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TITLE

Relationship between O-antigen chain length and resistance to colicin E2 in *Shigella flexneri*

Running Title

Shigella flexneri $WzzB_{SF}$ *confers resistance to colicin*

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Microbial Pathogenicity

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Abbreviations

Col, colicin; ES, extremely short; IS, intermediate short; L, long; LPS, lipopolysaccharide; Oag, O antigen; OM, outer membrane; PCP1a, polysaccharide co-polymerases class 1a; RU, repeat units; S, short; VL, very long; VS, very short.

SUMMARY

The *Shigella flexneri* polysaccharide co-polymerase class 1a (PCP1a) protein, $WzzB_{SF}$, regulates lipopolysaccharide (LPS) O-antigen (Oag) chain length to confer short (S) type Oag chains of ~10 - 17 Oag repeat units (RUs). The S-type Oag chains affect *S. flexneri* virulence as they influence IcsA-mediated actin based motility. However, they do not confer resistance to complement; this is conferred by the very long (VL) type Oag chains determined by $WzzB_{pHS2}$. Colicins are bacterial proteins produced by some *Escherichia coli* strains to kill related strains. While the presence of Oag chains has been shown to shield outer membrane proteins from colicins, the impact of Oag chain length against colicins is unknown. In this study, initial testing indicated that a *S. flexneri* Y *wzz::kan*^{r} mutant was more sensitive to colicin (Col) E2 compared to the wild-type strain. A set of plasmids encoding Wzz mutant and wild-type PCP1a proteins conferring different Oag modal chain lengths were then expressed in the mutant background, and tested against purified Col E2. Analysis of swab and spot sensitivity assays showed that strains expressing either S-type or long (L) type Oag chains (16 – 28 Oag RUs) conferred greater resistance to Col E2 compared to strains having very short (VS) type (2 – 8 Oag RUs), intermediate short (IS) type (8 – 14 Oag RUs), or VL-type (>80 Oag RUs) Oag chains. These results suggest a novel role for LPS Oag chain length control that may have evolved due to selection pressure from colicins in the environment.

INTRODUCTION

Lipopolysaccharide (LPS) is a major virulence determinant of *Shigella flexneri* and is composed of three domains: the lipid A domain anchored to the outer membrane (OM), the core sugar domain, and the O-antigen (Oag) polysaccharide chains which extend out from the bacterium into the extracellular milieu (Raetz & Whitfield, 2002). Oag is a polymer of Oag repeat units (RUs) and the basic Oag RU of *S. flexneri* is a tetrasaccharide of three rhamnose sugars and one *N*-acetylglucosamine sugar. Synthesis of Oag is carried out by the Wzy-dependant polymerisation pathway (Morona *et al.*, 2009; Raetz & Whitfield, 2002; Samuel & Reeves, 2003; Tocilj *et al.*, 2008). The number of Oag RUs on a *S. flexneri* LPS structure is regulated by two Oag chain length regulators belonging to the polysaccharide co-polymerase class 1a (PCP1a) family of proteins, known as $WzzB_{SF}$ and Wzz_{pHS2}. The chromosomally encoded $WzzB_{SF}$ protein confers short (S) type Oag chains of ~10 - 17 Oag RUs (Morona *et al.*, 1995), while the plasmid encoded Wzz_{pHS2} protein confers very long (VL) type Oag chains of approximately >80 Oag RUs (Stevenson *et al.*, 1995).

The role of Oag chain length regulation is variable among different bacterial species. In *S. flexneri*, S-type Oag chains have been shown to contribute to IcsA mediated actin based motility (Van Den Bosch & Morona, 2003) and affect virulence (Van Den Bosch *et al.*, 1997), while loss of the VL-type Oag chains in *S. flexneri* has been shown to enhance bacterial sensitivity to complement (Hong & Payne, 1997). In *Pseudomonas aeruginosa,* absence of LPS with long (L) type Oag chains resulted in greater sensitivity to complement killing and reduced virulence in mice (Kintz *et al.*, 2008). Similarly, the presence and regulation of Oag in *Yersinia enterocolitica* O:8 is essential for virulence in orally infected mice and rabbits (Al-Hendy *et al.*, 1992; Najdenski *et al.*, 2003; Zhang *et al.*, 1997). In *Salmonella typhimurium,* LPS structure is regulated by WzzB and FepE which

74 confer the L-type Oag chains (16 – 28 Oag RUs) and VL-type Oag chains (>80 Oag RUs),
75 respectively. Both modal Oag chain lengths have been shown to confer resistance to complement
76 (Murray *et al.*, 2003, 2005), but appear to have conflicting roles in bile acid resistance. Expression
77 of the *Salmonella typhimurium* VL-type Oag chains is suggested to confer resistance to bile acid
78 (Crawford *et al.*, 2012), while expression of the L-type Oag chains in *Salmonella typhimurium*
79 appears to impair *Salmonella* growth in bile by interfering with enterobacterial common antigen
80 mediated bile resistance (May & Groisman, 2013). Recent data in *Salmonella enteric* suggests that
81 loss of VL-type Oag chains may optimize capsule mediated immune invasion (Crawford *et al.*,
82 2013). In *E. coli*, the presence of LPS Oag has been shown to shield OM protein receptors and
83 confer resistance to colicin (Van der Ley *et al.*, 1986), but the effect of Oag chain length regulation
84 against colicins is unknown.
85
86 Colicins are plasmid encoded bacterial proteins produced by strains of *E. coli* in response to stress
87 conditions (Riley & Gordon, 1999). They are lethal to other related strains of *E. coli* and the
88 production of small immunity proteins by the colicin-producing *E. coli* strains prevents the colicin
89 from killing the producing cell (Bowman *et al.*, 1971). There are various types of colicins with
90 different modes of action (Nomura, 1964) but they generally kill cells by four main mechanisms:
91 forming channels in the cytoplasmic membrane, inhibiting cell wall synthesis, degrading cellular
92 DNA or by inhibiting protein synthesis. Colicin (Col) E2 is a DNase that interacts with the OM
93 receptor BtuB to enter cells (Sharma *et al.*, 2007). Colicins have also been shown to be produced by
94 *Shigella* strains (Smajs *et al.*, 1997).
95
96 In this study, we investigated the effect of Oag modal chain length on sensitivity to colicins by
97 initially testing a *S. flexneri* Y and a *S. flexneri* Y *wzz::kan*^{r} mutant against various colicin

98 producing strains, and then tested a set of plasmids encoding a range of Wzz mutant and wild-type
99 PCP1a proteins conferring different Oag modal chain lengths against Col E2. Analysis of swab and
100 spot sensitivity assays performed with purified Col E2 protein suggests that the S-type and L-type
101 LPS modal lengths commonly present in *E. coli* strains (Franco *et al.*, 1998) are optimal for
102 conferring colicin resistance, and WzzB proteins conferring these Oag chain lengths may have
103 evolved as a result of selection pressures from colicins in the environment.
104

METHODS

Ethics Statement. The anti-$WzzB_{SF}$ antibody was produced under the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by the University of Adelaide Animal Ethics Committee.

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Strains were routinely grown on Luria-Bertani (LB) agar (10 g l^{-1} Tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl, 15 g l^{-1} agar) or in LB broth. Strains carrying pQE30 constructs requiring induction were grown in the presence of 0.01 mM IPTG at 37°C in LB broth with aeration for 16 h, subcultured 1/20 into fresh broth with 0.01 mM IPTG and grown for another 4 h. Strains carrying pRMCD77 and pWSK29 constructs did not require induction and were grown under the same conditions without IPTG. Antibiotics were used at the following concentrations: 100 μg ampicillin (Amp) ml^{-1}; 25 μg chloramphenicol (Cml) ml^{-1}; 50 μg kanamycin (Kan) ml^{-1}; and 100 μg streptomycin (Sm) ml^{-1}.

DNA methods. The plasmids used in this study are described in Table 1. Unless otherwise stated, plasmids constructs were extracted from *E. coli* DH5α or XL10-Gold (Stratagene) strains using the QIAprep Spin Miniprep kit (Qiagen). Electroporation and preparation of electrocompetent cells were as previously described (Purins *et al.*, 2008).

LPS PAGE and silver staining. LPS samples and gels were prepared as described previously (Murray *et al.*, 2003; Papadopoulos & Morona, 2010).

129 **SDS-PAGE and Western immunoblotting.** Bacteria grown and induced as described above were
130 harvested by centrifugation, resuspended in 2X sample buffer (Lugtenberg *et al.*, 1975) and heated
131 at 100°C for 5 min, prior to SDS-PAGE on 15% gels for 1 h at 200 V. Western transfers were
132 performed at 400 mA for 1 h. Protein gels were then subjected to Western immunoblotting on
133 nitrocellulose membrane (Medos) with either polyclonal $WzzB_{SF}$ antibodies (prepared by Daniels
134 & Morona, 1999) at 1:500 dilution or monoclonal BtuB antibodies (kindly provided by Prof.
135 Robert Nakamoto at the University of Virginia) at 1:1000 dilution in 2.5 % (w/v) skim milk.
136 Detection was performed with goat anti-rabbit horseradish-peroxidase-conjugated antibodies (KPL)
137 and chemiluminescence reagent (Sigma). BenchMark protein ladder (Invitrogen) was used as the
138 molecular mass standard.
139
140 **Preparation of OM samples.** Strains carrying pWSK29 constructs were grown as described above
141 in 100 ml LB, harvested by centrifugation (9,800x*g*, 10 min, 4°C, Beckman J2-21M Induction
142 Drive Centrifuge) and resuspended in 10 ml buffer (500 mM NaCl, 50 mM sodium phoshate, pH
143 7). Cells were then sonicated, re-centrifuged (to remove cell debris) and whole membrane (WM)
144 pellets were collected by ultracentrifugation (126,000x*g*, 1 h, 4°C, Beckman Coulter Optima L-100
145 XP Untracentrifuge). Pellets were solubilised in 1 ml buffer containing 2 % (v/v) Triton X-100 + 10
146 mM $MgCl_2$ for 1 h at room temperature and OM pellets were collected by re-ultracentrifugation (as
147 above). OM pellets (~2 μg) were resuspended in 2X sample buffer (Lugtenberg *et al.*, 1975) and
148 heated at 100°C for 5 mins, prior to SDS-PAGE on 15% gels. Gels were stained with Coomassie
149 Blue to visualise protein bands and Western immunoblotting was carried out as described above.
150
151 **Purification of Col E2.** *E. coli* BL21(DE3) carrying pET41b expressing C-terminal His_8-tagged
152 Col E2 (kindly provided by Prof. William Cramer at the Purdue University (Sharma *et al.*, 2009))

153 was grown at 37°C for 16 h with aeration, subcultured 1/20 into 1 L LB, and grown to an OD_{600} of
154 ~0.8 prior to induction with 1 mM IPTG for another 3 h. Cells were then harvested by
155 centrifugation (11,300x*g*, 10 min; Beckman J2-21M Induction Drive Centrifuge), resuspended in
156 30 ml buffer A (300 mM NaCl, 50 mM sodium phosphate, pH 8.0), passed through a cell disruptor
157 once at 30,000 psi (Constant Cell Disruptor System) and re-centrifuged to remove cell debris.
158 Soluble fractions were collected by ultracentrifugation (126,000x*g*, 1 h, 4°C; Beckman Coulter
159 Optima L-100 XP Ultracentrifuge) and purified using an AKTA prime plus (GE Life Sciences)
160 with a HisTrap column as described by the manufacturer. The column was washed with buffer A
161 containing 10 % (v/v) glycerol (pH 6.0) and protein was eluted with buffer A containing 10% (v/v)
162 glycerol and 500 mM imidazole (pH 6.0). Eluted protein was mixed 1:1 with 2X sample buffer
163 (Lugtenberg *et al.*, 1975), checked by 15% SDS-PAGE and Coomassie Blue staining for the
164 presence of the ~66 kDa Col E2 protein band. Dialysis was carried out with 20 mM Tris (pH 7.5)
165 and purified protein was mixed 1:1 with 100 % (v/v) glycerol and stored at - 80°C. Protein was >
166 95 % pure and the yield was 20 mg.
167
168 **Colicin sensitivity assays.** The double layer sensitivity assay was performed as described
169 previously (Davies & Reeves, 1975; Masi *et al.*, 2007). In brief, colicin producing strains (Table 1)
170 were grown at 37°C for 16 h and streaked across 20 ml LB agar plates with cotton swabs. Plates
171 were incubated overnight at 37°C, treated with 400 µl of chloroform (added to a piece of Whatman
172 paper placed inside the lid of the upturned plate) for 30 min and then overlaid with 20 ml LB agar.
173 The *S. flexneri* strains to be tested were streaked across the original colicinogenic streak line and
174 plates were incubated at 37°C for 16 h. Any inhibition of growth was recorded. For swab sensitivity
175 assays, 0.1 µg ml^{-1} of purified Col E2 was swabbed onto selective 25 ml LB agar plates with cotton
176 swabs and allowed to dry. Individual bacterial strains were then swabbed perpendicular to the Col

E2 streak and plates were incubated at 37°C for 16 h. The zone of growth inhibition was measured in mm. For spot sensitivity assays, bacteria (~5 x 10^8 cells) were spread onto selective 25 ml LB agar plates and spotted with 5 µl of purified Col E2 protein in Milli-Q water at the following concentrations (µg ml^{-1}): 0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.50, 1.0, 2.0, 4.0, 8.0, 16, 32, 64, 128, 256, 512 and 1024. Plates were incubated at 37°C for 16 h and the minimal inhibitory concentration (MIC), defined here as the lowest concentration that generated a clear zone of inhibition, was recorded. Each strain was assayed a minimum of three times.

Complement bactericidal assay. Complement assays were carried out as described previously (Murray *et al.*, 2005). Strains carrying pWSK29 constructs were grown as described above and 5 x 10^8 bacterial cells were collected by centrifugation. Cells were serially diluted to 1×10^6 cfu ml^{-1} in PBS + Cml (Cml was added to prevent growth during the assay) in 1.5 ml reaction tubes. Human pooled serum (Murray *et al.*, 2005) was added to 10% (v/v) final concentration and mixtures incubated at 37°C without agitation. For controls, serum was heat-inactivated by incubation at 56°C for 30 min. Viable counts were taken at 30 min intervals by plating on LB agar and expressed as a percentage of initial concentration (% survival). Each strain was assayed a minimum of three times.

Bacterial growth assay. Bacteria grown at 37°C with aeration for 16 h were subcultured 1/20 in LB and incubated at 37°C with shaking in a 96 well tray sealed with Breath easy membrane (Sigma) in Powerwave XS (BioTek). Absorbance readings at 600 nm were taken every 30 min for 22 h.

RESULTS

Sensitivity of *S. flexneri* Y to colicins. To investigate the effect of S-type LPS Oag chains on sensitivity to colicins, *S. flexneri* strains RMA2162 (*S. flexneri* Y) and RMA2163 (*S. flexneri* Y *wzz::kan*^{r} strain) were initially tested against different *E. coli* strains producing Col A, D, E1, E2, E3, Ia or K (Table 1) using the double layer sensitivity assay. *S. flexneri* strains were swabbed perpendicular to *E. coli* strains producing different colicins on a plate and strains that grew over the *E. coli* streak line were considered resistant (R) (0 mm), while strains that could not (> 0 mm) were considered sensitive (S). Some inhibition of growth (but not complete clearing) at the streak line was denoted as slightly sensitive (S/S). Analysis of the results obtained showed that wild-type *S. flexneri* Y was sensitive to *E. coli* strains producing Col E2 (15.0 mm) and Col E3 (8.0 mm) (Table 2), with the mutant *S. flexneri* Y *wzz::kan*^{r} strain showing higher sensitivity to Col E2 (20.0 mm) and Col E3 (14.5 mm) (Table 2). *S. flexneri* strains 2457T (*S. flexneri* 2a) and RMA696 (*S. flexneri* 2a *wzz::kan*^{r} strain) were also tested and a similar trend was observed. The *S. flexneri* 2a strain was sensitive to Col E2 (12.0 mm) and resistant to Col E3 (0.0 mm) (Table 2), while the *S. flexneri* 2a *wzz::kan*^{r} mutant was more sensitive to Col E2 (16.0 mm) and Col E3 (showing slight sensitivity, S/S) (Table 2). These results suggest that *wzz* confers some level of resistance to Col E2 and Col E3, and that the level of resistance (especially to Col E3) can differ between serotypes. Since both mutants showed higher sensitivity to Col E2 than Col E3, Col E2 was chosen for subsequent experiments.

Characterisation of the LPS profile conferred by Wzz mutant and wild-type PCP1a proteins in RMA2163 background. The effect of Oag modal chain length on sensitivity to Col E2 was further investigated by introducing plasmids encoding a range of Wzz mutant and wild-type PCP1a

proteins conferring different Oag modal chain lengths (Table 1), into either RMA2163 or RMA4053 (RMA2163 carrying pCDFDuet-1) (Table 1). Plasmid pCDFDuet-1 encoding *lacI*^{q} was chosen in preference to the F′ (*lacI*^{q}) plasmid used previously (Papadopoulos & Morona, 2010), as the latter was observed to confer some non-specific resistance to Col E2 (data not shown). Wzz mutant proteins were either expressed from pQE30 with IPTG induction (in the RMA4053 background), or constitutively expressed from pRMCD77 (in the RMA2163 background). Wild-type PCP1a proteins were expressed from pWSK29, a low copy number vector (in the RMA2163 background). Since strains RMA2163 and RMA4053 carrying these plasmids have not been published before, the LPS profiles conferred by these new strains were re-analysed by SDS-PAGE and silver staining. This allowed for a direct comparison of their LPS profiles which was critical to this study. The LPS Oag modal chain length distribution conferred by each construct is summarised in Table 3 and Fig. 1. The *wzz* mutant constructs (referred to by their Wzz mutant proteins in Table 3 and Fig. 1) were grouped into 5 different phenotypic classes (adapted from Papadopoulos & Morona (2010)): class I (non-modal Oag chain length), class II (very short [VS], 2 to 8 Oag RUs), class III (intermediate short [IS], 8 to 14 Oag RUs), class IV (short [S], 10 to 17 Oag RUs) and class V (long [L], 16 to 28 Oag RUs).

The LPS profiles shown in Fig. 1a are similar to that of Wzz mutant proteins expressed from pQE30 described previously (Papadopoulos & Morona, 2010). Class I profiles were observed for strains encoding Wzz mutant proteins i32, i163, i290, i161, i279, i199 and i66 (Fig. 1a, lanes 3 - 9), class II profiles for Wzz mutant proteins i231, i191, i255, i247 and i219 (Fig. 1a, lanes 10 - 14), class III profiles for Wzz mutant proteins i92 and i138 (Fig. 1a, lanes 15 - 16), class IV profiles for Wzz mutant proteins i80 and i81 (Fig. 1a, lanes 17 - 18), and class V profiles for strains encoding Wzz mutant proteins i128 and i131 (Fig. 1a, lanes 19 - 20). The strain carrying pRMCD30

246 encoding wild-type $WzzB_{SF}$ had LPS with S-type Oag chains (Fig. 1a, lane 1) and the strain
247 carrying pQE30 had LPS with unregulated Oag chains, as expected (Fig. 1a, lane 2).
248
249 Inspection of the LPS profiles of Wzz mutant proteins expressed constitutively from the pRMCD77
250 vector (Daniels & Morona, 1999) showed that strains encoding Wzz mutant proteins K31A and
251 P292A were in class I, Wzz mutant proteins G305A/G311A and I35C/M32T were in class II, Wzz
252 mutant protein M32T was in class III, and Wzz mutant proteins I35C, G305A, P283A, G311A,
253 G305A/G209A and K267N were in class IV (Table 3 & Fig. 1b, lanes 3 - 13). The strain carrying
254 pRMCD78 encoding wild-type $WzzB_{SF}$ had LPS with S-type Oag chains (Table 3 & Fig. 1b, lane
255 2), and the vector control strain carrying pRMCD77 had LPS with unregulated Oag chains, as
256 expected (Table 3 & Fig. 1b, lane 1).
257
258 Investigation of the final set of plasmid constructs encoding different wild-type PCP1a proteins
259 expressed from pWSK29 showed that LPS with extremely short (ES)-type Oag chains of 1 – 4 Oag
260 RUs was conferred by the strain carrying pWSK29-Wzz_{O139}, LPS with S-type Oag chains of 10 -
261 17 Oag RUs was conferred by the strain carrying pWSK29-$WzzB_{SF,}$ LPS with L-type Oag chains of
262 16 – 28 Oag RUs was conferred by the strain carrying pWSK29-$WzzB_{ST}$, and LPS with VL-type
263 Oag chains of >80 Oag RUs were conferred by the strains pWSK29-Wzz_{pHS2} and pWSK29-$FepE_{ST}$
264 (Table 3 & Fig. 1b, lanes 14 - 18). The vector control strain carrying pWSK29 had LPS with
265 unregulated Oag chains, as expected (Table 3 & Fig. 1b, lane 19). Western immunoblotting
266 performed on whole cell lysates from *S. flexneri* strains expressing the above Wzz mutant and wild-
267 type PCP1a proteins with anti-Wzz antibodies detected a band consistent with the size of the wild-
268 type $WzzB_{SF}$ protein (~37 kDa) for all strains expressing Wzz mutant proteins (Table 3 & Fig. S1).
269 No band was detected for control strains (carrying pQE30, pRMCD77 or pWSK29), and strains

expressing the FepE, Wzz_{O139} and $WzzB_{pHS2}$ proteins (pWSK29-$FepE_{ST}$, pWSK29-Wzz_{O139} and pWSK29-$WzzB_{pHS2}$), as expected (Table 3 & Fig. S1). A band of ~37 kDa was however detected for the strain carrying pWSK29-$WzzB_{ST}$ (Table 3 & Fig. S1), suggesting that as the WzzB proteins from *S. flexneri* and *S. typhimurium* are similar, the rabbit anti-Wzz antibody used was cross-reactive and detected shared epitopes.

Effect of LPS modal chain length on sensitivity to Col E2. Having thoroughly characterised the LPS profiles conferred by the various Wzz mutant and wild-type PCP1a proteins, sensitivity to Col E2 was then investigated using swab sensitivity assays with purified Col E2. Strains with different LPS Oag modal chain lengths were swabbed perpendicular to a 0.1 μg ml^{-1} Col E2 streak on a plate and those that grew over the Col E2 streak line were considered resistant (R) (0 mm), while strains that could not (> 0 mm) were considered sensitive (S). Representative data for Wzz mutants proteins expressed from pQE30 is shown in Fig. 2. The strain carrying pRMCD30 encoding wild-type $WzzB_{SF}$ (Fig. 2 and Table 3) and strains encoding class IV and V (S-type and L-type) Wzz mutant proteins (i80, i81, i128 and i131) were resistant to Col E2, while strains encoding class I – III Wzz mutant proteins (i199, i279, i32, i163, i161, i290, i66, i231, i255, i247, i191, i219, i92 and i138) (conferring non-modal, VS-type and IS-type Oag chains) were sensitive (Fig. 2 and Table 3). The vector control strain carrying pQE30 was sensitive to Col E2 as expected (Fig. 2 and Table 3). A similar trend was also observed for Wzz mutant proteins expressed constitutively from pRMCD77. The strain carrying pRMCD78 encoding wild-type $WzzB_{SF}$, and strains encoding all class IV Wzz mutant proteins showed resistance to Col E2 (Table 3), while strains encoding class I – II Wzz mutant proteins showed sensitivity to Col E2 (Table 3). When strains encoding the different wild-type PCP1a proteins were tested, pWSK29-$WzzB_{SF}$ (conferring S-type Oag chains) and pWSK29-$WzzB_{ST}$ (conferring L-type Oag chains) were resistant to Col E2, while strains with

294 either pWSK29-Wzz_{O139} (ES-type Oag chains) or pWSK29-$FepE_{ST}$ and pWSK29-$WzzB_{pHS2}$ (VL-
295 type Oag chains) were sensitive (Table 3). These results indicated that expression of S-type and L-
296 type LPS Oag chains confer resistance to Col E2.
297
298 In addition to the Col E2 swab sensitivity assays described above, spot sensitivity assays were also
299 carried out as an alternative method to quantitatively investigate the sensitivity of strains with
300 different LPS Oag modal chain lengths. The lowest concentration that generated a clear zone of
301 inhibition was defined as the minimal inhibitory concentration (MIC). Spot sensitivity assays were
302 repeated three times and the average MIC is shown in Table 3. The strain carrying pRMCD30
303 encoding wild-type $WzzB_{SF}$ showed resistance to Col E2 (MIC = 32 μg ml^{-1}) (Fig. 3a & h and
304 Table 3), while the control strain expressing pQE30 was sensitive to Col E2 (MIC = 0.25 μg ml^{-1})
305 (Fig. 3b and Table 3). Hence, LPS Oag modal length control by $WzzB_{SF}$ conferred a 128 fold
306 increase in resistance to Col E2. Strains encoding class I Wzz mutant proteins i32, i163, i290, i161,
307 i279, i199 and i66 (represented by i32 in Fig. 3c) and class II Wzz mutant proteins i231, i191, i255,
308 i247 and i219 (represented by i219 in Fig. 3d) conferring non-modal or VS-type Oag chains were
309 all sensitive to Col E2 (MIC ≤ 0.5 μg ml^{-1}) (Table 3 and Fig. 4a). Strains encoding class III Wzz
310 mutant proteins i92 and i138 (represented by i92 in Fig. 3e) conferring IS-type Oag chains were
311 slightly more resistant to Col E2 (MICs of 2 μg ml^{-1} and 8 μg ml^{-1}, respectively) (Table 3 and Fig.
312 4a). Strains encoding class IV Wzz mutant proteins i80 and i81 (represented by i81 in Fig. 3f & i)
313 and class V Wzz mutant proteins i128 and i131 (represented by i131 in Fig. 3g & j), conferring S-
314 type and L-type Oag chains respectively, were resistant to Col E2 (MICs = 32 μg ml^{-1} for class IV,
315 and MIC = 64 μg ml^{-1} for class V) (Table 3 and Fig. 4a). These results suggest that expression of
316 class IV and class V Wzz proteins confer resistance to Col E2.
317

318 Spot sensitivity assays carried out on strains expressing wild-type $WzzB_{SF}$ and Wzz mutant proteins
319 expressed from pRMCD77 showed a similar trend. The strain carrying pRMCD78 encoding wild-
320 type $WzzB_{SF}$ was resistant to Col E2 (MIC = 32 μg ml^{-1}) (Table 3 and Fig. 4b), while the control
321 strain carrying pRMCD77 was sensitive to Col E2 (MIC = 2 μg ml^{-1}) (Table 3 and Fig. 4b) but at a
322 higher concentration than that observed for the strain carrying pQE30 above (MIC = 0.25 μg ml^{-1})
323 (Table 3 and Fig. 4b), suggesting that the pRMCD77 background confers some resistance to Col
324 E2. Despite this, LPS Oag modal length control by $WzzB_{SF}$ conferred a 16 fold increase in
325 resistance to Col E2 in the pRMCD77 background. In comparison to the control strain carrying
326 pRMCD77, strains encoding class I Wzz mutant proteins K31A and P292A, and the class II Wzz
327 mutant protein I35C/M32T were sensitive to Col E2 (MICs = 2 μg ml^{-1}) (Table 3 and Fig. 4b),
328 while one class II Wzz mutant protein G305A/G311A showed slight resistance (MIC = 4 μg ml^{-1})
329 (Table 3 and Fig. 4b). However, strains encoding class IV Wzz mutant proteins M32T, I35C,
330 G305A, P283A, G311A, G305A/G209A and K267N were more resistant to Col E2 (MICs ranging
331 from 8 - 32 μg ml^{-1}), conferring at least a 4 fold increase in resistance to Col E2 (Table 3 and Fig.
332 4b) when compared to the control strain carrying pRMCD77. Similar to the trend observed for Wzz
333 mutants expressed from pQE30, only class IV mutant proteins in the pRMCD77 background
334 showed resistance to Col E2.
335
336 Spot sensitivity assays were then carried out on strains expressing wild-type PCP1a proteins
337 expressed from the low copy number vector pWSK29. Strains with pWSK29-$WzzB_{SF}$ (conferring
338 S-type Oag chains) and pWSK29-$WzzB_{ST}$ (conferring L-type Oag chains) showed resistance to Col
339 E2 (MICs of 32 and 64 μg ml^{-1} respectively) (Table 3 and Fig. 4c), while strains with pWSK29-
340 $WzzB_{pHS2}$ and pWSK29-$FepE_{ST}$ (conferring VL-type Oag chains) were sensitive to Col E2 (MICs =
341 0.5 μg ml^{-1}) (Table 3 and Fig. 4c), supporting the observation made above that S-type and L-type

342 Oag chains confer resistance to Col E2. The control strain carrying pWSK29 was sensitive to Col
343 E2 (MIC = 0.25 μg ml^{-1}) (Table 3 and Fig. 4c), as expected. Interestingly, the strain with pWSK29-
344 Wzz_{O139} (conferring ES-type Oag chains of 1 – 4 Oag RUs) was more sensitive to Col E2 (MIC =
345 0.031 μg ml^{-1}) (Table 3 and Fig. 4c) than the pWSK29 control strain.
346
347 **Detection of the Col E2 receptor.** Since the primary *E. coli* OM receptor which allows import of
348 Col E2 into cells is BtuB (Sharma *et al.*, 2007), expression of the BtuB receptor was also
349 investigated to determine if the level of BtuB protein was affected by expression of the different
350 Wzz proteins. Since different LPS profiles might cause changes in the OM composition and hence
351 affect insertion of BtuB, OM samples were prepared from *S. flexneri* strains expressing wild-type
352 PCP1a proteins in the pWSK29 background and analysed by Western immunoblotting (Fig. S2).
353 All strains grew at the same growth rate (Fig. S2a) and a band consistent with the size of the BtuB
354 protein (~66 kDa) was detected for all strains with monoclonal anti-BtuB antibodies (Fig. S2c,
355 lanes 2 - 7). Equivalent levels of other major OM proteins OmpF+OmpC and OmpA were also
356 observed by Coomassie staining (Fig. S2b, lanes 2 - 7). These results suggest that the differences in
357 colicin resistance observed is due to the length of the LPS Oag modal chain length conferred by
358 each Wzz PCP1a protein, and not due to differences in the OM expression levels of the BtuB
359 receptor. Whole cell lysates from *S. flexneri* strains expressing Wzz mutant proteins in the pQE30
360 and pRMCD77 backgrounds were also investigated for expression of the BtuB receptor and
361 Western immunoblotting with monoclonal anti-BtuB antibodies showed a band consistent with the
362 size of the BtuB protein (~66 kDa) for all strains (Table 3).
363
364 **Effect of LPS modal chain length on serum resistance.** Strains expressing wild-type PCP1a
365 proteins expressed from pWSK29 were also subjected to complement sensitivity assays in 10%

366 human serum over a period of 1 h. Strains with pWSK29-$FepE_{ST}$ and pWSK29-$WzzB_{pHS2}$ (both
367 conferring VL-type Oag chains) showed serum resistance with >90% and >50% survival,
368 respectively (Fig. 5a), while strains with pWSK29-$WzzB_{SF}$, pWSK29-$WzzB_{ST}$ and pWSK29-
369 Wzz_{O139} all showed sensitivity to serum (with 0% survival) (Fig. 5a). Incubation with heat-
370 inactivated serum (HIS) as a control showed that all strains demonstrated > 90% survival, with the
371 exception of pWSK29-Wzz_{O139} which demonstrated ~30% survival (Fig. 5b), suggesting that other
372 active factors (not inactivated by heat) in the serum may effect strains expressing ES-type Oag
373 chains.
374

DISCUSSION

In this study, we used a range of plasmids encoding various Wzz mutant proteins and wild-type PCP1a proteins to confer different LPS Oag modal chain lengths in a common background (*S. flexneri* RMA2163/RMA4053), and performed assays with Col E2 to investigate the effect of LPS Oag modal chain length on *Shigella* sensitivity to this colicin. The results showed that strains with S-type and L-type LPS Oag profiles due to class IV and V proteins, respectively, had higher resistance to Col E2 than strains expressing the shorter Oag chain lengths due to Class I – III proteins (non-modal, VS-type and IS-type), suggesting that expression of near wild-type $WzzB_{SF}$ Oag modal chain lengths is essential for resistance to Col E2 (Fig. 2 – 3 and Table 3). Furthermore, when various constructs expressing different PCP1a proteins were assayed, strains with pWSK29-$WzzB_{SF}$ (conferring S-type Oag chains) and pWSK29-$WzzB_{ST}$ (conferring L-type Oag chains) were also more resistant to Col E2 (Table 3 and Fig. 4c) than strains with plasmids conferring ES-type or VL-type Oag chains. The resistance observed was also not due to differences in the level of BtuB receptor present (Fig. S2). These results confirm that these LPS Oag modal chain lengths are optimal for conferring Col E2 resistance. We hence hypothesise that the VL-type Oag chains have potentially different polysaccharide interactions which may allow Col E2 to move more readily between the LPS molecules. Interestingly, the strain with pWSK29-Wzz_{O139} (conferring ES-type Oag chains of 1 – 4 Oag RUs) was more sensitive to Col E2 than the pWSK29 control strain (Table 3 and Fig. 4c), and we speculate that the expression of these ES-type Oag chains provide no protection against Col E2 as the colicin is readily able to come into close contact with the bacterial OM surface and its receptor BtuB. In comparison, the smooth (though unregulated) LPS expressed by the strain carrying pWSK29 may still provide some hindrance to Col E2. Notably, the pWSK29-Wzz_{O139} strain was also more sensitive to incubation with HIS than the pWSK29 control strain (Fig.

398 5), and this may be due to other active factors besides complement in the serum which are not
399 inactivated by heat.
400
401 Loss of the S-type Oag chains in *S. flexneri* $WzzB_{SF}$ has been shown to result in a defect in *S.*
402 *flexneri* virulence (Van Den Bosch *et al.*, 1997) and its presence allows IcsA to function in actin
403 based motility (Morona *et al.*, 2003). Our data suggests that S-type LPS Oag chains may also act to
404 impede the access of colicins into bacteria and hence reduce their susceptibility towards Col E2
405 killing. We hypothesise that colicins such as Col E2 present in the environment may be the
406 selective pressure that has resulted in the evolution of Wzz proteins that confer S-type and L-type
407 Oag modal chain lengths. While LPS Oag has previously been shown to shield OM proteins from
408 colicin in *E. coli* (Van der Ley *et al.*, 1986), the impact of Oag chain length against colicins has not
409 been investigated. We propose here that *S. flexneri* regulation of S-type Oag chains is required for
410 conferring resistance to colicin, while *S. flexneri* regulation of the VL-type Oag chains is required
411 for conferring resistance to complement (Hong & Payne, 1997) as shown by our serum killing
412 assay (Fig. 5). Interestingly, growth-phase regulation of certain serovars of *Salmonella* expressing
413 L-type Oag chains can also confer resistance to serum but it is the expression of the VL-type Oag
414 chains that confers higher levels of resistance (Bravo *et al.*, 2008). Our study suggests that
415 expression of both S- and VL-type modal chain lengths by *S. flexneri* is essential for optimal
416 virulence (Morona *et al.*, 2003) and for survival inside the host environment, as well as in the
417 external environment, as a result of competition with colicin producing *Enterobacteriaceae*. Our
418 data also presents a novel and effective method for screening *S. flexneri* (or *S. typhimurium*) for the
419 presence of S-type and L-type modal Oag chain lengths using the Col E2 sensitivity streak assay, as
420 only strains conferring ~10 to 17 Oag RUs and ~16 to 28 Oag RUS were observed to be resistant to
421 Col E2 (Fig. 2).

During the course of this study, the crystal structure of *S. flexneri* $WzzB_{SF}$ was recently solved (Kalynych *et al.*, 2012). Analysis of the Wzz mutant proteins expressed from pQE30 (18 in total) on this 3-dimensional structure of $WzzB_{SF}$ (PBD 4E2H; Fig. S3) showed that almost all mutants had insertion mutations located on the inside curvature of the $WzzB_{SF}$ open trimer, with the exception of three class I Wzz mutant proteins (i161, i163 and i279), one class II Wzz mutant protein (i247) and one class III Wzz mutant protein (i138). Two Wzz mutant proteins (i32 and i290) were located in undefined regions of the crystal structure so their location could not be determined. Since class I mutant proteins confer non-modal Oag chain length, this suggests that the majority of the Wzz mutations conferring an effect on LPS modal Oag chain length are located inside the curvature of the $WzzB_{SF}$ trimer. This supports our recent study which showed that residues inside the *E. coli* FepE PCP1a protein oligomer were essential for LPS Oag modal chain length determination (Tran & Morona, 2013).

In summary, our results show that the specific S-type (~10 - 17 Oag RUs) and L-type (~16 - 28 Oag RUs) LPS Oag chains determined by *S. flexneri* $WzzB_{SF}$ and *Salmonella typhimurium* $WzzB_{ST}$, respectively, confer Col E2 resistance in *S. flexneri*, and most likely explains why these modal Oag chain lengths are common in bacteria. The biophysical basis for this function requires further investigation as the VL-type (>80 Oag RUs) LPS Oag chains determined by Wzz_{pHS2} and $FepE_{ST}$ were unable to confer resistance to colicin, although they do provide a high level of resistance to complement.

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447
448

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562 Table 1

TABLE 1. Bacterial strains and plasmids

Strain/plasmid	Description	Source/reference
Escherichia coli		
XL10 Gold	*end*A1 *gln*V44 *rec*A1 *thi*-1 *gyr*A96 *rel*A1 *lac* Hte Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *tet*^{R} F'[*pro*AB *lac*I^{q}ZΔM15 Tn10(Tet^R Amy Cm^R)]	Stratagene
AB1133	*thi argE his proA thr leu ara mtl xyl galK lacy supE* Str^R λ $^-$ F $^-$	(Davies & Reeves, 1975)
E92	*E. coli* BZB2101 carrying a plasmid encoding Colicin A	(Achtman *et al.*, 1983)
E94	*E. coli* BZB2103 carrying a plasmid encoding Colicin D	(Achtman *et al.*, 1983)
E95	*E. coli* BZB2104 carrying a plasmid encoding Colicin E1	(Achtman *et al.*, 1983)
E96	*E. coli* BZB2125 carrying a plasmid encoding Colicin E2	(Achtman *et al.*, 1983)
E97	*E. coli* BZB2106 carrying a plasmid encoding Colicin E3	(Achtman *et al.*, 1983)
E103	*E. coli* BZB2279 carrying a plasmid encoding Colicin Ia	(Achtman *et al.*, 1983)
E105	*E. coli* BZB2116 carrying a plasmid encoding Colicin K	(Achtman *et al.*, 1983)
Shigella flexneri		
RMA2162	*S. flexneri* PE860 serotype Y, cured of virulence plasmid and pHS-2	Laboratory stock
RMA2163	*S. flexneri* PE860 serotype Y *wzz::kan*^{r}, cured of virulence plasmid and pHS-2*;* Kan^R	Laboratory stock
RMA4053	RMA2163 carrying pCDFDuet-1	Laboratory stock
2457T	*S. flexneri* serotype 2a	Laboratory stock
RMA696	*S. flexneri* serotype 2a *wzz::kan*^{r}, Kan^R	(Van Den Bosch *et al.*, 1997)
Plasmids		
pCDFDuet-1	expression vector carrying *lacI* ^{q}, Sm^R	Novagen
pQE30	IPTG inducible, expression vector, Amp^R	Qiagen
pRMCD30	pQE30 with *S. flexneri* 2a *wzzB*$_{SF}$ gene, Amp^R	(Daniels & Morona, 1999)
pMPRMA45	pQE30 encoding Wzz mutant $_i$32	(Papadopoulos & Morona, 2010)
pMPRMA41	pQE30 encoding Wzz mutant $_i$231	(Papadopoulos & Morona, 2010)
pMPRMA50	pQE30 encoding Wzz mutant $_i$219	(Papadopoulos & Morona, 2010)
pMPRMA38	pQE30 encoding Wzz mutant $_i$92	(Papadopoulos & Morona, 2010)
pMPRMA52	pQE30 encoding Wzz mutant $_i$138	(Papadopoulos & Morona, 2010)
pMPRMA53	pQE30 encoding Wzz mutant $_i$80	(Papadopoulos & Morona, 2010)
pMPRMA39	pQE30 encoding Wzz mutant $_i$128	(Papadopoulos & Morona, 2010)
pMPRMA48	pQE30 encoding Wzz mutant $_i$131	(Papadopoulos & Morona, 2010)
pMPRMA40	pQE30 encoding Wzz mutant $_i$163	(Papadopoulos & Morona, 2010)
pMPRMA42	pQE30 encoding Wzz mutant $_i$290	(Papadopoulos & Morona, 2010)
pMPRMA43	pQE30 encoding Wzz mutant $_i$191	(Papadopoulos & Morona, 2010)
pMPRMA44	pQE30 encoding Wzz mutant $_i$161	(Papadopoulos & Morona, 2010)
pMPRMA46	pQE30 encoding Wzz mutant $_i$279	(Papadopoulos & Morona, 2010)

pMPRMA47	pQE30 encoding Wzz mutant $_{i}$255	(Papadopoulos & Morona, 2010)
pMPRMA49	pQE30 encoding Wzz mutant $_{i}$81	(Papadopoulos & Morona, 2010)
pMPRMA51	pQE30 encoding Wzz mutant $_{i}$247	(Papadopoulos & Morona, 2010)
pMPRMA55	pQE30 encoding Wzz mutant $_{i}$199	(Papadopoulos & Morona, 2010)
pMPRMA56	pQE30 encoding Wzz mutant $_{i}$66	(Papadopoulos & Morona, 2010)
pRMCD77	Modified pET17b (Novagen) vector, Amp^R	(Daniels & Morona, 1999)
pRMCD78	pRMCD77 with *S. flexneri* 2a $wzzB_{SF}$ gene	(Daniels & Morona, 1999)
pRMCD108	pRMCD77 encoding Wzz mutant K267N	(Daniels & Morona, 1999)
pRMCD109	pRMCD77 encoding Wzz mutant P283A	(Daniels & Morona, 1999)
pRMCD111	pRMCD77 encoding Wzz mutant G311A	(Daniels & Morona, 1999)
pRMCD112	pRMCD77 encoding Wzz mutant G305A/G309A	(Daniels & Morona, 1999)
pRMCD113	pRMCD77 encoding Wzz mutant G305A/G311A	(Daniels & Morona, 1999)
pRMCD116	pRMCD77 encoding Wzz mutant P292A	(Daniels & Morona, 1999)
pRMCD119	pRMCD77 encoding Wzz mutant K31A	(Daniels & Morona, 1999)
pRMCD121	pRMCD77 encoding Wzz mutant I35C	(Daniels & Morona, 1999)
pRMCD122	pRMCD77 encoding Wzz mutant I35C/M32T	(Daniels & Morona, 1999)
pRMCD125	pRMCD77 encoding Wzz mutant G305A	(Daniels & Morona, 1999)
pRMCD127	pRMCD77 encoding Wzz mutant M32T	(Daniels & Morona, 1999)
pWSK29	Low copy number cloning vector, Amp^R	(Wang & Kushner, 1991)
pWSK29-$WzzB_{SF}$	pWSK29 with *S. flexneri* 2a $wzzB_{SF}$ gene	(Murray *et al.*, 2006)
pWSK29-$WzzB_{ST}$	pWSK29 with *Salmonella typhimurium* $wzzB_{ST}$ gene	(Murray *et al.*, 2006)
pWSK29-$FepE_{ST}$	pWSK29 with *Salmonella typhimurium* $fepE_{ST}$ gene	(Murray *et al.*, 2006)
pWSK29-Wzz_{O139}	pWSK29 with *Vibrio cholerae* wzz_{O139} gene	(Murray *et al.*, 2006)
pWSK29-Wzz_{pHS2}	pWSK29 with *S. flexneri* wzz_{pHS2} gene	(Murray *et al.*, 2006)

563

564 Table 2

565

TABLE 2. *S. flexneri* sensitivity to colicins in double layer assays with colicin producers

Colicin producer	RMA2162	RMA2163	2457T	RMA696	*E. coli* K-12 AB1133
E92 (Col A)	R	R	R	R	S (9.0)
E94 (Col D)	R	R	R	R	S (11.0)
E95 (Col E1)	R	R	R	R	S (12.5)
E96 (Col E2)	S (15.0)	S (20.0)	S (12.0)	S (16.0)	S (26.0)
E97 (Col E3)	S (8.0)	S (14.5)	R	S/S (-)	S (19.0)
E103 (Col Ia)	R	R	R	R	S (8.0)
E105 (Col K	R	R	R		S (6.0)

Sensitivity to *E. coli* colicin producer streak line: S, sensitive (0 mm); R, resistant (> 0 mm); S/S, slightly sensitive (some inhibition of growth observed on streak line).

566 Table 3
567

TABLE 3. Summary of LPS profile, Col E2 sensitivity and protein detection

Plasmid	Wzz mutant protein	LPS Oag modal chain length	Wzz mutant class*	Col E2 sensitivity assays‡		Protein detection§	
				Streak assay† (mm, S/R)	Spot assay (μg ml^{-1})	αWzz	αBtuB‖
pQE30	-	Non-modal	na	S (7.0)	0.25	-	(+++)
pRMCD30	-	10 - 17	na	R	32.00	+++	(+++)
pMPRMA55	i199	Non-modal	I	S (7.0)	0.25	++	(+++)
pMPRMA46	i279	Non-modal	I	S (5.0)	0.33	+	(+++)
pMPRMA45	i32	Non-modal	I	S (8.0)	0.33	+++	(+++)
pMPRMA40	i163	Non-modal	I	S (6.0)	0.33	++	(+++)
pMPRMA44	i161	Non-modal	I	S (5.0)	0.50	++	(+++)
pMPRMA42	i290	Non-modal	I	S (6.0)	0.50	+++	(+++)
pMPRMA56	i66	Non-modal	I	S (4.0)	0.50	+++	(+++)
pMPRMA41	i231	2 - 6	II	S (7.0)	0.33	++	(+++)
pMPRMA47	i255	2 - 8	II	S (6.0)	0.25	+	(+++)
pMPRMA51	i247	2 - 8	II	S (7.0)	0.25	++	(+++)
pMPRMA43	i191	2 - 8	II	S (7.0)	0.50	+	(+++)
pMPRMA50	i219	2 - 10	II	S (8.0)	0.50	+++	(+++)
pMPRMA38	i92	8 - 13	III	S (3.0)	2.00	+++	(+++)
pMPRMA52	i138	8 - 14	III	S (3.5)	8.00	++	(+++)
pMPRMA53	i80	10 - 20	IV	R	32.00	++	(+++)
pMPRMA49	i81	10 - 20	IV	R	32.00	+++	(+++)
pMPRMA39	i128	16 - 20	V	R	64.00	+++	(+++)
pMPRMA48	i131	16 - 22	V	R	64.00	+++	(+++)
pRMCD77	-	Non-modal	na	S (5.0)	2.00	-	(+++)
pRMCD78	-	10 - 17	na	R	32.00	+++	(+++)

pRMCD119	K31A	Non-modal	I	S (5.0)	2.00	+++	(+++)
pRMCD116	P292A	Non-modal	I	S (5.0)	2.00	+++	(+++)
pRMCD122	I35C/M32T	2 - 8	II	S (5.0)	2.00	+++	(+++)
pRMCD113	G305A/G311A	2 - 8	II	S (5.0)	4.00	+++	(+++)
pRMCD127	M32T	9 - 16	IV	R	16.00	+++	(+++)
pRMCD112	G305A/G309A	10 - 17	IV	R	8.00	+++	(+++)
pRMCD111	G311A	10 - 17	IV	R	8.00	+++	(+++)
pRMCD125	G305A	10 - 17	IV	R	16.00	+++	(+++)
pRMCD109	P283A	10 - 17	IV	R	16.00	+++	(+++)
pRMCD121	I35C	10 - 17	IV	R	32.00	+++	(+++)
pRMCD108	K267N	10 - 20	IV	R	16.00	+++	(+++)
pWSK29	-	Non-modal	na	S (5.0)	0.25	-	+++
pWSK29-Wzz_{O139}	-	1 - 4	na	S (8.0)	0.031	-	+++
pWSK29-$WzzB_{SF}$	-	10 - 17	na	R	32.00	+++	+++
pWSK29-$WzzB_{ST}$	-	16 - 28	na	R	64.00	++	+++
pWSK29-$WzzB_{pHS2}$	-	>80	na	S (5.0)	0.50	-	+++
pWSK29-$FepE_{ST}$	-	>80	na	S (5.0)	0.50	-	+++

* Wzz mutant classification adapted from Papadopoulus & Morona (2010); Class I, non-modal Oag chain length; class II, very short (VS) (~2 to 8 Oag RUs); class III, intermediate short (IS) (~8 to 14 Oag RUs); class IV, short (S) (~10 to 17 Oag RUs); class V, long (L) (~16 to 28 Oag RUs); na, not applicable.

† Sensitivity to 0.1 μg ml^{-1} Col E2 streak: S, sensitive; R, resistant; S/S, slightly sensitive.

‡ Spot sensitivity assays were repeated three times and the average MIC (defined as the lowest concentration that generated a clear zone of inhibition) is shown in the column above.

§ +++, wild type; +, less than wild type; -, not detected.

‖(+++), detected from whole cells only.

FIGURE LEGENDS

Fig. 1. LPS profile conferred by Wzz mutant proteins expressed from pQE30/pRMCD77 and wild-type PCP1a proteins. LPS was isolated and detected from whole cell lysates of *S. flexneri* strains carrying plasmids encoding (a) Wzz mutant proteins expressed from pQE30, or (b) Wzz mutant proteins expressed from pRMCD77, or wild-type PCP1a proteins, as indicated above. The first 15 Oag RUs and the positions of the L-type and VL-type Oag chains are indicated on the side of the gel. Mutant proteins conferring similar Oag modal chain lengths are grouped as Class I (non-modal Oag chain length), Class II (~2 to 8 Oag RUs), Class III (~8 to 14 Oag RUs), Class IV (~10 to 17 Oag RUs) and Class V (~16 to 28 Oag RUs). Each lane contains ~2 x 10^8 bacterial cells of each strain.

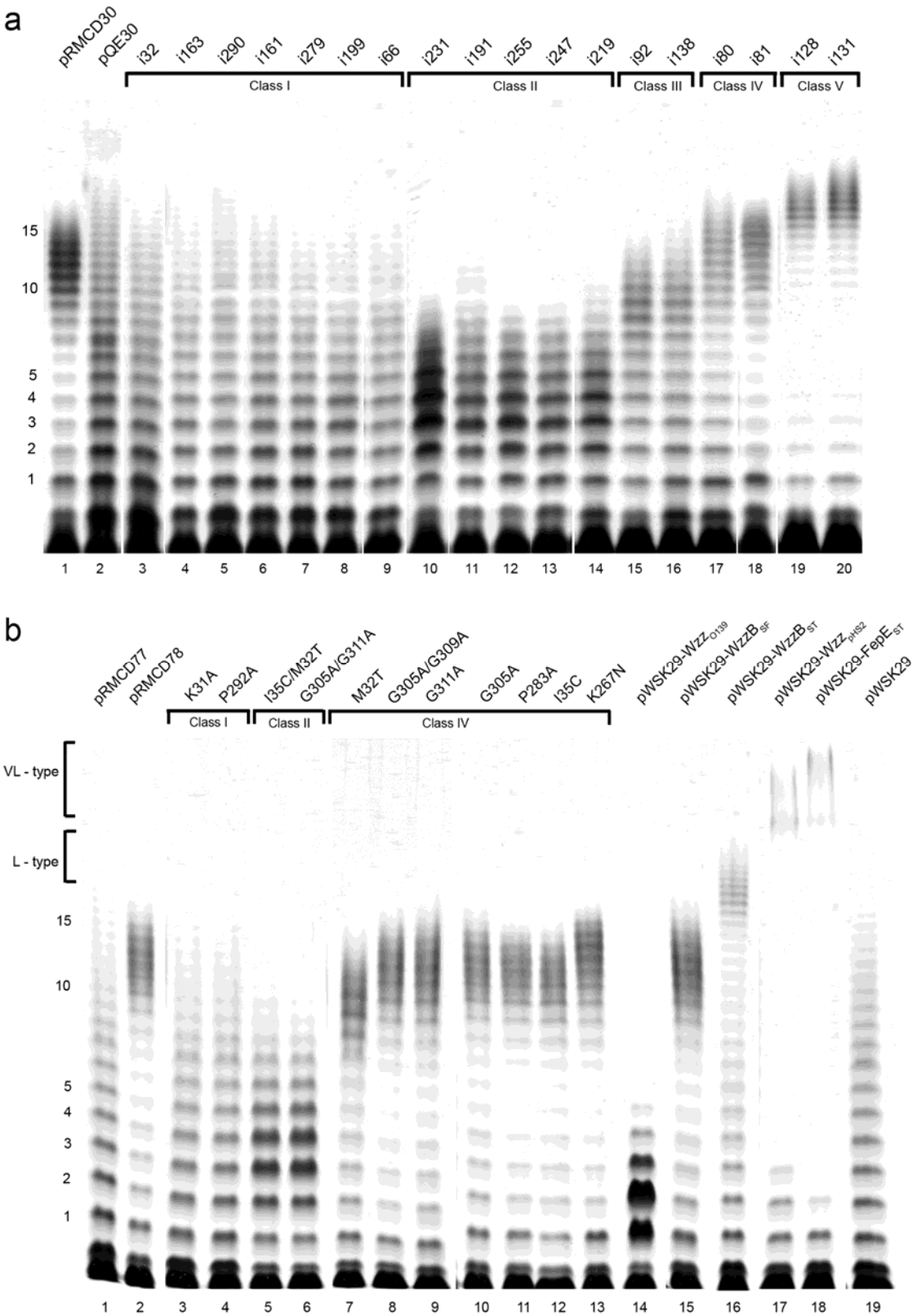
Fig. 2. Col E2 swab sensitivity assays for Wzz mutant proteins expressed from pQE30. Purified Col E2 (0.1 μg ml^{-1}) was swabbed onto LB agar plates and individual *S. flexneri* strains carrying pRMCD30, pQE30 or expressing the Wzz mutant proteins indicated above were swabbed perpendicular to the Col E2 streak. Strains that grew over the Col E2 streak line were considered resistant (R), and strains that did not were considered sensitive (S). Half plates are shown above, and the strain carrying pRMCD30 was used as a control on each plate.

Fig. 3. Col E2 spot sensitivity assays for different classes of Wzz mutants. *S. flexneri* strains carrying pRMCD30, pQE30 or expressing the Wzz mutant proteins indicated above were spread onto selective LB agar plates and spotted with different concentrations of purified Col E2 protein (μg ml^{-1}) as indicated.

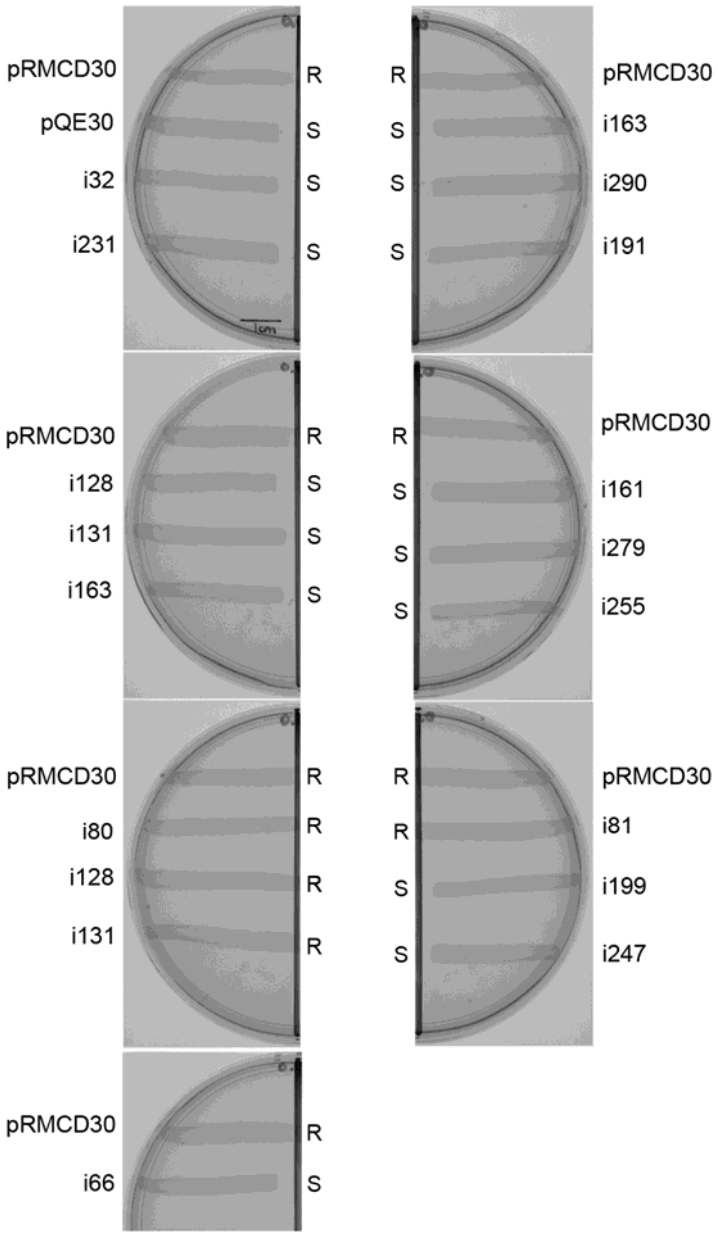
Fig. 4. Analysis of Colicin E2 sensitivity. *S. flexneri* strains expressing the indicated (a) Wzz mutants proteins from pQE30 or (b) Wzz mutants proteins from pRMCD77 or (c) wild-type PCP1a proteins, are

592 shown on the *x* axis. The MIC of Col E2 (in μg ml^{-1}) required to generate a clear zone of bacterial
593 growth inhibition is shown on the *y* axis (*n* = 3). Columns bars shaded in black in (a) and (b) refer to
594 strains carrying pRMCD30 and pRMCD78, respectively.
595
596 **Fig. 5. Survival of strains expressing wild-type PCP1a proteins in 10% human serum.** *S. flexneri*
597 strains expressing the indicated wild-type PCP1a proteins were incubated in (a) 10% (v/v) human
598 serum or (b) heat inactivated human serum (HIS) for 1 h at 37°C. Samples were taken at 30 min
599 intervals for viable counts. Data points represent percentage survival (mean ± S.D., *n* = 3 assays).
600

Figure 1


a
pRMCD30
pQE30
i32
i163
i290
i161
i279
i199
i66
i231
i191
i255
i247
i219
i92
i138
i80
i81
i128
i131
Class I
Class II
Class III
Class IV
Class V
15
10
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
b
pRMCD77
pRMCD78
K31A
P292A
I35C/M32T
G305A/G311A
M32T
G305A/G309A
G311A
G305A
P283A
I35C
K267N
pWSK29-Wzz$_{O139}$
pWSK29-WzzB$_{SF}$
pWSK29-WzzB$_{ST}$
pWSK29-Wzz$_{pHS2}$
pWSK29-FepE$_{ST}$
pWSK29
Class I
Class II
Class IV
VL - type
L - type
15
10
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

603 **Figure 2**



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605

606 **Figure 3**

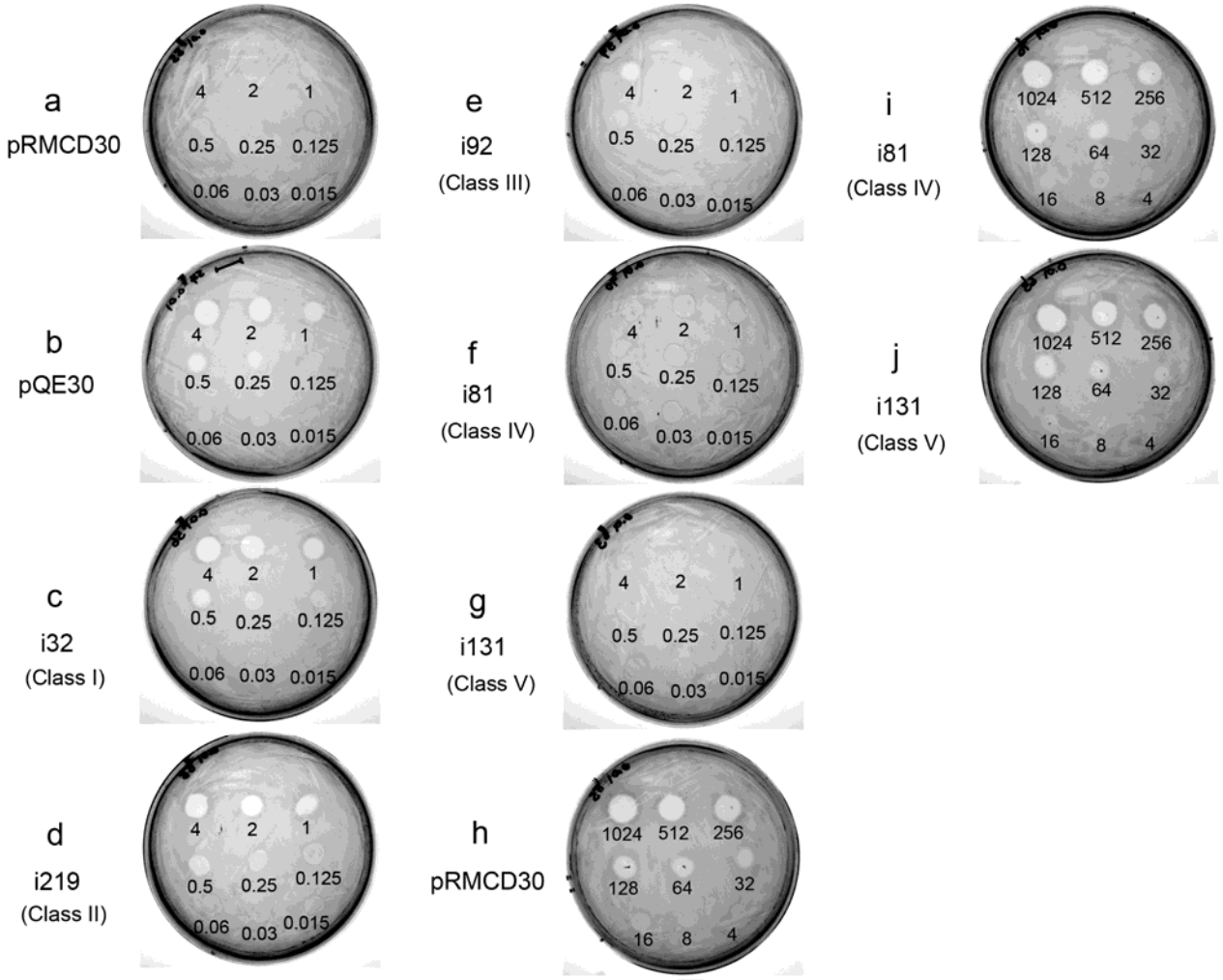

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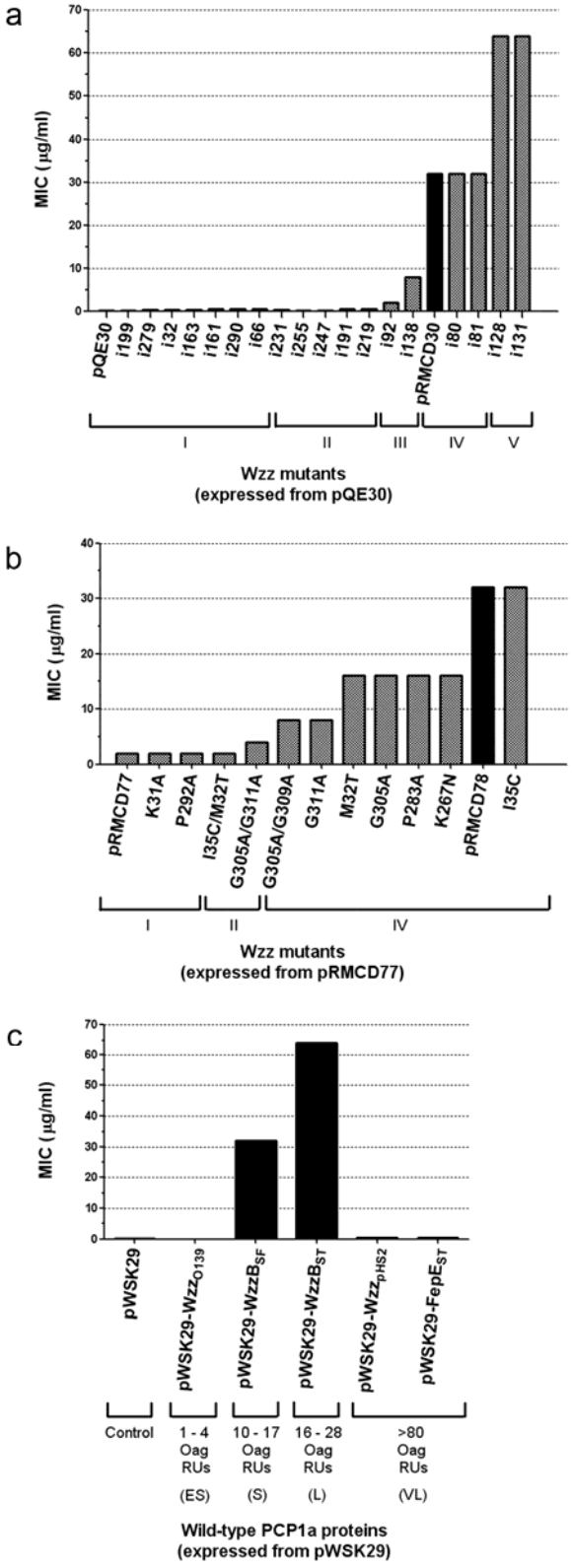
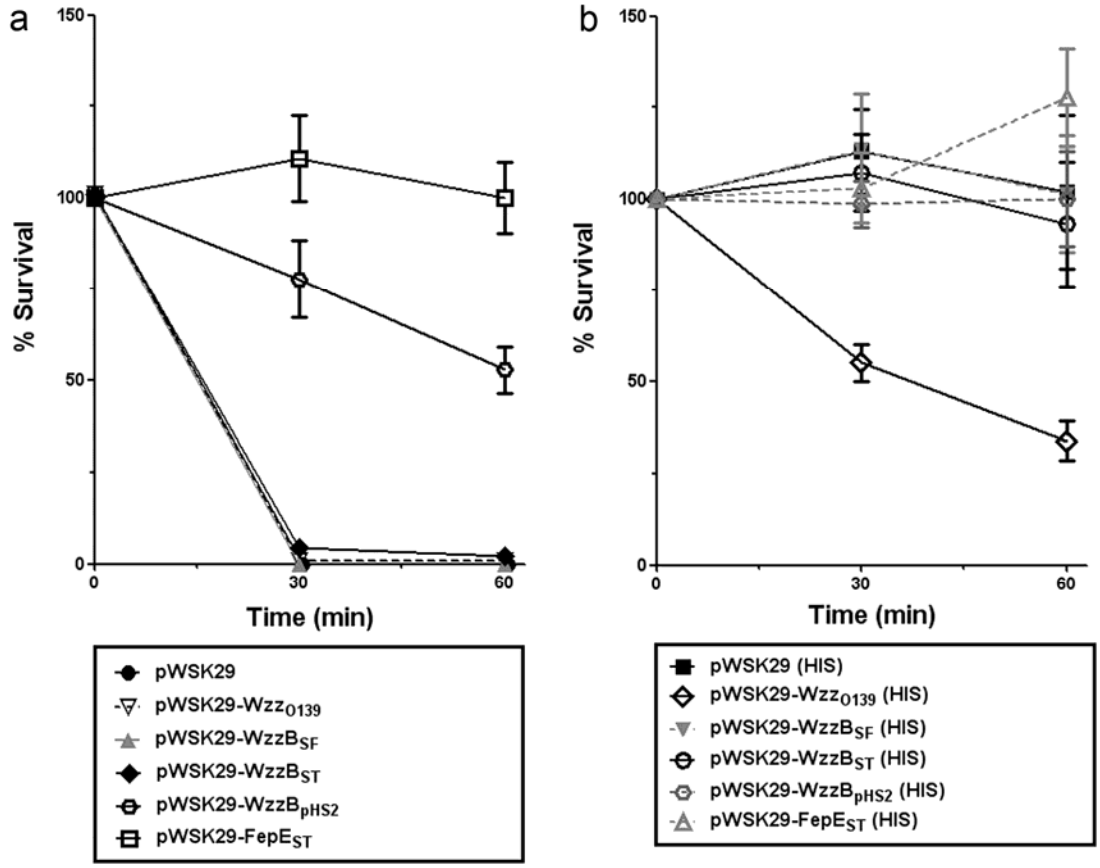
Figure 4


a
MIC (µg/ml)
pQE30
i199
i279
i32
i163
i161
i290
i66
i231
i255
i247
i191
i219
i92
i138
pRMCD30
i80
i81
i128
i131
I
II
III
IV
V
Wzz mutants
(expressed from pQE30)
b
MIC (µg/ml)
pRMCD77
K31A
P292A
I35C/M32T
G305A/G311A
G305A/G309A
G311A
M32T
G305A
P283A
K267N
pRMCD78
I35C
I
II
IV
Wzz mutants
(expressed from pRMCD77)
c
MIC (µg/ml)
pWSK29
pWSK29-WzzO139
pWSK29-WzzBSF
pWSK29-WzzBST
pWSK29-WzzpHS2
pWSK29-FepEST
Control
1 - 4 Oag RUs (ES)
10 - 17 Oag RUs (S)
16 - 28 Oag RUs (L)
>80 Oag RUs (VL)
Wild-type PCP1a proteins
(expressed from pWSK29)

611 **Figure 5**



612

Fig. S1

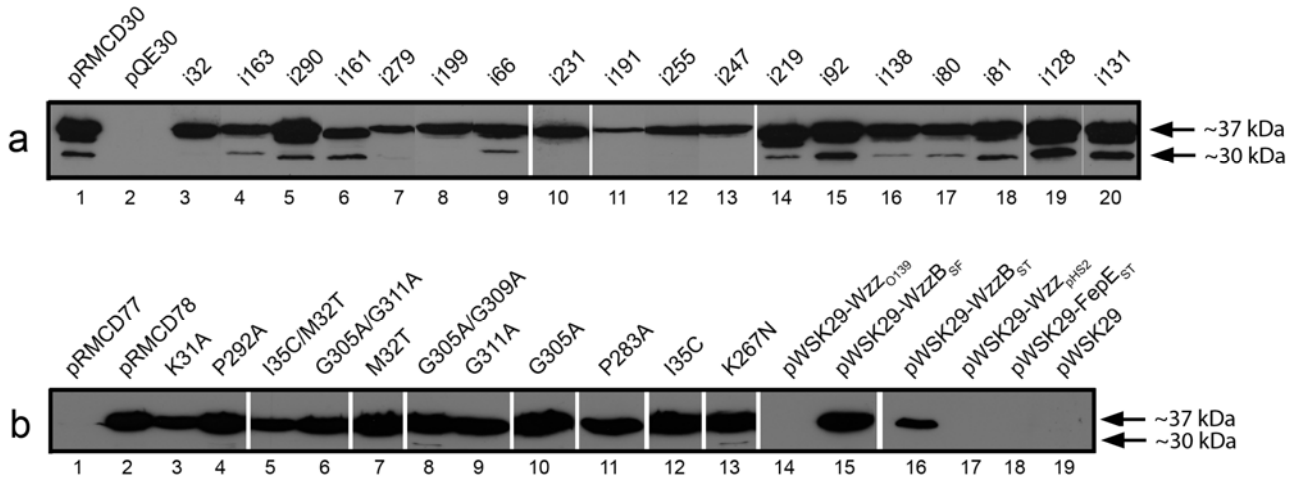


Fig. S1. Western immunoblotting with anti-Wzz antisera. Western blots on whole cell lysates of *S. flexneri* strains carrying plasmids encoding (a) Wzz mutant proteins expressed from pQE30, or (b) Wzz mutant proteins expressed from pRMCD77, or wild-type PCP1a proteins (as indicated above), were probed with rabbit anti-Wzz antisera. The size of the full length His_6-$WzzB_{SF}$ protein (~37 kDa) and degraded/altered His_6-$WzzB_{SF}$ (~30 kDa) is indicated. Each lane contains 5 x 10^7 bacterial cells of each strain.

Fig. S2

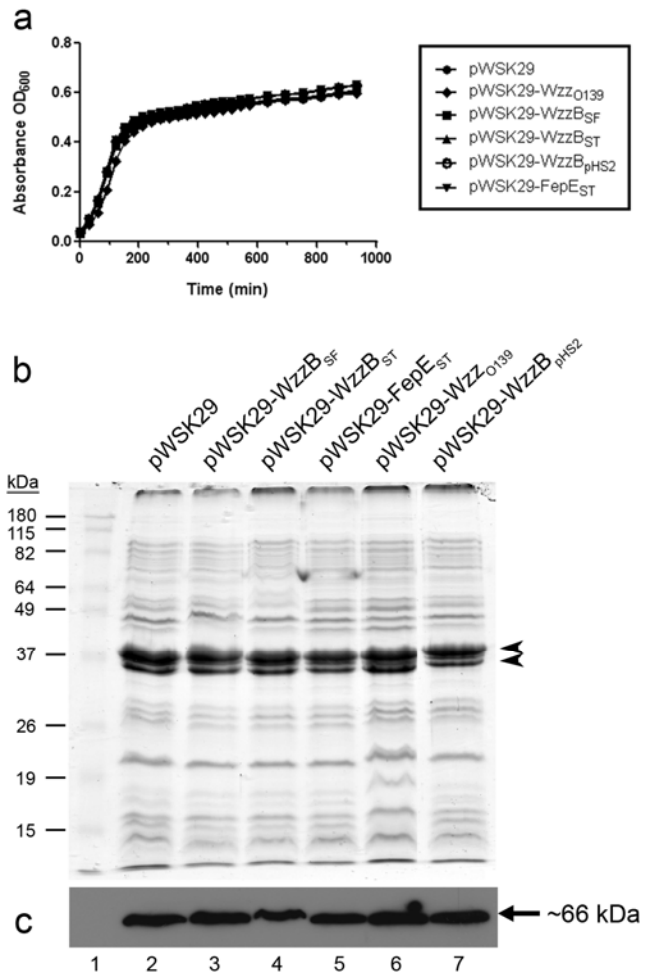


Fig. S2. Growth curve and analysis of OM samples from strains carrying wild-type PCP1a proteins. (a) Growth curves of *S. flexneri* strains carrying plasmids encoding wild-type PCP1a proteins (as indicated) sub 1/20, incubated at 37°C with aeration, and OD_{600} readings taken at 30 min intervals for 22 h; (b & c) OM samples prepared from the strains indicated above were analysed by (b) Coomassie staining and (c) Western immunoblotting with mouse anti-BtuB antisera. The migration positions of the Benchmark Prestained Marker (M) Standards (Invitrogen) are indicated on the left in kDa. The major OM proteins OmpF+OmpC and OmpA are indicated by the two arrowheads in (b) and the size of the full length BtuB protein (~66 kDa) is indicated in (c).

Fig. S3

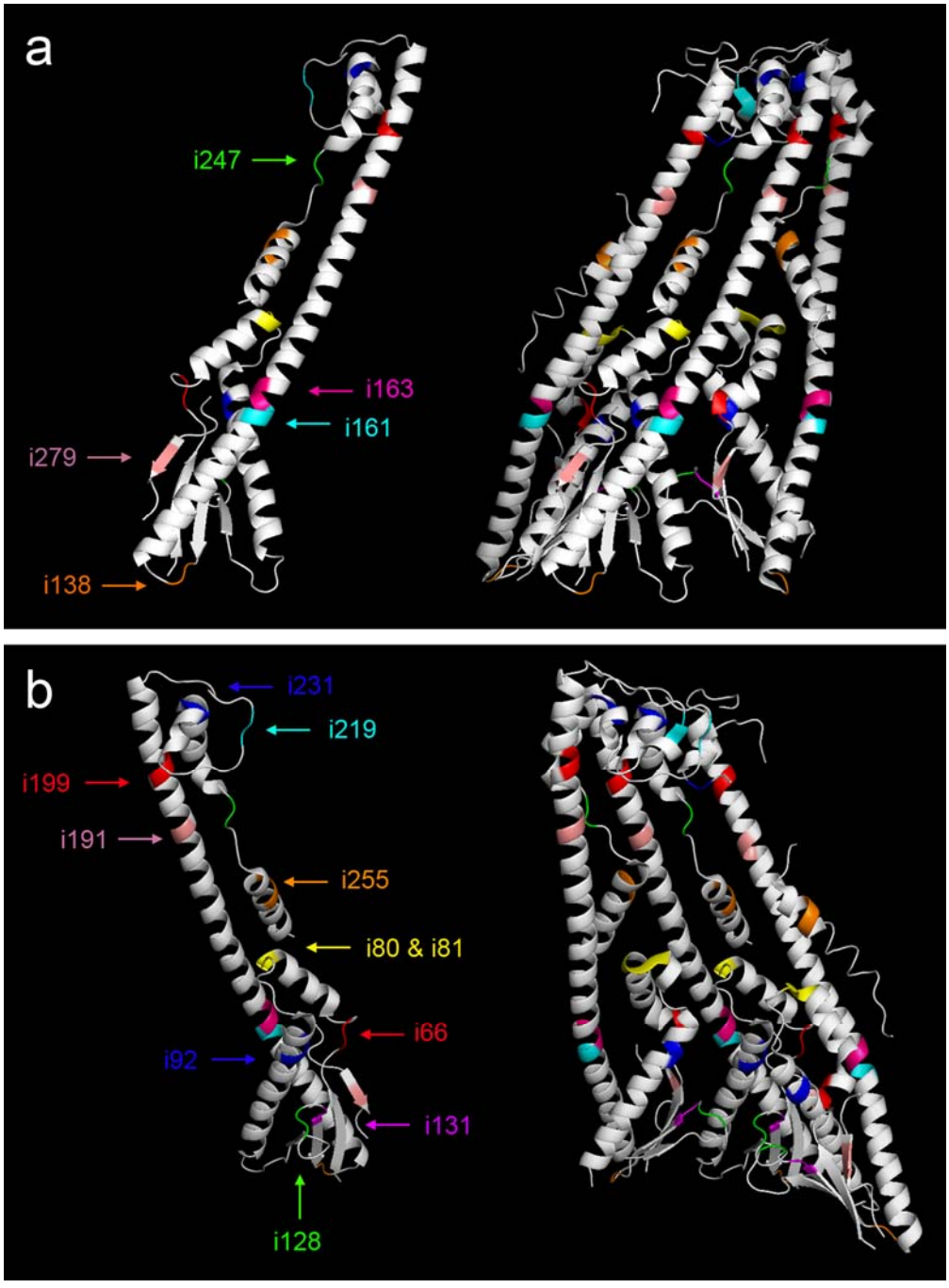


Fig. S3. Location of Wzz mutations on the 3D structures of $WzzB_{SF}$. The Wzz mutations located (a) outside and (b) inside the $WzzB_{SF}$ structure (PDB 4E2H) are mapped on the $WzzB_{SF}$ monomer (chain B) and $WzzB_{SF}$ open trimer, respectively.