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Mutational analysis of the major periplasmic loops of Shigella flexneri Wzy; identification of the residues affecting O antigen modal chain length control, and Wzz dependent polymerisation activity
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Abbreviations: Oag, O antigen; S-LPS, smooth LPS; SR-LPS, Semi rough LPS; R-LPS, rough LPS; PL, periplasmic loop; TM, transmembrane; Cytoplasmic loop, CL; RU, repeat units; Rha, Rhamnose; GlcNAc, *N*-acetylglucosamine; PEtN, phosphoethanolamine; OM, outer membrane; IM, inner membrane; GlcNAc-1-P, *N*-acetylglucosamine phosphate; UDP-GlcNAc, uridine diphosphate-GlcNAc; Und-P, undecaprenol phosphate; PCP, polysaccharide co-polymerase; S, short; V, very long; WT, wild type; GFP, green fluorescent protein; ColE2, Colicin E2; Rif, rifampicin; Km, kanamycin; Cm, chloramphenicol; Tet, tetracycline; LB, lysogeny broth.

Summary

The O antigen (Oag) component of lipopolysaccharide (LPS) is a major *S. flexneri* virulence determinant. Oag is polymerised by Wzy_{Sf}, and its modal chain length is determined by Wzz_{Sf} and Wzz_{pHS2}. Site-directed mutagenesis was performed on wzy_{Sf} in pWaldo-wzy_{Sf}-TEV-GFP to alter Arg residues in Wzy_{Sf}'s two large periplasmic loops (PL) (PL3 and PL5). Analysis of the LPS profiles conferred by mutated Wzy_{Sf} proteins in the wzy_{Sf} deficient (*Δwzy*) strain identified residues that affect Wzy_{Sf} activity. The importance of the guanidium group of the Arg residues was investigated by altering the Arg residues to Lys and Glu, which generated Wzy_{Sf} mutants conferring altered LPS Oag modal chain lengths. The dependence of these Wzy_{Sf} mutants on Wzz_{Sf} was investigated by expressing them in a wzy_{Sf} and wzz_{Sf} deficient (*Δwzy Δwzz*) strain. Comparison of the LPS profiles identified a role for the Arg residues in the association of Wzy_{Sf} and Wzz_{Sf} during Oag polymerisation. Colicin E2 and bacteriophage Sf6c susceptibility supported this conclusion. Comparison of the expression levels of different mutant Wzy_{Sf}-GFP proteins with the wild type (WT) Wzy_{Sf}-GFP showed that certain Arg residues affected production levels of Wzy_{Sf} in a Wzz_{Sf} dependent manner. To our knowledge, this is the first report of *S. flexneri* Wzy_{Sf} mutants having an effect on LPS Oag modal chain length, and identified functionally significant Arg residues in Wzy_{Sf}.

Introduction

Shigella flexneri is the main causative agent for the disease shigellosis or bacterial dysentery. 125 million shigellosis cases occur annually in Asia, with approximately 14000 fatalities (Bardhan *et al.*, 2010). The O antigen (Oag) component of the lipopolysaccharide (LPS) of *Shigella flexneri* plays an important role in the pathogenesis of the bacteria. Oag is composed of oligosaccharide repeat units (RUs) or O units. Oag is linked to the hydrophobic anchor of the LPS (Lipid A) by the non-repeating oligosaccharide domain known as the core sugar region (Raetz & Whitfield, 2002; Sperandeo *et al.*, 2009). The complete LPS structure with Oag chains is termed smooth LPS (S-LPS). However, the LPS structure devoid of Oag is termed rough LPS (R-LPS), and LPS with only one O unit is termed semi-rough LPS (SR-LPS) (Morona *et al.*, 1994).

Oag is the serotype determinant and also the protective antigen of the bacteria (Jennison & Verma, 2004; Stagg *et al.*, 2009; Sun *et al.*, 2013). Depending on the composition of Oag *S. flexneri* is divided into 17 serotypes (Sun *et al.*, 2013). Except serotype 6, all the serotypes share a basic polysaccharide backbone containing three L-rhamnoses (Rha), and one *N*-acetylglucosamine (GlcNAc). This basic Oag structure is known as serotype Y. The differences between the serotypes are conferred by addition of glucosyl, O-acetyl, or phosphoethanolamine (PEtN) functional groups by various linkages to the sugars of the basic tetrasaccharide RU (Allison & Verma, 2000; Sun *et al.*, 2012; Wang *et al.*, 2010). Oag restricts the

accessibility of the colicin to their outer membrane (OM) receptor protein (Tran *et al.*, 2014; van der Ley *et al.*, 1986). In addition, the bacteriophage Sf6 uses Oag as a receptor and forms plaques on serotype Y and X strains (Lindberg *et al.*, 1978).

S. flexneri LPS biosynthesis occurs mainly by two separate pathways: 1) lipid A and core biosynthesis and 2) Oag biosynthesis. Oag biosynthesis occurs on either side of the inner membrane (IM) and it is mediated by the Wzy-dependent pathway (Allison & Verma, 2000; Morona *et al.*, 1995). *S. flexneri* Oag biosynthesis starts by the transfer of *N*-acetylglucosamine phosphate (GlcNAc-1-P) from an uridine diphosphate-GlcNAc (UDP-GlcNAc) to undecaprenol phosphate (Und-P) at the cytoplasmic side of the IM by WecA (Guo *et al.*, 2008; Liu *et al.*, 1996; Wang *et al.*, 2010). Then the rhamnosyl transferases (RfbG and RfbF) add sequential Rha residues to the GlcNAc to form the O unit (McKinney *et al.*, 2002; Morona *et al.*, 1994). Translocation of the O unit to the periplasmic side is mediated by the protein Wzx. At the periplasmic side O units are polymerised by Wzy to form the Oag. The chain length of the Oag is regulated by Wzz (Daniels *et al.*, 1998; Morona *et al.*, 1994). Finally, the Oag chains are transferred to the core-lipid A by the ligase WaaL. The Lpt proteins (Lpt A - G) facilitate the transport of the LPS from the IM to the OM (Ruiz *et al.*, 2008; Sperandeo *et al.*, 2009).

S. flexneri Wzy (Wzy_{Sf}) is a 43.7 kDa hydrophobic integral membrane protein. It has 12 transmembrane (TM) segments and two large periplasmic (PL) domains (PL3 and PL5) (Daniels *et al.*, 1998; Morona *et al.*, 1994).

Previously we were able to identify some of the key functional amino acid residues of Wzy_{Sf}, providing insight into Wzy_{Sf} structure and function (Nath *et al.*, 2015). Wzy_{Sf} has a $RX_{15}G$ motif in both of the PL3 and PL5 starting from R164 in PL3 and R289 in PL5. It also has several Arg residues between these two motifs. In the *Pseudomonas aeruginosa* Wzy (Wzy_{Pa}), it was found that the PL3 and PL5 have $RX_{10}G$ motifs, which are important for Oag polymerisation activity. There are several Arg residues within these two motifs, which play an important role in the Oag polymerisation (Islam *et al.*, 2011). However, there is little sequence identity between Wzy_{Sf} and Wzy_{Pa}. Islam et al. performed extensive work on Wzy_{Pa} and conducted a "*jackhammer"* search to find the homologues of Wzy_{Pa}. However, their results showed that Wzy_{Pa} is not related to Wzy from Enterobacteriaceae (Islam *et al.*, 2013).

The modal length of the Oag chain is regulated by Wzz proteins, members of the polysaccharide co-polymerase (PCP) family (Morona *et al.*, 2000). *S. flexneri* 2a has S-LPS with two types of modal chain length: short (S) type (11 - 17 Oag RUs) and very long (VL) type (>90 Oag RUs), and the S-type and VL-type Oag chain lengths are determined by Wzz_{Sf} and Wzz_{pHS2}, respectively (Morona *et al.*, 2003; Morona & Van Den Bosch, 2003). Woodward et al. proposed that Wzy and Wzz have an interaction during Oag biosynthesis and according to them these two proteins are enough to shape the Oag modal chain length (Woodward *et al.*, 2010). Several other research groups have also suggested that Wzz and Wzy interact during Oag biosynthesis (Islam *et al.*, 2013; Marolda *et al.*, 2006; Taylor *et al.*, 2013; Tocilj

et al., 2008). However, there is a lack of direct evidence on the association of Wzz and Wzy in Oag polymerisation and chain length control. Recently, we identified the Wzz_{Sf} dependent Wzy_{Sf} mutants, and showed that Wzz_{Sf} has a novel role in the stability of Wzy_{Sf} and also in the Oag polymerisation activity of Wzy_{Sf} (Nath *et al.*, 2015).

In this study we performed site-directed mutagenesis on Arg residues in the PL3 and PL5 of Wzy_{Sf} and identified key Arg residues (R164, R250, R258, and R289) important for Wzy_{Sf} polymerisation activity and Oag modal chain length control. Several Arg residues have a role in the association of Wzz_{Sf} and Wzy_{Sf} during Oag biosynthesis and the Wzz_{Sf} dependent stability of Wzy_{Sf}.

Methods

Bacterial strains and plasmids

The strains and plasmids used in this study are shown in Table 1.

Growth media and growth conditions

The growth media used were lysogeny broth (LB) broth (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) and LB agar (LB broth, 15 g/liter bacto agar).

Strains were grown in LB broth with aeration for 18 h at 37°C. 18 h cultures were diluted 1/20 into fresh LB broth and grown to mid-exponential phase (optical density at 600 nm [OD_{600}] of 0.4 - 0.6). To suppress protein expression growth medium was supplemented with 0.2% (w/v) glucose where required. Cells were centrifuged (2200 x *g*, SIGMA 3K15 table top centrifuge, 10 min, 4°C) and washed twice with LB broth to remove glucose. To induce protein expression 0.2% (w/v) L-Arabinose was added to cultures and grown for 20 h at 20°C. Antibiotics were added as required to the media at the following final concentrations: 50 μg kanamycin (Km) ml^{-1}, and 25 μg chloramphenicol (Cm) ml^{-1}.

LPS method

LPS was prepared as described previously (Nath *et al.*, 2015). Cells ($1X10^9$) were harvested and resuspended in lysing buffer and incubated with proteinase K for approximately 16 h. The LPS samples were then separated by SDS-PAGE on 15% (w/v) gels for 16.5 h at 12 mA. Silver nitrate was used to stain the gels and finally the gels were developed with formaldehyde (Murray *et al.*, 2003).

Site-directed mutagenesis

Site-directed mutagenesis on wzy_{Sf} in plasmid pRMPN1 (Nath *et al.*, 2015; Waldo *et al.*, 1999) was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Catalog # 210518, Stratagene) following the manufacturer's instructions. Mutagenised plasmids were transformed into

XL10-Gold. Plasmids were isolated and the mutations within the coding region were identified by DNA sequencing (AGRF, Adelaide, Australia). The oligonucleotide primers used for site-directed mutagenesis are listed in Supplementary Table S1.

Detection of Wzy_{Sf} expression in *S. flexneri*

Procedure of Wzy_{Sf}-GFP expression in *S. flexneri* has been described previously (Nath *et al.*, 2015). Cells were harvested from the 50 ml L-arabinose induced culture. Then the cell pellet was resuspended in 4 ml sonication buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) and lysed by sonication. Cell debris was removed by centrifugation (2200 X *g*, SIGMA 3K15 table top centrifuge, 10 min, 4 °C). The whole membrane (WM) fraction was isolated by ultracentrifugation (Beckman Coulter Optima L-100 XP bench top ultracentrifuge, 126 000 X *g*, 1 h, 4 °C). The WM fraction was resuspended in PBS and then solubilized in Buffer A (200 mM Tris-HCl [pH 8.8], 20% [v/v] glycerol, 5 mM EDTA [pH 8.0], 0.02% [w/v] bromophenol blue, 4% [w/v] SDS, and 0.05 M DTT). Solubilized WM fractions (from 3 X 10^8 cells) were electrophoresed on SDS-15% (w/v) PAGE gels. Gels were rinsed with distilled water, and fluorescent imaging of the gels was performed to detect wild type (WT) and mutant Wzy_{Sf}-GFP protein expression with a Bio-Rad Gel Doc XR + System using Image Lab software (excitation at 485 nm and emission at 512 nm). Loading was checked by staining the gels with Coomassie Blue R-250. The intensity of WT and mutant Wzy_{Sf}-GFP expression in control and mutant strains was measured by Fiji image

processing package (http://fiji.sc/Fiji) and the percent relative Wzy_{Sf}-GFP intensity for each mutant was measured by comparing with WT Wzy_{Sf}-GFP intensity in the control strain PNRM13.

Colicin sensitivity assay

For the colicin sensitivity assay, His_6-colicin E2 (ColE2) with an initial concentration of 1 mg ml^{-1} was used (Tran *et al.*, 2014). The procedure of ColE2 spot assay has been described previously (Nath *et al.*, 2015). The ColE2 spot assay was performed for all strains expressing WT and mutant Wzy_{Sf}-GFP and the other control strains. The end point of the killing zones of mutant strains was compared with the controls.

Bacteriophage sensitivity assay

The procedures of phage propagation and phage stock preparation have been described previously (Mavris *et al.*, 1997; Morona *et al.*, 1994). The procedure of bacteriophage Sf6c sensitivity assay has been described previously (Nath *et al.*, 2015). Phage sensitivity of all strains expressing mutant Wzy_{Sf}-GFP was compared with the strains expressing WT Wzy_{Sf}-GFP and the other controls.

Results

Site-directed mutagenesis of Arg residues in PL3 and PL5 of Wzy_{Sf}

In a previous study on Wzy_{Sf}, random mutagenesis failed to detect any functional residue in PL5 (Nath *et al.*, 2015). However, Islam et al. found that the Arg residues in the two principal PLs (PL3 and PL5) of Wzy_{Pa} are important for Oag polymerisation activity (Islam *et al.*, 2011). The $RX_{10}G$ motifs of the PL3 and PL5 of Wzy_{Pa} (Islam *et al.*, 2011) are absent in Wzy_{Sf}. However, both PL3 and PL5 of Wzy_{Sf} contained $RX_{15}G$ motifs (starting from R164 in PL3 and R289 in PL5) (Fig. 1) (Table S2), and there are also several Arg residues between these two motifs. So, site-directed mutagenesis on wzy_{Sf} in the pRMPN1 was performed to change the basic polar and positively charged Arg residues (R164, R250, R258, R278, and R289) to Ala (nonpolar and neutral substitution), Lys (basic polar and positively charged substitution), and Glu (acidic polar and negatively charged substitution) (Fig. 1) (See Methods). Mutated plasmids were transformed into PNRM6 (RMM109 [pAC/pBADT7-1]) (Δ*wzy*) (Table 1) for phenotypic analysis (Nath *et al.*, 2015).

LPS phenotype conferred by the Wzy_{Sf} mutants

LPS profiling by SDS-PAGE and silver staining were used to detect the effect of the Wzy_{Sf} mutations on the LPS Oag polymerisation. Mutants were initially grouped into five different phenotypic classes (A, B, C, D, and F) (Fig. 2 and Table 2) by comparing the LPS profiles of the mutant strains with the WT positive control PNRM13. Based on the number of Oag RUs in the Oag modal

chain length (Wzz_{Sf} regulated S-type) the class A mutants were further subdivided into subclasses A1, A2, and A3 (Table 2). The mutational alteration R164A resulted in complete loss of Oag polymerisation activity (SR-LPS or class C) (Fig. 2, lane 3), R164K resulted in an S-LPS with reduced Oag polymerisation (< 30 Oag RUs) and lacking modal chain length control (class F LPS) (Fig. 2, lane 5; and Table 2), and R164E resulted in complete loss of polymerisation activity (SR-LPS or class C) (Fig. 2, lane 7), similar to R164A. Mutational alterations R250A and R250E resulted in decreased Oag polymerisation activity (LPS with < 11 Oag RUs or class B) (Fig. 2, lanes 9 and 13). However, the mutational alteration R250K resulted in an S-LPS with reduced Oag polymerisation, and the modal chain length was reduced to 8-11 RUs (Subclass A1) (Fig. 2, lane 11; and Table 2) and was just detectable compared to the positive control (PNRM13). Similar to mutational alterations of R164, both R258A and R258E resulted in an SR-LPS (class C) (Fig. 2, lanes 15 and 19). The mutational alteration R258K resulted in an S-LPS with reduced polymerisation and the modal chain length was reduced to 9-14 RUs (Subclass A2) (Fig. 2, lane 17; and Table 2). For the residue R278, the mutational alterations investigated had no detectable effect on the LPS profiles, and all strains had LPS profiles (class D) similar to the relevant WT control (PNRM13) (Fig. 2, lanes 21, 23, and 25). For residue R289, mutational alteration R289A resulted in a class A LPS profile and the LPS Oag modal chain length of this strain was similar to PNRM13 (Fig. 2, lane 27; and Table 2) but lacked S-LPS with Oag >22 RUs; the LPS profile of this strain was further classified as subclass A3. The mutational alteration R289E resulted in a class A LPS profile with an Oag modal length of 8-14 RUs (Subclass A2)

(Fig. 2, lane 31; and Table 2) that was shorter than that seen in the positive control (PNRM13). The mutational alteration R289K resulted in class D LPS profile (Fig. 2, lane 29). So, except R278 the other Arg residues (R164, R250, R258, and R289) were found to be important for Wzy_{Sf} Oag polymerisation activity. For the positions R164, R1250, and R258 the guanidium functional group of Arg is important as Lys substitution resulted in partial Wzy_{Sf} activity. In particular, certain substitutions [R164K (no modal chain length), R250K (8 - 11 RUs), R258K (9 - 14 RUs), and R289E (8 - 14 RUs)] (Table 2) resulted in an S-LPS with a decreased Oag modal chain length.

Wzz_{Sf} dependence and polymerisation activity

Previously we found an effect of Wzz_{Sf} on Wzy_{Sf} Oag polymerisation activity (Nath *et al.*, 2015). We investigated the dependence of the mutant Wzy_{Sf} proteins generated above on Wzz_{Sf} for their Oag polymerisation activity. All the plasmids encoding the mutated Wzy_{Sf} proteins were transformed into the strain PNRNM126 (RMA4337 [pAC/pBADT7]), which has both *wzy_{Sf}* and *wzz_{Sf}* genes inactivated. LPS profiles conferred in the PNRM126 background (Δ*wzy* Δ*wzz*) were directly compared with the LPS profiles conferred in the PNRM6 background (Δ*wzy*). The positive control strain in the Δ*wzy* Δ*wzz* background, PNRM134 (PNRM126 [pRMPN1]) (Table 1) had a class E LPS profile (S-LPS without Oag modal length control) (Fig.2, lane 2; and Table 2). Wzy_{Sf} with Ala, Lys, and Glu substitutions of R164 resulted in similar LPS profiles both in the Δ*wzy* and Δ*wzy* Δ*wzz* backgrounds (Fig. 2, lanes 3 - 4, 5 - 6, and 7 - 8). The Wzy_{Sf} mutations R250A, R250E, and R258A resulted in

similar LPS profiles both in the Δ*wzy* and Δ*wzy* Δ*wzz* backgrounds (Fig. 2, lanes 9 - 10, 13 - 14, and 15 - 16). Interestingly, WzyR250K resulted in LPS with greatly reduced Oag polymerisation (class B) in the Δ*wzy* Δ*wzz* background (Fig. 2, lane 12) compared to the Δ*wzy* background (class A1) (Fig. 2, lane 11). In contrast, WzyR258E resulted in dramatic increase in Oag polymerisation in the Δ*wzy* Δ*wzz* background (class E) compared to the Δ*wzy* background (Class C) (Fig. 2, lanes 19 - 20). However, WzyR258K resulted in a class F LPS profile (Fig. 2, lane 18) in the Δ*wzy* Δ*wzz* background. For residue R278, all changes resulted in class E LPS profiles (Fig. 2, lanes 22, 24, and 26) in the Δ*wzy* Δ*wzz* backgrounds, as expected. WzyR289A and WzyR289E resulted in class F LPS profiles (Fig. 2, lanes 28 and 32) in the Δ*wzy* Δ*wzz* background. In contrast, the Δ*wzy* Δ*wzz* strain with WzyR289K resulted in an S-LPS lacking Oag modal chain length control (class E LPS profile) (Fig. 2, lane 30) and was similar to the control PNRM134 (Fig. 2, lane 2). Hence, some of the Wzy_{Sf} mutants showed remarkably different LPS profiles in the absence of Wzz_{Sf}, indicating Wzz_{Sf} dependence of their Oag polymerisation activity as previously reported for other Wzy_{Sf} mutants (Nath *et al.*, 2015).

ColE2 sensitivity of strains with Wzy_{Sf} mutants

The ColE2 sensitivity of the strains expressing Wzy_{Sf} mutants was investigated to verify the LPS profiles determined by SDS-PAGE and silver staining. The ColE2 sensitivity (summarized in Table 3) was determined by spot testing as described in the Methods.

As expected, the negative control strains RMM109, PNRM6, PNRM11, RMA4337, and PNRM126 had the highest sensitivity to ColE2 (killing zone at a dilution of 1/256) (Table 3). The WT strain PE638 and the positive control with Wzy_{Sf}-GFP in the Δ*wzy* Δ*wzz* background (PNRM134) were resistant to the highest concentration of ColE2 used. However, the positive control with Wzy_{Sf}-GFP in the Δ*wzy* background (PNRM13) showed a killing zone at a dilution of 1/2 (Table 3) as previously reported (Nath *et al.*, 2015). Strains with a class A LPS profile in the Δ*wzy* background was sensitive to ColE2. Among them the strain with decreased Oag modal chain length (subclass A1) were relatively more sensitive to ColE2 (killing zone at 1/64). However, the strains with more Oag (subclass A2 and A3) showed a killing zone at 1/32. Strains with a class B LPS profiles (both Δ*wzy* and Δ*wzy* Δ*wzz* backgrounds) showed a killing zone at 1/128. As expected the strains with class C LPS profiles (SR-LPS) (both Δ*wzy* and Δ*wzy* Δ*wzz* backgrounds) showed the highest sensitivity to ColE2 (killing zone at 1/256), and their sensitivity to ColE2 was similar to the negative control strains. Strains with WT like class D LPS profiles (Δ*wzy* background) were more resistant to ColE2 (killing zone R - 1/4). Among them the Δ*wzy* strains with WzyR278A and WzyR278E showed ColE2 sensitivity similar to the WT strain PE638, greater than the relevant positive control PNRM13. Strains with a class E LPS profile (Δ*wzy* Δ*wzz* background) showed greater sensitivity to ColE2 (killing zone 1/16 - 1/64) compared to the relevant positive control PNRM134, suggesting that they had a decreased level of Oag polymerisation. Strains with a class F LPS profile in the Δ*wzy* and Δ*wzy* Δ*wzz* backgrounds were also very sensitive to ColE2, and showed a killing zone at 1/64 or 1/128. Hence, as reported previously (Nath *et*

al., 2015), the ColE2 assay detects subtle differences in LPS Oag chain length and density, which are consequences of difference in Oag polymerisation.

Bacteriophage Sf6c sensitivity of strains with Wzy_{Sf} mutants

The bacteriophage Sf6c sensitivity of the strains expressing Wzy_{Sf} mutants was investigated to further verify the LPS profiles determined by SDS-PAGE and silver staining. The bacteriophage Sf6c sensitivity of the strains (summarized in Table 3), carrying mutated *wzy*$_{Sf}$ plasmids were determined by spot testing (see Methods).

The negative control strains (with an SR-LPS profile) RMM109, PNRM6, PNRM11, RMA4337, and PNRM126 were all resistant to the highest concentration of bacteriophage Sf6c tested, as expected. The WT strain PE638 and the positive control with Wzy_{Sf}-GFP in the Δ*wzy* Δ*wzz* background (PNRM134) showed the highest sensitivity to bacteriophage Sf6c and showed plaques at 10^{-6}. However, for the positive control with Wzy_{Sf}-GFP in the Δ*wzy* background (PNRM13) the phage showed plaques at 10^{-5} (Table 3) (Nath *et al.*, 2015). Strains with class A, B, C, and F LPS profiles in the Δ*wzy* and Δ*wzy* Δ*wzz* backgrounds were resistant to the highest concentration of bacteriophage Sf6c tested, similar to the negative control strains. However, the strains with class D LPS profiles were very sensitive to bacteriophage Sf6c (plaques at 10^{-5} or 10^{-6}). Among them the Δ*wzy* strain with WzyR278A

374 and WzyR278E showed the highest sensitivity to Sf6c and their sensitivity to
375 bacteriophage Sf6c was greater than the relevant positive control PNRM13,
376 and similar to the positive control with Wzy_{Sf}-GFP in the Δ*wzy* Δ*wzz*
377 background (PNRM134). The strains with class E LPS profile were relatively
378 more resistant to bacteriophage Sf6c (resistant or plaques at 10^{-1} or N)
379 compared to the relevant positive control PNRM134, indicating a difference in
380 Oag density. Similar to our previous data (Nath *et al.*, 2015), the
381 bacteriophage Sf6c assays above indicated that the degree of Oag
382 polymerisation and density is correlated with bacteriophage Sf6c sensitivity.

383

384 **Protein expression levels of the Wzy_{Sf} mutants**

385 We measured parental and mutant Wzy_{Sf}-GFP expression in the Δ*wzy* and
386 Δ*wzy* Δ*wzz* backgrounds by in-gel fluorescence and then calculated the %
387 relative Wzy_{Sf}-GFP expression of all the mutant strains by comparing
388 expression levels of different Wzy_{Sf}-GFP mutants with the Wzy_{Sf}-GFP in
389 PNRM13 (100%) (See Methods). The positive control in the Δ*wzy* Δ*wzz*
390 background (PNRM134) had Wzy_{Sf}-GFP expression level (relative Wzy_{Sf}-GFP
391 level 17%) less than the positive control in the Δ*wzy* background (PNRM13)
392 (Fig. 3a, 3b, and 3c, lanes 1 and 2, Table 3).

393

394 Both in the Δ*wzy* and Δ*wzy* Δ*wzz* backgrounds, with some exceptions,
395 most of the Wzy_{Sf} mutants were expressed at a level less than 100%. The
396 Δ*wzy* strain with WzyR164A had expression of 132% (Fig. 3a, lane 3; and

Table 3) but the Δ*wzy* Δ*wzz* strain with WzyR164A had a relative Wzy_{Sf}-GFP level of 87% (Fig. 3a, lane 4; and Table 3). Both of these strains had an SR-LPS profile. The Δ*wzy* Δ*wzz* strain with WzyR258A had an SR-LPS but the relative Wzy_{Sf}-GFP level was 200% (Fig. 3a, lane 8; and Table 3), which is double than the Wzy_{Sf}-GFP level in PNRM13. The Δ*wzy* strain with WzyR258A had a relative Wzy_{Sf}-GFP level of 62% (Fig. 3a, lane 7; and Table 3). Both the Δ*wzy* and Δ*wzy* Δ*wzz* strains with WzyR164K expressed at a level more than 100% (120% and 125%, respectively) (Fig. 3b, lanes 3 and 4; and Table 3). In the Δ*wzy* Δ*wzz* background both WzyR250K and WzyR258K were expressed at a very low level (11% and 0.02%, respectively) (Fig. 3b, lane 8; Fig. 3C lane 4; and Table 3). However, in the Δ*wzy* strain, WzyR250K and WzyR258K were expressed at high levels (142% and 104%, respectively) (Fig. 3b, lane 7; Fig. 3c, lane 3; and Table 3). Interestingly, the Δ*wzy* and Δ*wzy* Δ*wzz* strains with WzyR250E had class B LPS profile but their relative Wzy_{Sf}-GFP levels were 133% and 143%, respectively (Fig. 3b, lanes 9 and 10; and Table 3). The Δ*wzy* strain with WzyR278E and the Δ*wzy* Δ*wzz* strain with WzyR289E had very high relative Wzy_{Sf}-GFP levels (135% and 140%, respectively) (Fig. 3c, lanes 9 and 14; and Table 3). However, the Δ*wzy* Δ*wzz* strain with WzyR278E and the Δ*wzy* strain with WzyR289E had low relative Wzy_{Sf}-GFP levels (28% and 21% respectively) (Fig. 3c, lanes 10 and 13; and Table 3). The comparison of % Wzy_{Sf}-GFP expression levels of different mutants in the Δ*wzy* and Δ*wzy* Δ*wzz* backgrounds indicates that the expression of certain Wzy_{Sf} mutant proteins was affected by Wzz_{Sf}.

Discussion

Wzy proteins are essential for the synthesis of many Oags that are virulence determinants of the Gram negative bacteria. Wzy_{Sf} has two large PLs (PL3 and PL5) (Daniels *et al.*, 1998). During mutational characterisation of Wzy_{Sf} we found that the amino acid P165 in PL3 is important for the stabilization of Wzy_{Sf} through interaction with Wzz_{Sf} (Nath *et al.*, 2015). However, through random mutagenesis we were unable to identify any other amino acid residues in PL3 and PL5 important for the Oag polymerisation activity and association with Wzz_{Sf}. In this study site-directed mutagenesis of these two loops generated mutants that were then characterised based on their LPS profiles, ColE2 and bacteriophage Sf6c sensitivities, and Wzy_{Sf}-GFP expression to reveal novel mutant phenotypes.

Islam et al. showed that the mutational alteration of Arg to Ala in the Wzy_{Pa} PL3 and PL5 within the two $RX_{10}G$ motifs resulted in either complete or partial loss of Oag polymerisation activity and alterations of some of the Arg residues to Lys resulted in LPS profiles similar to their Ala substitution (Islam *et al.*, 2011). In *S. flexneri,* site-directed mutation of R164, R250, R258, and R289 to Ala also resulted in the complete or partial loss of Oag polymerisation activity in the *Δwzy* background. The Arg residues in PL3 and PL5 were changed to Ala, Lys, and Glu to determine the importance of the guanidium functional group of Arg at these positions. In the Δ*wzy* background Ala, Lys, and Glu substitutions of R164, R250, and R258 resulted in complete or partial loss of polymerisation (Fig. 2). Lys substitutions at these three positions

resulted in S-LPS with reduced degree of Oag polymerisation (class F or class A) but with different Oag modal chain lengths [Class F (without modal chain), class A1 (8-11 RUs), class A2 (9-14 or 8-14 RUs)] (Fig. 2) compared to the relevant positive control PNRM13. These Wzy_{Sf} mutants (WzyR164K, WzyR250K, and WzyR258K) resulted in LPS with different Oag modal chain length. So, guanidium functional group of Arg residues at these positions had position specific effect on Oag polymerisation and modal chain length control. This effect has not been reported previously for *S. flexneri* wzy_{Sf} mutations.

Previously, we found Wzz-dependent Wzy_{Sf} mutants (Nath *et al.*, 2015). In this study we found several new examples of Wzz_{Sf}-dependent Wzy_{Sf} mutants. The Δ*wzy* Δ*wzz* strain with WzyR250K had decreased Oag polymerisation in the absence of Wzz_{Sf} and the Δ*wzy* Δ*wzz* strain with WzyR258E had increased Oag polymerisation in the absence of Wzz_{Sf}, even though WzyR258E was inactive in the Δ*wzy* background (Fig. 2). Hence, residues R250 and R258 have roles in the association of Wzy_{Sf} and Wzz_{Sf} during the Wzy_{Sf} mediated Oag polymerisation. These and our previous results (Nath *et al.*, 2015) suggest that the interactions between Wzy_{Sf} and Wzz_{Sf} are complex.

The ColE2 and bacteriophage Sf6c sensitivity assays supported the LPS profiles of the Wzy_{Sf} mutants. We found that the Wzy_{Sf} mutants with shorter Oag chains in the LPS were more sensitive to ColE2 (Table 3), consistent with our previous results (Nath *et al.*, 2015). Here we found that in

the Δ*wzy* background the strains with different Oag modal chain lengths showed different sensitivities to ColE2, with an increase in resistance correlated with an increase in Oag RUs in the LPS Oag modal chain. Strains with class A, B, and C LPS profiles in the Δ*wzy* background were resistant to bacteriophage Sf6c (Table 3). This result was consistent with our previous findings (Nath *et al.*, 2015) that bacteriophage Sf6c only infects if the S-LPS has WT or nearly WT level of Oag polymerisation. In our previous study we found that while the strains with class D LPS had S-LPS profiles very similar to the relevant positive control strain (PNRM13), they were more resistant to ColE2 and more sensitive to bacteriophage Sf6c compared to PNRM13 (Nath *et al.*, 2015). Here the Δ*wzy* strain with WzyR278A and WzyR278E showed a similar phenotype (Fig. 3, and Table 3).

Previously we found that Wzz_{Sf} is not only associated with Oag modal chain length control but also affects the level of Wzy_{Sf} (Nath *et al.*, 2015). In the Δ*wzy* and Δ*wzy* Δ*wzz* backgrounds most of the mutant Wzy_{Sf}-GFP proteins had expression levels less than the Wzy_{Sf}-GFP in PNRM13 (Table 3). However, in the Δ*wzy* background the expression level of WzyR164A and in the Δ*wzy* Δ*wzz* background the expression level of WzyR258A was greater than the Wzy_{Sf}-GFP in PNRM13 (Table 3). We speculate that residues R164 and R258 are important for the stabilization of Wzy_{Sf} through a potential interaction with Wzz_{Sf}. The Δ*wzy* strain with WzyR164A and the Δ*wzy* Δ*wzz* strain with WzyR258A had SR-LPS profiles (Fig. 2). So, the absence of Oag polymerisation activity is not due to a lack of protein expression.

493 The Δ*wzy* and Δ*wzy* Δ*wzz* strains with WzyR164K and WzyR250E had
494 a higher level of expression compared to Wzy_{Sf}-GFP in PNRM13 but the LPS
495 profiles of these strains indicated that the mutant proteins had decreased Oag
496 polymerisation activity compared to the relevant positive controls (Table 3 and
497 Fig. 3). So, these mutations in some way stabilized the protein, both in the
498 presence and absence of Wzz_{Sf}.
499
500 The Δ*wzy* strain with WzyR250K and WzyR258K had high level of
501 expression but Δ*wzy* Δ*wzz* strain with WzyR250K and WzyR258K had very
502 low level of expression (Fig. 3 and Table 3), suggesting that the presence of
503 Wzz_{Sf} stabilizes these Wzy_{Sf} mutants. The Δ*wzy* strain with WzyR250K had
504 LPS with an increased degree of Oag polymerisation compared to Δ*wzy* Δ*wzz*
505 strain with WzyR250K, and the Δ*wzy* and the Δ*wzy* Δ*wzz* strains with
506 WzyR258K had nearly similar LPS profiles (class A2 and class F) but the
507 Δ*wzy* strain with WzyR258K had LPS with an Oag modal chain length of 9-14
508 RUs (Fig. 2). However, all these strains had LPS with a decreased level of
509 Oag polymerisation compared to the relevant positive controls. These results
510 again suggest that Oag polymerisation activity of Wzy_{Sf} is not correlated with
511 the expression level of the protein. The Δ*wzy* strain with WzyR278E and the
512 Δ*wzy* Δ*wzz* strain with WzyR289E (Fig. 3 and Table 3) had higher level of
513 expression compared to the Wzy_{Sf}-GFP in PNRM13. Hence residues R278
514 and R289 are also important for the stabilization of Wzy_{Sf} through a potential
515 interaction with Wzz_{Sf}.
516

According to the model proposed by Islam et al. at a physiological pH, Wzy_{Pa} PL3 and PL5 possess a net positive charge and a net negative charge, respectively (Islam *et al.*, 2011). PL3, the “capture arm”, catches incoming negatively charged Oag subunit for subsequent transfer to PL5, which acts as a “retention arm”. It involves a relatively transient interaction with the Oag and is responsible for the constant binding and release of growing Oag chain. They proposed that these characteristics of PLs support their roles in the “catch- and-release” mechanism during Oag polymerisation by Wzy. PL3 and PL5 of Wzy_{Pa} have a high level of sequence equivalence (Islam *et al.*, 2011). Zhao et al. found that *Escherichia coli* O86 Wzy (Wzy_{Ec}) has a different number of TM and different amino acid sequence compared to Wzy_{Pa} (Zhao *et al.*, 2014) but the pI values of PL3 and PL4 (the two largest PLs) of Wzy_{Ec} are equivalent to PL3 and PL5 of Wzy_{Pa}. At a physiological pH, PL3 and PL4 of Wzy_{Ec} possess a net positive charge and a net negative charge, respectively, which led them to conclude that Wzy_{Ec} follows a similar catalytic mechanism to Wzy_{Pa} (Zhao *et al.*, 2014). For Wzy_{Sf}, we found that the pI values of PL3 and Pl5 were 4.65 and 5.09, respectively, using the ExPASy pI calculator (http://web.expasy.org/compute_pi/). Hence, at a physiological pH both the PL3 and PL5 of Wzy_{Sf} possess net negative charge. While the *P. aeruginosa* PAO1 Oag contains negatively charged uronic acid (Knirel *et al.*, 2006) *S. flexneri* Oag is neutral. So, the charge property of the substrate for Wzy_{Sf} is different from the Wzy_{Pa}. PL3 and PL5 of Wzy_{Sf} also lack shared conserved residues. Polymerisation of the Oag of all the serotypes of *S. flexneri* is conducted by a single type of Wzy_{Sf}, which defines the flexibility of substrate recruitment of Wzy_{Sf}. The $RX_{10}G$ motifs of Wzy_{Pa} contain several other Arg

542 residues within the motifs (R176, R180 in PL3 and R291 in PL5) (Islam *et al.*,
543 2011; Islam *et al.*, 2013) but the Wzy_{Sf} had no Arg residues within the $RX_{15}G$
544 motifs of PL3 and PL5 (Fig. 1). The $RX_{10}G$ motifs of Wzy_{Pa} starts in the PL
545 and ends in the PL (Islam *et al.*, 2011; Islam *et al.*, 2013) but the $RX_{15}G$ motifs
546 of Wzy_{Sf} starts in the PL and ends in the TM (Fig. 1). Nevertheless, we found
547 that the Arg residues in the PL3 and PL5 have roles in Oag polymerisation,
548 association with Wzz_{Sf}, and Wzy_{Sf} expression level. Hence, a modified version
549 of "catch-and-release" (Islam *et al.*, 2011) mechanism may exist for *S.*
550 *flexneri* Oag synthesis.

551

552 In conclusion, we identified key Arg residues in PL3 and PL5 of Wzy_{Sf}
553 that are important for the polymerisation activity, association with Wzz_{Sf} during
554 polymerisation, and Wzz_{Sf} dependent stabilization of the protein. The Wzy_{Sf}
555 mutants that confer altered Oag modal chain length suggest that Wzz_{Sf}
556 functions to alter the activity of Wzy_{Sf} and this is mimicked by certain
557 mutational alterations, leading to change in the Oag modal chain length. The
558 current findings extended the previous finding (Nath *et al.*, 2015). and we
559 conclude that a wider region (PL 2, 3, 5, 6 and TM 5, 8) is involved in the Oag
560 polymerisation activity and potential interaction with Wzz_{Sf}. We hypothesize
561 that these regions may contribute to the catalytic site of Wzy_{Sf}.

562

563

564

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References

Allison, G. E. & Verma, N. K. (2000). Serotype-converting bacteriophages and O-antigen modification in *Shigella flexneri*. *Trends Microbiol* **8**, 17-23.

Bardhan, P., Faruque, A. S., Naheed, A. & Sack, D. A. (2010). Decrease in shigellosis-related deaths without *Shigella* spp.-specific interventions, Asia. *Emerg Infect Dis* **16**, 1718-1723.

Daniels, C., Vindurampulle, C. & Morona, R. (1998). Overexpression and topology of the *Shigella flexneri* O-antigen polymerase (Rfc/Wzy). *Mol Microbiol* **28**, 1211-1222.

Guo, H., Yi, W., Song, J. K. & Wang, P. G. (2008). Current understanding on biosynthesis of microbial polysaccharides. *Curr Top Med Chem* **8**, 141-151.

Islam, S. T., Gold, A. C., Taylor, V. L., Anderson, E. M., Ford, R. C. & Lam, J. S. (2011). Dual conserved periplasmic loops possess essential charge characteristics that support a catch-and-release mechanism of O-antigen polymerisation by Wzy in *Pseudomonas aeruginosa* PAO1. *J Biol Chem* **286**, 20600-20605.

Islam, S. T., Huszczynski, S. M., Nugent, T., Gold, A. C. & Lam, J. S. (2013). Conserved-residue mutations in Wzy affect O-antigen polymerisation

608 and Wzz-mediated chain-length regulation in *Pseudomonas aeruginosa*
609 PAO1. *Sci Rep* **3**, 3441.

610

611 **Jennison, A. V. & Verma, N. K. (2004).** *Shigella flexneri* infection:
612 pathogenesis and vaccine development. *FEMS Microbiol Rev* **28**, 43-58.

613

614 **Knirel, Y. A., Bystrova, O. V., Kocharova, N. A., Zahringer, U. & Pier, G.**
615 **B. (2006).** Conserved and variable structural features in the
616 lipopolysaccharide of *Pseudomonas aeruginosa*. *J Endotoxin Res* **12**, 324-
617 336.

618

619 **Lindberg, A. A., Wollin, R., Gemski, P. & Wohlhieter, J. A. (1978).**
620 Interaction between bacteriophage Sf6 and *Shigella flexneri*. *J Virol* **27**, 38-44.

621

622 **Liu, D., Cole, R. A. & Reeves, P. R. (1996).** An O-antigen processing
623 function for Wzx (RfbX): a promising candidate for O-unit flippase. *J Bacteriol*
624 **178**, 2102-2107.

625

626 **Marolda, C. L., Tatar, L. D., Alaimo, C., Aebi, M. & Valvano, M. A. (2006).**
627 Interplay of the Wzx translocase and the corresponding polymerase and chain
628 length regulator proteins in the translocation and periplasmic assembly of
629 lipopolysaccharide o antigen. *J Bacteriol* **188**, 5124-5135.

630

631 **Mavris, M., Manning, P. A. & Morona, R. (1997).** Mechanism of
632 bacteriophage SfII-mediated serotype conversion in *Shigella flexneri*. *Mol*
633 *Microbiol* **26**, 939-950.

634

635 **McKinney, J., Guerrier-Takada, C., Galan, J. & Altman, S. (2002).** Tightly
636 regulated gene expression system in *Salmonella enterica* serovar
637 Typhimurium. *J Bacteriol* **184**, 6056-6059.

638

639 **Morona, R., Mavris, M., Fallarino, A. & Manning, P. A. (1994).**
640 Characterization of the *rfc* region of *Shigella flexneri*. *J Bacteriol* **176**, 733-
641 747.

642

643 **Morona, R., van den Bosch, L. & Manning, P. A. (1995).** Molecular, genetic,
644 and topological characterization of O-antigen chain length regulation in
645 *Shigella flexneri*. *J Bacteriol* **177**, 1059-1068.

646

647 **Morona, R., Van Den Bosch, L. & Daniels, C. (2000).** Evaluation of
648 Wzz/MPA1/MPA2 proteins based on the presence of coiled-coil regions.
649 *Microbiology* **146 (Pt 1)**, 1-4.

650

651 **Morona, R., Daniels, C. & Van Den Bosch, L. (2003).** Genetic modulation of
652 *Shigella flexneri* 2a lipopolysaccharide O antigen modal chain length reveals
653 that it has been optimized for virulence. *Microbiology* **149**, 925-939.

Morona, R. & Van Den Bosch, L. (2003). Lipopolysaccharide O antigen chains mask IcsA (VirG) in *Shigella flexneri*. *FEMS Microbiol Lett* **221**, 173-180.

Murray, G. L., Attridge, S. R. & Morona, R. (2003). Regulation of *Salmonella typhimurium* lipopolysaccharide O antigen chain length is required for virulence; identification of FepE as a second Wzz. *Mol Microbiol* **47**, 1395-1406.

Nath, P., Tran, E. N. & Morona, R. (2015). Mutational Analysis of the *Shigella flexneri* O-Antigen Polymerase Wzy: Identification of Wzz-Dependent Wzy Mutants. *J Bacteriol* **197**, 108-119.

Raetz, C. R. & Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71**, 635-700.

Ruiz, N., Gronenberg, L. S., Kahne, D. & Silhavy, T. J. (2008). Identification of two inner-membrane proteins required for the transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *Proc Natl Acad Sci U S A* **105**, 5537-5542.

Sperandeo, P., Deho, G. & Polissi, A. (2009). The lipopolysaccharide transport system of Gram-negative bacteria. *Biochim Biophys Acta* **1791**, 594-602.

Stagg, R. M., Tang, S. S., Carlin, N. I., Talukder, K. A., Cam, P. D. & Verma, N. K. (2009). A novel glucosyltransferase involved in O-antigen modification of *Shigella flexneri* serotype 1c. *J Bacteriol* **191**, 6612-6617.

Sun, Q., Knirel, Y. A., Lan, R. & other authors (2012). A novel plasmid-encoded serotype conversion mechanism through addition of phosphoethanolamine to the O-antigen of *Shigella flexneri*. *PLoS One* **7**, e46095.

Sun, Q., Lan, R., Wang, Y., Wang, J., Wang, Y., Li, P., Du, P. & Xu, J. (2013). Isolation and genomic characterization of SfI, a serotype-converting bacteriophage of *Shigella flexneri*. *BMC Microbiol* **13**, 39.

Taylor, V. L., Udaskin, M. L., Islam, S. T. & Lam, J. S. (2013). The D3 bacteriophage alpha-polymerase inhibitor (Iap) peptide disrupts O-antigen biosynthesis through mimicry of the chain length regulator Wzz in *Pseudomonas aeruginosa*. *J Bacteriol* **195**, 4735-4741.

Tocilj, A., Munger, C., Proteau, A. & other authors (2008). Bacterial polysaccharide co-polymerases share a common framework for control of polymer length. *Nat Struct Mol Biol* **15**, 130-138.

Tran, E. N., Papadopoulos, M. & Morona, R. (2014). Relationship between O-antigen chain length and resistance to colicin E2 in *Shigella flexneri*. *Microbiology* **160**, 589-601.

van der Ley, P., de Graaff, P. & Tommassen, J. (1986). Shielding of *Escherichia coli* outer membrane proteins as receptors for bacteriophages and colicins by O-antigenic chains of lipopolysaccharide. *J Bacteriol* **168**, 449-451.

Waldo, G. S., Standish, B. M., Berendzen, J. & Terwilliger, T. C. (1999). Rapid protein-folding assay using green fluorescent protein. *Nat Biotechnol* **17**, 691-695.

Wang, X., Zhang, C., Shi, F. & Hu, X. (2010). Purification and characterization of lipopolysaccharides. *Subcell Biochem* **53**, 27-51.

Woodward, R., Yi, W., Li, L. & other authors (2010). *In vitro* bacterial polysaccharide biosynthesis: defining the functions of Wzy and Wzz. *Nat Chem Biol* **6**, 418-423.

Zhao, G., Wu, B., Li, L. & Wang, P. G. (2014). O-antigen polymerase adopts a distributive mechanism for lipopolysaccharide biosynthesis. *Appl Microbiol Biotechnol* **98**, 4075-4081.

Tables

TABLE 1. Bacterial strains and plasmids used in this study

Strains or Plasmids	Characteristics*	Reference
Strains		
S. flexneri		
PE638	*S. flexneri* Y *rpoB* (Rif^r)	(Morona *et al.*, 1994)
RMM109	PE638*Δwzy*, Rif^r	(Morona *et al.*, 1994)
RMA4337	RMM109 *Δwzz* (Rif^r, Tet^r)	(Nath *et al.*, 2015)
PNRM6	RMM109 [pAC/pBADT7-1]	(Nath *et al.*, 2015)
PNRM13	PNRM6 [pRMPN1]	(Nath *et al.*, 2015)
PNRM16	PNRM6 [pRMPN2]	This study
PNRM17	PNRM6 [pRMPN3]	This study
PNRM18	PNRM6 [pRMPN4]	This study
PNRM19	PNRM6 [pRMPN5]	This study
PNRM20	PNRM6 [pRMPN6]	This study
PNRM126	RMA4337 [pAC/pBADT7-1]	(Nath *et al.*, 2015)
PNRM134	PNRM126 [pRMPN1]	(Nath *et al.*, 2015)
PMRM127	PNRM126 [pRMPN2]	This study
PMRM128	PNRM126 [pRMPN3]	This study
PMRM129	PNRM126 [pRMPN5]	This study
PMRM130	PNRM126 [pRMPN6]	This study
PMRM153	PNRM126 [pRMPN4]	This study
PNRM190	PNRM6 [pRMPN27]	This study
PNRM192	PNRM6 [pRMPN28]	This study
PNRM194	PNRM6 [pRMPN29]	This study
PNRM196	PNRM6 [pRMPN30]	This study
PNRM198	PNRM6 [pRMPN31]	This study
PNRM216	PNRM6 [pRMPN32]	This study
PNRM218	PNRM6 [pRMPN33]	This study
PNRM220	PNRM6 [pRMPN34]	This study
PNRM222	PNRM6 [pRMPN36]	This study
PNRM232	PNRM6 [pRMPN35]	This study
E.coli strains		
XL10-G	*Tet^{r}Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F´ proAB lacI^{q}ZΔM15Tn10 (Tet^r) Cam^r]*	Stratagene

Table 1: cont.

Strains or Plasmids	Characteristics*	Reference
Plasmids		
pAC/pBADT7-1	Source of T7 RNA polymerase; Cm^r	(McKinney *et al.*, 2002)
pWaldo-TEV-GFP	Cloning vector with GFP tag; Km^r	(Waldo *et al.*, 1999)
pRMPN1	pWaldo-wzy_{SF}-GFP; Km^r	(Nath *et al.*, 2015)
pRMPN2	pRMPN1 with WzyR164A	This study
pRMPN3	pRMPN1 with WzyR250A	This study
pRMPN4	pRMPN1 with WzyR258A	This study
pRMPN5	pRMPN1 with WzyR278A	This study
pRMPN6	pRMPN1 with WzyR289A	This study
pRMPN27	pRMPN1 with WzyR164K	This study
pRMPN28	pRMPN1 with WzyR250K	This study
pRMPN29	pRMPN1 with WzyR258K	This study
pRMPN30	pRMPN1 with WzyR278K	This study
pRMPN31	pRMPN1 with WzyR289K	This study
pRMPN32	pRMPN1 with WzyR164E	This study
pRMPN33	pRMPN1 with WzyR250E	This study
pRMPN34	pRMPN1 with WzyR258E	This study
pRMPN35	pRMPN1 with WzyR278E	This study
pRMPN36	pRMPN1 with WzyR289E	This study

* Rif^r, rifampicin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant; Tet^r, tetracycline resistant.

TABLE 2. LPS profiles of different Wzy_{Sf} mutant phenotypic classes

Wzy_{Sf} mutant Class	**LPS profile**
A1	S-LPS with reduced Oag polymerisation, and the modal chain length was reduced to 8-11 RUs
A2	S-LPS with reduced polymerisation and the modal chain length was reduced to 9-14 or 8-14 Oag RUs
A3	S-LPS with reduced polymerisation (< 22 Oag RUs) and the modal chain length was similar to the WT control (PNRM13)
B	LPS with few Oag RUs (< 11 Oag RUs)
C	SR-LPS
D	LPS profile similar to the WT control PNRM13
E	S-LPS lacking Oag modal chain length control
F	S-LPS with reduced Oag polymerisation and lacking Oag modal chain length control (< 30 Oag RUs)

764 **TABLE 3.** ColE2 and bacteriophage Sf6c sensitivities, and Wzy_{Sf}-GFP expression levels

Strain or mutants	Relevant details	Mutant class		Topology map location[#]	Sensitivity[*]				Relative Wzy_{Sf}-GFP (%)	
					ColE2		Sf6c			
		Δ*wzy* background	Δ*wzy* Δ*wzz* background		Δ*wzy* background	Δ*wzy* Δ*wzz* background	Δ*wzy* background	Δ*wzy* Δ*wzz* background	Δ*wzy* background	Δ*wzy* Δ*wzz* background
Strains										
RMM109	wzy_{Sf} mutant				1/256	-	R	-	-	-
PE638	Wild type				R	-	10^{-6}	-	-	-
PNRM13	Positive control				1/2	-	10^{-5}	-	100	-
PNRM6	Negative control				1/256	-	R	-	-	-
PNRM11	Negative control				1/256	-	R	-	-	-
RMA4337	wzy_{Sf} and wzz_{Sf} mutant				-	1/256	-	R	-	-
PNRM126	Negative control				-	1/256	-	R	-	-
PNRM134	Positive control				-	R	-	10^{-6}	-	17
Mutants										
R164A		C	C	PL3	1/256	1/256	R	R	132	87
R250A		B	B	PL5	1/128	1/128	R	R	81	55
R258A		C	C	PL5	1/256	1/256	R	R	62	200
R278A		D	E	PL5	R	1/16	10^{-6}	N	18	14
R289A		A3	F	PL5	1/32	1/128	R	R	14	82
R164K		F	F	PL3	1/64	1/128	R	R	120	125
R250K		A1	B	PL5	1/64	1/128	R	R	142	11
R258K		A2	F	PL5	1/32	1/64	N	R	104	0.02
R278K		D	E	PL5	1/2	1/16	10^{-5}	10^{-1}	46	36
R289K		D	E	PL5	1/4	1/64	10^{-5}	N	60	38
R164E		C	C	PL3	1/256	1/256	R	R	80	99
R250E		B	B	PL5	1/128	1/128	R	R	133	143
R258E		C	E	PL5	1/256	1/64	R	R	16	38
R278E		D	E	PL5	R	1/64	10^{-6}	R	135	28
R289E		A2	F	PL5	1/32	1/64	N	R	21	140

765

766 [#]PL - Periplasmic loop (See Fig. 1).

767 [*]R, Resistant; N, plaques detected with undiluted Sf6c stock; the numbers represent the highest dilution showing the zone of inhibition or plaques formation.

Figures

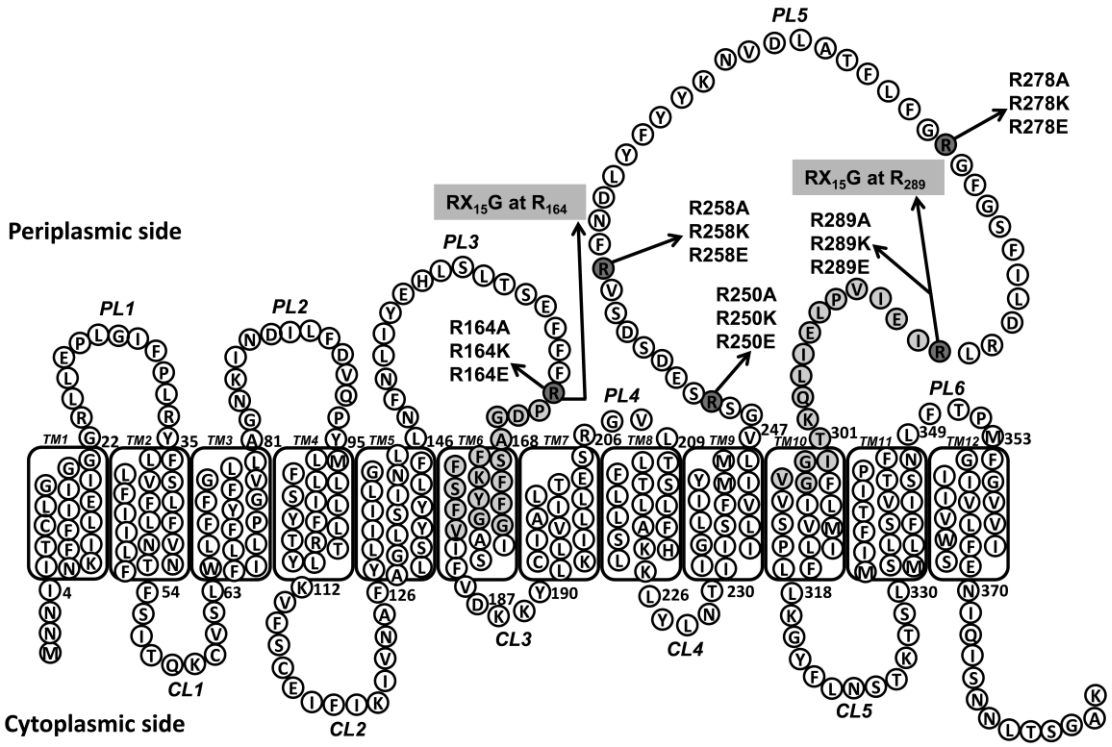
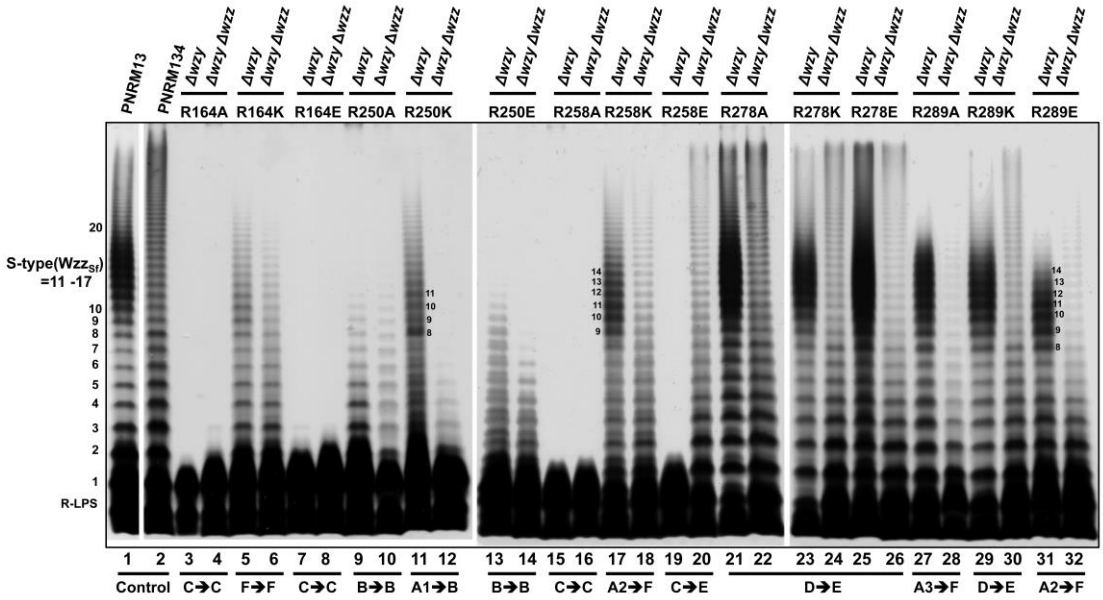


Fig. 1. Location of the mutations constructed in this study on the topology map of Wzy_{Sf}.

Mutational alterations were indicated by arrows on the Wzy_{Sf} topology map [adapted from (Daniels *et al.*, 1998)]. The position of the periplasmic loops (PL) 1-5, transmembrane regions (TM) 1-12, and cytoplasmic loops (CL) 1-5 are indicated. The residues mutated in this study (dark shaded circles) were located in the PL3 and 5. The position of $RX_{15}G$ motifs (light shaded circles) in the PL3 and PL5 of Wzy_{Sf}, starting from R_{164} and R_{289} respectively, are indicated.



781

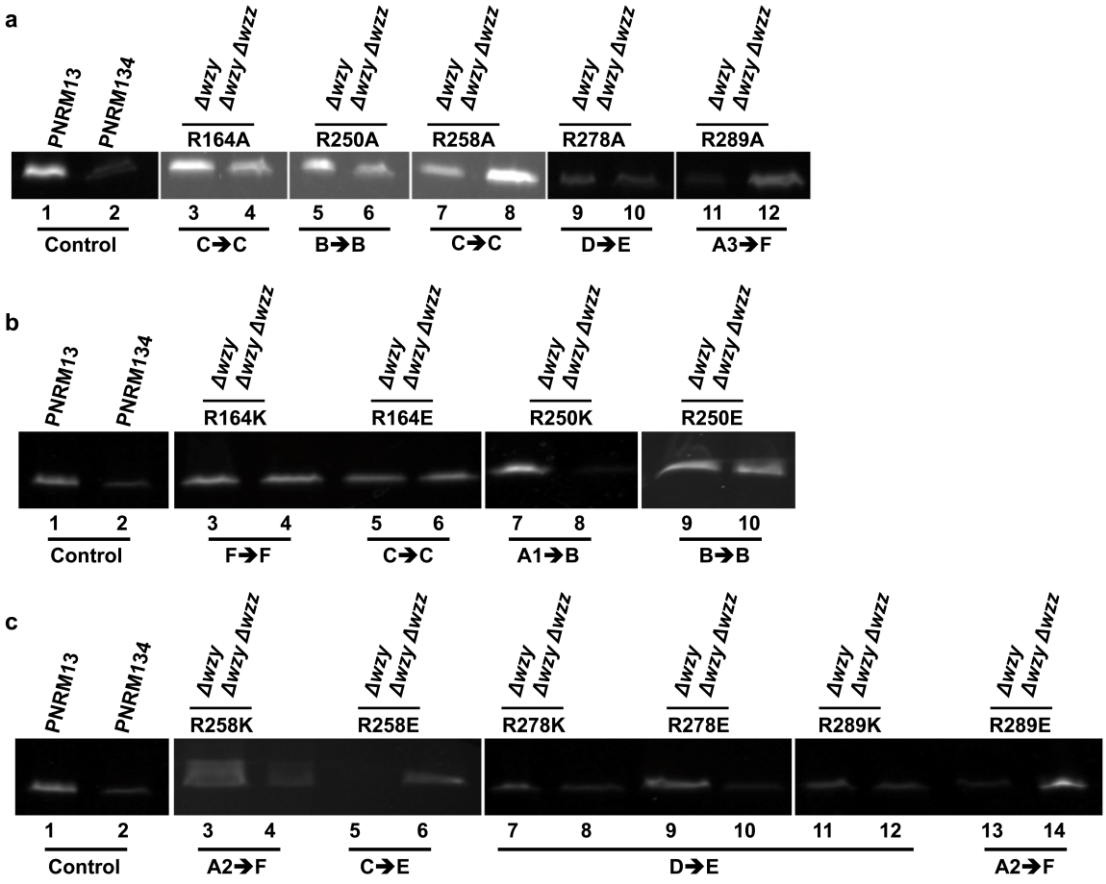
782 **Fig. 2. Comparison of the LPS phenotype conferred by the Wzy$_{Sf}$**
783 **mutants expressed in the Δ*wzy* and Δ*wzy* Δ*wzz* backgrounds.**

784 The plasmids encoded mutant and WT Wzy$_{Sf}$ proteins were expressed in
785 PNRM6 (RMM109 [pAC/pBADT7-1]) and PNRM126 (RMA4337
786 [pAC/pBADT7-1]). Strains were grown and induced as described in the
787 Methods. LPS samples were electrophoresed on a SDS-15% (w/v) PAGE gel
788 and silver stained (See Methods). Strains were grouped into various mutant
789 classes (A, B, C, D, E, and F) and subclasses (A1, A2, and A3) based on their
790 LPS profiles as described in the text (Table 2).

791 Lanes 1 - 2 are: 1. PNRM13 (PNRM6 [pRMPN1]), and 2. PNRM134
792 (PNRM126 [pRMPN1]). Lanes 3 - 32 are the *Δwzy* or *Δwzy Δwzz* strains with
793 plasmids encoding mutated Wzy$_{Sf}$ proteins. The Wzy$_{Sf}$ mutants in each lane
794 are as follows: 3. R164A (*Δwzy*), 4. R164A (*Δwzy Δwzz*), 5. R164K (*Δwzy*), 6.
795 R164K (*Δwzy Δwzz*), 7. R164E (*Δwzy*), 8. R164E (*Δwzy Δwzz*), 9. R250A
796 (*Δwzy*), 10. R250A (*Δwzy Δwzz*), 11. R250K (*Δwzy*), 12. R250K (*Δwzy Δwzz*),

13. R250E (*Δwzy*), 14. R250E (*Δwzy Δwzz*), 15. R258A (*Δwzy*), 16. R258A (*Δwzy Δwzz*), 17. R258K (*Δwzy*), 18. R258K (*Δwzy Δwzz*), 19. R258E (*Δwzy*), 20. R258E (*Δwzy Δwzz*), 21. R278A (*Δwzy*), 22. R278A (*Δwzy Δwzz*), 23. R278K (*Δwzy*), 24. R278K (*Δwzy Δwzz*), 25. R278E (*Δwzy*), 26. R278E (*Δwzy Δwzz*), 27. R289A (*Δwzy*), 28. R289A (*Δwzy Δwzz*), 29. R289K (*Δwzy*), 30. R289K (*Δwzy Δwzz*), 31. R289E (*Δwzy*), 32. R289E (*Δwzy Δwzz*).

The position of R-LPS is indicated. The numbers on the left and right indicate the Oag RUs. Letters (A1, A2, A3, B, C, D, E, and F) at the bottom indicate the mutant class (Table 2).



818

819 **Fig. 3. Protein expression level of the Wzy_{Sf}-GFP mutants.**

820 The strains were grown in LB and induced as described in the Methods. In-gel
821 fluorescence samples were prepared from the mutants in the *Δwzy* and *Δwzy*
822 *Δwzz* backgrounds, and electrophoresed on SDS 15% (w/v) PAGE gels (See
823 Methods).

824 **a.** Strains in lane 1-2 are as follows: 1. PNRM13 (PNRM6 [pRMPN1]), 2.
825 PNRM134 (PNRM126 [pRMPN1]). Lanes 3- 12 are the *Δwzy* or *Δwzy Δwzz*
826 strains expressing mutated Wzy_{Sf}-GFP proteins. The Wzy_{Sf} mutants in each
827 lane are as follows: 3. R264A (*Δwzy*), 4. R164A (*Δwzy Δwzz*), 5. R250
828 (*Δwzy*), 6. R250 (*Δwzy Δwzz*), 7. R258A (*Δwzy*), 8. R258A (*Δwzy Δwzz*), 9.
829 R278A (*Δwzy*), 10. R278A (*Δwzy Δwzz*), 11. R289A (*Δwzy*), 12. R289A (*Δwzy*
830 *Δwzz*).

b. Strains in lane 1 - 2 are as follows: 1. PNRM13, 2. PNRM134. Lanes 3- 10 are the *Δwzy* or *Δwzy Δwzz* strains expressing mutated Wzy_{Sf}-GFP proteins. The Wzy_{Sf} mutants in each lane are as follows: 3. R164K (*Δwzy*), 4. R164K (*Δwzy Δwzz*), 5. R164E (*Δwzy*), 6. R164E (*Δwzy Δwzz*), *7.* R250K (*Δwzy*), 8. R250K (*Δwzy Δwzz*), 9. R250E (*Δwzy*), 10. R250E (*Δwzy Δwzz*).

c. Strains in lane 1 - 2 are as follows: 1. PNRM13, 2. PNRM134. Lanes 3- 14 are the *Δwzy* or *Δwzy Δwzz* strains expressing mutated Wzy_{Sf}-GFP proteins. The Wzy_{Sf} mutants in each lane are as follows: 3. R258K (*Δwzy*), 4. R258K (*Δwzy Δwzz*), 5. R258E (*Δwzy*), 6. R258E (*Δwzy Δwzz*), *7.* R278K (*Δwzy*), 8. R278K (*Δwzy Δwzz*), 9. R278E (*Δwzy*), 10. R278E (*Δwzy Δwzz*), 11. R289K (*Δwzy*), 12. R289K (*Δwzy Δwzz*), 13. R298E (*Δwzy*), 14. R289E (*Δwzy Δwzz*).

In each panel, the relative Wzy_{Sf}-GFP level of all the mutants were measured by considering the Wzy_{Sf}-GFP in PNRM13 in lane 1 as 100%. Letters (A1, A2, A3, B, C, D, E, and F) at the bottom indicate the mutant class (Table 2).