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The moonlighting protein fructose-1, 6-bisphosphate aldolase of *Neisseria meningitidis*: surface localization and role in host cell adhesion

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Summary

Fructose-1, 6-bisphosphate aldolases (FBA) are cytoplasmic glycolytic enzymes, which despite lacking identifiable secretion signals, have also been found localized to the surface of several bacteria where they bind host molecules and exhibit non-glycolytic functions. *Neisseria meningitidis* is an obligate human nasopharyngeal commensal, which has the capacity to cause life-threatening meningitis and septicemia. Recombinant native *N. meningitidis* FBA was purified and used in a coupled enzymic assay confirming that it has fructose bisphosphate aldolase activity. Cell fractionation experiments showed that meningococcal FBA is localized both to the cytoplasm and the outer membrane. Flow cytometry demonstrated that outer membrane-localized FBA was surface-accessible to FBA-specific antibodies. Mutational analysis and functional complementation was used to identify additional functions of FBA. An FBA-deficient mutant was not affected in its ability to grow *in vitro*, but showed a significant reduction in adhesion to HBME and HEp-2 cells compared to its isogenic parent and its complemented derivative. In summary, FBA is a highly conserved, surface exposed protein that is required for optimal adhesion of meningococci to human cells.
Introduction

*Neisseria meningitidis* remains an important cause of septicemia and meningitis and is associated with high morbidity and mortality (Stephens *et al.*, 2007). As an obligate human commensal, it colonizes the nasopharyngeal mucosa of a substantial proportion of the population in an asymptomatic manner. In susceptible hosts, hyper-invasive strains of meningococci possess the ability to invade the nasopharyngeal sub-mucosa and enter the bloodstream, where they can multiply rapidly to high levels. Meningococci may also translocate across the brain vascular endothelium, proliferate in the cerebral-spinal fluid (CSF) and cause meningitis (Stephens, 2009). To reach the meninges, *N. meningitidis* must therefore interact with two cellular barriers and adhesion to both epithelial and endothelial cells are crucial stages of infection. Various bacterial factors including lipooligosaccharide (LOS), capsule, type IV pili, and outer membrane adhesins such as Opa, Opc, NhhA, App, NadA and MspA have been shown to have a role in meningococcal adhesion and invasion of epithelial and/or endothelial cells (reviewed in Virji, 2009).

Glycolytic enzymes, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), enolase and fructose bisphosphate aldolase (FBA) have been reported as potential virulence factors in a variety of organisms (Pancholi & Chhatwal, 2003). Although long recognized for their cytosolic role in glycolysis and gluconeogenesis, additional or ‘moon-lighting’ functions have been increasingly recognized. In particular, despite lacking identifiable secretion signals, glycolytic enzymes have been found on the bacterial cell surface where they interact directly with host soluble proteins and surface ligands. In *Mycoplasma genitalium*, surface-associated GAPDH was shown to be important for adhesion to human mucin (Alvarez *et al.*, 2003). In *Streptococcus pyogenes* and *Candida albicans* surface-associated GAPDH was shown to bind to fibronectin
(Pancholi & Fischetti, 1992; Gozalbo et al., 1998) and in *Staphylococcus aureus* the cell wall transferrin-binding protein was found to be GAPDH (Modun & Williams, 1999). GAPDH was also reported to be a virulence-associated immunomodulatory protein in *Streptococcus agalactiae* (Madureira et al., 2007). Surface-associated enolase has been reported as a plasminogen-binding protein in *S. pyogenes* (Pancholi & Fischetti, 1998), a fibronectin and plasminogen-binding protein in *Streptococcus suis* (Esgleas et al., 2008; Tian et al., 2009), and a plasminogen and laminin-binding protein in *Bacillus anthracis* (Agarwal et al., 2008). In *Streptococcus pneumoniae*, surface-associated FBA was shown to bind to a large 7-transpass transmembrane receptor belonging to the cadherin superfamily (Blau et al., 2007). FBA and GAPDH were also shown to be immunogenic in humans and capable of inducing a protective immune response against *S. pneumoniae* in mice (Ling et al., 2004). In addition, FBA was found to be a surface-localized immunogenic protein in *S. suis* (Zongfu et al., 2008) and a possible role for FBA in immunity to *Onchocerca volvulus* has also been reported (McCarthy et al., 2002).

Fructose-1, 6-bisphosphate aldolase catalyses the reversible cleavage of fructose-1, 6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Zgiby et al., 2000; Wehmeier, 2001; Ramsaywak et al., 2004). Aldolases can be divided into two groups with different catalytic mechanisms, designated Class-I and Class-II, respectively (Thomson et al., 1998; Arakaki et al., 2004). Class-I FBAs utilize an active site lysine residue to stabilize a reaction intermediate via Schiff-base formation, and are usually found in higher eukaryotic organisms (animals and plants). Class-II FBAs, have an absolute requirement for a divalent ion, usually zinc (Zgiby et al., 2000) and are commonly found in bacteria, archae and lower eukaryotes including fungi and some green algae grown under heterotrophic conditions (Plater et al., 1999; Sauvé & Sygusch, 2001; Ramsaywak et al., 2004). Most organisms contain only one class of FBA, although
a few possess enzymes of both classes. *Escherichia coli* (Alefounder *et al.*, 1989; Thomson *et al.*, 1998), *S. pneumoniae* (Isabel *et al.*, 1999) and *Synechocystis* sp. PCC 6803 (Nakahara *et al.*, 2003) amongst others have been reported to express both types of the enzyme. The Class-II FBAs can be sub-divided into two groups, Type A and B, depending on their amino acid sequences (Sauvé & Sygusch, 2001; Nakahara *et al.*, 2003). Since Class-II FBAs are not found in animals, it has been suggested that they could provide a possible therapeutic or vaccine target (Blom *et al.*, 1996; Ramsaywak *et al.*, 2004).

In *N. meningitidis*, it is noteworthy that, due to the absence of the enzyme phosphofructokinase, the Embden-Meyerhof-Parnas (EMP) glycolytic pathway is rendered non-functional (Baart *et al.*, 2007). Instead, the catabolism of glucose has been shown to be carried out through the Entner-Doudoroff (ED) and Pentose Phosphate pathways (PP) (Baart *et al.*, 2007). Nevertheless, the meningococcal genome retains functional genes for other glycolytic pathway enzymes, presumably for alternative (non-EMP pathway) functions. Furthermore, GAPDH expression (GapA1, but not GapA2) was found to be up-regulated on the meningococcal cell surface following contact with human epithelial cells (Grifantini *et al.*, 2002), although no biological function has so far been ascribed to this observation. In addition, enolase, has recently been shown to be a surface-localized protein in *N. meningitidis*, where it acts to recruit plasminogen onto the bacterial surface (Knaust *et al.*, 2007). The available *N. meningitidis* genome sequences contain a single, putative Class II FBA-encoding gene (*cbbA*), which has not previously been characterized. The aim of this study was to characterize the enzymatic function, sub-cellular localization and putative role of FBA in the pathogenesis of meningococcal infection.
Results

Sequence analysis of the cbbA gene, flanking DNA and FBA protein

In *N. meningitidis* strain MC58, the 1,065-bp *cbbA* gene (locus tag NMB1869) has a G+C content of 55.18% and encodes a predicted protein of 354 amino acids (estimated molecular weight 38.3 kDa). The *cbbA* gene is downstream of, and in the opposite orientation to, *xerC* (NMB1868) encoding the XerC integrase/recombinase and upstream of, and in the same orientation as, NMB1870, which encodes factor H-binding protein, fHbp (Madico *et al*., 2006). A similar genomic arrangement is present in the serogroup A meningococcal strain Z2491 (NMA0588, NMA0587 and NMA0586 encoding XerC, FBA and fHbp, respectively; Parkhill *et al*., 2000), the serogroup C strain FAM18 (Bentley *et al*., 2007) and the ST-4821 strain 053442 (Peng *et al*., 2008), suggesting that this is a conserved arrangement. In these three genomes, the *cbbA* sequences are >94% identical to the MC58 *cbbA* gene. Additionally, sequences >92% identical to MC58 *cbbA* are found in the gonococcal strain FA1090 (94% identical) and *N. lactamica* strain ATCC 23970 (93% identical) confirming that *cbbA* is highly conserved across *Neisseria* species.

At the amino acid level, FBA sequences from meningococcal strains MC58, FAM18, 053442, Z2491 and the gonococcal strain FA1090 are >99% identical. By alignment, the neisserial FBA protein (NMB1869) was 70, 67, 65 and 40% identical to Class-IIB FBA enzymes from *Cupriavidus metallidurans*, *Xanthobacter flavus*, *Synechocystis* sp., and *S. pneumoniae*, respectively, but was only 21 and 29% identical to the *E. coli* and *Haemophilus influenzae* Class-IIA FBA enzymes, respectively, indicating that the neisserial FBA belongs to Class-IIB. Furthermore, the neisserial FBA enzyme contains a 21-amino acid insertion sequence (S^{236}-Y^{256}), which is unique to two subclasses of the Class IIB enzymes. The presence of this insertion sequence suggests that the neisserial FBA enzyme may have a tetrameric quaternary structure, rather than the dimeric...
structure, which is typical of Class II FBA enzymes that lack this sequence (Sauvé & Sygusch, 2001; Izard & Sygusch, 2004). As expected for a Class-II FBA, a conserved putative zinc/cobalt-binding site (Berry & Marshall, 1993) was also identified ($H^{81}$-$XX$-$H^{84}$). FBA of *N. meningitidis* was predicted to be a non-secreted protein by the SignalP-HMM program; although a possible 13-amino acid signal peptide (predicted cleavage site $^{11}$DHA-AE$^{15}$) was identified by SignalP-NN. A signal peptide was similarly predicted for the Class-IIB FBA homologue in *X. flavus*, but not for the homologue in *Synechocystis* sp. or the *E. coli* Class-IIA FBA.

**Cloning, expression and purification of recombinant FBA**

To examine the aldolase function of meningococcal FBA, and to raise FBA-specific antibodies, the *cbbA* gene from MC58 was cloned into the expression vector pQE70 to facilitate the expression and subsequent purification of 6× histidine-tagged recombinant FBA. After induction of *E. coli* cells harboring the FBA expression plasmid, a recombinant protein with an apparent molecular mass consistent with the predicted mass of the tagged protein was strongly expressed, affinity-purified under non-denaturing conditions (Fig. 1A) and used to generate rabbit anti-FBA-specific polyclonal antiserum (RαFBA). Immunoblot analysis confirmed that RαFBA and anti-pentahistidine antibodies both reacted to the purified recombinant FBA (Fig. 1B & C).

**Meningococcal FBA has fructose bisphosphate aldolase activity**

A previously described coupled enzymic assay (Berry & Marshall, 1993) was used to confirm that the purified native meningococcal FBA was active as a fructose bisphosphate aldolase (Fig. 2). Kinetic parameters of the purified enzyme for cleavage of fructose bisphosphate (FBP) were estimated as $K_m$ (FBP) = 0.05 mM and $k_{cat}$ = 126 min$^{-1}$. 
These values are similar to those found for Class-II FBA enzymes from a variety of sources such as *E. coli* (*K*_m (FBP) ~ 0.19 mM and *k*_cat ~ 490 min\(^{-1}\)) (Plater *et al.*, 1999).

**Mutagenesis of cbbA and strain survey**

To examine any additional roles of FBA, a *cbbA* knockout derivative of *N. meningitidis* MC58 was generated. To achieve this, the *cbbA* gene plus flanking DNA was amplified and cloned, and inverse PCR was employed to remove the open reading frame. The product was then ligated to a kanamycin resistance marker and the resulting plasmid used to transform *N. meningitidis* MC58. Using this strategy, the *cbbA* gene was successfully mutated to yield MC58ΔcbbA. The genotype of this mutant was confirmed by PCR and sequencing (data not shown). Immunoblotting using RαFBA showed that a ca. 38-kDa protein corresponding to FBA could be detected in whole cell lysates of wild-type but not MC58ΔcbbA (Fig. 3, lanes 1 & 2) confirming that FBA is expressed under the conditions used and that expression had been abolished in the mutant. In addition to the strongly reactive FBA band, immunoblot analysis showed an additional cross-reactive band at ca. 50 kDa (Fig. 3). However, this band was also present in preparations of the ΔcbbA mutant demonstrating that this protein was not FBA. To further confirm that the ca. 38-kDa immuno-reactive protein was FBA, a wild-type copy of *cbbA* was introduced *in trans* into MC58ΔcbbA using the pYHS25-based plasmid pSAT-12 (Table 1). Introduction of *cbbA* at an ectopic site restored FBA expression (Fig. 3, lane 3). Further immunoblot analyses using a panel of 25 *N. meningitidis* strains (Table 2) including representatives of differing serogroups and sequence types showed that FBA expression was conserved across all strains (data not shown). Expression was also conserved in representative examples of *N. lactamica, N. polysacchareae* and *N. gonorrhoeae* examined (data not shown). These
data complement in silico predictions that cbbA is universally present and constitutively-expressed across Neisseria strains including commensal species.

Meningococcal FBA is localized to the cytoplasm and outer membrane

The sub-cellular localization of FBA was investigated by sub-cellular fractionation followed by immunoblot analysis of the fractions. FBA was predominately detected in outer membrane and cytosolic protein-enriched fractions, but was absent from the cytoplasmic membrane-enriched fraction (Fig. 4). FBA could also be detected in the periplasmic protein-enriched fraction, possibly representing transient FBA during translocation to the outer membrane (Fig. 4). FBA was not detected in concentrated culture supernatants (data not shown). Immunoblotting experiments with antisera against PorA, a known outer membrane protein of N. meningitidis, gave an identical profile except that PorA was absent in the cytosolic fraction (data not shown). These results demonstrate that meningococcal FBA is predominantly a cytosolic protein that is also found in the outer membrane.

Meningococcal FBA is surface accessible to antibodies

In order to investigate whether the outer membrane-localized FBA was accessible on the bacterial cell surface, RaFBA antibodies were used to probe intact meningococcal cells which were then analyzed by flow cytometry. MC58 cells treated with RaFBA alone or secondary antibody alone did not produce high fluorescence signals (3.4 and 4.4 mean fluorescence intensities, respectively), whilst cells treated with RaFBA followed by anti-rabbit IgG-Alexa Flour 488 conjugate demonstrated a clear shift in fluorescence signal (55.2 mean fluorescence intensity) confirming the cell surface localization of FBA (Fig. 5A). No shift in fluorescence signal was observed when MC58ΔcbbA cells were
examined under identical conditions (Fig. 5B; mean fluorescence intensity of 12.1 compared to samples treated with primary or secondary alone [3.6 and 6.7, respectively]). From the wild-type cells probed with both antibodies, 79.05% were found in the M1 region (Fig. 5A), suggesting that the majority of the population had FBA present on the cell surface. Pre-immune sera showed no reactivity against wild-type MC58 in immunoblot experiments confirming that the binding of RαFBA to wild-type MC58 observed by flow cytometry was FBA-specific.

FBA is required for efficient adhesion to host cells

Viable counts of bacteria associated with homogenized infected monolayers were used to compare the capacity of the wild-type, FBA mutant and complemented mutant strains to associate with, and invade human brain microvascular endothelial (HBME) cells. FBA-deficient meningococci had a significantly reduced capacity to adhere to monolayers of HBME cells (Fig. 6A). No statistically significant reduction was observed in the ability of the FBA mutant to invade monolayers of HBME cells (Fig. 6B). Similar results were also obtained using HEp-2 (human larynx carcinoma) cells, confirming that the effect was not cell-type specific. To confirm that the observed effects were not due to an impairment in in vitro growth, the growth rate of the strains was compared by measuring the optical density at 600 nm (OD$_{600}$) and determining the viable counts of broth cultures sampled during exponential growth over 24 h in triplicate on three separate occasions. No significant difference between strains was observed (data not shown). To further exclude the possibility that mutation of cbbA affected expression of the downstream gene encoding factor H binding protein, whole cell lysates of MC58, MC58ΔcbbA and MC58ΔcbbA cbbAEct were probed with anti-fHbp. Expression levels of this protein were
similar in the three strains. In summary, these experiments show that FBA plays a role in the adherence of *N. meningitidis* to human cells.

**Discussion**

An increasing number of reports show that classical cytoplasmic house-keeping enzymes without identifiable secretion signals may be localized to the surface of microbial pathogens, where they exhibit various functions, unrelated to glycolysis (Pancholi & Chhatwal, 2003). One such protein, fructose-1, 6-bisphosphate aldolase (FBA) has been previously reported to be localized to the surface of some Gram-positive bacteria. In *S. pneumoniae*, for example, surface-exposed FBA (Class IIB) was demonstrated to act as an adhesin, specifically binding to a large 7-transpass transmembrane receptor belonging to the cadherin superfamily (Blau *et al.*, 2007). In *N. meningitidis*, two cytoplasmic house-keeping enzymes, GAPDH and enolase have been shown to be surface-localized, and enolase has been suggested to act as a plasminogen receptor (Grifantini *et al.*, 2002; Knaust *et al.*, 2007). The non-glycolytic role(s) of GAPDH on the bacterial surface is undefined. We undertook to investigate whether FBA was also surface-localized in *N. meningitidis*, as has been described for GAPDH and enolase, and to determine whether FBA plays a role in meningococcal pathogenesis.

In the published meningococcal and gonococcal genome sequences, there is only one gene, *cbbA*, encoding a putative FBA enzyme. Despite being predicted to be part of a non-functional metabolic pathway, *cbbA* has not acquired spontaneous mutations in any of the Neisserial genomes examined, which suggests that the gene was acquired recently, that the glycolytic pathway became non-functional recently, or that the protein has one or more additional functions. Sequence analysis shows that FBA is highly-conserved at the amino acid level, and is a Class-IIB enzyme. A 21-amino acid insertion sequence, which
is present in two subclasses of the Class IIB FBA enzymes, was also present in the Neisserial sequences. The presence of this sequence suggests that the neisserial FBA enzyme is a tetrameric, rather than a dimeric enzyme; a feature which is present in extremophiles, and which has been suggested to confer thermal stability (Sauvé & Sygusch, 2001; Izard & Sygusch, 2004). Unexpectedly for a presumed cytosolic protein, a possible signal sequence was predicted for the neisserial FBA enzyme, suggesting a possible means of translocation across the cytoplasmic membrane.

In this study, recombinant FBA (rFBA) was expressed and purified under non-denaturing conditions. Purified rFBA was shown to have aldolase activity confirming that the enzyme was in native conformation after purification. The purified protein was also used to generate rabbit polyclonal anti-FBA antiserum (RαFBA), which was used to confirm that FBA was expressed in vitro in each of a range of Neisserial strains tested including commensal species. This suggests that FBA plays an important role which is required by both non-pathogenic and pathogenic lineages. FBA was shown to be present in both the cytosol and to be exposed at the cell surface of wild-type meningococci in a form that was accessible to antibodies, suggesting that, similar to GAPDH and enolase, FBA is translocated (or diverted) to the outer membrane. An alternative hypothesis is that FBA is released from lysed cells and recruited back onto the surface of intact meningococci; however we have probed lysates of cbbA-deficient meningococci following co-incubation with recombinant FBA and found no reactivity with RαFBA (data not shown). In M. genitalium, only a small proportion of the total cellular GAPDH is trafficked to the bacterial surface, however, this is sufficient to impart a biologically-significant phenotype (mucin-binding) on this organism (Alvarez et al., 2003). For organisms with relatively small genomes, multi-functional proteins may be advantageous
to optimize the potential of the genome. To our knowledge, this is the first report
demonstrating that a proportion of FBA is found on the cell surface of meningococci.

An FBA-deficient mutant grew at the same rate (in broth culture and on solid
media) as the wild-type and the complemented mutant strains, demonstrating that FBA is
not required for growth of the meningococcus under the in vitro conditions used. No
differences in either colony or bacterial cell morphology (using light microscopy) were
observed. The FBA-deficient mutant strain exhibited a significantly reduced capacity to
adhere to both HBME and HEp-2 cells. This phenotype was completely restored in a
complemented strain. Our observation that FBA is involved in adhesion to both epithelial
and endothelial cells, and that FBA expression is conserved in non-pathogenic strains
(such as *N. polysacchareae*) may suggest a role for FBA during colonization of the
nasopharyngeal mucosa by commensal Neisserial species.

Although we have shown that FBA is present on the meningococcal cell surface
and is required for optimal adhesion, the role of FBA in this process is unknown. It is a
possible that the enzymatic activity of FBA plays an indirect role that is required for
optimal adhesion. However, given that other FBA homologues (such as FBA in *S.
pneumoniae*) have been shown to directly bind to host cell ligands it is also possible that
the meningococcal protein has a direct host receptor binding activity. Determining the
ability of meningococci expressing a non-enzymatically functional FBA to adhere to host
cells would address this. *S. pneumoniae* FBA was shown to bind to a cadherin
superfamily receptor (Flamingo cadherin receptor, FCR) on the surface of host epithelial
cells, but it is unknown which FBA residues participate in this interaction (Blau *et al*.,
2007). Meningococcal FBA is only 40% identical to the pneumococcal enzyme at the
amino acid level, so it is unclear whether meningococcal FBA binds the same receptor.
Work is currently in progress to determine whether meningococcal FBA binds to FCR or a different host cell receptor.

Pneumococcal FBA has been shown to be immunogenic in humans and capable of eliciting a partially protective immune response against lethal *S. pneumoniae* intranasal challenge in mice (Ling *et al.*, 2004). Given that meningococcal FBA is: highly conserved, expressed by a wide range of isolates, surface-accessible to antibodies and structurally and antigenically unrelated to the human (Class I) FBA protein, meningococcal FBA is worthy of future study as a possible candidate vaccine component against this important human pathogen.

**Experimental procedures**

*Bacterial strains and growth conditions*

*Escherichia coli* TOP10F' and BL21(DE3) pLysS (Table 1) were used for the expression of 6 × histidine-tagged recombinant FBA encoded by plasmid pSAT-FBA (Table 1). *E. coli* JM109 was used as a host strain for the construction of mutagenic and complementation plasmids, pSAT-4 and pSAT-12, respectively. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth or on LB agar supplemented, where appropriate, with ampicillin (100 µg ml⁻¹), kanamycin (30 µg ml⁻¹) or erythromycin (200 µg ml⁻¹). Strains of *Neisseria* (Tables 1 and 2) were grown at 37°C, air plus 5% CO₂, on Brain Heart Infusion (BHI) agar supplemented with 1% Vitox (Oxoid) and kanamycin (50 µg ml⁻¹) or erythromycin (5 µg ml⁻¹) where appropriate.

**DNA manipulation**

Genomic DNA was extracted from *N. meningitidis* using the DNeasy Tissue kit (Qiagen). Plasmid DNA was prepared by using the QIAprep Spin kit (Qiagen). Restriction enzymes
and T4 DNA ligase were purchased from Roche. All enzymatic reactions were carried out according to the manufacturer’s instructions. DNA sequencing was carried out at the School of Biomedical Sciences (University of Nottingham) on an ABI 377 automated DNA sequencer.

**Preparation of recombinant FBA**

The *cbbA* gene was amplified from *N. meningitidis* MC58 using oligonucleotide primers FBA_pQE70 (F) and FBA_pQE70 (R) (Table 3) using the Expand High Fidelity PCR system (Roche). The resulting amplicon was digested with SphI and BglII, before being ligated into similarly treated pQE70, and the resulting plasmid, pSAT-FBA, used to transform *E. coli* BL21 (DE3) pLysS. Transformants were grown to log phase, induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h, and harvested by centrifugation. Recombinant 6 × histidine-tagged FBA was then affinity-purified under native conditions. Briefly, the culture pellet from an IPTG-induced culture of *E. coli* BL21 (DE3) pLysS (pSAT-FBA) was dissolved in 20 ml lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 7.4) and disrupted by sonication using a MSE Soniprep 150 for 10 cycles (each cycle consisting of a 10 s burst followed by a 10 s cooling period). The cell lysate was then mixed with 1 ml HisPur™ Cobalt Resin (Pierce) and incubated overnight at 4°C. The lysate-resin mixture was then applied to a column, and washed with 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 15 mM imidazole, pH 7.4. Bound protein was then eluted in elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 300 mM imidazole, pH 7.4).

**Production of a rabbit antiserum against purified recombinant FBA**
New Zealand White female rabbits were immunized subcutaneously four times at 2-week intervals with 30 µg of recombinant FBA protein emulsified in Freud’s complete (first immunization only) or incomplete adjuvant. After three injections, the animals were tested, boosted once more and sacrificed 10 days later.

**SDS-PAGE and immunoblotting**

Proteins were electrophoretically separated using 10% polyacrylamide gels (Mini-Protean III; Bio-Rad) and were stained using SimplyBlue Safestain™ (Invitrogen) or transferred to nitrocellulose membranes as previously described (Kizil et al., 1999). Membranes were probed with mouse anti-pentahistidine antibody (Qiagen) or rabbit primary antibody diluted 1:10,000 or 1:1,000 respectively in blocking buffer (5% [wt/vol] non fat dry milk, 0.1% [vol/vol] Tween 20 in 1 × phosphate-buffered saline [PBS]) and incubated for 2 h. After being washed in PBS with 0.1% Tween 20 (PBST), membranes were incubated for 2 h with 1:30,000-diluted goat anti-mouse (or anti-rabbit) IgG-alkaline phosphatase conjugate (Sigma). After washing with PBST, blots were developed using BCIP/NBT-Blue liquid substrate (Sigma).

**Kinetic analysis of fructose bisphosphate aldolase activity**

This was done using a previously described methodology (Berry & Marshall, 1993). Briefly, the assay was performed at 30°C in 1 ml 50 mM Tris-HCl supplemented with 0.1 M potassium acetate buffer (pH 8.0) containing 0.1 - 5 mM fructose 1, 6-bisphosphate (FBP), 0.2 mM NADH and 2 µl of a 10 mg ml⁻¹ mixture of glycerol phosphate dehydrogenase/triose phosphate isomerase (coupling enzymes). The reagents were added in the order: buffer; FBP; NADH; coupling enzymes. Finally, the reaction was started by adding 0.26 nmol of purified native FBA. A decrease in absorbance at 340 nm was
recorded as the measure of enzyme activity on an Uvikon 930 spectrophotometer. Activities were calculated using the molar extinction coefficient for NADH as 6220 M$^{-1}$ cm$^{-1}$. One unit of aldolase activity was defined as the amount of enzyme which catalyzed the oxidation of 2 µmol NADH/min. Kinetic parameters were determined using Origin Pro 7.5 software.

**Construction of MC58∆cbbA**

A 2.3-kb fragment of DNA consisting of the *cbbA* gene and flanking DNA was amplified using FBA_M1(F) and FBA_M2(R) (Table 3) from *N. meningitidis* MC58 chromosomal DNA. The amplified DNA was TA cloned into the pGEM-T Easy vector to generate pSAT-2. This was then subject to inverse PCR using primers FBA_M3(IR) and FBA_M4(IF) (Table 3) resulting in the amplification of a 5-kb amplicon in which the *cbbA* coding sequence was deleted and a unique BglII site had been introduced. The BglII site was used to introduce a kanamycin resistance cassette, BamHI-digested from pJMK30 (Table 1), in place of *cbbA*. One of the resulting plasmids, pSAT-4, containing the resistance cassette in the same orientation as the deleted gene, was confirmed by restriction digestion and sequencing and subsequently used to mutate the meningococcal strain MC58 by natural transformation and allelic exchange as previously described (Hadi *et al.*, 2001). The deletion in the resulting mutant (MC58∆cbbA) was confirmed by PCR analysis and immunoblotting. Growth curve assays carried out using liquid cultures showed no significant differences between MC58∆cbbA and the wild-type strain (data not shown).

**Complementation of cbbA**
A fragment corresponding to the cbbA coding sequence and upstream promoter was amplified from chromosomal DNA of strain MC58 with High Fidelity Expand Taq (Roche) using the primers FBA_COM(F) and FBA_COM(R) (Table 3) incorporating BamHI-sites into the amplified fragment. The BamHI-digested fragment was then introduced into a unique BamHI-site in pYHS25. This vector contains an erythromycin resistance gene flanked by the MC58 genes NMB0102 and NMB0103 (Winzer et al., 2002). The resulting plasmid pSAT-12 was used to transform MC58ΔcbbA by natural transformation, thus introducing a single chromosomal copy of cbbA and the downstream erythromycin resistance cassette in the intergenic region between NMB0102 and NMB0103. Insertion of the cbbA gene and erythromycin resistance cassette at the ectopic site was confirmed by PCR analysis and sequencing. Expression of FBA was confirmed by immunoblotting.

Sub-cellular localization of FBA

Cells from 100 ml overnight BHI broth cultures were harvested at 13,000 × g for 2 min and the pellet re-suspended in 1 ml of EB buffer (10 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 25% sucrose), and washed twice in the same buffer. Finally, the pellet was re-suspended in EB buffer and incubated for 10 min on ice. The preparation was centrifuged at 13,000 × g for 4 min; following re-suspension in 0.4 ml of ice cold water, the mixture was incubated on ice for a further 10 min, followed by centrifugation at 13,000 × g for 2 min. The supernatant, containing periplasmic proteins, was removed and stored at -20°C. The remaining cell pellet (spheroplasts) were re-suspended into 0.4 ml Tris-HCl (pH 7.5) and sonicated using a MSE Soniprep 150 for 10 cycles (each cycle consisting of a 10 s burst followed by a 10 s cooling period) to release the cytoplasmic contents. Non-disrupted cells were removed by centrifugation at 5,000 × g for 1 min. The upper clear
supernatant was transferred to a fresh tube and centrifuged at 17,000 × g for 30 min. The supernatant (representing the cytosolic fraction) was removed and stored at -20°C. The remaining pellet was re-suspended in 0.4 ml of 10 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 2% Triton X-100. The sample was incubated at 37°C for 30 min and then centrifuged at 17,000 × g for 30 min. The supernatant, enriched for cytoplasmic membrane proteins, was removed and stored at -20°C. The final pellet (enriched for outer membrane proteins) was re-suspended by brief sonication in 1 ml 10 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1% Triton X-100, incubated at 37°C for 30 min and then centrifuged at 17,000 × g for 30 min. The pellet was re-suspended in 0.2 ml 10 mM Tris-HCl pH 7.5 and stored at -20°C.

**Flow cytometry**

*N. meningitidis* strains were grown to mid-log phase (OD₆₀₀ approximately 0.7). 1 × 10⁷ CFU aliquots were centrifuged at 5,000 × g for 5 min and resuspended in 0.2 µm-filtered PBS. The cells were incubated for 2 h with αR-FBA (1:500 diluted in PBS containing 0.1% BSA, 0.1% sodium azide and 2% foetal calf serum) and untreated cells were used as a control. Cells were washed three times with PBS and incubated for 2 h in the dark with goat anti-rabbit IgG-Alexa Flour 488 conjugate (Invitrogen; diluted 1:50 in PBS containing 0.1% BSA, 0.1% sodium azide and 2% foetal calf serum). Again, untreated cells were used as a control. Finally, the samples were washed in PBS twice before being re-suspended in 1 ml PBS containing 0.5% formaldehyde to fix the cells. Samples were analyzed for fluorescence using a Coulter Altra Flow Cytometer. Cells were detected using forward and log-side scatter dot plots, and a gating region was set to exclude cell debris and aggregates of bacteria. A total of 50,000 bacteria (events) were analyzed.

**Association and invasion assays**
Association and invasion assays were performed essentially as previously described (Oldfield et al., 2007). Briefly, human brain microvascular endothelial (HBME) or larynx carcinoma (HEp-2) cells were grown to confluence in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen) and 2% antibiotic anti-mycotic solution (Invitrogen) in 24-well tissue culture plates (Costar) at 37°C in an atmosphere of 5% CO\(_2\). Prior to all experiments, mono-layers were transferred to DMEM supplemented with 2% FCS to remove the antibiotics. Meningococci were cultured in MHB for 2 h and monolayers were infected with 1 × 10\(^6\) CFU of meningococci and incubated for 2 h (association) or 4 h (invasion) in 5% CO\(_2\) at 37°C. To assess total cell association, monolayers were washed four times with 1 ml PBS per well. To assess invasion, monolayers were further incubated in DMEM containing gentamicin (100 µg ml\(^{-1}\)) for 2 h. Prior to further steps, aliquots of the gentamicin-containing supernatants were plated out to confirm killing of extra-cellular bacteria. Furthermore, the susceptibility of all meningococcal strains to gentamicin at 100 µg ml\(^{-1}\) was confirmed prior to testing. Monolayers were then washed four times with 1 ml PBS. In both association and invasion experiments, monolayers were then disrupted and homogenized in 1 ml 0.1% saponin in PBS. Meningococci were enumerated by serial dilution of the homogenized suspensions and subsequent determination of colony-forming units by plating 50 µl aliquots from appropriate dilutions of the lysates on agar. All association and invasion assays were repeated at least three times. Statistical significance was measured using a two-tailed Student \(t\)-test.

**Protein and nucleic acid sequence analysis**

Public databases containing previously published protein and DNA sequences were searched using the BLAST and PSI-BLAST algorithms available at...
http://blast.ncbi.nlm.nih.gov/Blast.cgi. The genome database of *N. meningitidis* MC58 was interrogated at [http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=gnm](http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=gnm). Sequence homology data were obtained using the CLUSTALX software ([http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX](http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX)). Protein secretion signals were analyzed using the SignalP 3.0 server available at [http://www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/) (Emanuelsson *et al.*, 2007). GenBank accession numbers for the *cbbA* sequences analyzed in this study are as follows: YP_974462 (FAM18), YP_001598513 (ST-4821 strain 053442), YP_002342063 (Z2491), YP_207215 (gonococcal strain FA1090) and ZP_03723075 (*N. lactamica* ATCC 23970).
Acknowledgements

We wish to thank Dr. A Robins for assistance with the flow cytometry experiments, and Prof. Kim (John Hopkins University School of Medicine, Baltimore, US) for providing HBME cells.
### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<td><em>E. coli</em></td>
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<td></td>
</tr>
<tr>
<td>JM109</td>
<td>endA1 recA1 gyrA96 thi hsdR17 (rK’/rK) relA1 supE44 Δ(lac-proAB) [F’ traD36 proAB laqI’ZΔM15]</td>
<td>Promega</td>
</tr>
<tr>
<td>TOP10F</td>
<td>F’lacIqTn10(TetR) mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG</td>
<td>Invitrogen</td>
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<tr>
<td>BL21(DE3)pLysS</td>
<td>F- ompT hsdSB (rB-mB-) gal dcm (DE3) pLysS (CamR)</td>
<td>Invitrogen</td>
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<td><strong>N. meningitidis</strong></td>
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<tr>
<td>MC58</td>
<td>wild-type serogroup B strain</td>
<td>(Tettelin <em>et al.</em>, 2000)</td>
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<tr>
<td>MC58ΔcbbA</td>
<td>cbbA deletion and replacement with kanamycin cassette</td>
<td>This study</td>
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<tr>
<td>MC58ΔcbbA cbbAEct</td>
<td>MC58ΔcbbA complemented with an ectopic copy of cbbA</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pQE70</td>
<td>Cloning vector encoding resistance to ampicillin</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pSAT-FBA</td>
<td>MC58 cbbA gene cloned in pQE70</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>Cloning vector encoding resistance to ampicillin</td>
<td>Promega</td>
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<tr>
<td>pSAT-2</td>
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<td>This study</td>
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<tr>
<td>pJMK30</td>
<td>Source of kanamycin resistance cassette</td>
<td>(van Vliet <em>et al.</em>, 1998)</td>
</tr>
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<td>pSAT-4</td>
<td>pSAT-2 containing the kanamycin resistance cassette in the same orientation as the deleted cbbA gene</td>
<td>This study</td>
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<tr>
<td>pYHS25</td>
<td>Ectopic complementation vector encoding resistance to erythromycin</td>
<td>(Winzer <em>et al.</em>, 2002)</td>
</tr>
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<td>pSAT-12</td>
<td>pYHS25 containing cbbA</td>
<td>This study</td>
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Table 2. Isolates of *N. meningitidis* examined for the expression of FBA

<table>
<thead>
<tr>
<th>Strain</th>
<th>Country of origin</th>
<th>Date of isolation</th>
<th>Disease</th>
<th>Serogroup</th>
<th>Sequence type</th>
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<td>Z1001</td>
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<td>Z1035</td>
<td>Pakistan</td>
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<td>meningitis and septicaemia</td>
<td>A</td>
<td>1</td>
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<tr>
<td>Z1054</td>
<td>Finland</td>
<td>1975</td>
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<td>A</td>
<td>5</td>
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<tr>
<td>Z1213</td>
<td>Ghana</td>
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<tr>
<td>Z1269</td>
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<td>Z1503</td>
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<td>UK</td>
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<tr>
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Further details of strains are available at [http://pubmlst.org/](http://pubmlst.org/).
<table>
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<th>Primer</th>
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<th>Restriction site</th>
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<tr>
<td>FBA_pQE70 (R)</td>
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<td>BglII</td>
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<td>FBA_M1(F)</td>
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<td>FBA_M2(R)</td>
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<td></td>
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<td>FBA_M4(IF)</td>
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<td>FBA_COM(R)</td>
<td>CGCGGATCCCGCATTTTGTACAGGCAACCTG</td>
<td>BamHI</td>
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\(^a\) All primers were designed from the *N. meningitidis* MC58 genome sequence. Sequences in bold identify restriction enzyme sites.
References


Figure Legends

**Fig. 1.** SDS-PAGE analysis confirms the purity of the recombinant FBA purified under native conditions (A). Immunoblot analysis shows that recombinant FBA is recognized by RαFBA (B) and anti-pentahistidine antibodies (C).

**Fig. 2.** Coupled enzymic assay to measure the activity of meningococcal fructose 1, 6-bisphosphate aldolase. Cleavage of fructose 1, 6-bisphosphate (FBP) was coupled to α-glycerophosphate dehydrogenase and NAD oxidation. One unit of aldolase activity was defined as the amount of enzyme which catalyzed the oxidation of 2 µmol NADH/min.

**Fig. 3.** Immunoblot analysis of whole cell proteins from the *N. meningitidis* MC58 wild-type, ΔcbbA mutant derivative and complemented mutant reveals the absence of FBA in the ΔcbbA mutant preparation.

**Fig. 4.** Sub-cellular localization of FBA. Cytosolic protein-enriched (CP), periplasmic protein-enriched (PP), cytoplasmic membrane protein-enriched (CM) and outer membrane protein-enriched (OM) fractions of MC58 were separated on a 10% acrylamide gel and probed in immunoblotting experiments with RαFBA.

**Fig. 5.** Flow cytometric analysis of MC58 wild-type (A) or MC58ΔcbbA cells (B) for FBA surface localization. Cells were stained with RαFBA (primary alone), anti-rabbit IgG-Alexa Flour 488 conjugate (secondary alone) or both. Fluorescence was displayed as a histogram. The histogram area in M1 represents the population of fluorescently labeled meningococci.
Fig. 6. FBA-deficient meningococci have a reduced ability to associate with (A) but not invade into (B) HBME cells compared to the wild-type or complemented strains. The number of FBA-deficient meningococci associating was significantly lower than the wild-type (*$P = 0.0011$). Numbers of mutant cells invading was not significantly lower compared to the wild-type ($P = 0.13$). Similar experiments were also carried out using HEp-2 cells with consistent results. Mean levels shown from three independent experiments, each using triplicate wells. Bars denote standard deviation. Cfu denotes colony forming units.
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