms. Intact sorbarod filters were fixed in 4% formaldehyde overnight, dehydrated by passage through sequential alcohol/xylene mixtures, and then embedded in paraffin wax. Sections were stained with the Gram stain and were photographed at $\times 400$ magnification with an Olympus BH2-RFCA.

DNA microarray hybridization and analysis. RNA samples were quantified using NanoDrop 1000 (NanoDrop Technologies) and confirmed by electrophoresis on agarose gels. Labelling of RNA and DNA samples (5 µg total RNA, 1 µg DNA), microarray hybridization and washing were carried in accordance with a method previously published by the Bacterial Microarray Group at St George's (Bugs) (Bacon *et al.*, 2004). The Pan-Neisseria array slides (obtained from Bugs) have previously been described (Stabler *et al.*, 2005). The array design is available in Bug@Sbase (accession no. E-BUGS-75; <http://bugs.sgul.ac.uk/E-BUGS-75>) and also ArrayExpress (accession no. E-BUGS-75). Each array slide was co-hybridized with one of the Cy5-labelled experimental samples and Cy3-labelled genomic DNA from *N. meningitidis* MC58 (universal control). Slides were scanned using GenePix-4000B (Axon Instruments) and the scanned images were analysed with GenePix Pro 6.0 (Axon). Statistical analysis was carried out using Genespring GX 7.3 (Agilent). Background-subtracted spot intensities resulting from hybridization of Cy5-labelled cDNA derived from meningococcal RNA (signal channel) were divided by the intensity of the signal derived from Cy3-labelled genomic DNA (control channel). Log-transformed ratios were normalized by applying the intensity-dependent data analysis technique LOWESS, using 20% of the data for smoothing. Only genes not marked absent by GenePix Pro analysis were included. If the value for the reference channel was less than 10 then a value of 10 was used instead. Data arising from the five biological replicate experiments were pooled as follows.

For each gene, the normalized log-transformed ratio arising from samples harvested from the sorbarod system at 48 h (T_{48}) was normalized to the median value, as was that arising from the initial inoculum samples (T_0). These were combined to give a ratio T_{48}/T_0 . Differentially expressed genes were selected using Student's *t*-test, with multiple testing correction (Benjamini and Hochberg False Discovery Rate), at a *P*-value cut-off 0.01. Fully annotated microarray data have been deposited in Bug@Sbase (accession number E-BUGS-75; <http://bugs.sgul.ac.uk/E-BUGS-75>) and also ArrayExpress (accession number E-BUGS-75).

Validation of microarray results using quantitative real-time RT-PCR. RNA samples from three fresh biological replicates were

prepared as described above, and were reverse-transcribed to first-strand cDNA using Superscript III and random primers (Invitrogen). Real-time PCR was performed using TaqMan technologies, TaqMan Universal PCR Master Mix and the ABI 7700 system (Applied Biosystems). The primers were designed and manufactured using Assay-by-Design (Applied Biosystems) and are listed in Table 1. Reporter dye and quencher were FAM and NFQ, respectively. We used 10 ng total RNA per reaction. Three controls were chosen: NMB0107 and NMB1347 (not differentially expressed in our study), and 16S rRNA coding sequence. Statistical analysis was as described in Applied Biosystems User Bulletin no. 2 (P/N 4303859).

LPS preparation and electrophoresis. *N. meningitidis* MC58 was harvested from a sorbarod at 48 h or from a 16 h plate culture of *N. meningitidis* and resuspended in PBS to an OD₆₀₀ of 0.9. The protein and DNA concentration in each sample was measured [Pierce Micro BCA Protein Assay; NanoDrop 1000 (NanoDrop Technologies)], and cells corresponding to 10 µg protein were pelleted, resuspended in 20 µl lysis buffer (0.1 M Tris pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10%, v/v, glycerol and bromophenol blue) and boiled for 10 min. Samples were run on a Tris/Tricine gel (Schagger & von Jagow, 1987) and bands corresponding to LOS visualized by silver staining (Tsai & Frasch, 1982).

Analysis of meningococcal RNA extracted from bacteria closely associated with epithelial cell monolayers using quantitative real-time RT-PCR. Preparation of monolayers of 16HBE14 cells, bacterial infection and co-incubation, intermediate washing and refreshing with culture medium were as described for the light microscopy studies, except that cultures were incubated in tissue culture flasks (75 cm²) and the final wash was carried out at 28 h post-infection. In four independent experiments (biological replicates) the human cells were lysed with 1% saponin (Sigma) in ice-cold medium and incubated on ice for 10 min, followed by vigorous pipetting. An equal volume of RNAProtect (Qiagen) was added, and the bacteria were pelleted by centrifugation and subjected to RNA extraction and DNase I treatment as described above. The quality and quantity of RNA samples were analysed using the NanoDrop 1000 and Bioanalyser 2100 (Agilent). All RNA samples were found to be contaminated with eukaryotic RNA, and the concentration of neisserial RNA was calculated using the intensity ratio of 16S/18S peaks. Input neisserial RNA of 15 ng was used for each PCR. TaqMan real-time PCR was carried out as described above. Ribosomal 16S RNA was used as internal reference to achieve a normalized value for each gene in a given sample. Levels of gene up- and downregulation were calculated by comparing the normalized values from 28 h post-infection and initial inoculum samples.

RESULTS

Establishing biofilms in static and continuous-flow systems with wild-type and capsule-null meningococci

We initially tested the serogroup B wild-type strain *N. meningitidis* MC58 and an isogenic *siaD*-null (cap^-) strain for the ability to form a mature biofilm in a static abiotic-surface model. The assay is based on visualization of bacterial adherence to the polystyrene surface of a microtitre plate and provides a means of screening many strains or different cultures simultaneously. While *N. meningitidis* MC58 wild-type and cap^- both demonstrated biofilm formation, MC58 cap^- produced twice as much

Table 1. Sequence of primers and probes used for TaqMan real-time RT-PCR

Gene number/name	Primer type	Primer/probe sequence
NMB0070/ <i>siaA</i>	Forward primer	GCCGTTTTAGGCAATACCATTACG
	Reverse primer	CCGTGAATCATGACCATATCAGGTT
	Reporter probe	ATCAGATAGACGAGAGATAAA
NMB0072/ <i>ctrB</i>	Forward primer	CTGGATCAGGTGAAAGCAGTCA
	Reverse primer	ACGCGCCTGCAAACC
	Reporter probe	ATCTGCGGATTCTCC
NMB1870/ <i>ponA</i>	Forward primer	AAACGCTTTTACCGGCATTGG
	Reverse primer	GCCGGACACGACATTGC
	Reporter probe	AACACCCCAAACATCC
NMB0107/ <i>pyrI</i>	Forward primer	GTGCAGAAGCGGCATTTGA
	Reverse primer	TCGGACAGCGGAACACTTC
	Reporter probe	ACCTGCCCAGCGAAAT
NMB1347/ <i>SuhB</i>	Forward primer	CGAACGCAACGACGTATATATGG
	Reverse primer	GTTTCGATGCGGTTGAAAACG
	Reporter probe	AAAGGCGCGTTGCTCA
NMB0663/ <i>nspA</i>	Forward primer	GCCGGCTACCGCTACAA
	Reverse primer	GCCGGAACGGACGTTTT
	Reporter probe	CTACATCGGCAAAGTC
NMB1053/ <i>opcA</i>	Forward primer	ATTGCCCTGACCGGTACTG
	Reverse primer	CGGTGTGGACGGTAAACTCATTA
	Reporter probe	ACAAGAGCTTCAAACCGC
NMB0586	Forward primer	CCGTCAAACAAAGCAAAGTATCCT
	Reverse primer	GCCTTCTTCTTCGGCTTTGAG
	Reporter probe	AAGCGACCAAAGGCA
NMB0390/ <i>mapA</i>	Forward primer	GCGTTTCAGACAGTCTCTCCTA
	Reverse primer	TCCACGCCGAAGGGATTG
	Reporter probe	CATTGCCGCCCAAACC
NMB0345	Forward primer	GACAAGAAACCGTCCTTCAAAACC
	Reverse primer	GGCGATATGCAATGCGTATGC
	Reporter probe	CCGTTCAAGCCATATTT
NMB1875	Forward primer	GTACAACCCAGCCCGATATTCC
	Reverse primer	GGGCGGTACGGTAACCT
	Reporter probe	CCGCCACCCCTGCTG
NMB1279	Forward primer	CTATGCGGGCGCAATGG
	Reverse primer	TCCGTCCCCGTCATAATCCA
	Reporter probe	TCGAGCTACCGGAAATG
NMB1533	Forward primer	TGCCAAAGCTGAAGACATGGA
	Reverse primer	ATCAGGTTTGACATAGTCGGTATCG
	Reporter probe	ACGCCGTCTTTAAATAC
16S	Forward primer	GCTAATACCGCATACGTCTTGAGA
	Reverse primer	TCGGCCGCTCGAATAGC
	Reporter probe	AAGTCCCCTGCTTTCT

biofilm as the wild-type strain, adjudged by the microtitre plate assay (data not shown). The sorbarod model system was exploited to establish a culture with continuous flow of medium using conditions previously described for *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Lactobacillus acidophilus* and *Gardnerella vaginalis* (Budhani & Struthers, 1998; Muli & Struthers, 1998). After 48 h, sorbarods were examined by light microscopy. Substantial accumulations of meningococci were seen between the fibres of the cellulose matrix (not shown), very similar to those seen with *L. acidophilus* or *G. vaginalis* (Muli & Struthers, 1998).

Transcriptome analysis to identify genes differentially regulated in formation of a mature biofilm

RNA samples extracted from the culture used to inoculate the sorbarod, or from a mature (48 h) biofilm, were labelled with Cy5 dye and co-hybridized to arrays with Cy3-labelled genomic DNA for comparative transcriptome profiling. Five independent biological replicates were analysed for each strain at each time point. Results were amalgamated for statistical analysis and are summarized in Supplementary Table S1 for *N. meningitidis* MC58 wild-type and Supplementary Table S2 for *N. meningitidis* MC58

cap⁻. The distribution of genes with altered regulation was found to be asymmetrical: a larger number were altered in MC58 wild-type compared to the cap⁻ strain. In the wild-type genome, 31.5 % (702 genes) showed altered transcription [16.2 % (361 genes) upregulated, 15.3 % (341 genes) downregulated], compared with 8.6 % (191 genes) of the cap⁻ genome [3.7 % (82 genes) upregulated, 4.9 % (109 genes) downregulated]. Of the 150 genes that were differentially regulated in both wild-type and cap⁻ strains, 34.2 % of the wild-type gene complement was altered in regulation (17.8 % up-, 16.8 % downregulated), and 9.3 % of the cap⁻ gene complement (4 % up-, 5.3 % downregulated). Sixty-nine of the upregulated and 81 of the downregulated genes were altered in the same direction in wild-type and cap⁻ strains.

The three largest functional groups of genes which were upregulated in wild-type *N. meningitidis* forming a biofilm were those classified as encoding proteins involved in amino acid synthesis, energy metabolism and cell wall/membrane biogenesis (Table 2). Unsurprisingly, genes encoding the many proteins still annotated as hypothetical were also strongly represented. The most significant up/downregulated genes are listed in Tables 3 and 4 respectively. We have chosen to focus on the expression profile of *N. meningitidis* MC58 wild-type; and in particular on the effect biofilm formation

has on capsule, outer-membrane vesicle and LOS production.

Biofilm development is associated with downregulation of capsulation genes in MC58 wild-type

The elaboration of meningococcal capsule depends on expression of genes directing polysaccharide biosynthesis, modification and transport. Polysaccharide biosynthesis genes as follows were found to be significantly downregulated in the wild-type strain (fold change in parentheses): *synX* (*siaA*) (-2.64, $P=0.001589$), *siaB* (-2.47, $P=0.001624$) and *siaC* (-2.22, $P=0.001369$). *siaD* was not represented on the microarray. Polysaccharide export genes *ctrC* (-1.93, $P=0.0000133$) and *ctrD* (-1.66, $P=0.001208$) were significantly downregulated; *ctrA* (-1.68, $P=0.0101$) and *ctrB* (-1.66, $P=0.0165$) appear also to be downregulated, although the result in each case is on the borderline of the chosen *t*-test *P*-value cut-off of 0.01. Synthesis of polysaccharide capsule is abrogated in *N. meningitidis* MC58 cap⁻ through inactivation of *siaD* with an antibiotic resistance cassette. In striking contrast to the situation in the wild-type, only a single one of the remaining capsulation genes – *ctrD*, involved in polysaccharide export – was found to be downregulated (-1.86, $P=0.0000836$). None were upregulated.

Table 2. Significantly up- and downregulated genes in each functional group during biofilm growth

Functional classification	Upregulated genes in biofilm		Downregulated genes in biofilm		No. of genes
	No.	%	No.	%	
Amino acid transport and metabolism	33	26	7	5	129
Carbohydrate transport and metabolism	19	40	3	6	48
Cell cycle control, mitosis and meiosis	9	35	1	4	26
Cell motility	3	15	4	20	20
Cell wall membrane biogenesis	24	21	12	10	117
Coenzyme transport and metabolism	15	21	0	0	73
Defence mechanism	2	13	1	7	15
Energy production and conversion	37	35	10	9	106
Inorganic ion transport and metabolism	12	15	6	8	80
Intracellular trafficking and secretion proteins	7	11	15	24	62
Lipid transport and metabolism	15	33	1	2	46
Nucleotide transport and metabolism	9	23	2	5	39
Post-translational modification, protein turnover, chaperones	12	18	10	15	65
Replication, recombination and repair	10	8	12	9	131
RNA processing and modification	0	0	1	100	1
Secondary metabolites biosynthesis, transport and catabolism	6	27	4	18	22
Signal transduction mechanisms	6	19	5	16	31
Transcription	7	11	20	31	64
Translation	16	13	16	13	126
Not in COGs*	94	13	182	25	715
Function unknown	26	21	18	14	126
General function prediction only	27	17	19	12	162

*COGs, clusters of orthologous groups of proteins.

Table 3. Top 50 upregulated genes of *N. meningitidis* MC58 during biofilm growth on a sorbarod compared with growth on an agar plateGenes with a >2.0 fold change in expression are shown ($P < 0.01$).

Gene and functional group	Function and/or product	Fold change	P-value
Energy production and conversion			
NMB2052	Ubiquinol-cytochrome <i>c</i> reductase, cytochrome <i>b</i>	6.14	1.17E-06
NMB1968	Aldehyde dehydrogenase A*	4.08	0.001822
NMB1613	Fumarate hydratase, class I	3.99	3.97E-05
NMB2053	Ubiquinol-cytochrome <i>c</i> reductase, iron-sulfur subunit	3.99	1.24E-05
NMB2051	Ubiquinol-cytochrome <i>c</i> reductase, cytochrome <i>c</i> ₁	3.82	7.19E-05
NMB1724	Cytochrome <i>c</i> oxidase, subunit II	3.55	2.22E-06
NMB1518	Acetate kinase*	3.39	7.41E-04
NMB1677	Cytochrome <i>c</i> ₅	3.32	4.18E-07
NMB1341	Pyruvate dehydrogenase, E1 component*	3.22	2.38E-05
NMB1723	Cytochrome <i>c</i> oxidase, subunit III	3.14	1.27E-05
NMB0631	Phosphate acetyltransferase Pta, putative*	2.98	0.001426
NMB0543	L-Lactate permease, putative	2.91	0.002419
NMB2060	Glycerol-3-phosphate dehydrogenase (NAD ⁺)	2.76	9.56E-05
Carbohydrate transport and metabolism			
NMB1457	Transketolase*	5.22	2.96E-06
NMB0390	Maltose phosphorylase*	4.95	2.14E-05
NMB0389	Aldose 1-epimerase	3.47	1.28E-04
Amino acid transport and metabolism			
NMB0763	Cysteine synthase*	4.52	5.52E-09
NMB0540	Aspartate aminotransferase	3.34	3.26E-05
NMB0944	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase*	2.97	2.33E-04
NMB1498	Aspartokinase, α and β subunits	2.88	9.56E-07
NMB1609	Trans-sulfuration enzyme family protein	2.67	1.79E-06
Cell wall/membrane biogenesis			
NMB0182	Outer-membrane protein Omp85*	4.26	1.42E-05
NMB0663	Outer-membrane protein NspA*	2.72	3.20E-04
Inorganic ion transport and metabolism			
NMB0257	NADH dehydrogenase I, L subunit	3.40	5.19E-06
NMB0784	Phage shock protein E precursor, putative	3.27	6.65E-07
NMB0586	Adhesin, putative	3.04	6.18E-06
NMB1946	Outer-membrane lipoprotein*	2.84	2.06E-05
NMB1271	Mercury transport periplasmic protein, putative	2.78	2.72E-04
Post-translational modification, protein turnover, chaperones			
NMB1972	Chaperonin, 60 kDa*	2.90	5.89E-04
Nucleotide transport and metabolism			
NMB1855	Carbamoyl-phosphate synthase, large subunit*	2.74	7.52E-04
NMB1439	Phosphoribosylaminoimidazole carboxylase, catalytic subunit	2.71	2.83E-04
Cell motility			
NMB0051	Twitching motility protein	2.70	4.29E-04
Unknown, general prediction			
NMB0391	β -Phosphoglucomutase	6.00	5.80E-04
NMB1557	Conserved hypothetical protein*	5.09	4.72E-04
NMB0783	Conserved hypothetical protein*	3.95	4.92E-05
NMB1286	Conserved hypothetical protein	3.88	6.21E-07
NMB0388	Sugar transporter, putative	3.84	1.04E-04
NMB0604	Alcohol dehydrogenase, zinc-containing*	3.41	9.02E-04
NMB0010	Phosphoglycerate kinase*	3.35	1.52E-04
NMB1053	Class 5 outer-membrane protein*	3.32	2.67E-05
NMB1035	Hypothetical protein	3.31	0.001628
NMB2091	Haemolysin, putative*	3.15	2.10E-04

Table 3. cont.

Gene and functional group	Function and/or product	Fold change	P-value
NMB1870	Hypothetical protein*	3.10	3.26E-05
NMB0035	Conserved hypothetical protein*	2.97	2.74E-06
NMB1073	Conserved hypothetical protein*	2.93	9.23E-05
NMB1523	Hypothetical protein	2.82	5.20E-05
NMB2005	Glutamate <i>N</i> -acetyltransferase/amino-acid acetyltransferase	2.79	0.00111
NMB0278	Thiol: disulfide interchange protein DsbA*	2.73	0.001977
NMB1533	H.8 outer-membrane protein*	2.67	5.34E-06
NMB1438	Conserved hypothetical protein	2.67	4.11E-06

*Asterisk indicates genes encoding proteins which are present in OMVs.

Genes encoding proteins found in outer-membrane vesicles are significantly upregulated in a mature biofilm

Aggregates of the outer-membrane vesicles (OMVs) elaborated by many Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Shewanella oneidensis*, *Escherichia coli* and *Azotobacter* sp., are found within their respective biofilm matrices (Schooling & Beveridge, 2006). Three recent studies have set out to identify the set of proteins contained in meningococcal OMVs from different serogroup B strains (Ferrari *et al.*, 2006; Vaughan *et al.*, 2006; Williams *et al.*, 2007). Nearly half of the set was found to be differentially expressed in the sorbarod biofilm: 41.9% of genes identified as encoding constituents of detergent-extracted OMVs of *N. meningitidis* MC58 (Williams *et al.*, 2007) (39/112 genes upregulated, 8/112 genes downregulated), 42.8% of genes identified as encoding constituents of detergent-extracted OMVs of *N. meningitidis* K454 (Vaughan *et al.*, 2006) (41/126 genes upregulated, 13/126 genes downregulated) and 50.7% of genes identified as encoding constituents of OMVs spontaneously released into culture supernatant by *N. meningitidis* MC58 ΔNMB0033 (*gna33*) (Ferrari *et al.*, 2006) (32/65 genes upregulated, 1/65 genes downregulated). Many of the upregulated genes were found to be highly expressed during growth of the meningococcal biofilm (24/50 genes); these are highlighted with an asterisk in Table 3.

Biofilm development is associated with downregulation of genes encoding components of core LOS

Heptose biosynthesis in *N. meningitidis* is complex, with several proposed pathways (Shih *et al.*, 2001). Of seven gene products so far considered to be involved in heptose synthesis, and in adding L-glycero-D-manno-heptose to the Kdo₂-lipid A substrate which forms the inner-core structure of meningococcal LOS, four demonstrated altered expression in our meningococcal biofilm model. Three genes were downregulated during growth of *N. meningitidis* wild-type biofilm (fold change in parentheses)

– *rfaE*/NMB0825 (−1.90971, *P*=0.000282), *rfaD*/NMB0828 (−1.93049, *P*=0.0000428) and *aut*/NMB2076 (−1.5889, *P*=0.002796) – while *gmhA*/NMB2090 (1.845019, *P*=0.0000132) was upregulated. In silver-stained Tris/Tricine gels, organisms recovered from biofilms showed a substantial reduction in LOS compared to the amount produced by the input organisms (Fig. 2). Results were normalized to constant protein concentration, rather than DNA, which was present in anomalous (1.4-fold) excess in the biofilm samples.

Validation of microarray results using real-time RT-PCR

Real-time RT-PCR was used to confirm our findings of transcriptional alteration using microarrays. Nine genes of interest were selected: capsulation genes *synX* (*siaA*) (NMB0070) and *ctrA* (NMB0072); genes encoding proteins known to be present in OMVs, *opcA* (NMB1053), *mapA* (NMB0390) and the putative cell-binding factor NMB0345; genes encoding candidate vaccine antigens *nspA* (NMB0663) and lipoprotein NMB1870; and genes encoding a putative adhesin (NMB0586) and a hypothetical protein (NMB1875). RNA samples from three fresh biological replicates (independent of samples used for microarray analysis) were studied. The relative levels of gene expression in mature biofilm samples compared to initial inoculum samples were calculated, and the results confirmed congruence of the two approaches (Fig. 3).

After extended growth in contact with human epithelial cells, the transcription of informative genes in wild-type meningococci mirrors that found in a sorbarod biofilm

Grifantini *et al.* (2002) reported the transcriptional profile of meningococci co-cultivated with human epithelial cells for periods of up to 3 h, a period which must be considered too brief for a biofilm to become established. In the light of observations by Greiner *et al.* (2005) on the ability of gonococci to form biofilms on primary urinary tract

Table 4. Top 50 downregulated genes of *N. meningitidis* MC58 during biofilm growth on a sorbarod compared with growth on an agar plateGenes with a >2.0 fold change in expression are shown ($P < 0.01$). Asterisk indicates genes encoding proteins which are present in OMVs.

Gene and functional group	NMB product	Fold change	P-value
Transcription			
NMB1650	Leucine-responsive regulatory protein	-21.6	1.02E-10
NMB0398	Transcriptional regulator, ArsR family	-6.94	1.11E-07
NMB0712	RNA polymerase sigma-32 factor	-6.10	8.81E-11
NMB1007	Transcriptional regulator	-5.94	4.15E-11
Replication, recombination and repair			
NMB1368	ATP-dependent RNA helicase, putative*	-7.95	2.91E-05
NMB0399	ExoDNase III	-3.49	3.91E-08
NMB0725	Modification methylase HgaI-1	-3.31	8.00E-05
NMB0727	N ⁶ -Adenine-specific DNA methylase	-2.88	6.33E-06
Inorganic ion transport and metabolism			
NMB1207	Bacterioferritin A*	-6.13	1.61E-04
NMB1206	Bacterioferritin B*	-4.01	2.28E-04
Amino acid transport and metabolism			
NMB1379	NifS protein	-4.72	6.60E-09
NMB2012	Transcriptional regulator, HTH_3 family	-3.10	1.58E-05
Intracellular trafficking and secretion			
NMB1587	Protease, putative	-4.70	1.84E-05
Cell wall/membrane biogenesis			
NMB1651	Alanine racemase	-4.32	1.04E-06
Energy production and conversion			
NMB1380	NifU protein	-3.32	2.17E-09
Post-translational modification, protein turnover, chaperones			
NMB1383	Chaperone protein HscB	-2.93	1.79E-07
Unknown, general prediction			
NMB1857	Modulator of drug activity B	-9.23	4.04E-07
NMB1008	Hypothetical protein	-9.10	1.35E-05
NMB1010	Hypothetical protein	-7.41	7.01E-07
NMB1011	Hypothetical protein	-7.10	3.40E-10
NMB0977	Modulator of drug activity B, putative	-6.75	1.41E-05
NMB1009	Conserved hypothetical protein	-5.61	4.72E-09
NMB0753	Conserved hypothetical protein	-5.05	1.18E-10
NMB1403	FrpA/C-related protein	-4.67	1.77E-05
NMB1402	Hypothetical protein	-4.45	1.48E-07
NMB1404	Hypothetical protein	-4.23	3.84E-05
NMB2013	Hypothetical protein	-4.20	4.28E-05
NMB0864	Hypothetical protein	-4.09	7.01E-05
NMB0674	Conserved hypothetical protein	-3.81	0.001247
NMB1079	Hypothetical protein	-3.79	3.27E-04
NMB0968	Hypothetical protein	-3.79	9.68E-04
NMB0507	Hypothetical protein	-3.66	1.03E-06
NMB0852	Essential GTPase	-3.62	9.71E-06
NMB1378	Conserved hypothetical protein	-3.48	2.23E-09
NMB1382	Conserved hypothetical protein	-3.44	6.34E-06
NMB1048	Hypothetical protein	-3.40	1.42E-05
NMB0104	Hypothetical protein	-3.40	1.61E-05
NMB0519	Hypothetical protein	-3.35	3.00E-04
NMB1410	Hypothetical protein	-3.23	4.74E-05
NMB1408	Hypothetical protein	-3.22	3.91E-05
NMB1666	Hypothetical protein	-3.20	6.28E-06
NMB1409	FrpA/C-related protein	-3.18	5.30E-06
NMB1858	Hypothetical protein	-3.17	2.97E-05
NMB0850	Hypothetical protein	-3.12	5.62E-04
NMB1208	Hypothetical protein	-3.12	1.02E-04

Table 4. cont.

Gene and functional group	NMB product	Fold change	P-value
NMB0726	Type II restriction enzyme Hgal	-3.11	9.54E-07
NMB0858	Hypothetical protein	-3.05	1.97E-04
NMB1757	Hypothetical protein	-3.04	3.45E-07
NMB1393	Phosphogluconate dehydratase	-2.91	6.16E-04
NMB0100	Hypothetical protein	-2.77	1.78E-05

epithelial cells, we attempted to establish biofilms of *N. meningitidis* MC58 over confluent monolayers of the human bronchial epithelial cell line 16HBE14, the experimental system used for host-pathogen interactive studies by Grifantini *et al.* (2002). Our aim was to establish the relevance (or otherwise) of our sorbarod system to studies of colonization. Confluent epithelial monolayers were stained with Giemsa solution for examination by light microscopy at different intervals after infection. A dense layer of bacteria was seen forming over the epithelial cells after 24 h (Fig. 4a), but this was not seen at earlier time points (data not shown).

Despite repeated attempts, we have not yet succeeded in isolating enough meningococcal RNA of adequate quality from this co-culture system to be able to carry out definitive microarray studies. Instead, meningococcal RNA recovered after 28 h co-cultivation was used for targeted assessment, by RT-PCR, of the transcription of four informative genes (NMB0070, NMB0586, NMB1279 and NMB1533), comparing transcription to that seen in a mature sorbarod biofilm on the one hand, and after short epithelial association (Grifantini *et al.*, 2002) on the other. NMB0070 (*synX*) (*siaA*), involved in capsule formation, and NMB0586, annotated as a putative adhesin gene, were reported to be unaltered in transcription in the course of the short co-culture experiment of Grifantini *et al.* (2002), but were significantly down- and upregulated respectively in the sorbarod model after 48 h. NMB1279, annotated as a putative membrane-bound lytic murein transglycosylase B, and NMB1533, encoding the H.8 outer-membrane protein, were selected as examples of genes that showed opposite transcriptional regulation in the two models. NMB1279 is upregulated in the sorbarod 48 h model, but downregulated after the first two time points in the co-

cultivation data of Grifantini *et al.* (2002). NMB1533 is upregulated in the sorbarod 48 h model, but reported to be downregulated at all time points in the system of Grifantini *et al.* (2002). Meningococcal RNA from four independent co-cultivation experiments was harvested for RT-PCR. The pattern of transcriptional regulation of all four genes was found in each case to be similar to that seen in a sorbarod 48 h biofilm (Fig. 4b).

DISCUSSION

The term biofilm to describe the state in which bacteria establish prolonged colonization of epithelial surfaces of the upper respiratory tract has gained currency but has also attracted scepticism (Moxon *et al.*, 2008). Acknowledging that these surface-associated bacteria may comprise a mixture of organisms in microcolonies and in a classic biofilm, we have chosen to adopt the term in our work with meningococci, recognizing the body of evidence that neisserial species can form such structures on abiotic surfaces, and probably on epithelia (Greiner *et al.*, 2005; Lappann *et al.*, 2006; Yi *et al.*, 2004). We have characterized the transcriptional profile of serogroup B *N. meningitidis* biofilms using a sorbarod system, which is simple, readily reproducible, and well suited to studying this important pathogen under safe containment conditions.

In transcriptomal studies of other organisms, the relationship between planktonic cells and those grown in biofilms has been delineated, revealing large differences – scarcely surprising as microbes harvested from these growth conditions are so very different. We have instead pragmatically chosen to compare biofilm to plate-grown organisms as, had our 48 h biofilms shown relatively little change from the starting transcriptome, we would have concluded that going to the lengths of preparing samples in the sorbarod system was not worthwhile. As it is, the striking changes we have found, with alterations in transcription in capsulation, for example, corresponding to observations made by others of organisms recovered from the nasopharynx, encourages continued exploration of the sorbarod experimental system. We report the potential for this tractable, adaptable system to model the biologically relevant, but far more experimentally challenging, colonizing state, encouraged by our preliminary data which demonstrate that the expression profile of *N. meningitidis* MC58 upon extended contact with epithelial

Fig. 2. *N. meningitidis* MC58 LOS profiles. Bacteria harvested from (A) an agar plate and (B) a sorbarod. Samples normalized for protein content were electrophoresed on a SDS-PAGE gel using the Tricine buffering system and silver-stained to reveal LOS.

(a) Microarray

Gene	NMB1053 <i>opcA</i>	NMB0390 <i>mapA</i>	NMB0663 <i>nspA</i>	NMB0072 <i>ctrB</i>	NMB0070 <i>siaA</i>	NMB1870	NMB0586	NMB0345	NMB1875
Fold change	3.32	4.95	2.72	-1.67	-2.64	3.10	3.04	2.07	1.79
<i>t</i> -test <i>P</i> -value	2.67E-05	2.14E-05	3.20E-04	0.0165	0.001589	3.26E-05	6.18E-06	0.003526	3.06E-04
SEM	0.05	0.04	0.05	0.06	0.06	0.04	0.05	0.05	0.04

(b) Real-time RT-PCR

Fig. 3. Confirmation of microarray results (a) by real-time RT-PCR (b). Following RT-PCR, relative increase/reduction of RNA levels was calculated for each gene from three independent biological samples and three technical replicates. Means $\pm$ SEM are plotted.

cells mirrors that of the mature sorbarod biofilm. However, we readily acknowledge that while in our view the model holds considerable promise, it cannot reproduce important aspects of bacterial gene regulation in response to host factors, and further work will be needed to establish with greater confidence that the sorbarod system mimics aspects of natural, long-term epithelial colonization.

Complete, genome-wide datasets of transcription are available in the BμG@Sbase and ArrayExpress databases. Here we discuss our findings in relation to some important components of the cell envelope, central to host–pathogen interactive biology and the induction of protective immunity.

The biofilm matrix characterized in studies of Gram-negative pathogens colonizing the gastrointestinal tract,

Fig. 4. Meningococcal/epithelial cell co-cultivation studies. (a) *N. meningitidis* MC58 biofilm on a confluent monolayer of the cell line 16HBE14. Monolayers were examined by light microscopy after staining with Giemsa solution ($\times 1000$). (b) Real-time RT-PCR comparing meningococcal gene expression after 28 h contact with epithelial cells to that in a mature sorbarod biofilm. Relative increase/reduction of RNA levels was calculated for each gene from four independent biological samples. Means $\pm$ SEM are plotted.

such as *E. coli* and *Pseudomonas* spp., contains abundant exopolysaccharide (Danese *et al.*, 2000; Davies *et al.*, 1993), but the matrix of meningococcal biofilms is clearly different. Searches of complete, annotated neisserial genome sequences from various strains have not identified genes homologous to those encoding production of colanic acid (*E. coli*), alginate (*Pseudomonas*), or poly-*N*-acetylglucosamine (*Actinobacillus* and *Aggregatibacter*; Kaplan *et al.*, 2004) and the only exopolysaccharide produced – the capsular antigen – appears positively to inhibit biofilm formation. Thus, in their studies, Lappann *et al.* (2006) were only able to establish meningococcal biofilms in a flow-chamber system with acapsulate meningococci, of limited value for the study of an organism in which polysaccharide capsule is of primary importance as a determinant of invasive virulence in humans. Yi *et al.* (2004), comparing the biofilm-forming properties of a range of meningococcal strains in a non-flow system, suggested that disease-causing strains were less able than carriage isolates to form a biofilm, a difference they speculated was due to a higher level of capsulation, and refined on this observation with a study of isogenic wild-type and capsule-deficient mutant strains. The latter were substantially more proficient in biofilm formation. The loss of capsule production has been shown to enhance biofilm formation in other bacteria such as *Porphyromonas gingivalis* (Davey & Duncan, 2006) and *Vibrio vulnificus* (Joseph & Wright, 2004).

Expression of capsule in serogroup B meningococcus is a phase-variable phenotype, slip-strand mispairing within the reading frame of *siaD* (polysialyltransferase) leading to premature translational arrest. We considered, but reject, the proposition that biofilms generated from the wild-type might be established with such *cap*[−] phase variants, noting the striking difference in the pattern of transcription of capsulation genes in organisms recovered from the sorbates to the situation found with the constitutive *siaD*-negative strain. In the wild-type, 5/7 capsulation genes were significantly downregulated, and the remaining two downregulated also (borderline significant); in *cap*[−], there was no significant change in transcription in all except one export gene. That organisms recovered from wild-type biofilms were expressing capsule (at reduced level) was confirmed by latex agglutination (data not shown). Unlike Lappann *et al.* (2006) then, we have succeeded in generating biofilms with serogroup B capsulate meningococci in our flow system, a reassuring achievement if the experimental system is to mimic natural colonization. Our findings complement those of previous investigators, as we have found that in the mature biofilm, as others have reported on epithelial colonization *in vivo* (Deghmane *et al.*, 2002), a wide range of capsulation genes were downregulated, inferentially facilitating biofilm maintenance.

LOS, another prominent component of the Gram-negative enteric biofilm, has also been reported to be comparatively detrimental to neisserial biofilm formation (Yi *et al.*, 2004). Here too our transcriptional and biological observations complement these investigators' findings, with down-

regulation of several genes in the key heptose pathway in the mature biofilm and a reduction in the quantity of LOS produced by organisms in biofilms.

Extracellular DNA may be an important constituent of the biofilm matrix *in vivo* (Jurcisek & Bakaletz, 2007; Lappann *et al.*, 2008). It is of note in this regard that we found 40 % excess of DNA per µg of bacterial protein extracted from biofilm compared to plate-grown organisms.

Greiner *et al.* (2005) have reported that where gonococci form biofilms on cultivation in a continuous-flow chamber, the matrix is characterized by prominent membranous structures. The bacterial outer leaflet is promiscuously overproduced by many neisserial species, blebbing OMVs, which are considered central to the provocation of the massive inflammatory response characterizing meningococcal septicaemia (Namork & Brandtzaeg, 2002). OMVs have recently been identified as previously unacknowledged components of the biofilm matrices elaborated by strains of *Pseudomonas aeruginosa*, *Shewanella oneidensis*, *Escherichia coli* and *Azotobacter* sp. (Schooling & Beveridge, 2006) and we speculate that fused, disintegrated OMVs may be an important component of the meningococcal biofilm. In contrast to the situation with capsulation, the evidence from transcriptional analysis of widespread upregulation of genes encoding envelope proteins is consistent with membrane blebbing persisting at least for a while as the biofilm is established. It is notable that when considering the wider range of protein constituents identified in blebs produced without detergent extraction from *N. meningitidis* ΔNMB0033 (a hyper-blebbing strain: Ferrari *et al.*, 2006) an even larger proportion was found to be upregulated in the wild-type biofilm state.

Finally, our data show that among genes encoding protein constituents of OMVs (which are substantially over-represented among those significantly – and in many cases highly – upregulated in the biofilm state), are several of the outer-membrane proteins currently considered the most promising constituents of proposed vaccines to prevent serogroup B meningococcal disease. This lends support to their vaccine candidacy. NMB1870 and NMB2091 – in the top 50 upregulated genes (Table 3) – are two components of the Novartis candidate vaccine 5CVMB (Giuliani *et al.*, 2006), together with two more (NMB1030 and NMB2132), which are also upregulated (Supplementary Table S1). Only the NadA component of 5CVMB was not found to be upregulated in the biofilm. NspA (another favoured vaccine candidate: Martin *et al.*, 1997) was also significantly and substantially upregulated. Other outer-membrane proteins identified in this work as upregulated in the biofilm state may accordingly hold promise in the search for a serogroup B meningococcal vaccine.

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