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



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

4  
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15 Running title: **Meningococcal aldolase has a role in adhesion**

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17 pathogenesis

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## 23 Summary

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

## 39 Introduction

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

54 Glycolytic enzymes, such as glyceraldehyde 3-phosphate dehydrogenase  
55 (GAPDH), enolase and fructose bisphosphate aldolase (FBA) have been reported as  
56 potential virulence factors in a variety of organisms (Pancholi & Chhatwal, 2003).  
57 Although long recognized for their cytosolic role in glycolysis and gluconeogenesis,  
58 additional or 'moon-lighting' functions have been increasingly recognized. In particular,  
59 despite lacking identifiable secretion signals, glycolytic enzymes have been found on the  
60 bacterial cell surface where they interact directly with host soluble proteins and surface  
61 ligands. In *Mycoplasma genitalium*, surface-associated GAPDH was shown to be  
62 important for adhesion to human mucin (Alvarez *et al.*, 2003). In *Streptococcus pyogenes*  
63 and *Candida albicans* surface-associated GAPDH was shown to bind to fibronectin

64 (Pancholi & Fischetti, 1992; Gozalbo *et al.*, 1998) and in *Staphylococcus aureus* the cell  
65 wall transferrin-binding protein was found to be GAPDH (Modun & Williams, 1999).  
66 GAPDH was also reported to be a virulence-associated immunomodulatory protein in  
67 *Streptococcus agalactiae* (Madureira *et al.*, 2007). Surface-associated enolase has been  
68 reported as a plasminogen-binding protein in *S. pyogenes* (Pancholi & Fischetti, 1998), a  
69 fibronectin and plasminogen-binding protein in *Streptococcus suis* (Esgleas *et al.*, 2008;  
70 Tian *et al.*, 2009), and a plasminogen and laminin-binding protein in *Bacillus anthracis*  
71 (Agarwal *et al.*, 2008). In *Streptococcus pneumoniae*, surface-associated FBA was shown  
72 to bind to a large 7-transpass transmembrane receptor belonging to the cadherin  
73 superfamily (Blau *et al.*, 2007). FBA and GAPDH were also shown to be immunogenic  
74 in humans and capable of inducing a protective immune response against *S. pneumoniae*  
75 in mice (Ling *et al.*, 2004). In addition, FBA was found to be a surface-localized  
76 immunogenic protein in *S. suis* (Zongfu *et al.*, 2008) and a possible role for FBA in  
77 immunity to *Onchocerca volvulus* has also been reported (McCarthy *et al.*, 2002).

78 Fructose-1, 6-bisphosphate aldolase catalyses the reversible cleavage of fructose-  
79 1, 6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate  
80 (Zgiby *et al.*, 2000; Wehmeier, 2001; Ramsaywak *et al.*, 2004). Aldolases can be divided  
81 into two groups with different catalytic mechanisms, designated Class-I and Class-II,  
82 respectively (Thomson *et al.*, 1998; Arakaki *et al.*, 2004). Class-I FBAs utilize an active  
83 site lysine residue to stabilize a reaction intermediate via Schiff-base formation, and are  
84 usually found in higher eukaryotic organisms (animals and plants). Class-II FBAs, have  
85 an absolute requirement for a divalent ion, usually zinc (Zgiby *et al.*, 2000) and are  
86 commonly found in bacteria, archae and lower eukaryotes including fungi and some  
87 green algae grown under heterotrophic conditions (Plater *et al.*, 1999; Sauvé & Sygusch,  
88 2001; Ramsaywak *et al.*, 2004). Most organisms contain only one class of FBA, although

89 a few possess enzymes of both classes. *Escherichia coli* (Alefounder *et al.*, 1989;  
90 Thomson *et al.*, 1998), *S. pneumoniae* (Isabel *et al.*, 1999) and *Synechocystis* sp. PCC  
91 6803 (Nakahara *et al.*, 2003) amongst others have been reported to express both types of  
92 the enzyme. The Class-II FBAs can be sub-divided into two groups, Type A and B,  
93 depending on their amino acid sequences (Sauvé & Sygusch, 2001; Nakahara *et al.*,  
94 2003). Since Class-II FBAs are not found in animals, it has been suggested that they  
95 could provide a possible therapeutic or vaccine target (Blom *et al.*, 1996; Ramsaywak *et*  
96 *al.*, 2004).

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

113

## 114 Results

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

116 In *N. meningitidis* strain MC58, the 1,065-bp *cbbA* gene (locus tag NMB1869) has a G+C  
117 content of 55.18% and encodes a predicted protein of 354 amino acids (estimated  
118 molecular weight 38.3 kDa). The *cbbA* gene is downstream of, and in the opposite  
119 orientation to, *xerC* (NMB1868) encoding the XerC integrase/recombinase and upstream  
120 of, and in the same orientation as, NMB1870, which encodes factor H-binding protein,  
121 fHbp (Madico *et al.*, 2006). A similar genomic arrangement is present in the serogroup A  
122 meningococcal strain Z2491 (NMA0588, NMA0587 and NMA0586 encoding XerC,  
123 FBA and fHbp, respectively; Parkhill *et al.*, 2000), the serogroup C strain FAM18  
124 (Bentley *et al.*, 2007) and the ST-4821 strain 053442 (Peng *et al.*, 2008), suggesting that  
125 this is a conserved arrangement. In these three genomes, the *cbbA* sequences are >94%  
126 identical to the MC58 *cbbA* gene. Additionally, sequences >92% identical to MC58 *cbbA*  
127 are found in the gonococcal strain FA1090 (94% identical) and *N. lactamica* strain ATCC  
128 23970 (93% identical) confirming that *cbbA* is highly conserved across *Neisseria* species.  
129 At the amino acid level, FBA sequences from meningococcal strains MC58, FAM18,  
130 053442, Z2491 and the gonococcal strain FA1090 are >99% identical. By alignment, the  
131 neisserial FBA protein (NMB1869) was 70, 67, 65 and 40% identical to Class-IIB FBA  
132 enzymes from *Cupriavidus metallidurans*, *Xanthobacter flavus*, *Synechocystis* sp., and *S.*  
133 *pneumoniae*, respectively, but was only 21 and 29% identical to the *E. coli* and  
134 *Haemophilus influenzae* Class-IIA FBA enzymes, respectively, indicating that the  
135 neisserial FBA belongs to Class-IIB. Furthermore, the neisserial FBA enzyme contains a  
136 21-amino acid insertion sequence (S<sup>236</sup>-Y<sup>256</sup>), which is unique to two subclasses of the  
137 Class IIB enzymes. The presence of this insertion sequence suggests that the neisserial  
138 FBA enzyme may have a tetrameric quaternary structure, rather than the dimeric

139 structure, which is typical of Class II FBA enzymes that lack this sequence (Sauvé &  
140 Sygusch, 2001; Izard & Sygusch, 2004). As expected for a Class-II FBA, a conserved  
141 putative zinc/cobalt-binding site (Berry & Marshall, 1993) was also identified (H<sup>81</sup>-XX-  
142 H<sup>84</sup>). FBA of *N. meningitidis* was predicted to be a non-secreted protein by the SignalP-  
143 HMM program; although a possible 13-amino acid signal peptide (predicted cleavage site  
144 <sup>11</sup>DHA-AE<sup>15</sup>) was identified by SignalP-NN. A signal peptide was similarly predicted for  
145 the Class-IIB FBA homologue in *X. flavus*, but not for the homologue in *Synechocystis*  
146 sp. or the *E. coli* Class-IIA FBA.

147

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

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

158

#### 159 *Meningococcal FBA has fructose bisphosphate aldolase activity*

160 A previously described coupled enzymic assay (Berry & Marshall, 1993) was used to  
161 confirm that the purified native meningococcal FBA was active as a fructose  
162 bisphosphate aldolase (Fig. 2). Kinetic parameters of the purified enzyme for cleavage of  
163 fructose bisphosphate (FBP) were estimated as  $K_m$  (FBP) = 0.05 mM and  $k_{cat}$  = 126 min<sup>-1</sup>.

164 These values are similar to those found for Class-II FBA enzymes from a variety of  
165 sources such as *E. coli* ( $K_m$  (FBP)  $\sim$  0.19 mM and  $k_{cat}$   $\sim$  490 min<sup>-1</sup>) (Plater *et al.*, 1999).

166

#### 167 *Mutagenesis of cbbA and strain survey*

168 To examine any additional roles of FBA, a *cbbA* knockout derivative of *N. meningitidis*  
169 MC58 was generated. To achieve this, the *cbbA* gene plus flanking DNA was amplified  
170 and cloned, and inverse PCR was employed to remove the open reading frame. The  
171 product was then ligated to a kanamycin resistance marker and the resulting plasmid used  
172 to transform *N. meningitidis* MC58. Using this strategy, the *cbbA* gene was successfully  
173 mutated to yield MC58 $\Delta$ *cbbA*. The genotype of this mutant was confirmed by PCR and  
174 sequencing (data not shown). Immunoblotting using R $\alpha$ FBA showed that a *ca.* 38-kDa  
175 protein corresponding to FBA could be detected in whole cell lysates of wild-type but not  
176 MC58 $\Delta$ *cbbA* (Fig. 3, lanes 1 & 2) confirming that FBA is expressed under the conditions  
177 used and that expression had been abolished in the mutant. In addition to the strongly  
178 reactive FBA band, immunoblot analysis showed an additional cross-reactive band at *ca.*  
179 50 kDa (Fig. 3). However, this band was also present in preparations of the  $\Delta$ *cbbA* mutant  
180 demonstrating that this protein was not FBA. To further confirm that the *ca.* 38-kDa  
181 immuno-reactive protein was FBA, a wild-type copy of *cbbA* was introduced *in trans* into  
182 MC58 $\Delta$ *cbbA* using the pYHS25-based plasmid pSAT-12 (Table 1). Introduction of *cbbA*  
183 at an ectopic site restored FBA expression (Fig. 3, lane 3). Further immunoblot analyses  
184 using a panel of 25 *N. meningitidis* strains (Table 2) including representatives of differing  
185 serogroups and sequence types showed that FBA expression was conserved across all  
186 strains (data not shown). Expression was also conserved in representative examples of *N.*  
187 *lactamica*, *N. polysacchareae* and *N. gonorrhoeae* examined (data not shown). These

188 data complement *in silico* predictions that *cbbA* is universally present and constitutively-  
189 expressed across *Neisseria* strains including commensal species.

190

191 *Meningococcal FBA is localized to the cytoplasm and outer membrane*

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

203

204 *Meningococcal FBA is surface accessible to antibodies*

205 In order to investigate whether the outer membrane-localized FBA was accessible on the  
206 bacterial cell surface, R $\alpha$ FBA antibodies were used to probe intact meningococcal cells  
207 which were then analyzed by flow cytometry. MC58 cells treated with R $\alpha$ FBA alone or  
208 secondary antibody alone did not produce high fluorescence signals (3.4 and 4.4 mean  
209 fluorescence intensities, respectively), whilst cells treated with R $\alpha$ FBA followed by anti-  
210 rabbit IgG-Alexa Flour 488 conjugate demonstrated a clear shift in fluorescence signal  
211 (55.2 mean fluorescence intensity) confirming the cell surface localization of FBA (Fig.  
212 5A). No shift in fluorescence signal was observed when MC58 $\Delta$ *cbbA* cells were

213 examined under identical conditions (Fig. 5B; mean fluorescence intensity of 12.1  
214 compared to samples treated with primary or secondary alone [3.6 and 6.7, respectively]).  
215 From the wild-type cells probed with both antibodies, 79.05% were found in the M1  
216 region (Fig. 5A), suggesting that the majority of the population had FBA present on the  
217 cell surface. Pre-immune sera showed no reactivity against wild-type MC58 in  
218 immunoblot experiments confirming that the binding of R $\alpha$ FBA to wild-type MC58  
219 observed by flow cytometry was FBA-specific.

220

221 *FBA is required for efficient adhesion to host cells*

222 Viable counts of bacteria associated with homogenized infected monolayers were used to  
223 compare the capacity of the wild-type, FBA mutant and complemented mutant strains to  
224 associate with, and invade human brain microvascular endothelial (HBME) cells. FBA-  
225 deficient meningococci had a significantly reduced capacity to adhere to monolayers of  
226 HBME cells (Fig. 6A). No statistically significant reduction was observed in the ability  
227 of the FBA mutant to invade monolayers of HBME cells (Fig. 6B). Similar results were  
228 also obtained using HEP-2 (human larynx carcinoma) cells, confirming that the effect  
229 was not cell-type specific. To confirm that the observed effects were not due to an  
230 impairment in *in vitro* growth, the growth rate of the strains was compared by measuring  
231 the optical density at 600 nm (OD<sub>600</sub>) and determining the viable counts of broth cultures  
232 sampled during exponential growth over 24 h in triplicate on three separate occasions.  
233 No significant difference between strains was observed (data not shown). To further  
234 exclude the possibility that mutation of *cbbA* affected expression of the downstream  
235 gene encoding factor H binding protein, whole cell lysates of MC58, MC58 $\Delta$ *cbbA* and  
236 MC58 $\Delta$ *cbbA* *cbbA*<sup>Ect</sup> were probed with anti-fHbp. Expression levels of this protein were

237 similar in the three strains. In summary, these experiments show that FBA plays a role in  
238 the adherence of *N. meningitidis* to human cells.

239

## 240 **Discussion**

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

255 In the published meningococcal and gonococcal genome sequences, there is only  
256 one gene, *cbba*, encoding a putative FBA enzyme. Despite being predicted to be part of a  
257 non-functional metabolic pathway, *cbba* has not acquired spontaneous mutations in any  
258 of the Neisserial genomes examined, which suggests that the gene was acquired recently,  
259 that the glycolytic pathway became non-functional recently, or that the protein has one or  
260 more additional functions. Sequence analysis shows that FBA is highly-conserved at the  
261 amino acid level, and is a Class-IIB enzyme. A 21-amino acid insertion sequence, which

262 is present in two subclasses of the Class IIB FBA enzymes, was also present in the  
263 Neisserial sequences. The presence of this sequence suggests that the neisserial FBA  
264 enzyme is a tetrameric, rather than a dimeric enzyme; a feature which is present in  
265 extremophiles, and which has been suggested to confer thermal stability (Sauvé &  
266 Sygusch, 2001; Izard & Sygusch, 2004). Unexpectedly for a presumed cytosolic protein,  
267 a possible signal sequence was predicted for the neisserial FBA enzyme, suggesting a  
268 possible means of translocation across the cytoplasmic membrane.

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

286 to optimize the potential of the genome. To our knowledge, this is the first report  
287 demonstrating that a proportion of FBA is found on the cell surface of meningococci.

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

298 Although we have shown that FBA is present on the meningococcal cell surface  
299 and is required for optimal adhesion, the role of FBA in this process is unknown. It is a  
300 possible that the enzymatic activity of FBA plays an indirect role that is required for  
301 optimal adhesion. However, given that other FBA homologues (such as FBA in *S.*  
302 *pneumoniae*) have been shown to directly bind to host cell ligands it is also possible that  
303 the meningococcal protein has a direct host receptor binding activity. Determining the  
304 ability of meningococci expressing a non-enzymatically functional FBA to adhere to host  
305 cells would address this. *S. pneumoniae* FBA was shown to bind to a cadherin  
306 superfamily receptor (Flamingo cadherin receptor, FCR) on the surface of host epithelial  
307 cells, but it is unknown which FBA residues participate in this interaction (Blau *et al.*,  
308 2007). Meningococcal FBA is only 40% identical to the pneumococcal enzyme at the  
309 amino acid level, so it is unclear whether meningococcal FBA binds the same receptor.

310 Work is currently in progress to determine whether meningococcal FBA binds to FCR or  
311 a different host cell receptor.

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

319

## 320 **Experimental procedures**

### 321 *Bacterial strains and growth conditions*

322 *Escherichia coli* TOP10F' and BL21(DE3) pLysS (Table 1) were used for the expression  
323 of 6 × histidine-tagged recombinant FBA encoded by plasmid pSAT-FBA (Table 1). *E.*  
324 *coli* JM109 was used as a host strain for the construction of mutagenic and  
325 complementation plasmids, pSAT-4 and pSAT-12, respectively. *E. coli* strains were  
326 grown at 37°C in Luria-Bertani (LB) broth or on LB agar supplemented, where  
327 appropriate, with ampicillin (100 µg ml<sup>-1</sup>), kanamycin (30 µg ml<sup>-1</sup>) or erythromycin (200  
328 µg ml<sup>-1</sup>). Strains of *Neisseria* (Tables 1 and 2) were grown at 37°C, air plus 5% CO<sub>2</sub>, on  
329 Brain Heart Infusion (BHI) agar supplemented with 1% Vitox (Oxoid) and kanamycin  
330 (50 µg ml<sup>-1</sup>) or erythromycin (5 µg ml<sup>-1</sup>) where appropriate.

331

### 332 *DNA manipulation*

333 Genomic DNA was extracted from *N. meningitidis* using the DNeasy Tissue kit (Qiagen).

334 Plasmid DNA was prepared by using the QIAprep Spin kit (Qiagen). Restriction enzymes

335 and T4 DNA ligase were purchased from Roche. All enzymatic reactions were carried  
336 out according to the manufacturer's instructions. DNA sequencing was carried out at the  
337 School of Biomedical Sciences (University of Nottingham) on an ABI 377 automated  
338 DNA sequencer.

339

#### 340 *Preparation of recombinant FBA*

341 The *cbbA* gene was amplified from *N. meningitidis* MC58 using oligonucleotide primers  
342 FBA\_pQE70 (F) and FBA\_pQE70 (R) (Table 3) using the Expand High Fidelity PCR  
343 system (Roche). The resulting amplicon was digested with SphI and BglII, before being  
344 ligated into similarly treated pQE70, and the resulting plasmid, pSAT-FBA, used to  
345 transform *E. coli* BL21 (DE3) pLysS. Transformants were grown to log phase, induced  
346 with 1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) for 3 h, and harvested by  
347 centrifugation. Recombinant 6  $\times$  histidine-tagged FBA was then affinity-purified under  
348 native conditions. Briefly, the culture pellet from an IPTG-induced culture of *E. coli*  
349 BL21 (DE3) pLysS (pSAT-FBA) was dissolved in 20 ml lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>,  
350 300 mM NaCl, 10 mM imidazole, pH 7.4) and disrupted by sonication using a MSE  
351 Soniprep 150 for 10 cycles (each cycle consisting of a 10 s burst followed by a 10 s  
352 cooling period). The cell lysate was then mixed with 1 ml HisPur™ Cobalt Resin (Pierce)  
353 and incubated overnight at 4°C. The lysate-resin mixture was then applied to a column,  
354 and washed with 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 15 mM imidazole, pH 7.4. Bound  
355 protein was then eluted in elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 300 mM  
356 imidazole, pH 7.4).

357

#### 358 *Production of a rabbit antiserum against purified recombinant FBA*

359 New Zealand White female rabbits were immunized subcutaneously four times at 2-week  
360 intervals with 30  $\mu\text{g}$  of recombinant FBA protein emulsified in Freud's complete (first  
361 immunization only) or incomplete adjuvant. After three injections, the animals were test  
362 bled, boosted once more and sacrificed 10 days later.

363

#### 364 *SDS-PAGE and immunoblotting*

365 Proteins were electrophoretically separated using 10% polyacrylamide gels (Mini-Protean  
366 III; Bio-Rad) and were stained using SimplyBlue Safestain<sup>TM</sup> (Invitrogen) or transferred  
367 to nitrocellulose membranes as previously described (Kizil *et al.*, 1999). Membranes  
368 were probed with mouse anti-pentahistidine antibody (Qiagen) or rabbit primary antibody  
369 diluted 1:10,000 or 1:1,000 respectively in blocking buffer (5% [wt/vol] non fat dry milk,  
370 0.1% [vol/vol] Tween 20 in 1  $\times$  phosphate-buffered saline [PBS]) and incubated for 2 h.  
371 After being washed in PBS with 0.1% Tween 20 (PBST), membranes were incubated for  
372 2 h with 1:30,000-diluted goat anti-mouse (or anti-rabbit) IgG-alkaline phosphatase  
373 conjugate (Sigma). After washing with PBST, blots were developed using BCIP/NBT-  
374 Blue liquid substrate (Sigma).

375

#### 376 *Kinetic analysis of fructose biphosphate aldolase activity*

377 This was done using a previously described methodology (Berry & Marshall, 1993).  
378 Briefly, the assay was performed at 30°C in 1 ml 50 mM Tris-HCl supplemented with 0.1  
379 M potassium acetate buffer (pH 8.0) containing 0.1 - 5 mM fructose 1, 6-bisphosphate  
380 (FBP), 0.2 mM NADH and 2  $\mu\text{l}$  of a 10 mg  $\text{ml}^{-1}$  mixture of glycerol phosphate  
381 dehydrogenase/triose phosphate isomerase (coupling enzymes). The reagents were added  
382 in the order: buffer; FBP; NADH; coupling enzymes. Finally, the reaction was started by  
383 adding 0.26 nmol of purified native FBA. A decrease in absorbance at 340 nm was

384 recorded as the measure of enzyme activity on an Uvikon 930 spectrophotometer.  
385 Activities were calculated using the molar extinction coefficient for NADH as 6220 M<sup>-1</sup>  
386 cm<sup>-1</sup>. One unit of aldolase activity was defined as the amount of enzyme which catalyzed  
387 the oxidation of 2 μmol NADH/min. Kinetic parameters were determined using Origin  
388 Pro 7.5 software.

389

#### 390 *Construction of MC58ΔcbbA*

391 A 2.3-kb fragment of DNA consisting of the *cbbA* gene and flanking DNA was amplified  
392 using FBA\_M1(F) and FBA\_M2(R) (Table 3) from *N. meningitidis* MC58 chromosomal  
393 DNA. The amplified DNA was TA cloned into the pGEM-T Easy vector to generate  
394 pSAT-2. This was then subject to inverse PCR using primers FBA\_M3(IR) and  
395 FBA\_M4(IF) (Table 3) resulting in the amplification of a 5-kb amplicon in which the  
396 *cbbA* coding sequence was deleted and a unique BglII site had been introduced. The BglII  
397 site was used to introduce a kanamycin resistance cassette, BamHI-digested from  
398 pJMK30 (Table 1), in place of *cbbA*. One of the resulting plasmids, pSAT-4, containing  
399 the resistance cassette in the same orientation as the deleted gene, was confirmed by  
400 restriction digestion and sequencing and subsequently used to mutate the meningococcal  
401 strain MC58 by natural transformation and allelic exchange as previously described (Hadi  
402 *et al.*, 2001). The deletion in the resulting mutant (MC58Δ*cbbA*) was confirmed by PCR  
403 analysis and immunoblotting. Growth curve assays carried out using liquid cultures  
404 showed no significant differences between MC58Δ*cbbA* and the wild-type strain (data not  
405 shown).

406

#### 407 *Complementation of cbbA*

408 A fragment corresponding to the *cbbA* coding sequence and upstream promoter was  
409 amplified from chromosomal DNA of strain MC58 with High Fidelity Expand *Taq*  
410 (Roche) using the primers FBA\_COM(F) and FBA\_COM(R) (Table 3) incorporating  
411 BamHI-sites into the amplified fragment. The BamHI-digested fragment was then  
412 introduced into a unique BamHI-site in pYHS25. This vector contains an erythromycin  
413 resistance gene flanked by the MC58 genes NMB0102 and NMB0103 (Winzer *et al.*,  
414 2002). The resulting plasmid pSAT-12 was used to transform MC58 $\Delta$ *cbbA* by natural  
415 transformation, thus introducing a single chromosomal copy of *cbbA* and the downstream  
416 erythromycin resistance cassette in the intergenic region between NMB0102 and  
417 NMB0103. Insertion of the *cbbA* gene and erythromycin resistance cassette at the ectopic  
418 site was confirmed by PCR analysis and sequencing. Expression of FBA was confirmed  
419 by immunoblotting.

420

#### 421 *Sub-cellular localization of FBA*

422 Cells from 100 ml overnight BHI broth cultures were harvested at 13,000  $\times$  g for 2 min  
423 and the pellet re-suspended in 1 ml of EB buffer (10 mM Tris-HCl pH 7.5, 10 mM  
424 MgCl<sub>2</sub>, 25% sucrose), and washed twice in the same buffer. Finally, the pellet was re-  
425 suspended in EB buffer and incubated for 10 min on ice. The preparation was centrifuged  
426 at 13,000  $\times$  g for 4 min; following re-suspension in 0.4 ml of ice cold water, the mixture  
427 was incubated on ice for a further 10 min, followed by centrifugation at 13,000  $\times$  g for 2  
428 min. The supernatant, containing periplasmic proteins, was removed and stored at -20°C.  
429 The remaining cell pellet (spheroplasts) were re-suspended into 0.4 ml Tris-HCl (pH 7.5)  
430 and sonicated using a MSE Soniprep 150 for 10 cycles (each cycle consisting of a 10 s  
431 burst followed by a 10 s cooling period) to release the cytoplasmic contents. Non-  
432 disrupted cells were removed by centrifugation at 5,000  $\times$  g for 1 min. The upper clear

433 supernatant was transferred to a fresh tube and centrifuged at  $17,000 \times g$  for 30 min. The  
434 supernatant (representing the cytosolic fraction) was removed and stored at  $-20^{\circ}\text{C}$ . The  
435 remaining pellet was re-suspended in 0.4 ml of 10 mM Tris-HCl pH 7.5, 10 mM  $\text{MgCl}_2$ ,  
436 2% Triton X-100. The sample was incubated at  $37^{\circ}\text{C}$  for 30 min and then centrifuged at  
437  $17,000 \times g$  for 30 min. The supernatant, enriched for cytoplasmic membrane proteins,  
438 was removed and stored at  $-20^{\circ}\text{C}$ . The final pellet (enriched for outer membrane proteins)  
439 was re-suspended by brief sonication in 1 ml 10 mM Tris-HCl pH 7.5, 10 mM  $\text{MgCl}_2$ ,  
440 1% Triton X-100, incubated at  $37^{\circ}\text{C}$  for 30 min and then centrifuged at  $17,000 \times g$  for 30  
441 min. The pellet was re-suspended in 0.2 ml 10 mM Tris-HCl pH 7.5 and stored at  $-20^{\circ}\text{C}$ .

442

443 *Flow cytometry*

444 *N. meningitidis* strains were grown to mid-log phase ( $\text{OD}_{600}$  approximately 0.7).  $1 \times 10^7$   
445 CFU aliquots were centrifuged at  $5,000 \times g$  for 5 min and resuspended in 0.2  $\mu\text{m}$ -filtered  
446 PBS. The cells were incubated for 2 h with  $\alpha\text{R-FBA}$  (1:500 diluted in PBS containing  
447 0.1% BSA, 0.1% sodium azide and 2% foetal calf serum) and untreated cells were used  
448 as a control. Cells were washed three times with PBS and incubated for 2 h in the dark  
449 with goat anti-rabbit IgG-Alexa Flour 488 conjugate (Invitrogen; diluted 1:50 in PBS  
450 containing 0.1% BSA, 0.1% sodium azide and 2% foetal calf serum). Again, untreated  
451 cells were used as a control. Finally, the samples were washed in PBS twice before being  
452 re-suspended in 1 ml PBS containing 0.5% formaldehyde to fix the cells. Samples were  
453 analyzed for fluorescence using a Coulter Altra Flow Cytometer. Cells were detected  
454 using forward and log-side scatter dot plots, and a gating region was set to exclude cell  
455 debris and aggregates of bacteria. A total of 50,000 bacteria (events) were analyzed.

456

457 *Association and invasion assays*

458 Association and invasion assays were performed essentially as previously described  
459 (Oldfield *et al.*, 2007). Briefly, human brain microvascular endothelial (HBME) or larynx  
460 carcinoma (HEp-2) cells were grown to confluence in DMEM supplemented with 10%  
461 heat-inactivated fetal calf serum (FCS; Invitrogen) and 2% antibiotic anti-mycotic  
462 solution (Invitrogen) in 24-well tissue culture plates (Costar) at 37°C in an atmosphere of  
463 5% CO<sub>2</sub>. Prior to all experiments, mono-layers were transferred to DMEM supplemented  
464 with 2% FCS to remove the antibiotics. Meningococci were cultured in MHB for 2 h and  
465 monolayers were infected with  $1 \times 10^6$  CFU of meningococci and incubated for 2 h  
466 (association) or 4 h (invasion) in 5% CO<sub>2</sub> at 37°C. To assess total cell association,  
467 monolayers were washed four times with 1 ml PBS per well. To assess invasion,  
468 monolayers were further incubated in DMEM containing gentamicin (100 µg ml<sup>-1</sup>) for 2  
469 h. Prior to further steps, aliquots of the gentamicin-containing supernatants were plated  
470 out to confirm killing of extra-cellular bacteria. Furthermore, the susceptibility of all  
471 meningococcal strains to gentamicin at 100 µg ml<sup>-1</sup> was confirmed prior to testing.  
472 Monolayers were then washed four times with 1 ml PBS. In both association and invasion  
473 experiments, monolayers were then disrupted and homogenized in 1 ml 0.1% saponin in  
474 PBS. Meningococci were enumerated by serial dilution of the homogenized suspensions  
475 and subsequent determination of colony-forming units by plating 50 µl aliquots from  
476 appropriate dilutions of the lysates on agar. All association and invasion assays were  
477 repeated at least three times. Statistical significance was measured using a two-tailed  
478 Student *t*-test.

479

#### 480 *Protein and nucleic acid sequence analysis*

481 Public databases containing previously published protein and DNA sequences were  
482 searched using the BLAST and PSI-BLAST algorithms available at

483 <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. The genome database of *N. meningitidis* MC58  
484 was interrogated at <http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=gnm>.  
485 Sequence homology data were obtained using the CLUSTALX software ([http://www-](http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX)  
486 [igbmc.u-strasbg.fr/BioInfo/ClustalX](http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX)). Protein secretion signals were analyzed using the  
487 SignalP 3.0 server available at <http://www.cbs.dtu.dk/services/SignalP/> (Emanuelsson *et*  
488 *al.*, 2007). GenBank accession numbers for the *cbbA* sequences analyzed in this study are  
489 as follows: YP\_974462 (FAM18), YP\_001598513 (ST-4821 strain 053442),  
490 YP\_002342063 (Z2491), YP\_207215 (gonococcal strain FA1090) and ZP\_03723075 (*N.*  
491 *lactamica* ATCC 23970).

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495 HBME cells.

For Peer Review

496 **Table 1.** Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
<i>E. coli</i>		
JM109	<i>endA1 recA1 gyrA96 thi hsdR17 (r<sub>K</sub><sup>-</sup>r<sub>K</sub><sup>-</sup>) relA1 supE44 Δ(lac-proAB) [F' traD36 proAB laq1<sup>q</sup>ZΔM15]</i>	Promega
TOP10F'	F' <i>lacIqTn10</i> (TetR) <i>mcrA</i> Δ( <i>mrr-hsdRMS-mcrBC</i> ) Φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG</i>	Invitrogen
BL21(DE3)pLysS	F- <i>ompT hsdSB (rB-mB-)</i> <i>gal dcm</i> (DE3) pLysS (CamR)	Invitrogen
<i>N. meningitidis</i>		
MC58	wild-type serogroup B strain	(Tettelin <i>et al.</i> , 2000)
MC58Δ <i>cbbA</i>	<i>cbbA</i> deletion and replacement with kanamycin cassette	This study
MC58Δ <i>cbbA cbbA</i> <sup>Ect</sup>	MC58Δ <i>cbbA</i> complemented with an ectopic copy of <i>cbbA</i>	This study
Plasmids		
pQE70	Cloning vector encoding resistance to ampicillin	Qiagen
pSAT-FBA	MC58 <i>cbbA</i> gene cloned in pQE70	This study
pGEM-T Easy	Cloning vector encoding resistance to ampicillin	Promega
pSAT-2	2.3-kb fragment spanning the MC58 <i>cbbA</i> region cloned in pGEM-T Easy	This study
pJMK30	Source of kanamycin resistance cassette	(van Vliet <i>et al.</i> , 1998)
pSAT-4	pSAT-2 containing the kanamycin resistance cassette in the same orientation as the deleted <i>cbbA</i> gene	This study
pYHS25	Ectopic complementation vector encoding resistance to erythromycin	(Winzer <i>et al.</i> , 2002)
pSAT-12	pYHS25 containing <i>cbbA</i>	This study

497 **Table 2.** Isolates of *N. meningitidis* examined for the expression of FBA

Strain <sup>a</sup>	Country of origin	Date of isolation	Disease	Serogroup	Sequence type	Clonal complex
Z1001	USA	1937	invasive (unspecified)	A	4	ST-4 complex/subgroup IV
Z1035	Pakistan	1967	meningitis and septicaemia	A	1	ST-1 complex/subgroup I/II
Z1054	Finland	1975	invasive (unspecified)	A	5	ST-5 complex/subgroup III
Z1213	Ghana	1973	invasive (unspecified)	A	4	ST-4 complex/subgroup IV
Z1269	Burkina Faso	1963	invasive (unspecified)	A	4	ST-4 complex/subgroup IV
Z1503	China	1984	invasive (unspecified)	A	5	ST-5 complex/subgroup III
Z3771	UK	1987	invasive (unspecified)	A	5	ST-5 complex/subgroup III
Z3842	Norway	1976	invasive (unspecified)	B	32	ST-32 complex/ET-5 complex
Z4181	Mali	1989	carrier	C	11	ST-11 complex/ET-37 complex
Z4323	Israel	1988	invasive (unspecified)	C	11	ST-11 complex/ET-37 complex
Z4662	Netherlands	1967	invasive (unspecified)	B	8	ST-8 complex/Cluster A4
Z4667	Netherlands	1963	invasive (unspecified)	B	48	ST-41/44 complex/Lineage 3
Z4673	Netherlands	1986	invasive (unspecified)	B	41	ST-41/44 complex/Lineage 3
Z4676	Denmark	1962	invasive (unspecified)	B	37	ST-37 complex
Z4678	East Germany	1985	invasive (unspecified)	B	19	ST-18 complex
Z4684	Norway	1988	invasive (unspecified)	B	13	ST-269 complex
Z4685	Norway	1988	carrier	B	14	ST-269 complex
Z4701	Norway	1969	invasive (unspecified)	B	11	ST-11 complex/ET-37 complex
Z5826	China	1992	invasive (unspecified)	A	7	ST-5 complex/subgroup III
Z6413	South Africa	1990	invasive (unspecified)	C	8	ST-8 complex/Cluster A4
Z6414	New Zealand	1994	invasive (unspecified)	C	66	ST-8 complex/Cluster A4
Z6417	UK	1993	invasive (unspecified)	C	11	ST-11 complex/ET-37 complex
Z6418	Cuba	1992	invasive (unspecified)	B	33	ST-32 complex/ET-5 complex
Z6419	Austria	1991	invasive (unspecified)	B	40	ST-41/44 complex/Lineage 3
Z6420	Greece	1992	invasive (unspecified)	B	41	ST-41/44 complex/Lineage 3

498

499 <sup>a</sup> Further details of strains are available at <http://pubmlst.org/>

500 **Table 3.** List of primers used in this study

Primer	DNA sequence <sup>a</sup>	Restriction site
Expression		
FBA_pQE70 (F)	<b>CGCGGATCC</b> ATGGCACTCGTATCCATGCG	SphI
FBA_pQE70 (R)	<b>CGCGGTACC</b> GTCGTCCGAACGGCGG	BglII
Mutagenesis		
FBA_M1(F)	CTGCTGTGCCCGAGC	
FBA_M2(R)	CCGCTGCTGCAGGCG	
FBA_M3(IR)	<b>GCGAGATCT</b> TGTGTCTCCTTGGGCAATAGG	BglII
FBA_M4(IF)	<b>GCGAGATCT</b> GCTCCATCCAACCTGGG	BglII
Complementation		
FBA_COM(F)	<b>CGCGGATCC</b> ATGAGCTGTTTATGGTTTTTTTGCTG	BamHI
FBA_COM(R)	<b>CGCGGATCC</b> GGCATTGTTTACAGGCAACCTG	BamHI

501

502 <sup>a</sup> All primers were designed from the *N. meningitidis* MC58 genome sequence. Sequences in

503 bold identify restriction enzyme sites.

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629 **Figure Legends**

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

633

634 **Fig. 2.** Coupled enzymic assay to measure the activity of meningococcal fructose 1, 6-  
635 bisphosphate aldolase. Cleavage of fructose 1, 6-bisphosphate (FBP) was coupled to  $\alpha$ -  
636 glycerophosphate dehydrogenase and NAD oxidation. One unit of aldolase activity was  
637 defined as the amount of enzyme which catalyzed the oxidation of 2  $\mu$ mol NADH/min.

638

639 **Fig. 3.** Immunoblot analysis of whole cell proteins from the *N. meningitidis* MC58 wild-type,  
640  $\Delta$ *cbbA* mutant derivative and complemented mutant reveals the absence of FBA in the  $\Delta$ *cbbA*  
641 mutant preparation.

642

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

647

648 **Fig. 5.** Flow cytometric analysis of MC58 wild-type (A) or MC58 $\Delta$ *cbbA* cells (B) for FBA  
649 surface localization. Cells were stained with R $\alpha$ FBA (primary alone), anti-rabbit IgG-Alexa  
650 Flour 488 conjugate (secondary alone) or both. Fluorescence was displayed as a histogram.  
651 The histogram area in M1 represents the population of fluorescently labeled meningococci.

652

653 **Fig. 6.** FBA-deficient meningococci have a reduced ability to associate with (A) but not  
654 invade into (B) HBME cells compared to the wild-type or complemented strains. The number  
655 of FBA-deficient meningococci associating was significantly lower than the wild-type ( $*P =$   
656  $0.0011$ ). Numbers of mutant cells invading was not significantly lower compared to the wild-  
657 type ( $P = 0.13$ ). Similar experiments were also carried out using HEp-2 cells with consistent  
658 results. Mean levels shown from three independent experiments, each using triplicate wells.  
659 Bars denote standard deviation. Cfu denotes colony forming units.

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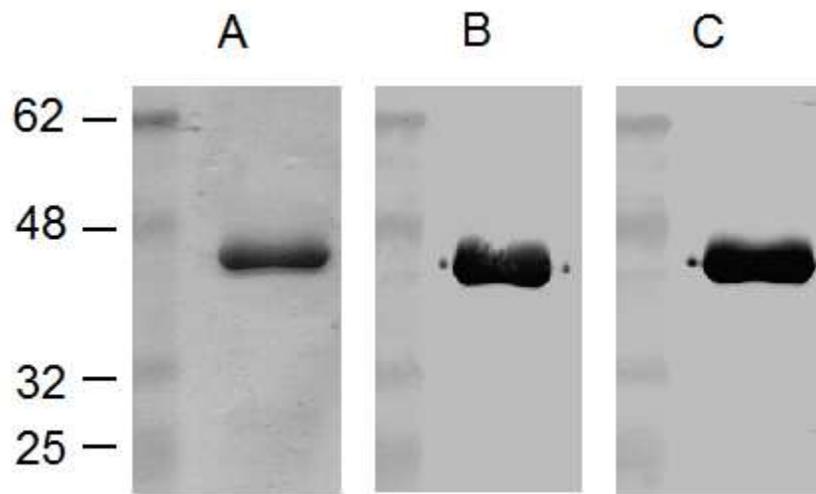


Fig. 1. SDS-PAGE analysis confirms the purity of the recombinant FBA purified under native conditions (A). Immunoblot analysis shows that recombinant FBA is recognized by RoFBA (B) and anti-pentahistidine antibodies (C).

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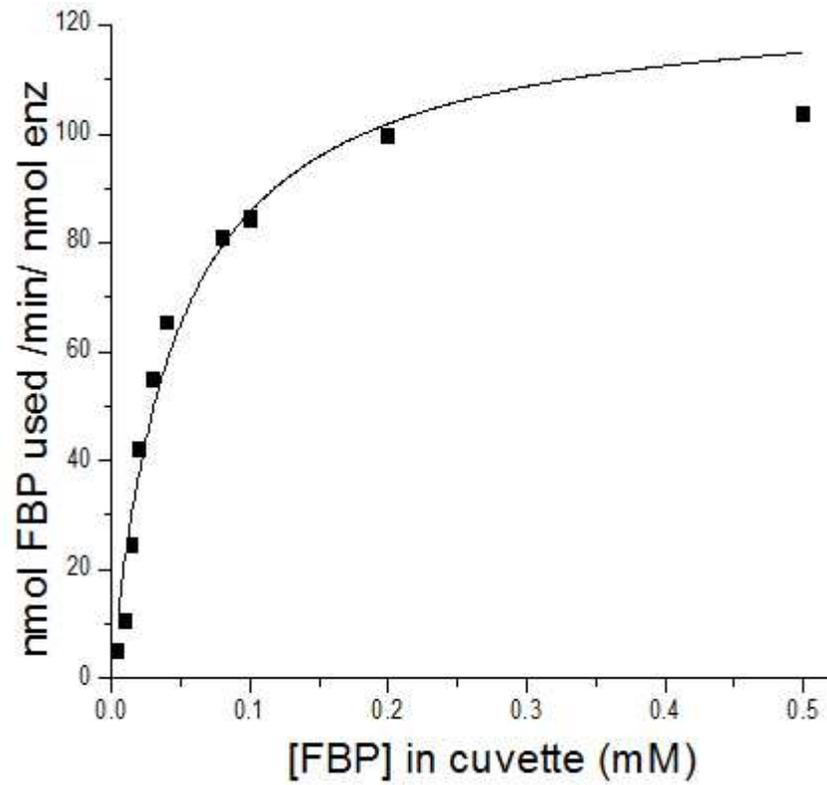


Fig. 2. Coupled enzymic assay to measure the activity of meningooccal fructose 1, 6-bisphosphate aldolase. Cleavage of fructose 1, 6-bisphosphate (FBP) was coupled to alpha-glycerophosphate dehydrogenase and NAD oxidation. One unit of aldolase activity was defined as the amount of enzyme which catalyzed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

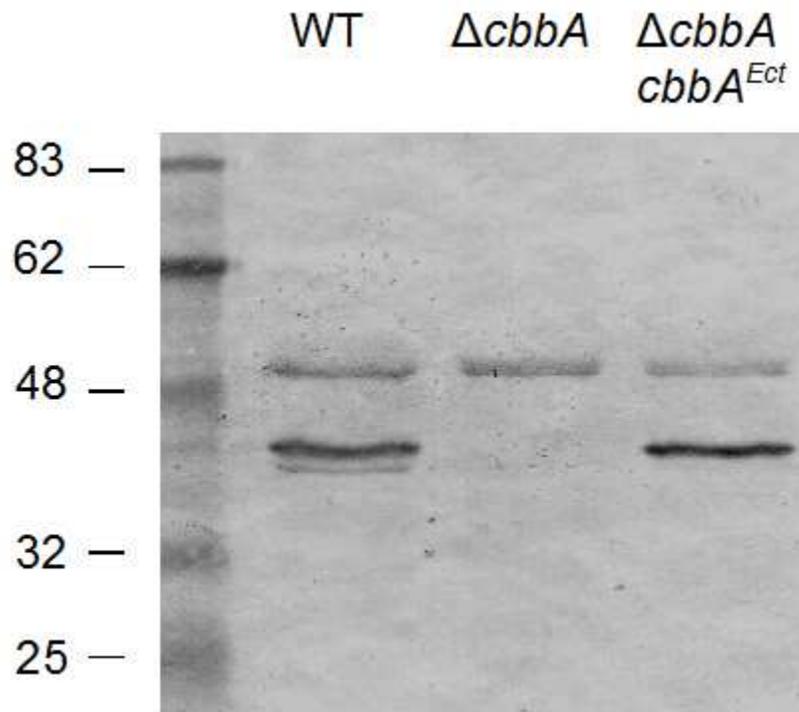


Fig. 3. Immunoblot analysis of whole cell proteins from the *N. meningitidis* MC58 wild-type,  $\Delta cbbA$  mutant derivative and complemented mutant reveals the absence of FBA in the  $\Delta cbbA$  mutant preparation.

view

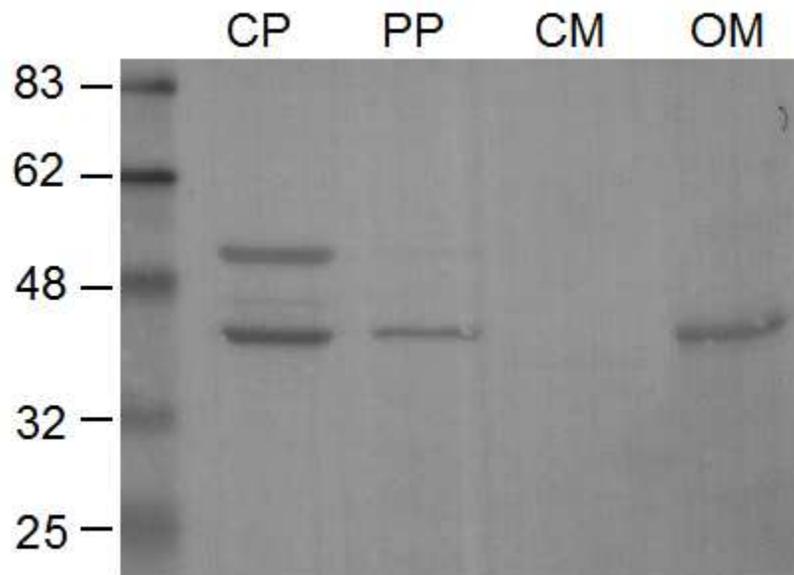


Fig. 4. Sub-cellular localization of FBA. Cytosolic protein-enriched (CP), periplasmic protein-enriched (PP), cytoplasmic membrane protein-enriched (CM) and outer membrane protein-enriched (OM) fractions of MC58 were separated on a 10% acrylamide gel and probed in immunoblotting experiments with RaFBA.

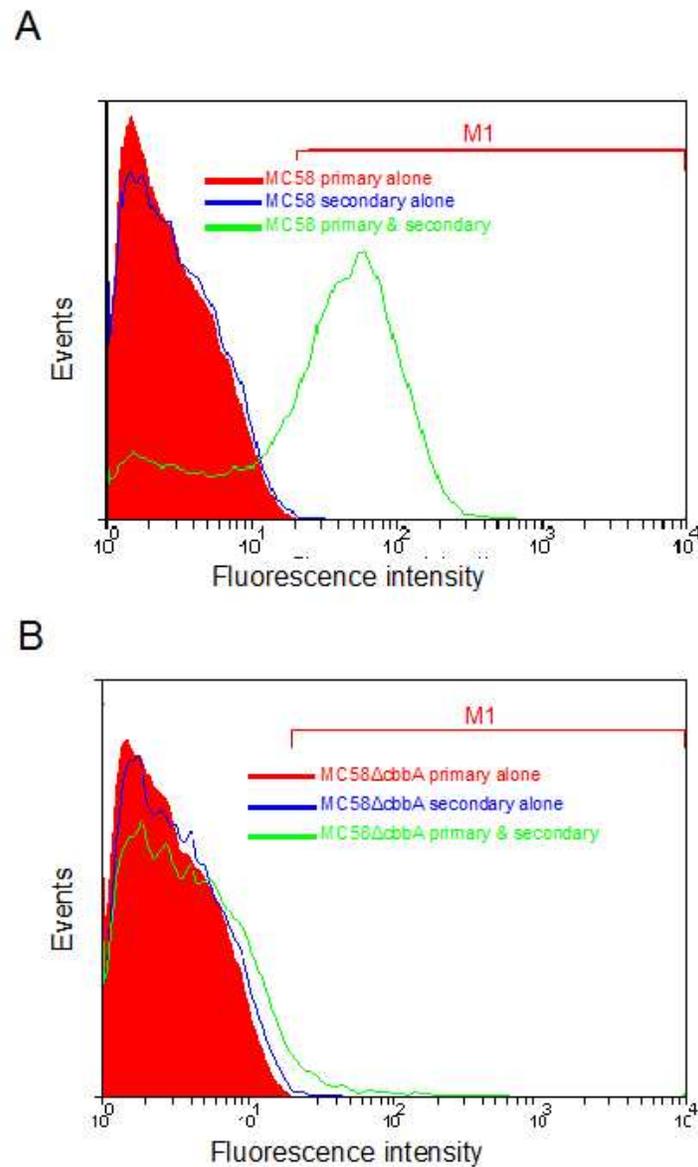


Fig. 5. Flow cytometric analysis of MC58 wild-type (A) or MC58 $\Delta$ cbbA cells (B) for FBA surface localization. Cells were stained with RaFBA (primary alone), anti-rabbit IgG-Alexa Flour 488 conjugate (secondary alone) or both. Fluorescence was displayed as a histogram. The histogram area in M1 represents the population of fluorescently labeled meningococci.

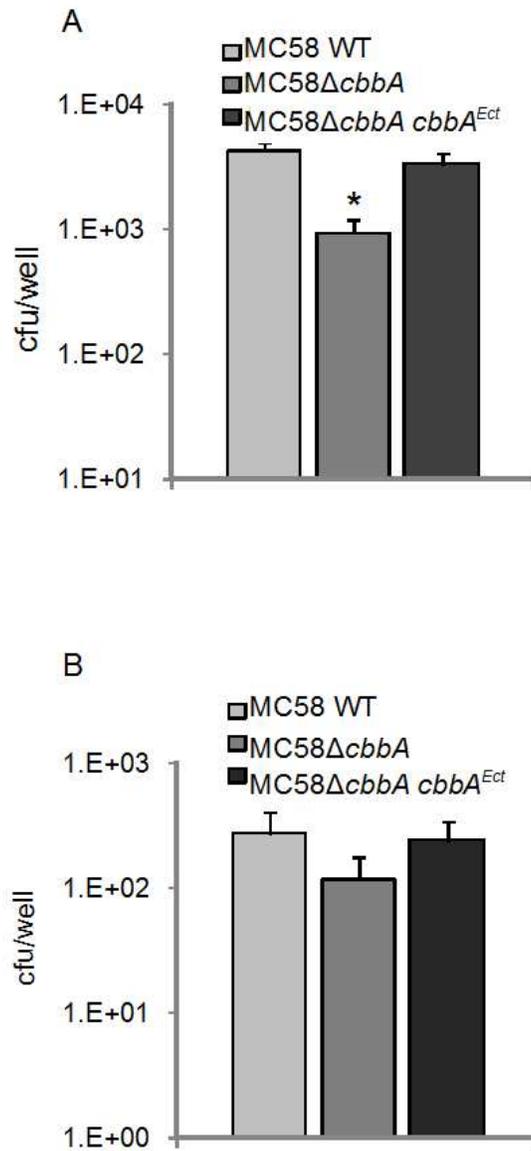


Fig. 6. FBA-deficient meningococci have a reduced ability to associate with (A) but not invade into (B) HBME cells compared to the wild-type or complemented strains. The number of FBA-deficient meningococci associating was significantly lower than the wild-type ( $*P = 0.0011$ ). Numbers of mutant cells 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.