Reduced DNA binding and uptake in the absence of DsbA1 and DsbA2 of *Neisseria meningitidis* due to inefficient folding of the outer-membrane secretin PilQ

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DsbA ensures the correct folding of many exported bacterial proteins by forming intramolecular disulphide bonds in the bacterial periplasm. The pathogen *Neisseria meningitidis* is unusual in its possession of three different *dsbA* genes (*dsbA1*, *dsbA2* and *dsbA3*), encoding two membrane-anchored (DsbA1 and DsbA2) and one periplasmic (DsbA3) thiol-disulphide oxidoreductase enzymes. In this study, the involvement of DsbA1 and DsbA2 in natural competence was confirmed and attributed to events in the early stages of the transformation process. Strains lacking both DsbA1 and DsbA2 were reduced in competence as a result of decreased DNA binding and uptake. Overexpression of DsbA3 could not overcome this defect, suggesting differences in substrate specificity and protein-folding abilities between the DsbA homologues. Competence in *Neisseria* is dependent on the expression of type IV pili, which are extruded and retracted through the outer-membrane secretin PilQ. Both DsbA1 and DsbA2 were able to specifically bind PilQ in solid-phase overlay assays. Consistent with this, deletion of both *dsbA1* and *dsbA2* resulted in reduced levels of PilQ, confirming inefficient folding of PilQ, while pilus expression was apparently unaffected. The secretin PilQ is involved in DNA binding and transport as well as pilus biogenesis, and the defect in PilQ folding resulting from the absence of DsbA1 and DsbA2 is revealed in the observed decreased DNA binding and uptake.

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INTRODUCTION

Neisseria meningitidis (the meningococcus) asymptotically colonizes the human upper respiratory tract but, for reasons that are not well understood, occasionally invades the bloodstream to cause life-threatening septicaemia and/or meningitis in previously healthy individuals. *N. meningitidis* represents a significant public health problem, responsible for deaths and disability worldwide.

N. meningitidis is naturally competent for DNA uptake by transformation, and genetic exchange contributes to the

extensive variety of phenotypes it can display. DNA uptake occurs through the correct operation of an incompletely characterized complex of membrane proteins and also requires the presence of the frequently repeated 10/12-nucleotide motif (AT)GCCGTCTGAA (the DNA uptake sequence; DUS) (Ambur *et al.*, 2007; Elkins *et al.*, 1991). Neisserial transformation further depends on the expression at the cell surface of type IV pili (T4P), which are also important for interactions with host cells during both colonization and invasion (Nassif *et al.*, 1994; Virji *et al.*, 1991). The abundant T4P fibres mainly consist of polymerized pilin (PilE) subunits, and their extrusion and retraction across the outer membrane occurs via the oligomeric outer-membrane secretin PilQ, which is naturally expressed at high levels (Tønnum *et al.*, 1998). PilQ has also recently been shown to bind DNA, confirming an involvement in natural transformation distinct from its role in pilus biogenesis (Assalkhou *et al.*, 2007).

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Abbreviations: DUS, DNA uptake sequence; Nal, nalidixic acid; T4P, type IV pilus/pili.

A supplementary table of primers is available with the online version of this paper.

The proper interactions and coordinate functioning of pilus components depend on their correct folding, a process that relies on enzymes including the thiol-disulphide oxidoreductase DsbA. DsbA has been linked to both pilus expression and DNA uptake in several organisms. Involvement in pilus formation was highlighted in enteropathogenic and uropathogenic *Escherichia coli*, where DsbA is required for the stability of the type IV pilin and the folding of PapD, a chaperone essential for the stability and functionality of the P pilus, respectively (Jacob-Dubuisson *et al.*, 1994; Zhang & Donnenberg, 1996). In both *Haemophilus influenzae* and *Bacillus subtilis*, the DsbA equivalent is involved in competence-induced DNA uptake, catalysing the formation of disulphide bond(s) in one or more protein(s) involved either in competence development or in the DNA-binding/uptake machinery (Bolhuis *et al.*, 1999; Tomb, 1992).

N. meningitidis unusually produces three different DsbA proteins. In the serogroup B strain MC58, two of these [DsbA1 (NMB0278) and DsbA2 (NMB0294)] share high sequence identity and are located in the inner membrane (Sinha *et al.*, 2004; Tinsley *et al.*, 2004). The third DsbA [DsbA3 (NMB0407)] is periplasmic and differs from the other two in sequence and in substrate specificity when expressed in *E. coli* (Sinha *et al.*, 2004; Tinsley *et al.*, 2004). Homologues of all three are to be found in the genome sequences of serogroup A and serogroup C strains.

Tinsley *et al.* (2004) have demonstrated a role for two DsbA proteins of *N. meningitidis* serogroup C – NMC0273 and NMC1885, homologues respectively of NMB0278 and

NMB0294 from the serogroup B strain MC58 – in the process of natural transformation (a strain lacking both these proteins having reduced ability to take up marked chromosomal DNA and to bind endothelial cells) and have established a role for these proteins in the folding of the T4P subunit, pilin. Somewhat confusingly, these NMC proteins (described in advance of the genome annotation) were respectively termed *dsbA2* and *dsbA1* by Tinsley *et al.* (2004), the opposite way to the NMB nomenclature.

In this work, we confirm the transformation defect in serogroup B strain MC58 lacking DsbA1 (NMB0278) and DsbA2 (NMB0294), and show that both of the membrane-associated DsbAs also directly interact with the outer-membrane secretin PilQ. While T4P production appears to be unimpaired, the transformation defect due to the absence of both DsbA proteins is reflected in a reduced level of DNA binding and uptake linked to inefficient folding of the secretin PilQ.

METHODS

Bacterial strains and growth conditions. *E. coli* strains (listed in Table 1) were grown on Luria–Bertani (LB) medium (Oxoid, Unipath). *N. meningitidis* strains (listed in Table 1) were cultured on gonococcal (GC) agar (Difco) supplemented with 1% (w/v) Vitox (sGC) (Oxoid), in rich Müller–Hinton (MH) broth (Oxoid) or in semi-defined Frantz medium (8.7 mM L-glutamic acid, 0.076 mM L-cysteine·HCl, 0.016 M NaH₂PO₄, 0.001 M KCl, 0.1 M NaCl, 0.02 M NH₄Cl, 0.002 M MgSO₄·7H₂O, 0.5% glucose, 0.2% yeast extract; pH 7.3). All bacteria were grown at 37 °C in 5% CO₂. When required, antibiotics were used at the following concentrations: for *E.*

Table 1. Bacterial strains used in this study

Strain name	Relevant genotype/phenotype	References/source
<i>E. coli</i>		
XL1	$\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacI^q \Delta M15 Tn10 (Tet^r)]$ – used as host for cloning experiments	Stratagene
BL21(DE3)	F [−] <i>ompT hsdS_B(r_B[−] m_B[−]) gal dcm</i> (DE3) – used for protein overexpression	Invitrogen
S17.1 λ pir	<i>recA pro hsdR RP4-2-Tc::Mu-Km::Tn7</i> – used for conjugation into meningococcus	Simon <i>et al.</i> (1983)
<i>N. meningitidis</i> serogroup B		
MC58	Wild-type	Masson & Holbein (1983)
M1	<i>dsbA1::aphA3</i>	This work
M2	<i>dsbA2::ermC</i>	This work
M3	<i>dsbA3::cat</i>	This work
M1M2	<i>dsbA1::aphA3 dsbA2::ermC</i>	This work
M1M3	<i>dsbA1::aphA3 dsbA3::cat</i>	This work
M2M3	<i>dsbA2::ermC dsbA3::cat</i>	This work
M1M2M3	<i>dsbA1::aphA3 dsbA2::ermC dsbA3::cat</i>	This work
MC58 NaI ^R	Resistant to nalidixic acid	Our strain collection
H44/76	Wild-type	Holten (1979)
<i>N. gonorrhoeae</i>		
MS11-B2	Spontaneous non-piliated variant of MS11	Segal <i>et al.</i> (1985)

coli, kanamycin 50 µg ml⁻¹, erythromycin 200 µg ml⁻¹ and chloramphenicol 20 µg ml⁻¹; and for *N. meningitidis*, kanamycin 50 µg ml⁻¹, erythromycin 10 µg ml⁻¹, chloramphenicol 2 µg ml⁻¹ and nalidixic acid (Nal) 20 µg ml⁻¹.

DTT sensitivity. DTT sensitivity assays were performed essentially as described elsewhere (Sinha *et al.*, 2004) except that 2 mM DTT was used as the concentration that inhibited growth of *dsbA* mutant but not wild-type *N. meningitidis*.

Molecular biology techniques. Unless otherwise stated, recombinant DNA techniques were carried out as described by Sambrook *et al.* (1989). Chromosomal DNA, plasmids and RNA were extracted using appropriate kits (Qiagen). RT-PCR reactions were performed using the OneStep RT-PCR kit, according to the manufacturer's instructions (Qiagen).

Inactivation of *dsbA* genes. *Neisseria* strains were transformed as described below using cloned DNA fragments designed to recombine and integrate in the host chromosome allowing each *dsbA* gene to be interrupted by an antibiotic-resistance gene. Primers used are listed in Supplementary Table S1 (available with the online version of this paper). For each gene, primer pairs *_1/_2* and *_3/_4* were used to amplify regions flanking *dsbA*, chosen to include at least one copy of the neisserial uptake sequence. These were then interrupted by the *Enterococcus faecalis aphA3* gene (Ménard *et al.*, 1993) for *dsbA1*, the *B. subtilis ermC'* gene (Monod *et al.*, 1986) for *dsbA2* and the *Staphylococcus aureus cat* gene for *dsbA3* (Jansen *et al.*, 1995), using relevant restriction sites. The products of these genes encode resistance to kanamycin, erythromycin and chloramphenicol, respectively. Transformants were selected by growth on the appropriate antibiotic(s) and gene disruption(s) confirmed by PCR and Southern blotting.

Complementation using *pdsbA3*. Plasmid pMIDG201 (O'Dwyer *et al.*, 2004) was modified to express *dsbA3* in *N. meningitidis* strain M1M2 from the neisserial promoter *ner*. This *E. coli/Neisseria* shuttle vector carries the *aphA3* gene. In order to enable selection in M1M2 (Kan^R Erm^R), an alternative plasmid (pMIDG301) was constructed, in which this gene was replaced by the *S. aureus cat* gene. For complementation, *dsbA3* was amplified from the genome of MC58, using primers *dsbA3_F* and *dsbA3_R* (Table S1), and cloned in pMIDG301. The resulting plasmid, named *pdsbA3*, was conjugated from *E. coli* S17.1 λ pir to strain M1M2 using methods described previously (O'Dwyer *et al.*, 2004). Transconjugants were selected on medium containing kanamycin, erythromycin and chloramphenicol.

Transformation of *N. meningitidis*. Transformation was performed as described elsewhere (Robertson *et al.*, 1993). For quantitative transformation assays, 300 µl bacterial suspension from plate-grown organisms at an OD₆₀₀ of 0.2 in pre-warmed MH broth containing 10 mM MgCl₂ was incubated at 37 °C with 1 µg transforming DNA in a flat-bottomed bijoux bottle. After 30 min, samples were treated with 40 µg DNase I and the bacteria left to incubate at 37 °C for 3.5 h. Serial dilutions were then plated on to both selective and non-selective media and allowed to grow overnight. Transformation frequencies were calculated as the ratio of the number of antibiotic-resistant c.f.u. to the number of c.f.u. obtained without antibiotic selection. The transforming DNA used was chromosomal DNA prepared from a Nal^R strain of MC58.

DNA binding/uptake. The donor DNA used in these assays was genomic DNA from *N. meningitidis* strain H44/76 or plasmid DNA containing zero or one copy of DUS (described in Ambur *et al.*, 2007). Genomic DNA, sheared using a sonicator, or linearized plasmid DNA were treated with ExoIII (New England Biolabs) and labelled using Klenow Exo⁻ (New England Biolabs) with 25 µM

dGTP/dTTP/dCTP and 1 µM [α -³²P]dATP [3000 Ci mmol⁻¹ (111 TBq mmol⁻¹); GE Healthcare]. GFX columns (GE Healthcare) were used to remove unincorporated nucleotides. Bacteria freshly restreaked from an overnight plate were suspended after 4 h growth to the equivalent of 5 × 10⁸ c.f.u. in GC medium containing 7 mM MgCl₂. Five hundred nanograms of labelled DNA was added to 1 ml bacterial suspension in duplicate and samples gently tumbled for 30 min at 37 °C. One of each sample was treated with 100 µg DNaseI ml⁻¹ and incubated for 5 min at room temperature. All samples were then washed four times and the pellet resuspended in 100 µl ice-cold GC medium. Radionuclide incorporation was measured in a Tri-Carb 2900TR liquid scintillation analyser (Packard) after mixing with 2 ml liquid scintillation cocktail (Ultima Gold MV, Perkin Elmer). Total DNA added, cell-associated DNA after washing (bound and taken up) and DNA taken up in a DNase I-resistant state were measured.

Electron microscopy. A suspension was prepared from plate-grown bacteria in PBS to OD₆₀₀ 0.1. Copper palladium grids were gently floated on to 5 µl of this suspension for 5 min and then air-dried. The bacteria were then fixed for 5 min in 0.5% (v/v) glutaraldehyde in PBS, washed with distilled water and stained with 1% (w/v) uranyl acetate for 5 min. The grids were finally rinsed with water, air-dried and viewed in an FEI Technai G² electron microscope.

Quantitative whole-cell ELISA. The method described by (Hélaine *et al.*, 2005) was followed, using a polyclonal rabbit antiserum directed against meningococcal pilin described elsewhere (Collins *et al.*, 2005) and used at 1:1000 dilution. Experiments were performed on three separate occasions in triplicate. Values given represent the mean of the separate assays ± standard deviation of the mean and are expressed as the ratio of the tested strain over wild-type.

Protein purification, extraction and analysis. Whole-cell lysates were prepared by resuspension of plate-grown bacteria in PBS and lysis with the FastPrep FP120 instrument (ThermoElectron) using two runs at setting 6.0 for 40 s each. Where necessary, iodoacetamide was added at a final concentration of 100 mM. T4P fibres were isolated using methods described by Collins *et al.* (2005). Protein concentrations were measured on a Nanodrop ND-1000 spectrophotometer). An Invitrogen XCell SureLock Mini-Cell and XCell II Blot Module were used for SDS-PAGE and Western blotting, according to the manufacturer's instructions. For colony blotting, the membrane was gently placed on the agar plate and left for a couple of minutes to absorb the bacteria. Following this, the membrane was washed several times in PBS and then processed as a Western blot. The polyclonal antisera raised against meningococcal PilE, PilQ and PilP are described elsewhere (Balasingham *et al.*, 2007; Collins *et al.*, 2005; Tønnum *et al.*, 1998) and were used at 1:1000 dilution. The secondary antibody used in all reactions was a horseradish-peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories), used at 1:10 000 dilution. Antibody detection was performed using ECL Plus (Amersham Pharmacia Biotech) or 3,3'-diaminobenzidine tablets (Sigma) according to the manufacturer's instructions. Purification of pili, PilQ complex and PilP from meningococcal cells and recombinant, full-length and partial PilQ was performed as previously described (Balasingham *et al.*, 2007; Collins *et al.*, 2005). Solid-phase overlay assays (far-Western analysis) were performed as described by Collins *et al.* (2005) and Balasingham *et al.* (2007).

Purification of DsbA1/2 and rabbit immunization. DsbA1 and DsbA2 were expressed in the periplasm of *E. coli* BL21 from the expression vector pET20b (Novagen) with a C-terminal hexahistidine tag. Bacteria were grown to mid-exponential phase in 500 ml LB broth and protein expression was induced with 1 mM IPTG. After a further 4 h growth, bacteria were pelleted and cells disrupted by two passages through a French press. The His-tagged

proteins were purified under native conditions by immobilized metal-affinity chromatography with Ni-NTA agarose (Qiagen). Samples were dialysed against 50 mM phosphate buffer containing 300 mM NaCl and 10 % (v/v) glycerol. These proteins were used to immunize rabbits in order to generate antisera for use in future experiments. Animals were given three injections of 300 ng protein each at 14 day intervals. The sera, obtained by exsanguination after euthanasia at 60 days, were used at 1:1000 dilution for protein detection. Recombinant DsbA1 and DsbA2 were also used in far-Western analyses.

RESULTS

Mutations in *N. meningitidis* *dsbA* genes affect growth and sensitivity to reducing agents

The genes *dsbA1* (*nmb0278*), *dsbA2* (*nmb0294*) and *dsbA3* (*nmb0407*) were interrupted with kanamycin-, erythromycin- and chloramphenicol-resistance cassettes, respectively, in the *N. meningitidis* serogroup B strain MC58. The *dsbA1* gene lies in a monocistronic operon between two diverging genes, while *dsbA2* is located at the 3' end of a gene cluster. The gene *nmb0408* lies directly downstream of *dsbA3* and is transcribed in the same direction, but disruption of *dsbA3* had no effect on its transcription (data not shown). A full set of *dsbA* mutants was created where *dsbA* genes were disrupted in all possible combinations, creating strains M1, M2, M3, M1M2, M1M3, M2M3 and M1M2M3 (Table 1). Apart from the *dsbA*-null strain M1M2M3, all strains grew as well as wild-type in rich and semi-defined liquid media (Fig. 1). All strains grew equally well on agar plates.

In order to assess DsbA functionality in these strains, sensitivity to reducing agents was examined on agar plates containing increasing concentrations of DTT. Only M1M2 and M1M2M3 were impaired in growth on plates containing 2 mM DTT, indicating that the presence of at least one of DsbA1 and DsbA2, but not DsbA3, is essential for maintaining the redox equilibrium of the bacterial periplasm.

Strain M1M2, lacking both DsbA1 and DsbA2, is impaired in competence for DNA uptake

To test for natural competence for DNA uptake, meningococcal *dsbA* mutants were transformed with marked chromosomal DNA and transformation frequencies established. As with DTT sensitivity, markedly reduced transformation frequencies were only observed for strains M1M2 and M1M2M3 (Fig. 2a). The double *dsbA1 dsbA2* mutant showed only 16 % of the wild-type transformation frequency, while single mutants in either of these genes were unaffected. Deletion of *dsbA3* in M1M2 did not result in any further reduction in competence.

Overexpression of DsbA3 cannot compensate for the lack of DsbA1 and DsbA2

We sought to understand whether the inability of DsbA3 alone to ensure wild-type levels of transformation was due to differences in expression levels or in substrate specificities between DsbA1/DsbA2 and DsbA3. We therefore attempted to complement the M1M2 transformation defect by overexpressing *dsbA3* from a plasmid (*pdsbA3*), where the gene cloned into plasmid pMIDG301 enabled overexpression in *N. meningitidis*. Semiquantitative RT-PCR was used to monitor expression of *dsbA3* and confirmed no difference between wild-type and M1M2 but significant overexpression in M1M2 carrying *pdsbA3* (Fig. 2b). The transformation frequency of this strain was comparable to that of strain M1M2 (Fig. 2a), confirming that DsbA3 is unable to replace DsbA1/2 in this respect even when it is produced at high levels. As DsbA3 has no apparent role in the transformation phenotype, results in the following experiments are shown only for M1M2 compared to wild-type.

M1M2 produces pili that are quantitatively and qualitatively indistinguishable from wild-type

As competence in *N. meningitidis* is dependent on the expression of T4P at the cell surface, it was possible that the

Fig. 1. Growth of *N. meningitidis* strains in liquid culture. Bacteria were grown in Müller–Hinton broth with agitation at 37 °C. A representative set of growth curves is shown.

Fig. 2. (a) Competence for transformation of DNA in wild-type MC58 and its *dsbA* mutants. Bacteria as indicated were transformed with chromosomal DNA from a *Nal*^R strain of MC58, enabling selection of positive transformants on medium containing 20 µg *Nal* ml⁻¹. In parallel, c.f.u. were determined on non-selective growth medium. Transformation frequencies were calculated for each strain by dividing the number of selective transformants by the number of c.f.u. (both adjusted to per ml). Assays were performed on three separate occasions and values given represent the mean ± SD of the separate assays. (b) Overexpression of *dsbA3* in M1M2 from plasmid *pdsbA3*: RT-PCRs on the RNA of *N. meningitidis* strains MC58, M1M2 and M1M2 carrying plasmid *pdsbA3* with primers nmb0407_5' and nmb0407_3'. Ten nanograms of template RNA was used for each strain. The amplified PCR product of 0.7 kb is shown with an arrow. No product was generated when the RNA samples were treated with RNase prior to the RT-PCR cycle, confirming that there was no DNA contamination (data not shown).

reduced transformation frequency observed in M1M2 was the result of a lack of pilus expression. To explore this, bacterial suspensions bound on copper palladium grids were examined by transmission electron microscopy using uranyl acetate as a contrasting staining agent. T4P were clearly visible on the surface of wild-type and M1M2 cells. These occurred both as individual pilus fibres (of 6–7 nm in width) and as aggregates of large pilus bundles. The strains were indistinguishable in visible pilus density. No surface structures were observed on cells from the non-piliated control *N. gonorrhoeae* MS11-B2. Wild-type and M1M2 produced the T4P pilin subunit at comparable levels, as shown by colony blotting using a polyclonal antibody raised against purified meningococcal pilin. Semi-quantification of pilus expression levels by whole-cell ELISA again showed no difference in M1M2 compared with wild-type, the ratio of M1M2/wild-type being 1.49 ± 0.30 . These experiments indicate that the transformation defect could not simply be attributed to differences in the quantity of pilus produced.

In order to investigate more subtle differences in pilus composition that could explain the transformation defect

in strain M1M2, extracellular pili were purified and their components compared by PAGE and immunoblotting. There were no differences in protein profiles on analysis of samples separated on 15 % gels under denaturing/non-denaturing and reducing/non-reducing conditions (data not shown), indicating that there were no substantial differences in the content and levels of proteins that fractionate with T4P. No differences were observed in levels of the T4P subunit pilin (PilE) when serial dilutions of pilus preparations were compared for the presence and relative levels of this protein (Fig. 3).

These experiments collectively indicate that the transformation defect cannot be attributed to gross differences in pilus composition.

The transformation defect in M1M2 stems from an inability to bind and take up DNA

Defects in one or more of the different step(s) in the process of natural transformation (DNA binding/uptake, transfer to the cytosol, processing and RecA-dependent recombination into the chromosome) could account for the diminished transformation ability of M1M2. The expression of fully functional pili is required at the initial stages of the transformation process. The ability of wild-type and *dsbA1 dsbA2* mutant meningococci to bind and take up DNA was investigated using ³²P-labelled plasmid DNA lacking (p0-DUS) or containing (p-12DUS) a single 12-mer copy of the extended neisserial DUS (Ambur *et al.*, 2007). In *Neisseria*, DNA uptake is DUS-dependent whilst binding is not (Aas *et al.*, 2002; Ambur *et al.*, 2007). Accordingly, both wild-type and M1M2 bound p0-DUS and p12-DUS but only p12-DUS was efficiently taken up. M1M2 was impaired in its ability to bind both p0-DUS and p12-DUS DNA compared with wild-type (Fig. 4a). DNA uptake was also reduced in this strain. In order to address the inability of M1M2 to fully bind DNA (which may directly result in decreased uptake), DNA uptake was quantified in relation to the amount of cell-associated DNA (rather than to the total amount of DNA added). While both strains took up comparable amounts of p0-DUS, uptake of p12-DUS was reduced in the absence of DsbA1 and DsbA2 (69 % of wild-type) (Fig. 4b). This

Fig. 3. Semiquantitative assessment of amounts of pili purified from *N. meningitidis* wild-type and M1M2. Sheared pilus preparations from identical numbers of bacteria were immunoblotted with polyclonal rabbit PilE antiserum, detecting the pilin subunits present. Lanes: 1–3, MC58; 4–6, M1M2. Concentrations loaded were 1:10, 1:2 and 1:1 for each strain.

Fig. 4. DNA binding and uptake in *N. meningitidis* strain M1M2. Assays using DNA lacking (p0-DUS) or containing (p12-DUS) the neisserial DNA uptake motif were performed on three separate occasions. Values shown correspond to the mean $\pm$ SEM of the separate assays. (a) Values given represent the amount of bound DNA as a percentage of total DNA added. DNA binding in a *pilQ*⁻ strain, calculated in the same way in one assay, is represented by dashed lines. (b) To factor in the reduced DNA-binding ability of M1M2, values given represent the amount of DNA taken up as a percentage of cell-associated DNA. DNA uptake in a *pilQ*⁻ strain, calculated in the same way in one assay, is represented by dashed lines.

confirms that M1M2 displays a reduced ability to take up DUS-containing DNA, separable from its reduced DNA binding. Experiments using labelled genomic DNA from *N. meningitidis* strain H44/76 (serogroup B) also showed that DNA uptake was reduced (47% of wild-type) in the absence of DsbA1 and DsbA2. This suggests that the influence of DsbA1/2 on competence for natural transformation is exerted at least in part at the initial transformation stages of DNA binding and uptake.

DsbA1 and DsbA2 interact specifically with the outer-membrane secretin PilQ

DsbA1 and DsbA2 were expressed in *E. coli* with C-terminal hexa-histidine tags and purified to homogeneity under native conditions. Recombinant DsbA proteins were used to immunize rabbits in order to generate specific antisera. Direct interactions between recombinant DsbA and two pilus biogenesis proteins (PilP and PilQ) were investigated by solid-phase overlay assays (far-Western analysis). The meningococcal outer-membrane secretin PilQ is essential for pilus biogenesis (Tønnum *et al.*, 1998) and has been shown to bind T4P fibres and DNA (Assalkhou *et al.*, 2007; Collins *et al.*, 2005). The C-terminal part of the PilQ monomer contains two cysteine residues that form a disulphide bond and is essential for the stability of the dodecameric PilQ complex (Collins *et al.*, 2005). The lipoprotein PilP also plays a role in pilus biogenesis (Drake *et al.*, 1997). The PilP polypeptide contains only one cysteine residue, indicating that this protein is not internally disulphide-bonded. As PilP and PilQ themselves directly interact (Balasingham *et al.*, 2007), these were used as positive controls in the solid-phase overlay assays. As shown in Fig. 5(a), the PilQ antiserum detected PilQ bound to PilP (lane 4) as well as to DsbA1 and DsbA2 (lanes 1 and 2). On the other hand, detection with the PilP antiserum was only positive with the PilQ protein (lane 8), confirming that PilP and DsbA1/2 do not interact. Further experiments using truncations of PilQ (as described by Collins *et al.*,

2005) showed that the DsbA–PilQ interaction was specific to the C-terminal part of the PilQ monomer (Fig. 5b, lane 9). This region of the PilQ monomer, extending over the last 420 amino acids of the protein, contains the two cysteine residues that form the PilQ disulphide bond. Purified T4P did not directly interact with DsbA1/2 in this solid-phase overlay assay (data not shown).

PilQ levels are reduced in the absence of DsbA1 and DsbA2

In order to investigate the effect of *dsbA* mutations on PilQ folding, whole-cell lysates were prepared in the presence of iodoacetamide, which modifies free thiol groups in cysteine residues, thus irreversibly blocking disulphide bond formation and preventing proteins from spontaneously refolding. Levels and migration of PilQ were then analysed by immunoblotting. Cell lysates from M1M2 showed reduced amounts of both the monomeric and multimeric forms of PilQ as compared to wild-type (Fig. 5c), consistent with ineffective PilQ folding in the absence of DsbA1 and DsbA2.

DISCUSSION

The meningococcus is extremely unusual in carrying three versions of *dsbA*. It is the only neisserial species to do so, and we have speculated that the acquisition of additional *dsbA* genes with specific protein-folding functions relevant to virulence has played a part in the evolution of this pathogen. A similar situation is seen in *Salmonella enterica* serovar Typhimurium, where in addition to the chromosomal copy, a *dsbA* homologue (*srgA*) is found on the virulence plasmid. *SrgA*, but not *DsbA*, has been shown to oxidize the disulphide bond in the main subunit of plasmid-encoded fimbriae (Bouwman *et al.*, 2003).

Mutagenesis of *dsbA* genes both singly and in combination in the serogroup B strain MC58 has confirmed a role for

Fig. 5. Interactions between meningococcal DsbA1/2 and PilQ. (a) Far-Western blot membranes overlaid with PilQ (left) or PilP (right). Purified recombinant proteins were loaded as follows: DsbA1 (lanes 1 and 5), DsbA2 (lanes 2 and 6), PilP (lane 4), PilQ (lane 8). One microgram of BSA was loaded as a negative control (lanes 3 and 7). Blots were incubated with purified PilQ (lanes 1–4) or purified PilP (lanes 5–8) as indicated, and these proteins detected with the corresponding polyclonal antibodies. Relevant bands are denoted with asterisks. (b) Far-Western blot with PilQ truncations (right) and the corresponding colloidal-blue-stained SDS-PAGE (left). Purified recombinant proteins were loaded as follows: PilQ full-length (lanes 1 and 6), PilQ N-terminus (lanes 2 and 7), PilQ central region (lanes 3 and 8), PilQ C-terminus (lanes 4 and 9). One microgram of BSA was loaded as a negative control (lanes 5 and 10). Blots were incubated with purified DsbA1, which was then detected with the corresponding polyclonal antibody. The same results were obtained using DsbA2 in place of DsbA1. Relevant bands are denoted with asterisks. (c) PilQ levels determined by Western blotting. Ten micrograms of whole-cell lysates prepared in the presence of iodoacetamide (see Methods) and separated on a 12% SDS-PAGE gel were transferred to nitrocellulose and immunoblotted with PilQ antisera. As expected, PilQ, shown with arrows, was detected both in the gel (monomer, bottom) and in the stacking (multimer, top). Lanes: 1, MC58; 2, M1M2.

the two membrane-bound lipoproteins (DsbA1 and DsbA2) but not the periplasmic (DsbA3) protein-folding enzyme, both in maintaining resistance to reducing agents and in natural competence. This is in agreement with the work of Tinsley *et al.* (2004), who found similar results with a serogroup C meningococcal strain. We further detected direct interaction between DsbA and the outer-membrane secretin PilQ and also showed that the reduced amount of PilQ in a DsbA1 and DsbA2 double mutant negatively affected transformation.

The growth in broth of our *dsbA*-null strain was reduced, as has been observed in many bacteria (Bardwell *et al.*, 1991; Turcot *et al.*, 2001), but the presence of any one copy of *dsbA* was sufficient to confer wild-type levels of growth. The expression of fully functional pilus and wild-type levels of competence, however, were dependent on the presence of at least one membrane-bound DsbA, confirming that substrate specificities of DsbA1 and DsbA2 overlap in this respect. The expression of *dsbA3* at higher levels in a strain devoid of DsbA1 and DsbA2 (strain M1M2) was unable to restore competence. This indicates a difference in substrate specificity between the periplasmic (DsbA3) and membrane-bound (DsbA1/2) enzymes, perhaps reflecting the difference between the DsbA3 active site (C-V-H-C), and that of DsbA1/2 (C-P-H-C). The membrane proteins also

contain an additional helix compared to DsbA3, potentially affecting protein structure and therefore also enzyme/substrate interactions (Ondo-Mbele *et al.*, 2005). Mutagenesis and crystallization experiments are in progress, which will shed light on this hypothesis.

Neisserial competence is coupled to type IV pilus expression, and the majority of mutations affecting transformation have been attributed to defects in pilus biogenesis (Aas *et al.*, 2002). Surprisingly, strain M1M2 in this study appears to produce intact pilus fibres at levels comparable to wild-type. Both binding of DNA and its uptake were substantially reduced in the absence of DsbA1 and DsbA2, suggestive of a defect in pilus functionality and/or that other competence-related proteins are more sensitive to the lack of DsbA1 and DsbA2 than PilE or PilQ.

Various structural components of the pilus are likely candidates for DsbA-facilitated folding, and mutants may be functionally impaired. Tinsley *et al.* (2004) suggested that DsbA is important for normal folding of the pilin major subunit (PilE) and demonstrated a competence as well as an adherence defect in a DsbA1/2 mutant. Here, our attention has been focused on the outer-membrane secretin PilQ. The PilQ monomer is known to form an internal disulphide bond essential for the structural integrity of the oligomeric outer-membrane complex through which the

pilus is extruded and retracted (Collins *et al.*, 2005). PilQ is normally expressed in large amounts, in a surplus compared to what is required to maintain wild-type levels of pili emanating from the bacterial surface (Tønnum *et al.*, 1998). Specific interactions were demonstrated between DsbA1/2 and the C-terminal domain of the PilQ monomer in solid-phase overlay assays. Reduced levels of both monomeric and multimeric PilQ were accordingly observed in the absence of DsbA1 and DsbA2, consistent with a reduced ability to fold PilQ correctly. The presence of intact pili on M1M2 suggests that enough disulphide-bonded PilQ is formed to produce the secretin without DsbA-catalysed folding, but it may be that the function of the pore or a separable PilQ activity in DNA binding and uptake is affected more profoundly. These findings are also in agreement with the recent discovery that the PilQ complex binds DNA in the core of its central channel (Assalkhou *et al.*, 2007; Frye *et al.*, 2006), explaining why DNA binding and uptake are reduced in the DsbA1/2 mutant. Assalkhou *et al.* (2007) showed that PilQ bound DNA irrespective of the presence of DUS, which was confirmed in our assays, as binding of plasmid DNA with and without DUS was comparably reduced in M1M2. DNA binding and uptake were much more severely reduced in the absence of *pilQ* than of *dsbA1* and *dsbA2* (Fig. 4), consistent with the finding that PilQ function is only partially impaired in the absence of the two protein-folding catalysts.

DsbA can act both as a chaperone and as a protein-folding catalyst, as has been demonstrated in uropathogenic *E. coli*, where it maintains the PapD protein in a folded conformation prior to catalysing disulphide-bond formation (Jacob-Dubuisson *et al.*, 1994). In a similar way, we propose that DsbA1/2 maintain PilQ in a folded state prior to multimer assembly and catalyse the formation of its disulphide bond. In the absence of DsbA1/2, the majority of PilQ formed will be unstable and prone to degradation, resulting in the reduced levels of both PilQ monomer and complex we observe. The folding of PilQ will not be totally abolished in M1M2, as disulphide bonds can still form *in vivo* in the absence of folding catalysts (albeit at a slower and possibly more error-prone rate). Enough native PilQ is produced in M1M2 to enable the formation of the PilQ complex through which T4P is secreted and retracted, but neither the T4P nor PilQ are fully functional: PilQ is likely to be aberrantly folded in the absence of DsbA, which would affect its function, as Tinsley *et al.* (2004) have suggested for the T4P subunit, PilE.

DNA uptake via interaction with PilQ is likely to be only one stage at which the lack of DsbA is revealed. The absence of DsbA1/DsbA2 is anticipated to affect many other proteins, themselves disulphide-bonded, or dependent on interaction with disulphide-bonded proteins for normal conformation or function. In the case of some of these, defective folding may affect different stages of the transformation process, each contributing to the overall competence defect we observe. For example, the folding of

minor pilins crucial for efficient transformation, as well as later stages of the uptake process, such as the passage of DNA across the periplasm and inner membrane, may also involve DsbA-folded proteins and their defective folding will further cumulate the inability for transformation.

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