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Neisseria meningitidis lacking the major porins PorA and PorB are viable and modulate apoptosis and the oxidative burst of neutrophils

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Abstract

The bacterial pathogen *Neisseria meningitidis* expresses two major outer-membrane porins. PorA expression is subject to phase-variation (high frequency, random, on-off switching) and both PorA and PorB are antigenically variable between strains. PorA expression is variable and not correlated with meningococcal colonisation or invasive disease, whereas all naturally-occurring strains express PorB suggesting strong selection for expression. We have generated *N. meningitidis* strains lacking expression of both major porins, demonstrating that they are dispensable for bacterial growth *in vitro*. The *porAB* mutant strain has an exponential growth rate similar to the parental strain, as do the single *porA* or *porB* mutants, but the *porAB* mutant strain does not reach the same cell density in stationary phase. Proteomic analysis suggests that the double mutant strain exhibits compensatory expression changes in proteins associated with cellular redox state, energy/nutrient metabolism, and membrane stability. On solid media, there is obvious growth impairment that is rescued by addition of blood or serum from mammalian species, particularly heme. These porin mutants are not impaired in their capacity to inhibit both staurosporine-induced apoptosis and a phorbol 12-myristate 13-acetate -induced oxidative burst in human neutrophils suggesting that the porins are not the only bacterial factors that can modulate these processes in host cells.

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Keywords

Neisseria meningitidis; Porins; PorA; PorB

Introduction

The pathogenic *Neisseria, N. meningitidis* and *N. gonorrhoeae* cause meningococcal disease and gonorrhoea, respectively. They are host-adapted organisms, residing only in humans. They must acquire all necessary nutrients from the host environment. Although closely related, there are significant differences between *N. meningitidis* and *N. gonorrhoeae*. One significant difference is that *N. meningitidis* expresses two major porins, PorA and PorB, whereas *N. gonorrhoeae* expresses a single major porin, PIB (most similar to PorB). Bacterial porins are integral major outer membrane proteins that allow small molecules to transit the outer membrane into the periplasm. PorA and PorB of *N. meningitidis* are trimeric voltage-gated pores that mediate ion exchange between the organism and its environment[1]. PorA is cation selective[2], and different PorA proteins differ in their cation selectivity, perhaps as the result of changes in net charge of an extracellular loop[3]. PorB also possesses cation and anion translocation pathways[4–5], and is reported to be more selective for anions (Cl^-) over cations (Na^+)[5]

A pseudogene ortholog of the *porA* gene of *N. meningitidis* is present in *N. gonorrhoeae* that is not expressed as a result of accumulated mutations, although rare strains of *N. gonorrhoeae* contain a meningococcal *porA* gene[6]. The PorA protein of *N. meningitidis* displays phase-variable expression (high frequency reversible on/off switching)[7]. Although most *N. meningitidis* strains express PorA, there are occasional reports of strains lacking PorA causing disease in humans[8–9]. There have been no reports of natural isolates of *N. meningitidis* or *N. gonorrhoeae* lacking PorB/PIB. Efforts to produce *N. gonorrhoeae* lacking PorB expression have been unsuccessful[10]. PorB expression has been experimentally abolished in *N. meningitidis*[2], but this strain is reported to exhibit slightly reduced growth rates *in vitro*, whereas PorA mutants appear not to have growth defects[2]. PorA-dependent uptake of sugars is size dependent, with demonstrated transport of arabinose, glucose, and n-acetylglucosamine[3]. This is similar to the uptake of sugars by PorB using liposomes, with translocation of (in order of fastest translocation): glucose>galactose>arabinose[11]. PorB also has a minor role in antibiotic influx, where loss of this protein decreases susceptibility to cefsulodin, tetracycline[2], doxycycline, ciprofloxacin, cefotaxime, ceftazidime, and cephalothin[12]. In contrast, PorA deletion has no effect on antimicrobial susceptibility[2, 12]. Taken together, both pathogens require PorB/PIB expression, but PorA expression by *N. meningitidis* is advantageous but not essential for human colonisation or disease.

In addition to their functions as voltage-gated pores, porins have also been shown to play additional roles in *Neisseria* biology. PorB purified from *Neisseria gonorrhoeae* was first proposed to induce apoptosis when HeLa and other cells were exposed to concentrations of 10 μg/mL purified PorB[13]. Purified gonococcal PorB, or PorB transferred into cells during bacterial infection by unknown mechanisms, was shown to target to the mitochondria and induce apoptosis[14]. Conversely, other studies have shown that purified meningococcal PorB

interacts with Jurkat cell mitochondria to protect cells from apoptosis[15]. Furthermore, following infection of HeLa cells with *N. meningitidis*, PorB was shown to be physically associated with mitochondria, but RmpM, another major *Neisseria* protein, was not[16]. It was inferred from these observations that the anti-apoptotic effect is PorB-dependent. The PorB-dependent anti-apoptotic effect of *N. meningitidis* infection on macrophage cells was further found to be dependent on the presence of nitrosative stress[17]. More recently, Chen and Seifert made a series of *N. gonorrhoeae* PorB mutant strains, demonstrating that almost all PorB amino acids are mutable[18]. A subset of these mutant strains was tested for their ability to protect against apoptosis, and none of the strains were altered in their capacity to inhibit apoptosis compared to the parental strain[18–19].

In this study, we generated meningococcal mutant strains lacking PorA, PorB or both PorA and PorB expression. We examined how depletion of both major porins affected growth rate, undertook proteomic and genomic analysis of these strains to assess compensatory regulatory or genetic changes, and examined their capacity to inhibit the proposed, porin-dependent PMN apoptosis and the oxidative burst *in vitro*.

Material and Methods

Bacterial strains and growth conditions: *Escherichia coli* was routinely grown on LB medium at 37 °C. Antibiotics were added as necessary at 100μg/mL (ampicillin), 10 μg/mL (tetracycline, chloramphenicol). *N. meningitidis* was routinely grown on BHI agar with 5% defibrinated horse blood, or with added Levinthal's supplement (BHI and defibrinated horse blood equal volume, autoclaved at 121 °C, cooled and centrifuged, and added at 10% v/v to BHI). Antibiotics were added as necessary at 5 μg/mL (tetracycline), and 3–5 μg/mL (chloramphenicol). For experiments investigating growth of mutant strains on different media, *N. meningitidis* was cultured on gonococcal medium base (GCB; Difco) agar plus various supplements. These supplements included Kellogg's supplements[20], IsoVitalex (Becton Dickinson), defibrinated animal blood (Lampire Biologicals), animal serum (Lampire Biologicals), 10 μM bovin hemin (Sigma), and 5% human hemoglobin (Sigma). Animal serum was also treated with either 5% Chelex 100 (BioRad), 500 ug/ml proteinase K (Invitrogen), 10 ug/ml DNase (Worthington), 10 ug/ml RNase (Sigma), or 5 mM PMSF (Sigma) before being added to the GCB. Strains were cultured on agar medium for 16 to 18 hours at 37°C with 5% CO_2.

Growth curves

N. meningitidis MC58 and derivatives from 16–18 hours growth on solid media were subcultured for 3–4 hours on solid media, resuspended to OD A600= 1, diluted 1:5 into 1ml Levinthal's supplemented BHI in triplicate into individual sealed 2 mL cuvettes, and then incubated with shaking at 37 °C. Growth was monitored at hourly intervals. The experiment was repeated twice. Exponential growth phase was defined as between 120 min and 180 min. Samples at OD A600=1 were analysed by coomassie staining and Western blotting, PorA expression was monitored with anti P1.7 mAb MN14C11.6, and PorB with anti P3.15 mAb 2-P-15 (obtained from NIBSC, UK). Samples at OD A600=1 were serially diluted and colonies counted after 30 hours growth on Levinthal's supplemented BHI agar.

Generation of plasmid constructs

The *porA* gene and flanking sequence was amplified from MC58 genomic DNA using primers PorAFor1 (5′-GTTTCGGTCGTTTCCGATAA-3′) and PorAR1 (5′-TTGAAACCCTGACCCTCTG-3′) and cloned into pGEMTeasy (Promega), to generate pPorA (fig 1A). Inverse PCR on pPorA using primers DelPorA-UP 5′-TCGCATATC GGCTTCCTTTTGTAAATTTGA-3′ and DelPorA-DOWN 5′-TCC GTC GGT TTG CGC CAC AAA TTC-3′ was performed to delete the *porA* ORF and to insert a *Sma*I site. The tetracycline resistance cassette from pGemTetMB[21] was cloned into the *Sma*I site of pPorA to generate pPorA::Tet (fig 1C). After amplification and cloning of *porB* from MC58 genomic DNA with PorBF1 (5′-GCCCTCCAATACCCTCCCGAGTA-3′) and PorBR1 (5′-TGCCGTCTGAAGACTTCAGACGGCCGACAGGCTTTTTGTTGATACC-3′) (see fig. 1C), a *Bgl*II site was introduced by PCR. The chloramphenicol resistance determinant was amplified from pCmGFP[22] with primers including *Bam*HI sites (Cat5′Bam: 5′-GTGGATCCACACAATCTGCCCTTT-3′, Cat3′Bam: 5′-GGATCCGCACCAATAACTGCCTTA-3′ and the *cat* gene was cloned into BglII digested pPorB to generate pPorB:Cat (fig 1D), and plasmid sequenced to confirm site of *cat* insertion. RNA was extracted from bacteria grown in Levinthal's supplemented BHI liquid media (grown to A600 OD1) using QIAGEN RNeasy kit, prior to conversion to cDNA, and quantitative real-time PCR, using primers Nm16S_For : 5′-CGTGGGTGCGAGCGTTAATC-3′, Nm16S_Rev: 5′-CTGCCTTCGCCATCGGTATTCCT-3′, PorA_RT_For: 5′-TAAGGGGAGTGAGGATTTGGGC-3′, PorA_RT_Rev: 5′-ATCAATGGCTTGGCTGGCATCG-3′, PorB_RT_For: 5′-TCAAACCGAAGTTGCCGCTACC-3′, and PorB_RT_Rev: 5′-TTGGAGAAGTCGTATTCCGCACC-3′. Abundance of transcripts for *porA* and *porB* was calculated for strains MC58, 58ΔA and 58ΔB relative to 16sRNA.

Protein analysis

Bacteria were harvested from liquid media growth at OD A600= 1 and resuspended in 10 mM Tris pH 8.0. After separation on 4–12% Bis-Tris acrylamide gels, proteins were analysed using coomassie staining or western blotting. PorA and PorB expression was confirmed using goat polyclonal anti-PorA (sc-17396, Santa Cruz Biotechhnology), or murine mAb 8B5-5-G9 (anti-PorB, P3.15, obtained from NIBSC, UK). Alkaline-phosphatase-conjugated donkey anti-goat, and goat anti-mouse antibodies (Sigma-Aldrich) were used with colorimetric reagents to detect binding. For protein identification of bands excised from coomassie-stained gels, outer-membrane proteins were enriched using 1% sarkosyl detergent extraction, and LC-MS/MS was carried out as previously described[23]

Mass spectrometry (MS)

For SWATH-MS, 3–5 colonies were harvested into 300 μL 6M guanidinium chloride, 50 mM Tris-HCl pH 8, 10 mM DTT and incubated at 30 °C for 30 min. Cysteines were alkylated by addition of acrylamide to 25 mM and incubation at 30 °C for 1 hour, followed by further addition of DTT to 5 mM. Proteins were precipitated by addition of 1.2 mL 1:1 methanol:acetone, incubation at −20 °C for 16 hours, and centrifugation at 18,000 rcf for 10

min. The air-dried protein pellet was resuspended in 100 μL 50 mM Tris-HCl pH 8 with 1 μg trypsin (proteomics grade, Sigma-Aldrich) and incubated at 37 °C for 16 hours. Peptides were desalted with C18 ZipTips (Millipore). Proteins were identified by information dependent acquisition LC-ESI-MS/MS analysis performed as previously described[24] using a Prominence nanoLC system (Shimadzu) and TripleTof 5600 mass spectrometer with a Nanospray III interface (AB SCIEX). Briefly, approximately 2 μg peptides were desalted on an Aglient C18 trap and then separated on a Vydac EVEREST reversed-phase C18 HPLC column at a flow rate of 1 μL/min. Separation used a gradient of 10–60% buffer B over 45 min, with buffer A (1 % acetonitrile and 0.1 % formic acid) and buffer B (80 % acetonitrile and 0.1 % formic acid). An MS TOF scan was performed from an *m/z* of 350–1800 for 0.5 s followed by information dependent acquisition of MS/MS of the top 20 peptides from *m/z* 40–1800 for 0.05 s per spectrum, with automated CE selection. Identical LC conditions were used for SWATH-MS (Sequential Window Acquisition of all THeoretical Mass Spectra)[25]. SWATH-MS of triplicate biological replicates was performed as previously described[26] with an MS-TOF scan from an *m/z* of 350–1800 for 0.05 s, followed by high sensitivity information-independent acquisition with 26 *m/z* isolation windows with 1 *m/z* window overlap each for 0.1 s across an *m/z* range of 400–1250. Collision energy was automatically assigned by Analyst software (AB SCIEX) based on *m/z* window ranges. Proteins were identified essentially as described[27] using ProteinPilot (AB SCIEX), searching a database with all predicted *N. meningitidis* MC58 proteins and common contaminants with standard settings: Sample type, identification; Cysteine alkylation, acrylamide; ID focus, biological modifications; Enzyme, Trypsin, Search effort, thorough ID. False discovery rate analysis was performed on all searches. ProteinPilot search results were used as ion libraries for SWATH analyses. The abundance of proteins was measured automatically using PeakView (AB SCIEX) with standard settings. Comparison of protein relative abundance was performed based on protein intensities[26], or ion intensities using a linear mixed-effects model with the MSstats package in R[28]. Proteins with greater than 30 % changes in abundance and with adjusted P-values < 0.05 were considered significant.

Genome sequencing and assembly

The genome assembly was carried out using the Spades Genome Assembler in pair end mode with – careful preset[29]. BayesHammer was used for read (150 bp) error correction prior to assembly with default parameters. In the Spades genome assembly module, k-mer lengths of 21, 33, 55 and 77 were used to build an iterative genome assembly. Post processing of assembled contigs/scaffolds for mismatch correction was done with Burrows Wheeler Aligner (BWA tool)[30]. For SNP analysis, paired-end reads were mapped against the *N. meningitidis* MC58 reference genome (NC_003112.2) using Bowtie2 with -sensitive preset. MarkDuplicates from Picard tool set was used to remove duplicate reads and SAMtools for transforming data into mpileup format. Single nucleotide polymorphisms were then called using VarScan 2.3.6[30–31] with only those reported that had evidence from reads in both orientations.

Eukaryotic cell assays

The ability of *N. meningitidis* porin mutants to inhibit apoptosis and ROS production was determined using HL-60 cells differentiated down the granulocytic pathway as previously

described [17]. HL-60 cells were differentiated in 0.7% dimethylformamide (Sigma) for a period of 5 days, after which cells were infected with *N. meningitidis* strains. For the apoptosis inhibition assays, differentiated HL-60 cells were infected at a multiplicity of infection (MOI) of 50 for 3 hours, followed by treatment of cells with 1 μM staurosporine for 3 hours to induce apoptosis. Cells were washed with phosphate-buffered saline (PBS) before being treated with 50 μL cell lysis buffer (BD Pharmingen) to harvest cell lysates. To measure caspase-3 activity in the lysates, 5 μL reconstituted caspase-3 substrate (N-acetyl–Asp–Glu—Val–Asp–7-amino-4-methylcoumarin [Ac-DEVD-AMC]; BD Pharmingen) at a concentration of 1 mg/ml was incubated with assay buffer and 25 μL cell lysates for 60 min at 37°C. 7-Amino-4-methylcoumarin (AMC) fluorescence was measured using an excitation wavelength of 380 nm, an emission wavelength of 440 nm, and a SpectraMax M5 plate reader (Molecular Devices). Luminol-dependent chemiluminescence (LDCL) assays were performed to examine ROS production by infected HL-60 cells [17]. Assays were carried out in a total volume of 0.2 ml PBS supplemented with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 7.5 mM glucose (PBSG) in black-bottom 96-well plates (Nunc). HL-60 cells were resuspended at a concentration of 4×10^7 cells/ml, and 10^6 cells were seeded in the presence of 100 μM luminol. The cells were then stimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) and/or *N. meningitidis* strains that had been grown overnight on GCB + 5% horse blood. Following stimulation, LDCL was measured every 2 min over a total period of 60 min at 37°C using a SpectraMax M5 plate reader (Molecular Devices).

Results

Generation and characterisation of *N. meningitidis* porin mutants

Mutations in each porin gene of *N. meningitidis* were made by allelic replacement via homologous recombination during natural DNA transformation with an inactivated allele carrying an antibiotic resistance marker. Plasmids pPorA::Tet and pPorB:Cat (Fig 1) were linearized and transformed into MC58 to generate strains 58ΔA and 58ΔB respectively. The linearized pGEMporB::Cat was subsequently transformed into 58ΔA to generate 58ΔAB. Acapsulate strains lacking expression of either PorA or PorB had been previously generated[12] designated ¢9DPorA and ¢9DPorB. Strain ¢9DPorAB was generated by transformation of ¢9DPorA with pPorB:Cat, demonstrating that capsular polysaccharide is not required to generate meningococci lacking both major porins, strain ¢9DPorAB.

Sequencing of PCR products from *porB* regions of the transformants confirmed replacement of the wild-type *porB* allele with the *porB*::*cat* allele, and full genome and sequence analysis confirmed no disruption or alteration in upstream or downstream genes, nor evidence of other chromosomal rearrangements or duplications: the double porin mutant strain 58ΔAB contained no unique SNPs or INDELs (data not shown). SDS-PAGE analysis confirmed that expression of PorA, PorB, or both had been abolished in the respective strains (Fig 2). Western immunoblot (Fig 2) and MS analysis of major bands confirmed the identity of the major bands (Supplementary Figure 1).

SWATH-MS proteomic analysis

We were able to generate *N. meningitidis* cells with abolition of expression of both major porins, and with no detectable genomic changes associated with deletion of PorA and PorB. In order to determine if any compensatory protein expression changes were apparent in the double porin mutant, and to gain insights into the normal role of PorA and PorB, we performed relative quantification of the global cellular proteomes of MC58, 58ΔA, 58ΔB and 58ΔAB using SWATH-MS. Whole cell proteomics of these strains identified a total of 413 unique proteins (Supplementary Table S1), 400 of which were used in SWATH-MS analysis. SWATH-MS data was analysed considering proteins which showed significant ($P<0.05$) and large (greater than 30%) changes in expression. Relative quantification of cellular proteomes using SWATH-MS identified 9 proteins (2.3%) with higher abundance, and 2 proteins (0.5%) with lower abundance in 58ΔA than MC58 (Supplementary Table S2, Fig 3A). In contrast, the 58ΔB strain showed substantially more changes in global proteome relative to MC58, with 12 proteins (3%) with higher abundance, and 14 proteins (3.5%) with lower abundance (Supplementary Table S3, Fig 3B). Deletion of both *porA* and *porB* (strain 58ΔAB) showed many large changes in expression relative to the parental MC58, with 33 proteins (8.3%) with higher abundance and 34 (8.5%) with lower abundance (Supplementary Table S4, Fig 3C). Consistent with this, 2 proteins (0.5%) had higher abundance and 9 proteins (2.2%) had lower abundance in 58ΔB compared with 58ΔA (Supplementary Table S5, Fig 3D). Untargeted SWATH-MS relative quantification of global cellular proteomes confirmed the absence of PorA and PorB expression where expected, as these proteins showed the largest fold change reductions in expression in the respective mutant strains (Supplementary Tables S2, S3 and S4, Fig 3F,G). Residual apparent detection of PorA or PorB protein in respective deletion strains was due to measurement of background inherent in SWATH-MS measurements. Expression of PorA and PorB were upregulated in the reciprocal mutants, suggesting partial functional complementation (Fig 3F, G). This was not an artefact due to the abundance of PorA and PorB, as these proteins contribute only ~5% of total MS ion intensity to the global peptide intensity count. Examination of RNA levels indicated that the *porA* transcript was 5.5 fold more abundant in strain 58ΔB than in the parental MC58 strain (relative to 16S RNA), whereas *porB* transcript was similar in MC58 and 58ΔA.

Amongst other proteins that were significantly more abundant ($P<10^{-10}$) in strains lacking PorB (58ΔB and 58ΔAB) relative to MC58 and 58ΔA strains, NMB1963 encodes a putative ABC transporter with similarity to a toluene tolerance family, and NMB1963 and its associated operon has been implicated in glutamate uptake in low sodium ion environments[32]. RmpM, a protein that forms complexes with porins, and stabilises *Neisseria* outer membrane proteins[33–34], also was over-represented in 58ΔB and 58ΔAB relative to MC58 and 58ΔA strains. Opc, a trimeric beta-barrel protein[35], was identified as being more abundant in the double porin knockout compared with all other strains. NMB0995, (macrophage infectivity potentiator-related protein) was also increased in strain 58ΔAB compared with all other strains, as was NMB1030 (Fig 3K), recently characterised as a ubiquinone binding protein[36].

Determination of growth requirement of porin mutants

Growth in Levinthal's-supplemented BHI broth was assessed. Abolition of both porins did not reduce growth rate to a large extent in this rich medium (which contains heated, clarified horse serum), with the growth rate in the exponential phase not significantly different between any of the strains when calculated by comparing slope of the curve (Fig 4). Calculation of generation times revealed small but significantly increased generation times for 58ΔB and 58ΔAB compared to MC58 and 58ΔA (supplementary table S6). At each time point the 58ΔAB strain exhibited lower OD than MC58 ($P<0.05$, independent t-Test), and reached lower OD at stationary phase (Fig 4). Bacterial numbers were similar in samples taken from these cultures at A600= 1.

To further characterise growth, bacterial colony growth was observed on solid media. Growth was poor on GCB plates containing Kellogg's supplements or IsoVitalex (Fig 5), which are both supplements that are routinely used for growth of *Neisseria*. As NMB1030, NMB0995, and Opc are all upregulated during growth in human blood[37], and these proteins were each upregulated in the 58ΔAB strain (Fig 4), we assessed growth with blood and blood derivatives. Qualitative observations indicated that strain 58ΔAB grew reasonably well on medium containing 5% equine blood or serum (Fig 5). Treatment of serum with chelex or nucleases to remove divalent cations and DNA/RNA, respectively did not reduce growth. Growth was similar on GCB when supplemented with porcine or bovine blood, and on media supplemented with human hemoglobin, porcine or fetal bovine serum (not shown), whereas colony size was not enhanced with bovine hemin (Fig 5). These observations suggest a nutrition acquisition defect for the 58ΔAB strain that is complemented by growth with blood or blood derivative.

Effect on host cells: modification of oxidative burst

Porin purified from *N. gonorrhoeae* has previously been demonstrated to directly affect the oxidative burst of human neutrophils[38], and gonococcal infection of HL-60 cells inhibits PMA-induced oxidative burst[39]. We tested the ability of meningococci lacking both PorA and PorB to inhibit luminol-dependent chemiluminescence induced by PMA in differentiated HL-60 cells, using *N. gonorrhoeae* as a positive control (Fig 6A). The porin mutant strain lacking both PorA and PorB did not differ in its ability to inhibit PMA-induced ROS production relative to wild-type *N. gonorrhoeae* or *N. meningitidis*. Meningococcal mutants lacking either PorA or PorB singly also inhibited PMA-induced ROS production to the same extent as the wild-type strain, and none of the strains tested induced ROS production from HL-60 cells in the absence of PMA (data not shown).

Effect on host cells: inhibition of apoptosis

PorB has been cited as having a role in either inducing[13–14] or protecting against apoptosis[15–18, 39]. We tested the effect of PorA and/or PorB mutation on the modulation of cellular apoptosis during infection. HL-60 cells were differentiated towards granulocyte phenotype for 5 days using 0.7% dimethylformamide as previously described[39] and the capacity of meningococci to inhibit staurosporine-induced apoptosis was assessed in granulocyte-differentiated HL-60 cells (Fig 6B). As expected[39], *N. gonorrhoeae* inhibited apoptosis induced by STS, indicated by decreased caspase-3 activity relative to STS-treated,

uninfected cells, as did the porin-replete meningococcal strain. The acapsulate meningococcal ¢9DAB strain lacking both PorA and PorB was equally effective at inhibiting staurosporine-induced caspase-3 activity relative to both of the wild-type strains.

Discussion

This study is the first report of the generation of a *N. meningitidis* strain that lacks both major porins. This was unexpected as it is not possible to generate a mutant of the closely related *N. gonorrhoeae* that lacks its single, major porin, PIB. A major difference between the two species is the presence of a capsular polysaccharide in *N. meningitidis*. However, capsule is not required for survival of *N. meningitidis* in the absence of major porins, as we were able to generate an acapsulate strain lacking both PorA and PorB expression (Supplementary Fig 1). Western immunoblot of capsulate strains indicated the absence of the porins (Fig 2), and MS analysis of total cellular protein confirmed that the major porins were absent (Fig 3). In the single porin mutant strains, the relative proportion of the reciprocal porin was higher, suggesting compensatory upregulation (Fig 3F, G). The increased transcript level of *porA* in the 58ΔB strain indicates transcriptional compensation. Conversely, in the 58ΔA strain, the slight increase in PorB protein level detected by SWATH-MS was not matched by an increased *porB* transcript level, suggesting that PorB protein content is influenced on a post-transcriptional mechanism.

We analysed the proteome of the strains to determine whether compensatory changes occur in the absence of porins. Lack of PorA resulted in few changes whereas deletion of PorB resulted in an increased set of differentially expressed proteins. Loss of both major porins resulted in even further changes. These results are consistent with the observation that PorA is more dispensable than PorB: naturally occurring PorB-lacking strains have not been reported, and PorB has an important *in vivo* function, whereas clinical isolates lacking PorA are described, and PorA is naturally phase-variable.

The *Neisseria* protein RmpM protein was over-represented in total cellular protein, as assessed by quantitative- SWATH-MS analysis, in strains lacking either PorB alone, or both PorB and PorA. This suggests that in the absence of the major porin PorB, RmpM is upregulated and may be required to stabilise other protein complexes, or may be required for membrane stabilisation. However, it is of note that RmpM is not apparent as a major band in sarkosyl-extracted outer-membrane proteins of a strain lacking both major porins (Supplementary figure 1). RmpM forms complexes with porins and other outer membrane proteins[3, 33, 40], and is associated with Omp85/β-barrel assembly machinery[34], and the absence of PorA and PorB may thus reduce the association of this protein with membrane proteins in this detergent. PorA, PorB, and RmpM are reported to be upregulated after prolonged growth with human epithelial cells[41], indicating an important role for these proteins in interactions with host cells. In strain 58ΔAB, Opc is more abundant. The Opc protein is phase-variably expressed through alterations in length of a poly(C) tract in its promoter[42]. Genome sequence analysis revealed no alterations in the promoter region of the *opc* gene in the 58ΔAB strain, suggesting that the increased abundance of this protein (Fig 4H) is the result of regulatory changes rather than the result of phase-variation. Indeed, *opc* is reported as induced during growth in serum[37].

NMB1963 is part of a glutamate uptake operon[32]. The protein encoded by this gene was more abundant in strains 58ΔB and 58ΔAB, both of which lack PorB. *N. meningitidis* does not contain a glutamine synthase gene, and must therefore rely on glutamate uptake. The upregulation of NMB1963 may be an indication of compensatory upregulation to rescue general nutrient stress when major porins are absent, or may suggest that PorB has an unrecognised role in amino acid uptake. Other proteins with altered abundance indicative of changes in nutrient and/or energy metabolism included SucA (NMB0995) and SucC (NMB0959), downregulated in the PorAB mutant strain, as well as reductions of PpsA (phosphoenolpyruvate synthase, NMB0618), AdhP (alcohol dehydrogenase, propanol-preferring, NMB0546), and AcnB (aconitate hydratase, NMB1572.) Cellular processes associated with proline also appear to be affected, with downregulation of ProS (NMB0133) and PutA (NMB0401) in the double mutant, strain 58ΔAB.

As an alternate role for the upregulation of NMB1963, glutamate has several cellular fates after uptake, including synthesis of glutathione, an important molecule in maintenance of intracellular redox state. A mutant of the glutamate GltT ABC type transporter (NMB1965, in the same operon as NMB1963) has recently been reported to have significantly reduced glutathione content. This strain was attenuated *in vivo*, and GltT was shown to have a significant role in bacterial resistance to neutrophil oxidative burst[43]. Mutants of GltT were significantly more susceptible to H_2O_2[43]. The protein encoded by NMB0995 was also over-represented in the total protein sample of strain 58ΔAB compared with all other strains. This protein belongs to the family of macrophage infectivity potentiator-related proteins. The exact function of these proteins are unclear, but MIP-related proteins contain conserved domains of the TIGR01926 family, some of which are known to act as peroxidases, or correlate with resistance to oxidative stress. Other proteins were upregulated in the double porins mutant strain that are indicative of an alteration of intracellular redox state: NMB1044 (*fpr*1), NMB0946 (encoding peroxiredoxin 2 family protein). The increased abundance of these proteins indicate that cells may be under additional oxidative stress as a result of PorAB abrogation, but this link remains to be experimentally assessed. DnaK (molecular chaperone, NMB0554) and DsbA2 were also upregulated, suggesting protein synthesis and stabilisation is affected.

In light of these obvious changes in protein expression (which were not attributable to selection of compensatory mutations in the double porin mutant), it was noted that growth on solid media was improved when blood products were added, specifically blood, serum, or human hemoglobin. Growth in a rich liquid medium containing Levinthal's supplement (which includes horse blood) is slightly reduced in the absence of PorB, and stationary phase was reached at a lower OD for 58ΔAB. PorB mutants have previously been described as having "slightly reduced" growth rates[2].

We next investigated the effect of porin deletion on interactions between *N. meningitidis* and granulocytes. Considerable evidence exists to suggest that porins of *Neisseria*, in particular PorB, can function to modulate apoptotic signaling in various eukaryotic cell types[13–16, 44–46]. Porin has also been shown to affect the oxidative burst in phagocytic cells[38–39, 47–48], and for these reasons we investigated the ability of the *N. meningitidis* double mutants to inhibit both apoptosis and the oxidative burst in granulocytic HL-60 cells.

Our results suggest that meningococci lacking both PorA and PorB are still able to inhibit both STS-induced apoptosis and a PMA-induced oxidative burst to the same extent as the parental strain, contrary to our expectations. Potential explanations for our lack of observed phenotype are either (i) an alternate neisserial outer membrane protein is complementing the function of PorB (and/or PorA) with respect its effects on eukaryotic cell survival and function, or (ii) PorA or PorB do not play a significant role for either apoptosis inhibition or regulation of ROS production. Future analysis will be required to test between these hypotheses.

In summary, we report that *N. meningitidis* lacking the major porins are viable. This differs from the related *N. gonorrhoeae* which is not viable without PorB. Proteomic analysis indicates that deletion of both major porins results in compensatory alterations in metabolism, specifically likely affecting intracellular redox state. Future studies will focus on further characterising the functional importance of porins in the biology of the pathogenic *Neisseria*, and their interaction with host cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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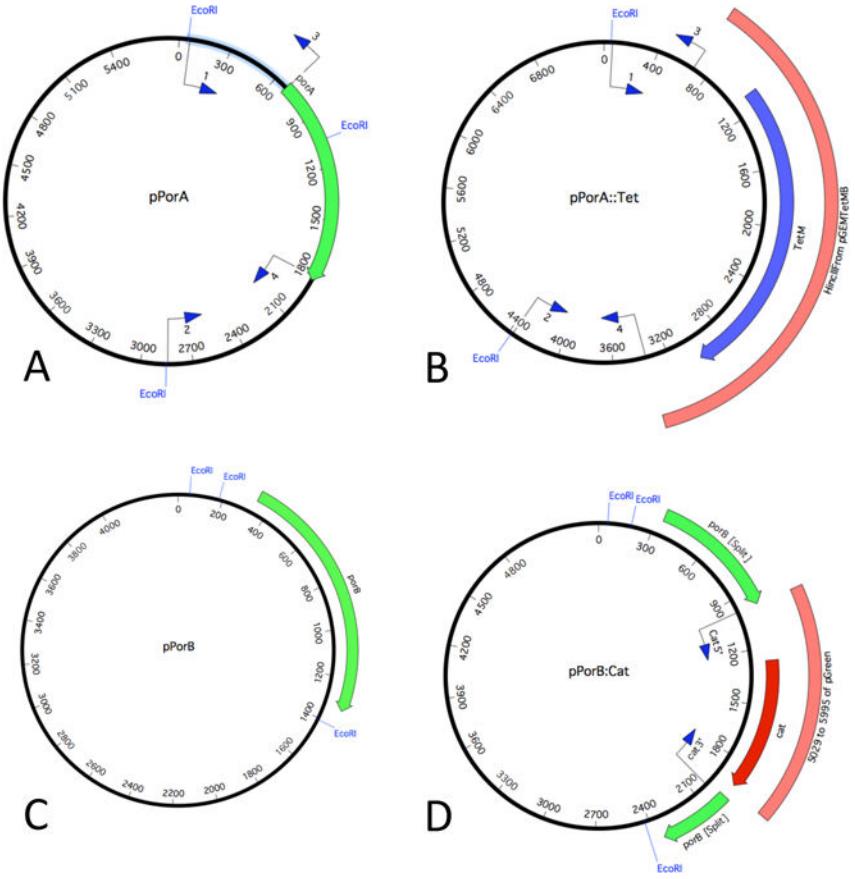

Figure 1. Plasmids generated for deletion of porin genes

The *porA* and *porB* genes were amplified and cloned into pGEMTeasy (A,C). B: After deletion of *porA* by PCR, an HincII fragment (orange box) from pGem-TetMB containing tetracycline resistance (blue gene)[21] was cloned into pPorA to generate pPorA::Tet. D: A fragment was amplified from pCmGFP (pGreen, orange box) containing the chloramphenicol acetyl transferase gene (red gene) and introduced into a PCR engineered site in *porB*, to generatepPorB:Cat.

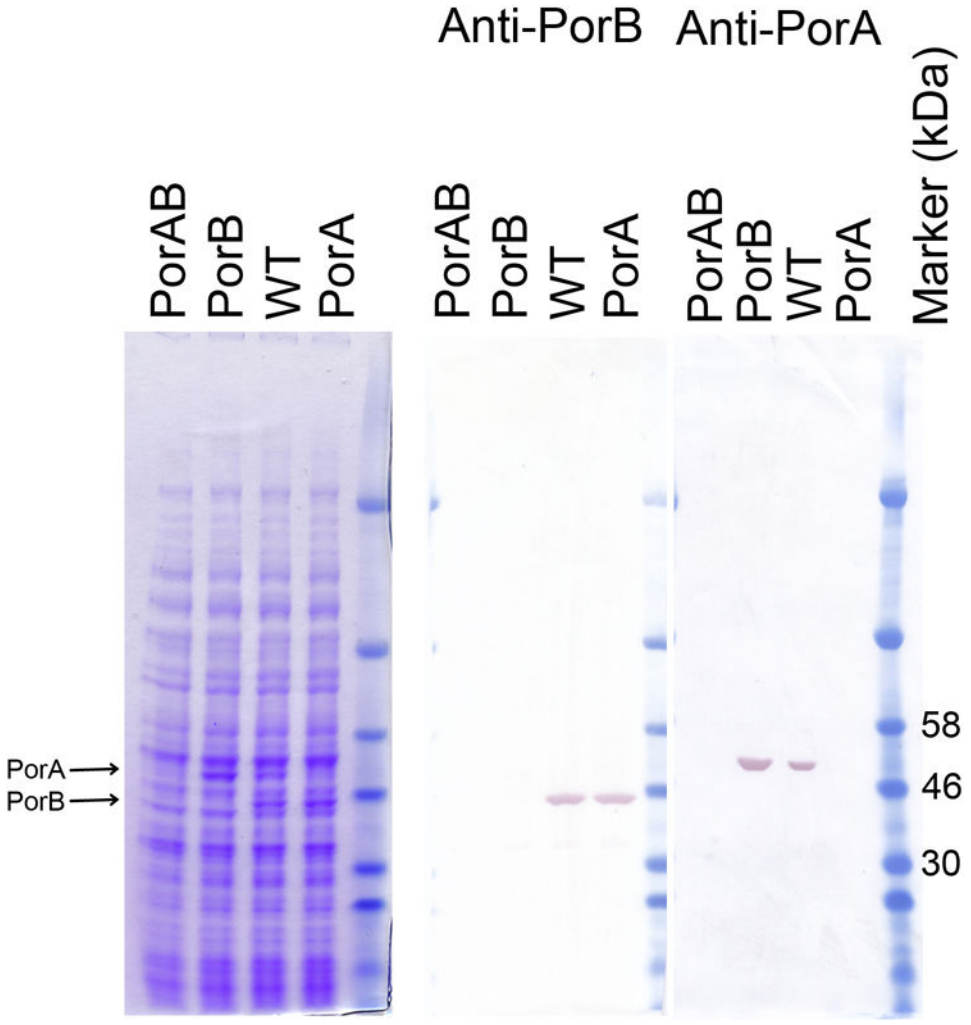

Figure 2. Confirmation of abolition of Porin expression

Bacteria were harvested from liquid cultures (OD A600=1) and separated electrophoretically. Gels containing total cellular protein from strains MC58, 58ΔPorA, 58ΔPorB and 58ΔPorAB (labelled WT, PorA, PorB, or PorAB respectively) were commassie stained, or western blotted and probed with antibodies specific for PorA or PorB.

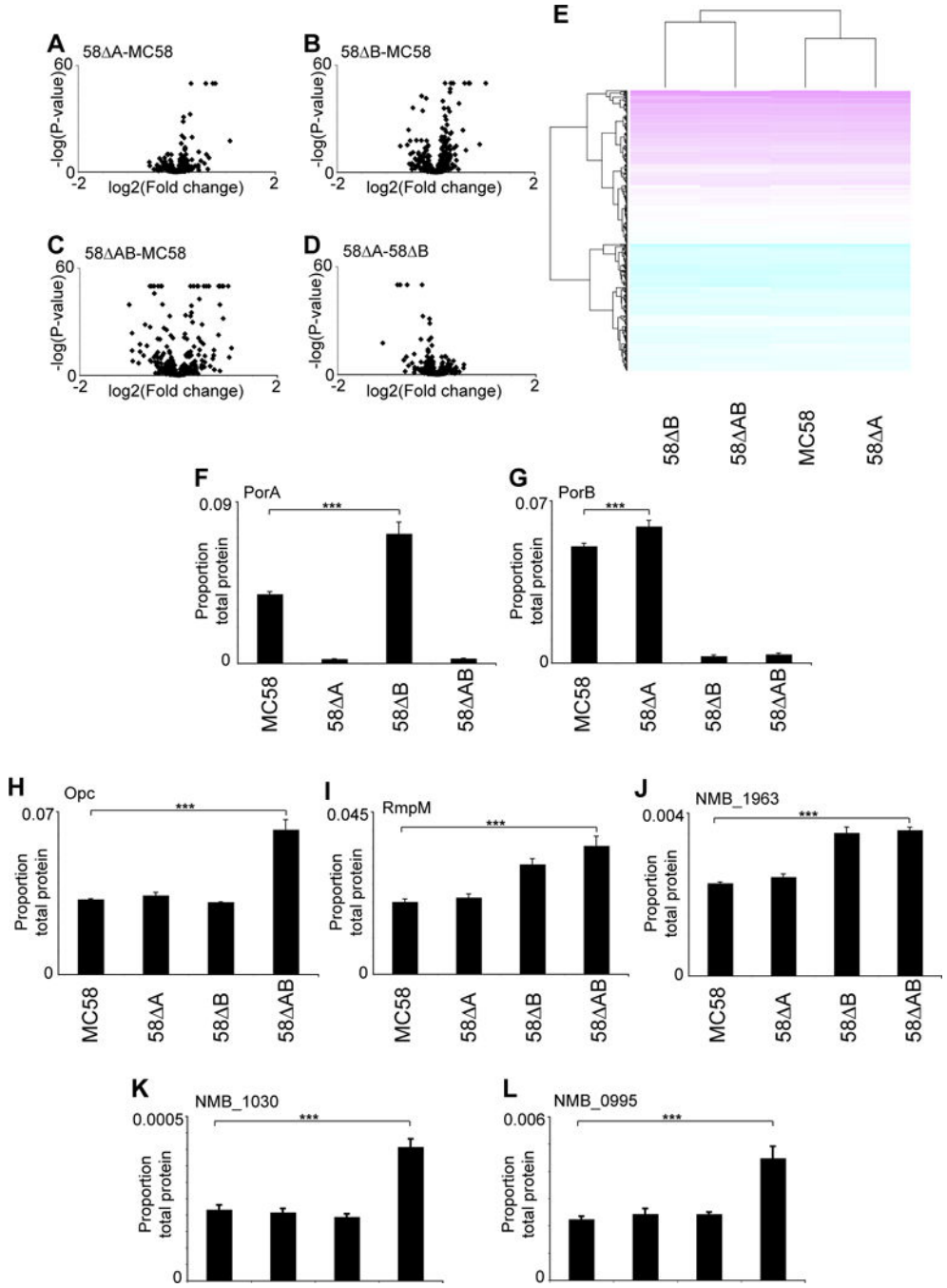

Figure 3.

SWATH-MS relative quantification of PorA and PorB-dependent changes in global meningococcal proteome. Volcano plots for (A) 58ΔA versus MC58, (B) 58ΔB versus MC58, (C) 58ΔAB versus MC58, (D) 58ΔA versus 58ΔB. (E) Clustered heat map of protein expression in MC58, 58ΔA, 58ΔB and 58ΔAB. Protein abundance in MC58, 58ΔA, 58ΔB and 58ΔAB for (F) PorA, (G) PorB, (H) Opc, (I) RmpM, (J) NMB1963, (K) NMB1030, and (L) NMB0995. For F-L, ***, P< 0.001

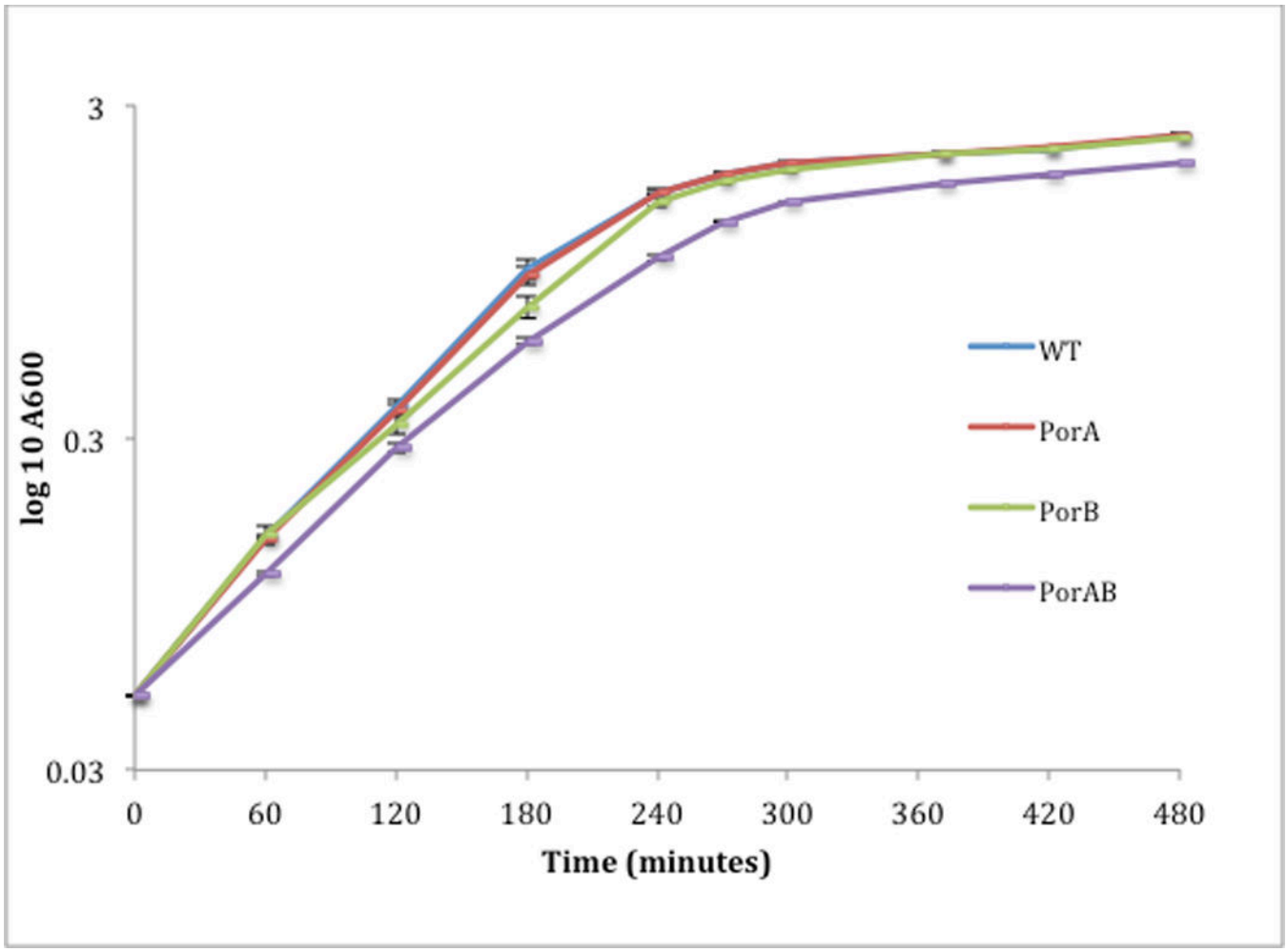

Figure 4.

Growth curve of parental and porin deficient strains in rich media. Each data point represents the mean of three replicate cultures, ± SD.

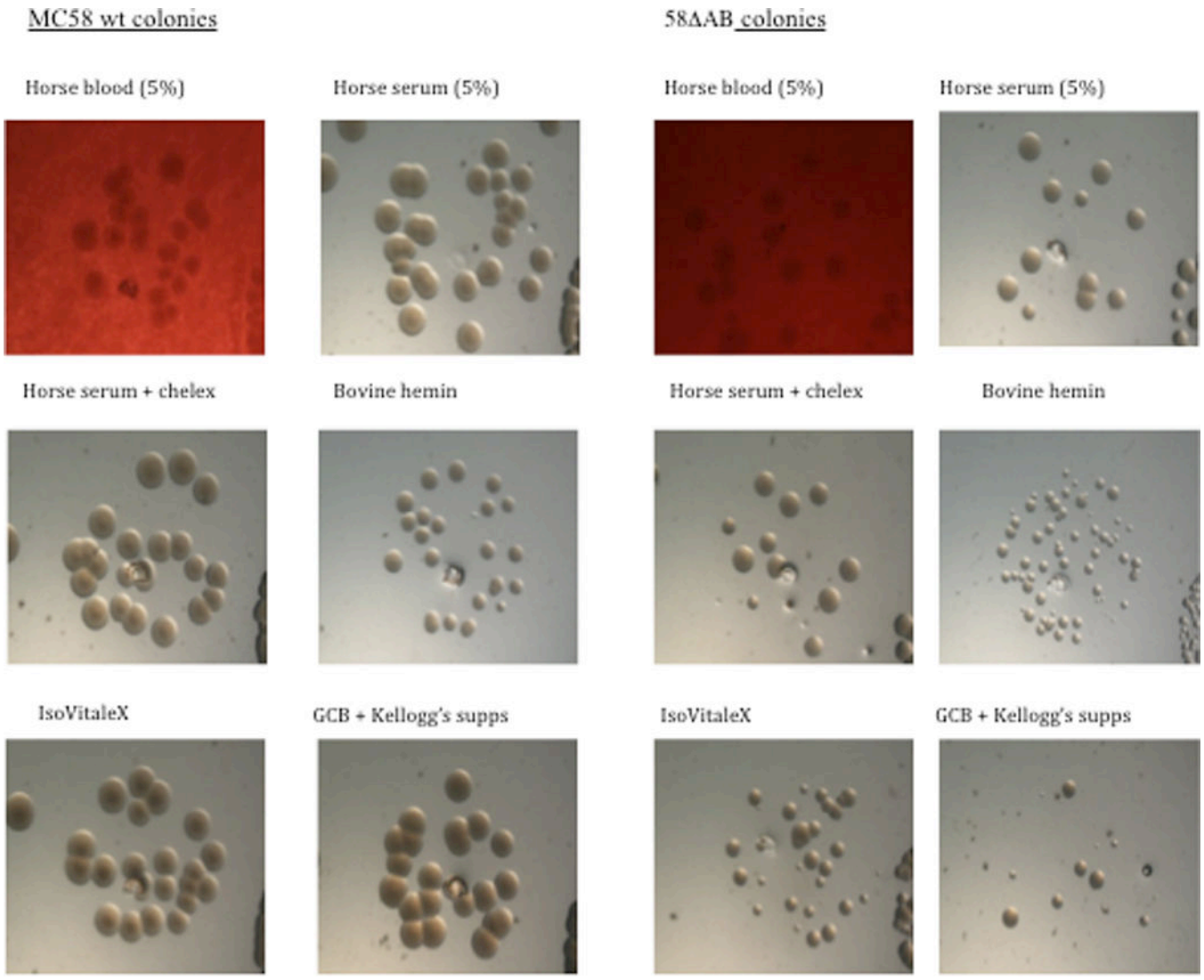

Figure 5.

Growth of *N. meningitidis* 58ΔAB on solid media. Bacteria were grown overnight on solid GCB that contained supplementation as indicated, and imaged using a stereoscope.

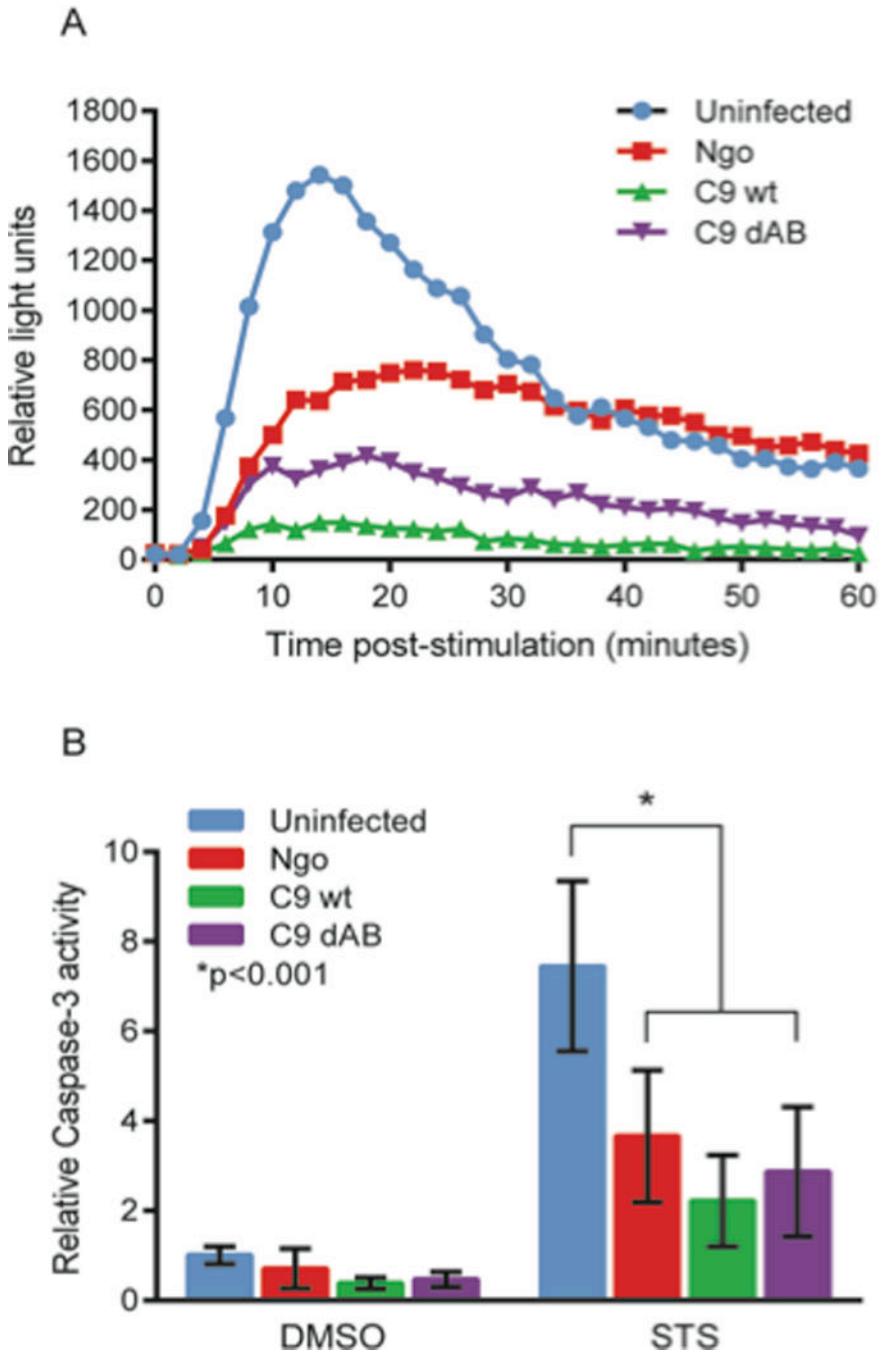

Figure 6.

Effect of porin mutations on (A) the PMA-induced oxidative burst, or (B) staurosporine (STS)-induced caspase-3 activity in differentiated HL-60 cells. Meningococcal strains ¢9 (labelled C9 wt) and ¢9ΔPorAB (labelled C9 dAB) were compared with *N. gonorrhoeae* (labelled Ngo). (A) Differentiated HL-60 cells were stimulated with PMA to induce an oxidative burst and infected with *N. gonorrhoeae* or *N. meningitidis* strains. Luminol-dependent chemiluminescence was measured over a period of 60 minutes. (B) Differentiated HL-60 cells were infected with *N. gonorrhoeae* or *N. meningitidis* strains for 3 hours, then

treated with either STS to induce apoptosis or DMSO as a control for a further 3 hours. Caspase-3 activity was measured using the fluorogenic substrate Ac-DEVD-AMC, and data are presented as the caspase-3 activity relative to that of uninfected, DMSO-treated controls.