Pratiti Nath, Elizabeth Ngoc Hoa Tran, Renato Morona
Mutational analysis of the Shigella flexneri O-antigen polymerase Wzy: Identification of Wzz-dependent Wzy mutants,
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Title: Mutational analysis of the *Shigella flexneri* O antigen polymerase Wzy; identification of Wzz-dependent Wzy mutants

Authors: Pratiti Nath[1], Elizabeth Ngoc Hoa Tran[1], and Renato Morona[1#]

Address: [1]Discipline of Microbiology and Immunology, School of Molecular and Biomedical Science, University of Adelaide, Adelaide, SA 5005, Australia.

Running title: Characterization of *S. flexneri* Wzy.

Corresponding author: Renato Morona,

Email: renato.morona@adelaide.edu.au, Phone: +61 8 8313 4151, Fax: +61 8 8313 7532

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Abstract

The O antigen (Oag) component of lipopolysaccharide (LPS) is a major virulence determinant of *Shigella flexneri* and is synthesized by the O antigen polymerase, Wzy_{Sf}. Oag chain length is regulated by chromosomally encoded Wzz_{Sf} and pHS-2 plasmid encoded Wzz_{pHS2}. To identify functionally important amino acid residues in Wzy_{Sf}, random mutagenesis was performed on the *wzy*$_{Sf}$ in a pWaldo-TEV-GFP plasmid followed by screening with colicin E2. Analysis of the LPS conferred by mutated Wzy_{Sf} proteins in the *wzy*$_{Sf}$ deficient (Δ*wzy*) strain identified 4 different mutant classes, with mutations found in the Periplasmic Loops (PL) - 1, 2, 3, and 6; Trans-membrane (TM) regions - 2, 4, 5, 7, 8, and 9; and Cytoplasmic Loops (CL) - 1 and 5. The association of Wzy_{Sf} and Wzz_{Sf} was investigated by transforming these mutated *wzy*$_{Sf}$ plasmids into a *wzy*$_{Sf}$ and *wzz*$_{Sf}$ deficient strain (Δ*wzy* Δ*wzz*). Comparison of the LPS profiles in the Δ*wzy* and Δ*wzy* Δ*wzz* backgrounds identified Wzy_{Sf} mutants whose polymerization activity was Wzz_{Sf}-dependent. Colicin E2 and bacteriophage Sf6c sensitivities were consistent with the LPS profiles. Analysis of the expression levels of the Wzy_{Sf}-GFP mutants in the Δ*wzy* and Δ*wzy* Δ*wzz* backgrounds identified a role for Wzz_{Sf} in Wzy_{Sf} stability. Hence, in addition to its role in regulating Oag modal chain length, Wzz_{Sf} also affects Wzy_{Sf} activity and stability.

Introduction

Shigella flexneri lipopolysaccharide (LPS) is crucial for pathogenesis **(1)**. LPS is exclusively located in the outer leaflet of the outer membrane (OM) and has three domains: 1) Lipid A - a hydrophobic domain which anchors LPS to the OM, 2) the core oligosaccharides - a non-repeating oligosaccharide domain, and 3) the O-antigen (Oag) polysaccharide - an oligosaccharide repeat domain **(1) (2)**. The complete LPS structure with Oag chains is termed smooth LPS (S-LPS). However, the LPS structure lacking the Oag is termed rough LPS (R-LPS), and LPS with a single Oag tetrasaccharide repeat unit (RU) attached to the Lipid A and core sugar is termed semi-rough LPS (SR-LPS) **(3)**. *S. flexneri* is subdivided into various serotypes depending on the differences in the composition of LPS Oag. So far there are 17 known serotypes of *S. flexneri* **(4)**. Except for *S. flexneri* serotype 6, the Oag of all other serotypes has the same polysaccharide backbone containing three L-rhamnose residues (Rha), and one *N*-acetylglucosamine (GlcNAc). This basic Oag structure is known as serotype Y. Addition of either glucosyl, O-acetyl, or phosphoethanolamine (PEtN) groups by various linkages to the sugars of the Y serotype tetrasaccharide repeat creates different *S. flexneri* serotypes **(5-7)**. Oag is the protective antigen as immunity against the *S. flexneri* infection is serotype specific **(8, 9)**. S-LPS confers resistance to complement **(10)** and colicins **(11, 12)**, and Y serotype Oag acts as a receptor to bacteriophage Sf6 **(13)**.

S. flexneri Oag biosynthesis occurs by the Wzy-dependent pathway. Most of the Oag biosynthesis genes (except *wecA*) of *S. flexneri* are located in the Oag biosynthesis locus between *galF* and *his* **(6, 14)**. *S. flexneri* Oag biosynthesis occurs on either side of the inner

membrane (IM). Initially N-acetylglucosamine phosphate (GlcNAc-1-P) is transferred from uridine diphosphate-GlcNAc (UDP-GlcNAc) by WecA to undecaprenol phosphate (Und-P) at the cytoplasmic side of the IM **(5, 15, 16)**. RfbG and RfbF then add Rhamnose (Rha) residues from thymidine diphospho-rhamnose (dTDP-Rha) to the GlcNAc **(3, 17)** to form the O unit. In the Wzy-dependent model of LPS assembly, the flippase protein Wzx translocates this O unit to the periplasmic side. At the periplasmic side, the O units are polymerized at the non-reducing end by the Oag polymerization protein Wzy via a block transfer mechanism to form the polymer. The chain length of the final Oag is regulated by the protein Wzz **(3, 18)**. Finally, the Oag ligase WaaL ligates the Oag chains to the previously synthesized core-lipid A. The Lpt proteins (Lpt A-G) then transport the LPS from the IM to the OM **(1, 19)**.

The Oag polymerization protein Wzy_{Sf} is encoded by the *rfc/wzy* gene. Wzy_{Sf} is a 43.7 kDa hydrophobic integral membrane protein. It has 12 transmembrane (TM) segments and two large periplasmic (PL) domains **(3, 18)**. Based on a topology model proposed by our group the amino and carboxy terminal ends are located on the cytoplasmic side of the IM. The wild type wzy_{Sf} gene lacks a detectable ribosome binding site, has a low G+C %, and a high percentage of minor codons in the first 25 amino acids, contributing to low expression and poor detection of the protein **(3, 18)**.

Islam et al. (2011) performed extensive work on *Pseudomonas aeruginosa* Wzy (Wzy_{Pa})

and showed that both PL3 and PL5 of Wzy_{Pa} contain $RX_{10}G$ motifs, which are important for the functioning of Wzy_{Pa}. They also found several Arg residues within these two motifs are also important for Wzy_{Pa} function **(20)**. However there is little sequence identity between Wzy_{Pa} and Wzy_{Sf}. So, it is not possible to predict the functional amino acid residues of Wzy_{Sf} from another model.

Wzz is a member of the polysaccharide co-polymerase (PCP) family. *S. flexneri* has two types of Wzz - chromosomally encoded Wzz_{Sf} and pHS-2 plasmid encoded Wzz_{pHS2}. *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. Controlling Oag chain length is crucial for bacterial virulence and loss of Wzz_{Sf} mediated Oag modal chain length regulation affects virulence due to masking of the OM protein IcsA **(21, 22)**. Daniels and Morona (1999) showed that Wzz_{Sf} forms a dimer *in vivo* and may oligomerise up to a hexamer **(23)**. Formation of these large complexes is consistent with the hypothetical complex formation between Wzz and other enzymes of the Oag biosynthesis pathway, including Wzy **(14, 24)**. Wzz_{Sf} and Wzz_{pHS2} compete for the available Wzy_{Sf} **(25)**.

Woodward et al. (2010) showed that Wzz and Wzy are sufficient to determine the Oag modal chain length **(26)**. There are several proposed mechanisms for modal length control by Wzz and its association with Wzy: a molecular clock model was proposed by Bastin et

al. (1993) **(24)** and a molecular chaperone model was proposed by Morona et al. (1995) **(14)**. Tocilj et al. (2008) suggested that Wzz may form a scaffold and recruits Wzy **(27)**. However, there is no direct evidence to date on how these proteins are associated with each other in Oag polymerization and chain length control.

In this study we were able to overexpress and detect Wzy_{Sf}-GFP expression in *S. flexneri*. We performed random mutagenesis on *wzy*$_{Sf}$ and following screening with colicin E2, for the first time identified amino acid residues important for Wzy_{Sf} function. We classified the *wzy*$_{Sf}$ mutants based on their LPS profile. We were able to determine mutant protein expression levels, and also characterised the mutants depending on the phage and colicin sensitivities they conferred. These findings provided insight to Wzy structure and function. We further identified Wzz_{Sf}-dependent Wzy_{Sf} mutants, and identified a novel role for Wzz_{Sf} in Wzy_{Sf} Oag polymerization activity and stability, in addition to its role in regulating Oag modal chain length.

Materials and 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 Luria-Bertani (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).

Unless otherwise stated strains were grown in LB broth with aeration for 18 h at 37°C, then 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). Where required growth medium was supplemented with 0.2% (w/v) glucose to suppress protein expression. Before induction cells were centrifuged (2200 x g, SIGMA 3K15 table top centrifuge, 10 min, 4°C) and washed twice with LB broth to remove glucose. During induction conditions, 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) or 0.2% (w/v) or 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: kanamycin (Km; 50 μg/ml), and chloramphenicol (Cm; 25 μg/ml).

Construction of expression vector and cloning of wzy_{Sf}

Primers PN1_*wzy*$_{Sf}$*Kpn*F and PN2_*wzy*$_{Sf}$*BamH*R (Supplementary Table S1) which incorporated *Kpn*I and *Bam*HI restriction sites, respectively, were used to amplify the previously mutated *wzy*$_{Sf}$ coding region (GenBank accession number X71970) in the pRMCD6 plasmid (Table 1), possessing three changes at codons 4, 9 and 23 **(18)**. The rare codons 4, 9 and 23 are present in the translation initiation site of Wzy_{Sf} and causes lower expression of the wild-type Wzy_{Sf}. The codons ATA, ATA and AGA at 4, 9 and 23 were changed to ATT, ATT and CGT, respectively, and resulted in increased expression of Wzy_{Sf}

(18). Following restriction digestion with enzymes *Bam*HI and *Kpn*I, the amplified wzy_{Sf} was ligated into similarly digested pWaldo-TEV-GFP **(28)**, resulting in the plasmid pWaldo-wzy_{Sf}-TEV-GFP (denoted as pRMPN1). PCR, restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation were performed as described previously **(3, 14)**.

Wzy_{Sf} expression in Lemo21(DE3) and In-gel fluorescence

For Wzy_{Sf} expression in Lemo21(DE3) cells (Table 1), strains were induced with IPTG and the over-expressed Wzy_{Sf}-GFP fusion protein was detected by In-gel fluorescence following the method of Drew et al. (2006) **(29)** with some modifications. $5X10^8$ cells were harvested from the induced culture and washed twice in 200 µl phosphate buffered-saline (PBS). The pellet was re-suspended in 20 µl PBS and 20 µl 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 DDT) was then added to the cell suspension. The solubilized cell suspension was incubated at 37°C for 5 min. Samples were then electrophoresed on SDS 15% (w/v) polyacrylamide gels (SDS-15% PAGE) and BenchMark™ Pre-Stained Protein Ladder (Invitrogen) was used as a molecular mass standard. The gel was rinsed with distilled water and fluorescent imaging of the gel was performed to detect 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).

LPS method

LPS was prepared as described previously **(30, 31)**. To prepare LPS samples, $1X10^9$ cells were harvested and resuspended in lysing buffer (Buffer B - 10% [w/v] glycerol, 2% [w/v] SDS, 4% [w/v] β-mercaptoethanol, 0.1% [w/v] bromophenol blue, 1 M Tris-HCl, pH 7.6) and incubated with 2 μg/ml of proteinase K for approximately 16 h. The LPS samples were then electrophoresed on a SDS-15% PAGE for 16 to 18 h at 12 mA. The gel was stained with silver nitrate, developed with formaldehyde **(30).**

Random mutagenesis

Random mutagenesis of *wzy*$_{Sf}$ was undertaken to obtain a wide range of *wzy*$_{Sf}$ mutations in a non-selected manner. For PCR random mutagenesis the *wzy*$_{Sf}$ coding region in plasmid pRMPN1 was mutagenized by an error prone DNA polymerase using the GeneMorp II EZ-Clone Domain Mutagenesis Kit (Catalog # 200552, Stratagene) as per the manufacturer's instructions with the primers PN1_*wzy*$_{Sf}$*Kpn*F and PN2_*wzy*$_{Sf}$*BamH*R (Supplementary Table S1). Mutagenized plasmids were transformed into competent *E. coli* cells, XL10-Gold (Agilent Technologies). Plasmid DNA was then isolated from randomly chosen transformed mutated colonies and transformed into strain PNRM6 (Table 1). Colicin swab assays were performed to screen the mutants (See below). Plasmid DNA was isolated from putative mutants, transformed into XL10G, and subjected to DNA sequencing (AGRF, Adelaide, Australia).

185 **Construction of the strain RMA4437 (Δ*wzy* Δ*wzz*)**

186 The *S. flexneri* Y PE638 Δ*wzy* Δ*wzz* mutant strain was constructed using allelic exchange
187 mutagenesis **(14)** to inactivate the wzz_{Sf} gene in RMM109 **(3)**. Initially, a tetracycline
188 resistance (tet^R) cartridge was inserted into the *Bgl*II site of pRMA577 **(14)** to inactivate
189 wzz_{Sf}, and the resulting pCACTUS-wzz_{Sf}::*tet* (Table 1) plasmid was transformed into
190 RMM109 via electroporation **(32)**. Allelic exchange mutagenesis was performed as
191 previously described **(14)**. The wzz_{Sf}::tet^R mutation in the chromosome was confirmed by
192 PCR with primers ET35 and ET36 (Supplementary Table S1) to give the PE638 Δ*wzy* Δ*wzz*
193 mutant RMA4337.

194

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

196 For the detection of Wzy_{Sf} expression in *S. flexneri*, cells were harvested from the 50 ml
197 0.2% (w/v) L-arabinose induced culture by centrifugation (9800 x g, Beckman J2-21M
198 Induction Drive Centrifuge, 10 min, 4 °C) and the cell pellet was resuspended in 4 ml
199 sonication buffer (Buffer C, 20 mM Tris-HCl, 150 mM NaCl, pH 7.5). The mixture was then
200 lysed by sonication, followed by centrifugation (2200 x g, SIGMA 3K15 table top centrifuge,
201 10 min, 4 °C) to remove debris. Ultracentrifugation was performed in a Beckman Coulter
202 Optima L-100 XP bench top ultracentrifuge (126000 x g for 1 h at 4 °C) to isolate the whole
203 membrane (WM) fraction. The WM fraction was resuspended in PBS and then solubilized in
204 Buffer A. Solubilized WM fraction from 3 X 10^8 cells was electrophoresed on a SDS-15%
205 PAGE. The gel was rinsed with distilled water and In-gel imaging was performed as

206 described above. Loading was checked by staining the gel with Coomassie Blue R-250.
207 The intensity of Wzy_{Sf}-GFP expression for mutant and control strains was measured by Fiji
208 image processing package (http://fiji.sc/Fiji) and the percent relative Wzy_{Sf}-GFP intensity for
209 each mutant strain was measured by comparing the Wzy_{Sf}-GFP intensity of each mutant
210 strain with Wzy_{Sf}-GFP intensity in the control strain PNRM13.

211

212 **Colicin sensitivity assay**

213 For the colicin sensitivity assay a solution of purified His_6-ColE2 (ColE2) with an initial
214 concentration of 1 mg/ml was used (11).

215

216 *ColE2 swab assay*

217 A two fold serial dilution of 10^{-3} dilution of ColE2 was swabbed onto antibiotic selective LB
218 agar plates containing 0.2% (w/v) L-arabinose with a cotton swab. Plates were left to dry for
219 1 h at room temperature (RT). Individual 0.2% (w/v) L-arabinose induced mutant and
220 control LB cultures were then swabbed perpendicular to the ColE2 streak and plates were
221 left to dry for another 1 h at RT. Plates were then incubated for 16 h at 37°C. The
222 susceptibility of the mutant strains compared to the control strain was recorded and the
223 image was taken using a Canon scanner (CanoScan 9000F) against a dark background.

224

ColE2 spot assay

The spot assay was performed using the serial dilutions of ColE2. 100 μl of the individual L-arabinose induced mutant and control strain cultures were spread onto LB agar plates with appropriate antibiotics and 0.2% (w/v) L-arabinose. Plates were left to dry for 2 h at RT. A 2-fold serial dilution of 10^{-3} dilution of ColE2 (denoted as Neat or [N]) was spotted on the dried plates, and plates were left to dry for another 3 h at RT. Plates were then incubated for 18 h at 37°C. The end point of the killing zones of mutant strains was compared with the controls. Images were recorded as above.

Bacteriophage sensitivity assay

Procedures of phage propagation and phage stock preparation have been described previously **(3, 33)**. The concentration of the bacteriophage Sf6c stock used was 8.6 x 10^7 p.f.u./ ml. Mutant and control strains were grown and induced with 0.2% (w/v) L-arabinose. 100 μl of the individual mutant and control LB cultures were spread onto LB agar plates with appropriate antibiotics and 0.2% (w/v) L-arabinose. Plates were left to dry for 2 h at RT. Serial dilutions of the bacteriophage Sf6c stock (undiluted bacteriophage Sf6c stock was denoted as N) were spotted on the dried plates and plates were dried for a further 3 h at RT. The plates were incubated for 18 h at 37°C. Phage sensitivity of the test strains were compared with the controls. Images were recorded as above.

Results

Construction of a Wzy_{Sf}-GFP expression plasmid

A suitable expression system was constructed to express *wzy*$_{Sf}$ and to detect Wzy_{Sf} by fusion to Green Fluorescent Protein (GFP) **(29)**. *S. flexneri* 2457T 2a *wzy*$_{Sf}$ with three modified codons at postions 4, 9, 23 in pRMCD6 plasmid **(18)** was PCR amplified and ligated into pWaldo-TEV-GFP (Table 1) (See Materials and Methods). To confirm the construct was able to express Wzy_{Sf}-GFP-His_8 protein, pWaldo-*wzy*$_{Sf}$-TEV-GFP-His_8 (denoted as pRMPN1) was transformed into Lemo21(DE3) cells. Whole cell In-gel fluorescence samples were then prepared from PNRM15 (Lemo21(DE3), [pRMPN1) and fluorescent imaging of the gel detected a fluorescent band at approximately 64 kDa which corresponded to the predicted size of the Wzy_{Sf}-GFP protein (Supplementary Fig. S1, lane 1), indicating that the construct was able to express Wzy_{Sf}-GFP.

Complementation of *wzy*$_{Sf}$ deficiency

A complementation assay was performed to confirm the functionality of Wzy_{Sf}-GFP. For this assay pRMPN1 was co-transformed along with pAC/pBADT7-1 **(34)** into a *wzy*$_{Sf}$ deficient strain RMM109 **(3)**. RMM109 has a frameshift mutation at position 9214 in the *wzy*$_{Sf}$ gene that results in premature termination of Wzy_{Sf} synthesis **(3)**. pAC/pBADT7-1 encodes T7 RNA polymerase which drives the expression of *wzy*$_{Sf}$*-GFP* in pRMPN1. LPS samples were prepared from these control strains. The silver-stained gel showed that PNRM6 (RMM109, pAC/pBADT7-1) had a SR-LPS profile (Fig. 1, lane 3). But the PNRM13 (PNRM6,

pRMPN1) had a S-LPS profile (Fig. 1, lane 5). Hence, pRMPN1 was able to complement the *wzy*$_{Sf}$ mutation in RMM109, and the LPS profile resembled that of the wild type strain PE638 (Fig. 1, lane 2).

Random mutagenesis of *wzy*$_{Sf}$

Nothing is known about the residues important for Wzy$_{Sf}$ function, and there is little sequence identity between Wzy proteins of different bacterial species. Hence, it is difficult to predict the functional amino acid residues of the *S. flexneri* Wzy$_{Sf}$. To obtain insight into the Wzy$_{Sf}$ residues needed for function, *wzy*$_{Sf}$ coding region in plasmid pRMPN1 was subjected to random mutagenesis using an error prone DNA polymerase (See Materials and Methods). The resulting mutagenized plasmid library was transformed into PNRM6. We screened the transformants to find mutants using ColE2. The basis for this is that the R-LPS strains are more susceptible to the killing by colicins than the S-LPS strains **(12)**, and we recently found that there is a strong correlation between LPS Oag modal chain length and susceptibility to ColE2 **(11)**. A ColE2 swab assay (See Materials and Methods) was used to screen and detect mutants that had a different sensitivity to ColE2 compared to the positive control strain PNRM13 (Supplementary Table S2). Interestingly the wild type strain PE638 was slightly more resistant to ColE2 compared to the complemented positive control strain PNRM13 (Supplementary Table S2) (Table 2). The *wzy*$_{Sf}$ mutant RMM109 (SR-LPS) was highly sensitive to ColE2 (Supplementary Table S2) (Table 2). Transformants that were either more resistant or more sensitive to ColE2 than PNRM13 were selected (Supplementary Table S2), and the plasmids isolated and transformed into the XL10G

strain. Plasmid DNA from these isolates was subjected to DNA sequencing to identify mutational alterations in *wzy*$_{Sf}$. The *wzy*$_{Sf}$ mutants had the following substitutions: P352H, V92M, Y137H, L214I, G130V, N147K, P165S, L191F, C60F, L49F/T328A, L28V, N86K, F54C, F52Y, L111I, G82C, and F52C/I242T (Supplementary Table S3). The mutations were present in PL1, 2, 3, and 6; TM 2, 4, 5, 7, 8, and 9; and Cytoplasmic Loops (CL) 1 and 5 of the Wzy$_{Sf}$ topology map (summarized in Fig. 2 and Table 2). After sequence confirmation, the mutated plasmids were transformed into PNRM6 (Table 1) for detailed characterization.

LPS phenotype conferred by Wzy$_{Sf}$ mutants

The effect of mutations on Wzy$_{Sf}$ LPS Oag polymerization activity was determined by SDS-PAGE and silver staining. Following comparison of the resulting degree of LPS Oag polymerization of the mutant strains with the relevant positive control PNRM13, the mutants were grouped into four different phenotypic classes: A, B, C, and D (Fig. 3 and Supplementary Fig. S2). Three of the 17 mutants had reduced degrees of polymerization compared to PNRM13 and were classified as class A. Class A mutants had following alterations in Wzy$_{Sf}$: P352H, V92M, Y137H (Fig. 3, lanes 2-4). One mutant (with L214I alteration in Wzy$_{Sf}$) exhibited LPS banding pattern with only a few Oag RUs and was classified as Class B (Fig. 3, lane 5). Another mutant had SR-LPS (with a G130V alteration in Wzy$_{Sf}$) and was categorized into Class C (Fig. 3, lane 6). The other 12 mutants conferred a LPS profile nearly similar to the positive control PNRM13 and were classified as Class D. Members of this class had the following mutations: N147K, P165S, L191F, C60F,

L49F/T328A, L28V, N86K, F54C, F52Y, L111I, G82C, F52C/I242T (Fig. 3, lanes 7-10 and Supplementary Fig. S2, Lanes 1-8). For rest of the paper the mutant Wzy_{Sf} proteins will be referred according to their conferred LPS class.

Wzz_{Sf} dependence

Since the Wzy-dependent model of LPS assembly suggests a potential interaction between Wzy and Wzz, we investigated if the LPS profile conferred by mutated Wzy_{Sf} proteins was dependent on the presence of Wzz_{Sf}. All the plasmids encoding mutated Wzy_{Sf} proteins were transformed into RMA4337 carrying pAC/pBADT7-1 (strain PNRM126) (Table 1). RMA4337 is a *wzy*$_{Sf}$ and *wzz*$_{Sf}$ double mutant. The LPS profiles conferred in the PNRM126 background (Δ*wzy* Δ*wzz*) were directly compared with the LPS profile conferred in the Δ*wzy* background (PNRM6). The control strain PNRM134 (PNRM126 [pRMPN1]) had S-LPS without Oag modal chain length control (Fig. 4, lane 3) as expected, and was classified as Class E for the purpose of comparison. PNRM126 with mutated *wzy*$_{Sf}$ plasmids that conferred a Class A LPS profile in the Δ*wzy* background had a Class E LPS profile similar to PNRM134, except PNRM126 expressing WzyP352H had LPS with few Oag RUs (Class B) (Fig. 4, lane 5). PNRM126 expressing WzyL214I had SR-LPS (Fig. 4, lane 11). However, in comparison PNRM6 (Δ*wzy*) expressing WzyL214I conferred a Class B LPS profile (Fig. 4, lane 10). PNRM126 with a mutated *wzy*$_{Sf}$ plasmid that conferred a Class C LPS profile in the Δ*wzy* background (PNRM6 expressing G130V), had S-LPS without modal length control and a reduced degree of polymerization (designated Class F) compared to PNRM134 (Fig. 4, lane 13). PNRM126 with mutated *wzy*$_{Sf}$ plasmids from the strains with

Class D LPS profile in the Δ*wzy* background, had Class E LPS profile, similar to PNRM134 (Fig. 4, lanes 14-21 and Supplementary Fig. S3, lanes 1-16). Hence certain Wzy_{Sf} mutants conferred dramatically different LPS profiles depending on the presence and absence of Wzz_{Sf}.

ColE2 and bacteriophage **Sf6c sensitivities**

To confirm the LPS profiles determined above using assays of LPS Oag function, the ColE2 and bacteriophage Sf6c sensitivities of the mutant strains were investigated. ColE2 sensitivity (summarized in Table 2) was determined by spot testing as described in the Materials and Methods. Strains RMM109, PNRM6, PNRM11, RMA4337, and PNRM126 showed killing zones at a dilution of 1/256. The wild type strain PE638 was resistant to the highest concentration of ColE2 used. The relevant positive control strain in the Δ*wzy* background (PNRM13) showed a killing zone at a dilution of 1/2 but the relevant positive control strain in the Δ***wzy*** Δ***wzz*** background (PNRM134) was resistant to the tested highest concentration of ColE2. Strains conferring Class A LPS profile in the Δ*wzy* background were sensitive to ColE2 (killing zone at 1/32 or 1/64). However, Δ***wzy*** Δ***wzz*** strains with mutated wzy_{Sf} plasmids conferring the Class A LPS profile in the Δ*wzy* background were two-fold more sensitive to ColE2 (killing zone at 1/64 or 1/128) compared to the Δ*wzy* background. The Δ*wzy* strain expressing WzyL214I (Class B) and the Δ***wzy*** Δ***wzz*** strain expressing WzyL214I (Class C) had similar ColE2 sensitivity (1/128). The strain with the Class C LPS profile (Δ*wzy* expressing WzyG130V) had the greatest sensitivity to ColE2 (1/512), greater than RMM109 and the negative control strains (PNRM6, PNRM11,

PNRM126). However, the Δ*wzy* Δ*wzz* strain expressing WzyG130V (Class F) was more resistant (1/128) to ColE2 compared to the Δ*wzy* background. Strains with Class D LPS profile in the Δ*wzy* background were resistant to the tested highest concentration of ColE2, except Δ*wzy* expressing WzyP165S showed slight sensitivity (1/4). Hence, almost all the strains with Class D LPS profile in the Δ*wzy* background were more resistant to ColE2 compared to PNRM13 and were similar to PE638. The Δ*wzy* Δ*wzz* strains with mutated wzy_{Sf} plasmids from the strains with Class D LPS profile in the Δ*wzy* background were more sensitive to ColE2 (killing zone at 1/8 to 1/64) compared to the control PNRM134 (Δ*wzy* Δ*wzz* [pRMPN1]), suggesting they have slight defect in Oag polymerization in the absence of Wzz_{Sf}. Hence, ColE2 resistance correlated with degree of Oag polymerization and degree of LPS capping with Oag.

The bacteriophage Sf6c sensitivity of the strains (summarized in Table 2), carrying mutated wzy_{Sf} plasmids, was determined by spot testing as described in the Materials and Methods. The SR-LPS strains RMM109, PNRM6, PNRM11, RMA4337, and PNRM126 were resistant to bacteriophage Sf6c. The S-LPS strains wild type PE638, and the controls PNRM13 and PNRM134 were bacteriophage Sf6c sensitive, and plaques were detected at 10^{-6}, 10^{-5}, and 10^{-6} dilutions, respectively. Strains with Class A, B, and C LPS profiles in the Δ*wzy* background were resistant to the highest concentration of bacteriophage Sf6c tested and were similar to the strains with SR-LPS (RMM109, PNRM6, PNRM11, RMA4337, and PNRM126). Similarly, the Δ*wzy* Δ*wzz* strains with mutated wzy_{Sf} plasmids from the strains with Class A, B, and C LPS profiles in the Δ*wzy* background were also resistant to the

highest concentration of bacteriophage Sf6c tested. Strains with Class D LPS profile in the Δ*wzy* background were bacteriophage Sf6c sensitive (plaques at 10^{-6}), except for the strain expressing WzyP165S was slightly less sensitive (plaque at 10^{-5}). Hence, the bacteriophage Sf6c sensitivity of the strains with Class D LPS profile in the Δ*wzy* background was greater than PNRM13 and similar to PE638. Δ*wzy* Δ*wzz* strains with mutated wzy_{Sf} plasmids from the strains with Class D LPS profile in the Δ*wzy* background were more resistant to bacteriophage Sf6c compared to the Δ*wzy* background. Although they have a Class E LPS profile, similar to PNRM134, they were more resistant to bacteriophage Sf6c than PNRM134. Hence, bacteriophage Sf6c correlated with degree of Oag polymerization, and degree of LPS capping with Oag.

Wzy_{Sf} expression level

We determined the level of parental and mutant Wzy_{Sf}-GFP expressed in Δ*wzy* and Δ*wzy* Δ*wzz S. flexneri* strains. To measure the protein expression level of the mutants, In-gel fluorescence was performed and the % relative Wzy_{Sf}-GFP expression was calculated (See Materials and Methods). The expression levels of different Wzy_{Sf}-GFP mutants were compared with that of Wzy_{Sf}-GFP in PNRM13 (100%). The Wzy_{Sf}-GFP expression level in PNRM134 was less than PNRM13 (Fig. 5A and B, lane 2), having a relative Wzy_{Sf}-GFP level of 17% (Table 2). In the Δ*wzy* background most of the Wzy_{Sf}-GFP mutants were expressed at a level less than 100% except the mutants L191F (Class D) had expression of 162% (Fig. 5B, lane 7 and Table 2). However, in the Δ*wzy* Δ*wzz* background the relative Wzy_{Sf}-GFP level of L191F was 64% (Fig. 5B, lane 8 and Table 2), which was less than the

control PNRM13. In the Δ*wzy* background, the relative Wzy_{Sf}-GFP levels of P165S, L214I, and G130V were very low (7%, 1.4%, and 1.6% respectively) (Fig. 5B, lane 5; Fig. 5A, lanes 9 and 11; and Table 2). However, in the Δ*wzy* Δ*wzz* background P165S and G130V had relative Wzy_{Sf}-GFP levels of 125% and 28.50%, respectively (Fig. 5B, lane 6; Fig 5A, lane 12; and Table 2) but the relative Wzy_{Sf}-GFP level of L214I was almost not detectable at 0.03% (Fig. 5A, lane 10 and Table 2). In the Δ*wzy* Δ*wzz* background, the relative Wzy_{Sf}-GFP level of P165S was higher than the control PNRM13 (100%) (Fig. 5B, lane 6 and Table 2). These data indicates that expression of certain Wzy_{Sf} mutants were affected by Wzz_{Sf}, but the effect was mutant specific.

Discussion

In this study, we constructed and characterized a collection of wzy_{Sf} mutants. We found that ColE2 screening was an effective method to detect wzy_{Sf} mutants conferring subtle effects on LPS structure (Table 2). The use of Wzy_{Sf}-GFP allowed comparison of the protein expression level of different mutants. We also found that bacteriophage Sf6c sensitivity was dependent on LPS phenotype structure. Strains with mutant Wzy_{Sf} conferring longer Oag chain and/or a greater degree of Oag polymerization were more sensitive to bacteriophage Sf6c; while bacteriophage Sf6c only infected strains with wild type (or nearly wild type) LPS, both in degree of Oag polymerization and apparent level of LPS capping with Oag chains.

Only a few studies have been conducted on Wzy proteins. During characterization of Wzy of *Francisella tularensis,* Kim et al. (2010) reported several amino acid residues important for Oag polymerization. Modification of these residues (G176, D177, G323, and Y324) led to a loss of Oag polymerization **(35)**. Islam et al. showed that the PL3 and PL5 of Wzy_{Pa} have net positive and net negative charges respectively and they established their "catch-and-release" model **(20)**. However, for Wzy_{Sf}, we found that at a physiological pH both the PL3 and PL5 possess net negative charge (pI of PL3 is 4.65 and PL5 is 5.09). In *Pseudomonas aeruginosa* PAO1 there is a uronic acid sugar in the Oag **(36)**, which is negatively charged. However, the Oag of Wzy_{Sf} is neutral. So, the charged property of the substrate for Wzy_{Sf} is different from the Wzy_{Pa}.The $RX_{10}G$ motifs of the PL3 and PL5 of Wzy_{Pa} **(20)** are also 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. 2). So, perhaps a modified version of the "catch-and-release" mechanism **(20)** exists for *S. flexneri*. Moreover, addition of either glucosyl or O-acetyl groups or PEtN residue by various linkages to the sugars within the tetrasaccharide repeats of serotype Y creates 17 different serotypes of the *S. flexneri* **(5-7)**. As the polymerization of all these Oags is done by Wzy_{Sf}, and we conclude that Wzy_{Sf} must be quite flexible for its substrate recruitment. Due to scant homology between *wzy* proteins of different bacterial species, we were unable to create any directly comparable mutation in Wzy_{Sf} with respect to other systems. This led us to perform random mutagenesis on wzy_{Sf} followed by screening with ColE2.

The mutations we found are present in a wide region (PL1, 2, 3, 5, and 6; TM 2, 4, 5, 7, 8, and 9; and CL 1 and 5) of the Wzy_{Sf} topological model experimentally determined by Daniels et al. (1998) **(18)**. We were able to locate a number of mutations in the TM regions and interestingly the G130V mutation, which resulted in the complete loss of polymerization activity of Wzy_{Sf} in the Δ*wzy* background, was also located in TM5. Topological models are not very accurate but due to a lack of crystal structure we used the Wzy_{Sf} topology model previously determined by our group to locate the mutational alterations. Furthermore, we reassessed our topological model using 5 different topology prediction programs (SPOCTOPUS, MEMSAT, HMMTOP, TOPCONS, and TMHMM) (Supplementary Table S4). The results showed that until trans-membrane (TM) region 6 all the programs are consistent with our topological model. Three of them also validate TM10 - TM11 of our topological model. So, the topological model of Wzy_{Sf} determined by our group is nearly consistent with the topological models predicted by the programs. The mutations, which resulted in the partial loss of polymerization in the Δ*wzy* background, were V92M, Y137H, L214I, and P352H. Among them V92M and Y137H are present in the PL2 and TM5. These regions were validated by 5 of the programs. P352H is present in the PL6 and this region was validated by 3 of the programs (MEMSAT, HMMTOP, and TOPCONS). L214I is present in the TM8 and this region (amino acid 209 - 226) was different compared to the computer prediction (amino acid 227 - 247). However, G130V is present in the TM5, which was validated by 5 of the programs. Recently, Reddy et al. (2014) reported that different topology prediction programs are suitable for different families of proteins **(37)**. So, non-prediction of TM7 - TM9 by some programs does not invalidate the presence of these regions in our model.

462 In the Δ*wzy* background, strains with the Class A LPS profile had S-LPS with reduced
463 degree of Oag polymerization (Fig. 3, lanes 2-4), mutants with the Class B LPS profile had
464 LPS with few Oag RUs (Fig. 3, lane 5), and the mutant with Class C LPS profile had SR-
465 LPS (Fig. 3, lane 6). As a result the Class A mutants were more sensitive to ColE2 than
466 PNRM13 and the Class B mutants were even more sensitive to ColE2 than the mutants
467 with the Class A LPS profile. The mutant with Class C LPS had the highest ColE2
468 sensitivity. From these three classes it is clear that the LPS with shorter Oag chains were
469 more sensitive to ColE2. Strains with Class A, B, and C LPS profiles in the Δ*wzy*
470 background were resistant to bacteriophage Sf6c. The lack of bacteriophage Sf6c
471 sensitivity indicates that bacteriophage Sf6c only infects if the LPS has wild type or close to
472 wild type level of Oag polymerization. In this study we found that the Wzy_{Sf} mutants
473 conferring the Class D LPS profile in the Δ*wzy* background, had a similar level of Oag
474 polymerization compared to the positive control strain PNRM13, as determined by SDS-
475 PAGE and silver staining (Fig. 3, lanes 7-10 and Supplementary Fig. S2, lanes 1-8) but
476 they were more resistant to ColE2 and more sensitive to bacteriophage Sf6c (Table 2)
477 (except Δ*wzy* strain with WzyP165S). The ColE2 and bacteriophage Sf6c sensitivities of
478 these strains were similar to wild type strain PE638. We conclude that the Oag
479 polymerization activity of the mutants with Class D LPS profile in the Δ*wzy* background, was
480 similar to the wild type strain (PE638). Hence, the wild type Wzy_{Sf}-GFP protein is not 100%
481 active, and the mutant proteins conferring the Class D LPS profile are more active and/or
482 are better exported or folded or assembled to the IM. The level of Oag polymerization of the
483 strains with Class D LPS profile was more than PNRM13, although this was not

484 distinguishable by silver staining. Hence, the ColE2 assay was more sensitive than silver
485 staining and SDS-PAGE in determining the degree of Oag polymerization.

486

487 Mutation in *wzz* resulted in Oag without modal chain length control in different organisms
488 **(14, 24)**. Recently, Kenyon and Reeves showed that Wzy of *Yersinia pseudotuberculosis*
489 also needs Wzz for complete Oag polymerization **(38)**. Here we examined the
490 polymerization activity of different Wzy_{Sf} mutants in the absence of Wzz_{Sf}. In the Δ***wzy*** Δ***wzz***
491 background, the strains with mutated wzy_{Sf} plasmids from the strains having Class B LPS
492 profiles in the Δ*wzy* background had a SR-LPS profile, and ColE2 and bacteriophage Sf6c
493 sensitivities were similar to the Δ*wzy* background. Interestingly, the Δ***wzy*** Δ***wzz*** strain with
494 WzyG130V had S-LPS without modal length control (Class F) where as the Δ*wzy* strain
495 with WzyG130V had SR-LPS (Class C) and this correlated with an increased resistance to
496 ColE2. In the Δ***wzy*** Δ***wzz*** background, strains with a Class E LPS profile (S-LPS without
497 modal length control) had increased sensitivity to ColE2 and increased or similar resistance
498 to bacteriophage Sf6c compared to PNRM134 (Δ***wzy*** Δ***wzz*** [pRMPN1]) and when in the
499 Δ*wzy* background (strains with Class A and Class D LPS profiles) (Table 2). We speculate
500 that these strains (strains with a Class E LPS profile) had subtle alterations in the level of
501 Oag polymerization and/or capping of LPS with Oag, and were unable to act efficiently as a
502 bacteriophage Sf6c receptor, and correspondingly were also more sensitive to ColE2. It is
503 known that phage adsorption to the cells of *Escherichia coli* K12 increases with increase in
504 density of receptor protein at the cell surface **(39)**. Based on our study, the relationship
505 between ColE2 and Oag density/concentration seems linear. Deceasing Oag progressively

increases sensitivity to ColE2. However, the relationship between bacteriophage Sf6c and Oag density/concentration seems non-linear. Decreasing Oag rapidly decreases sensitivity to bacteriophage Sf6c. This may be because bacteriophage Sf6c interaction with its receptor is complex and most likely requires multi-receptor binding to bacteriophage Sf6c tail spike proteins (TSPs) to achieve irreversible binding and hence activation of bacteriophage Sf6c.

According to the proposed molecular clock model, Wzz acts as a molecular clock and regulates Wzy activity between two states: “E” or extension state favors polymerization and “T” or transfer state favors the ligation reaction **(24)**. The molecular chaperone model describes Wzz as a typical molecular chaperone, which regulates the overall ratio of Wzy and WaaL in a complex, and controls the enzyme kinetics of the ligation reaction to define the modality **(14)**. However, Woodward et al. (2010) suggests that there is an interaction between Wzy and Wzz and they showed that these proteins are enough to shape the Oag modal chain length **(26)**. Islam et al. (2013) suggested that the chain length of the Oag is determined by the interaction of Wzz and Wzy **(40)**. Recent work of Taylor et al. (2013) also suggested the direct interaction of Wzz and Wzy in the Oag biosynthesis pathway **(41)**. In this study, for the first time we were able to give an insight in the association of Wzy_{Sf} and Wzz_{Sf} in the Oag biosynthesis. We found Wzy_{Sf} mutants where polymerization activity was dependent on Wzz_{Sf} (WzyP352H and WzyL214I), and some other mutants where polymerization activity was repressed in the presence of Wzz_{Sf} (WzyV92M, WzyY137H, and WzyG130V). The *Δwzy Δwzz* strain with WzyV92M or WzyY137H had S-LPS without

modal length control (Class E) but in the *Δwzy* background, they showed Class A LPS profile (a greatly reduced degree of Oag polymerization) (Fig 4, lanes 6-7 and lanes 8-9). The *Δwzy* strain with WzyG130V had Class C LPS profile (SR-LPS) but remarkably the *Δwzy Δwzz* strain with WzyG130V had S-LPS without modal length control albeit with a reduced degree of polymerization (Class F) (Fig 4 lanes 12-13). Oag polymerization by these Wzy_{Sf} mutants was repressed by Wzz_{Sf}, in the *Δwzy*$_{Sf}$ background; their LPS had shorter Oag chains. The *Δwzy* strain with WzyP352H had Class A LPS profile, and the *Δwzy* strain with WzyL214I had Class B LPS profile but in the *Δwzy/Δwzy* background they had shorter Oag chains (Class B and C respectively) compared to the *Δwzy* background (Fig. 4, lanes 4-5 and 4 lanes 10-11). So, these Wzy_{Sf} mutants need Wzz_{Sf} for their Oag polymerization activity. These findings suggest that Wzz_{Sf} is associated with Wzy_{Sf} not only for Oag modal chain length control but also for polymerization activity, and amino acids V92, G130, Y137, L214, and P352 have role in the association of Wzy_{Sf} and Wzz_{Sf} during the Oag polymerization mediated by Wzy_{Sf}.

The relative Wzy_{Sf}-GFP level in PNRM134 (*Δwzy Δwzz*) was lower than in PNRM13 (*Δwzy*) (Fig. 5A and B, lanes 1-2), suggesting that the wild type Wzy_{Sf}-GFP had better expression in the presence of Wzz_{Sf}. Wzy_{Sf} mutants conferring Class A LPS profile (*Δwzy* background) had Wzy_{Sf}-GFP levels less than the Wzy_{Sf}-GFP in PNRM13 (Fig. 5A, lanes 3, 5, 7 and Table 2). WzyL214I was not detectable either in the *Δwzy* (Class B) or *Δwzy Δwzz* (Class C) backgrounds (Fig. 5A, lanes 9-10 and Table 2). Hence, amino acid L214 is important for Wzy_{Sf}-GFP production. WzyG130V was not detectable in the *Δwzy* background (Class C)

550 but was detected in the *Δwzy Δwzz* background (Class F) (Fig. 5A, lanes 11-12 and Table
551 2). Except for the strain *Δwzy* with WzyL191F, all the mutants with Class D LPS profile
552 (*Δwzy* background) had a lower protein expression level than Wzy_{Sf}-GFP in PNRM13 (Fig.
553 5B and Table 2) by some unknown mechanism. However, the mutants with Class D LPS
554 profile (*Δwzy* background) had S-LPS and were more resistant to ColE2 and more sensitive
555 to bacteriophage Sf6c than PNRM13. In the *Δwzy Δwzz* background WzyL191F had an
556 expression level less than Wzy_{Sf}-GFP in PNRM13 (Fig. 5B, lane 8 and Table 2). WzyP165S
557 was not detectable in PNRM6 (*Δwzy*) but strain PNRM126 (*Δwzy Δwzz*) with WzyP165S
558 present at a greater level than Wzy_{Sf}-GFP in PNRM13 (Fig. 5B, lanes 5-6 and Table 2).
559 Apparently there are more than one underlying mechanism for the Class D (Δ*wzy*
560 background) to Class E (*Δwzy Δwzz* background) LPS profile conversion. Some of the
561 mutations (P165S and G130V) resulted in decreased Wzy_{Sf}-GFP levels in the presence of
562 Wzz_{Sf}. This suggested that the presence of Wzz_{Sf} destabilizes WzyP165S and WzyG130V,
563 which resulted in lower Wzy_{Sf}-GFP levels. The mutation L191F resulted in decreased
564 Wzy_{Sf}-GFP level in the absence of Wzz_{Sf}. So, in this case Wzz_{Sf} stabilizes the protein
565 WzyL191F resulting in better Wzy_{Sf}-GFP expression. This finding suggests that G130,
566 P165, and L191 are important for the stabilization of Wzy_{Sf} through interaction with Wzz_{Sf}.

567

568 In conclusion, our findings identified amino acid residues on the Wzy_{Sf} important for its
569 polymerization function and interaction with Wzz_{Sf}. Residues, which are important for the
570 polymerization and interaction of Wzz_{Sf} and Wzy_{Sf} are present in the PL 2, 6 and TM 5, 8.
571 These regions may contribute in the interaction of Wzy_{Sf} with substrates and also in the

572 interaction with Wzz_{Sf}. We identified a number of amino acids (G130, P165, L191), which
573 are important for the stabilization of Wzy_{Sf} in association with Wzz_{Sf}. Hence, our data
574 suggested that Wzz_{Sf} also has a role in Wzy_{Sf} stability.

575

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581

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Tables

Table 1. Bacterial strains and plasmids used in this study

Strains or Plasmids	Characteristics	Reference
***S. flexneri* Strains**		
PE638	*S. flexneri* Y *rpoB* (Rif^R)	(14)
RMM109	PE638*Δwzy*, Rif^R	(3)
RMA4337	RMM109 *Δwzz* (Rif^R, Tet^R)	This study
PNRM6	RMM109 [pAC/pBADT7-1]	This study
PNRM11	PNRM6 [pWaldo-TEV-GFP]	This study
PNRM13	PNRM6 [pRMPN1]	This study
PNRM75	PNRM6 [pRMPN7]	This study
PNRM76	PNRM6 [pRMPN8]	This study
PNRM77	PNRM6 [pRMPN9]	This study
PNRM78	PNRM6 [pRMPN10]	This study
PNRM79	PNRM6 [pRMPN11]	This study
PNRM80	PNRM6 [pRMPN12]	This study
PNRM81	PNRM6 [pRMPN13]	This study
PNRM82	PNRM6 [pRMPN14]	This study
PNRM83	PNRM6 [pRMPN15]	This study
PNRM84	PNRM6 [pRMPN16]	This study
PNRM85	PNRM6 [pRMPN17]	This study
PNRM119	PNRM6 [pRMPN19]	This study
PNRM120	PNRM6 [pRMPN21]	This study
PNRM121	PNRM6 [pRMPN22]	This study
PNRM122	PNRM6 [pRMPN23]	This study
PNRM123	PNRM6 [pRMPN24]	This study
PNRM124	PNRM6 [pRMPN25]	This study
PNRM126	RMA4337 [pAC/pBADT7-1]	This study
PNRM134	PNRM126 [pRMPN1]	This study
PNRM131	PNRM126 [pRMPN7]	This study
PNRM132	PNRM126 [pRMPN15]	This study
PNRM133	PNRM126 [pRMPN16]	This study
PNRM136	PNRM126 [pRMPN8]	This study
PNRM137	PNRM126 [pRMPN10]	This study
PNRM140	PNRM126 [pRMPN13]	This study
PNRM141	PNRM126 [pRMPN14]	This study
PNRM142	PNRM126 [pRMPN9]	This study
PNRM143	PNRM126 [pRMPN11]	This study
PNRM144	PNRM126 [pRMPN19]	This study
PNRM145	PNRM126 [pRMPN24]	This study
PNRM146	PNRM126 [pRMPN25]	This study
PNRM147	PNRM126 [pRMPN23]	This study
PNRM148	PNRM126 [pRMPN21]	This study

Table 1: cont.

Strains or Plasmids	Characteristics	Reference
***S. flexneri* Strains**		
PNRM149	PNRM126 [pRMPN22]	This study
PNRM150	PNRM126 [pRMPN12]	This study
PNRM151	PNRM126 [pRMPN17]	This study
E.coli strains		
XL10-G	*TetrD[mcrA]183 D[mcrCB-hsdSMR-mrr]173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F´ proAB lacIqZDM15 Tn10[Tetr] Camr]*	Stratagene
Lemo21[DE3]	*fhuA2 [lon] ompT gal [λ DE3] [dcm] ΔhsdS/ pLemo[CamR] λ DE3 = λ sBamHIo ΔEcoRI-B int:: [lacI :: PlacUV5 ::T7 gene1] i21 Δnin5* pLemo = pACYC184-*PrhaBAD-lysY*	New England Biolabs
PNRM15	Lemo21(DE3) [pRMPN1]	This study
Plasmids		
pRMCD6	Source of wzy_{Sf} [modified codons at positions 4, 9, and 23].	(18)
pAC/pBADT7-1	Source of T7 RNA polymerase; Cm^R	(34)
pWaldo-TEV-GFP	Cloning vector with GFP tag; Km^R	(28)
pRMPN1	pWaldo-wzy_{Sf}-GFP; Km^R	This study
pRMPN7	pRMPN1 with G130V point mutation in the wzy_{Sf} gene	This study
pRMPN8	pRMPN1 with L111I point mutation in the wzy_{Sf} gene	This study
pRMPN9	pRMPN1 with N86K point mutation in the wzy_{Sf} gene	This study
pRMPN10	pRMPN1 with L28V point mutation in the wzy_{Sf} gene	This study
pRMPN11	pRMPN1 with P165S point mutation in the wzy_{Sf} gene	This study
pRMPN12	pRMPN1 with G82C point mutation in the wzy_{Sf} gene	This study
pRMPN13	pRMPN1 with N147K point mutation in the wzy_{Sf} gene	This study
pRMPN14	pRMPN1 with L191F point mutation in the wzy_{Sf} gene	This study
pRMPN15	pRMPN1 with L214I point mutation in the wzy_{Sf} gene	This study
pRMPN16	pRMPN1 with P352H point mutation in the wzy_{Sf} gene	This study
pRMPN17	pRMPN1 with V92M point mutation in the wzy_{Sf} gene	This study
pRMPN19	pRMPN1 with F52Y point mutation in the wzy_{Sf} gene	This study
pRMPN21	pRMPN1 with F52C/I242T point mutations	This study

	in the *wzy*$_{Sf}$ gene

Table 1: cont.

Strains or Plasmids	Characteristics	Reference
Plasmids		
pRMPN22	pRMPN1 with C60F point mutation in the *wzy*$_{Sf}$ gene	This study
pRMPN23	pRMPN1 with Y137H point mutation in the *wzy*$_{Sf}$ gene	This study
pRMPN24	pRMPN1 with L49F/T328A point mutation in the *wzy*$_{Sf}$ gene	This study
pRMPN25	pRMPN1 with F54C point mutation in the *wzy*$_{Sf}$ gene	This study
pCACTUS	Suicide vector containing *sacB*, Cm^R, and *Ori*$_{ts}$	(14)
pRMA577	Suicide vector contatining *Sph*I-*Sph*I fragment with the *rol* gene	(14)
pCACTUS-*wzz*$_{Sf}$::Tc^R	Suicide mutagenesis construct to construct the strain RMA4337	This study

***** Km^R, Kanamycin-resistant; Cm^R, Chloramphenicol-resistant; Tc^R, Tetracycline-resistant.

715

716 **Table 2.** ColE2 sensitivity, Sf6c sensitivity, and Wzy_{Sf}-GFP expression of the controls and different classes of mutants.

Strain	**Relevant details**			**ColE2 sensitivity***		**Sf6c sensitivity***		**Relative Wzy_{Sf}-GFP**	
				(Δ*wzy* background)	**(Δ*wzy*/Δ*wzz* background)**	**(Δ*wzy* background)**	**(Δ*wzy*/Δ*wzz* background)**	**(Δ*wzy* background)**	**(Δ*wzy*/Δ*wzz* background)**
RMM109	wzy_{Sf} mutants			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 wzy_{Sf} mutant			-	1/256	-	R	-	-
PNRM126	Negative control			-	1/256	-	R	-	-
PNRM134	Positive control			-	R	-	10^{-6}	-	17%
Mutant	**Mutant class**	**Mutant class**	**Topology map location#**	**ColE2 sensitivity***		**Sf6c sensitivity***		**Relative Wzy_{Sf}-GFP**	
	(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)**
P352H	Class A	Class B	PL6	1/64	1/128	R	R	36%	38%
V92M	Class A	Class E	PL2	1/32	1/64	R	R	84%	76%
Y137H	Class A	Class E	TM5	1/32	1/64	R	R	87%	97%
L214I	Class B	Class C	TM8	1/128	1/128	R	R	1.4%	0.03%
G130V	Class C	Class F	TM5	1/512	1/128	R	R	1.60%	28.50%
N147K	Class D	Class E	PL3	R	1/16	10^{-6}	R	21%	52%
P165S	Class D	Class E	PL3	1/4	1/64	10^{-5}	N	7%	125%
L191F	Class D	Class E	TM7	R	1/16	10^{-6}	N	162%	64%
C60F	Class D	Class E	TM1	R	1/64	10^{-6}	N	30%	66%
L49F/T328A	Class D	Class E	TM2/CL5	R	1/64	10^{-6}	R	65%	68%
L28V	Class D	Class E	PL1	R	1/8	10^{-6}	N	55%	80%
N86K	Class D	Class E	PL2	R	1/64	10^{-6}	R	84%	82%
F54C	Class D	Class E	CL1	R	1/64	10^{-6}	R	42%	71%
F52Y	Class D	Class E	TM2	R	1/64	10^{-6}	R	47%	63%
L111I	Class D	Class E	TM4	R	1/8	10^{-6}	N	41%	51%
G82C	Class D	Class E	PL2	R	1/64	10^{-6}	R	40%	47%
F52C/I242T	Class D	Class E	TM2/TM9	R	1/64	10^{-6}	N	82%	81%

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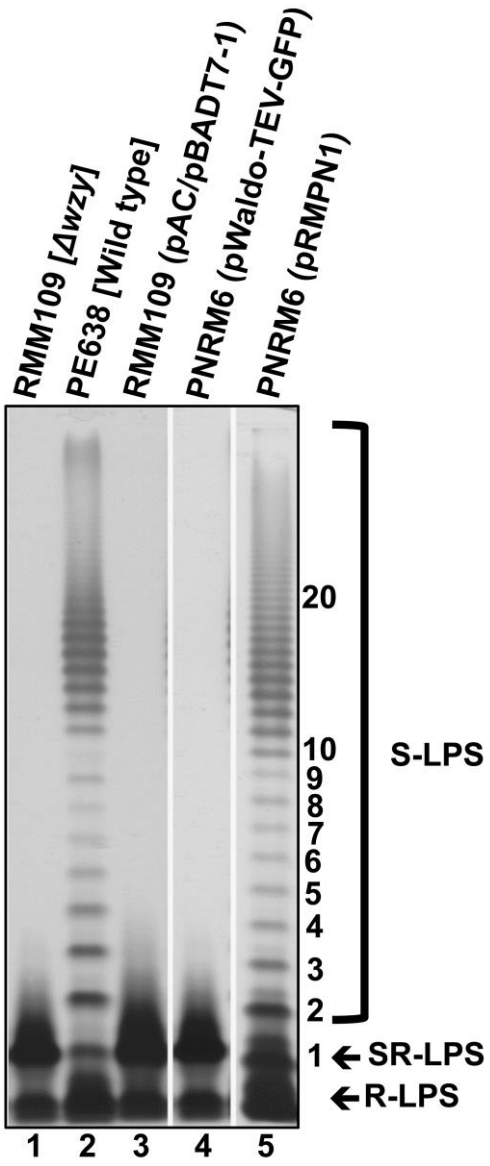
718 *R- Resistant, N- plaques detected with undiluted Sf6c stock; # PL - Periplasmic loop, TM - Trans-membrane region, CL – Cytoplasmic loop (See Fig. 2).

719 ## Figures

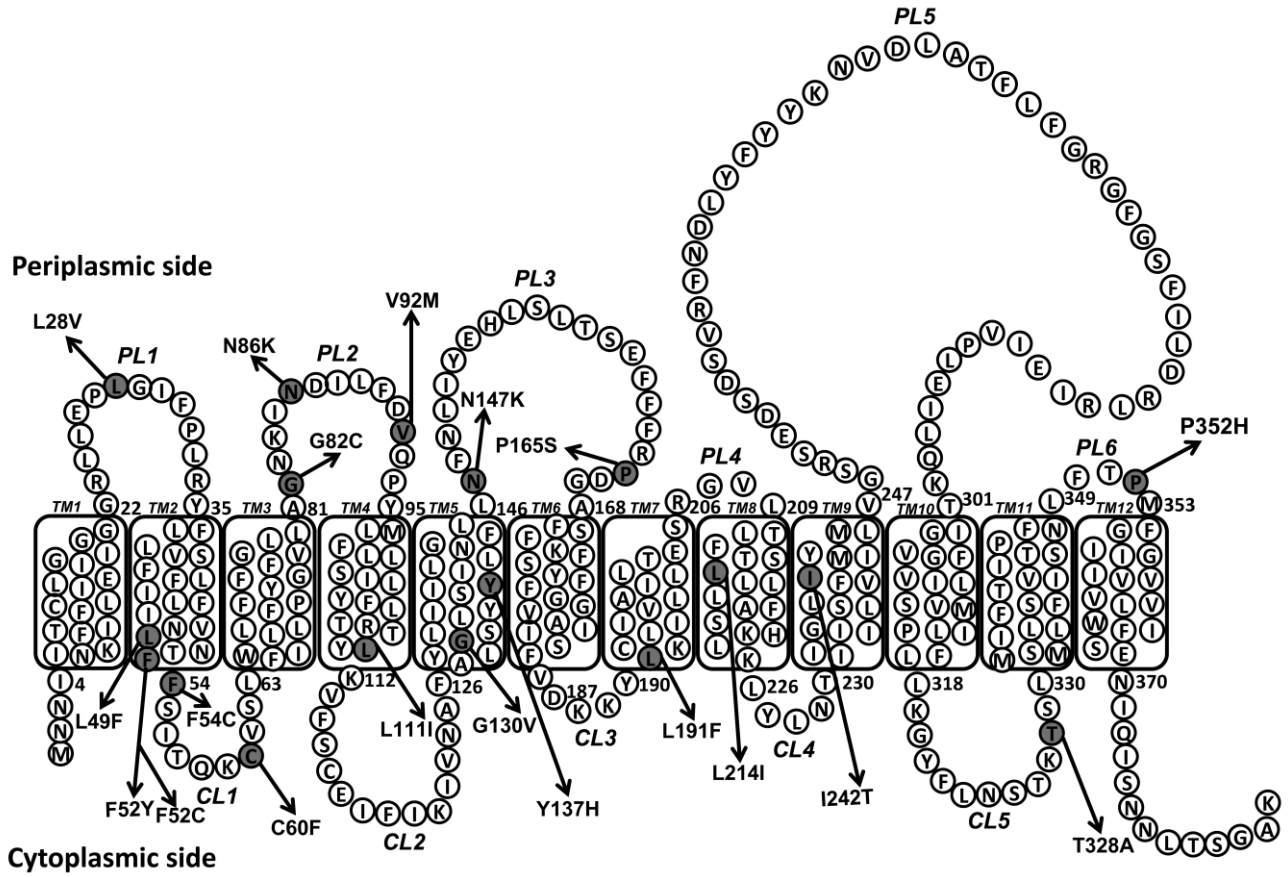


720

721 **Fig. 1. Complementation of *wzy*$_{Sf}$ deficiency by Wzy$_{Sf}$-GFP**

722 LPS samples (equivalent to 1 × 10^9 bacterial cells) were prepared from the strains indicated
723 in the figure by proteinase K treatment, electrophoresed on a SDS-15% (w/v) PAGE gel,
724 and silver stained (See Materials and Methods). Strains in each lane are as follows: 1.

RMM109, 2. PE638, 3. PNRM6 (RMM109 [pAC/pBADT7-1]), 4. PNRM6 [pWaldo-TEV-GFP], 5. PNRM13 (PNRM6 [pRMPN1]). Positions of S-LPS, SR-LPS, and R-LPS are indicated. The numbers on the RHS indicate the Oag RU.



735

736 **Fig. 2. Location of the mutations on the topology map of Wzy_{Sf}.**

737 Mutational alterations were indicated by arrows on the Wzy_{Sf} topology map (adapted from
738 Daniels et al. 1998). The position of the periplasmic loops (PL) 1-5, transmembrane regions
739 (TM) 1-12, cytoplasmic loops (CL) 1-5, and inner membrane (IM) are indicated. Mutations
740 (shaded circles) were located in the PL – 1, 2, 3, 6; TM – 2, 4, 5, 7, 8, 9; and CL – 1, 5.

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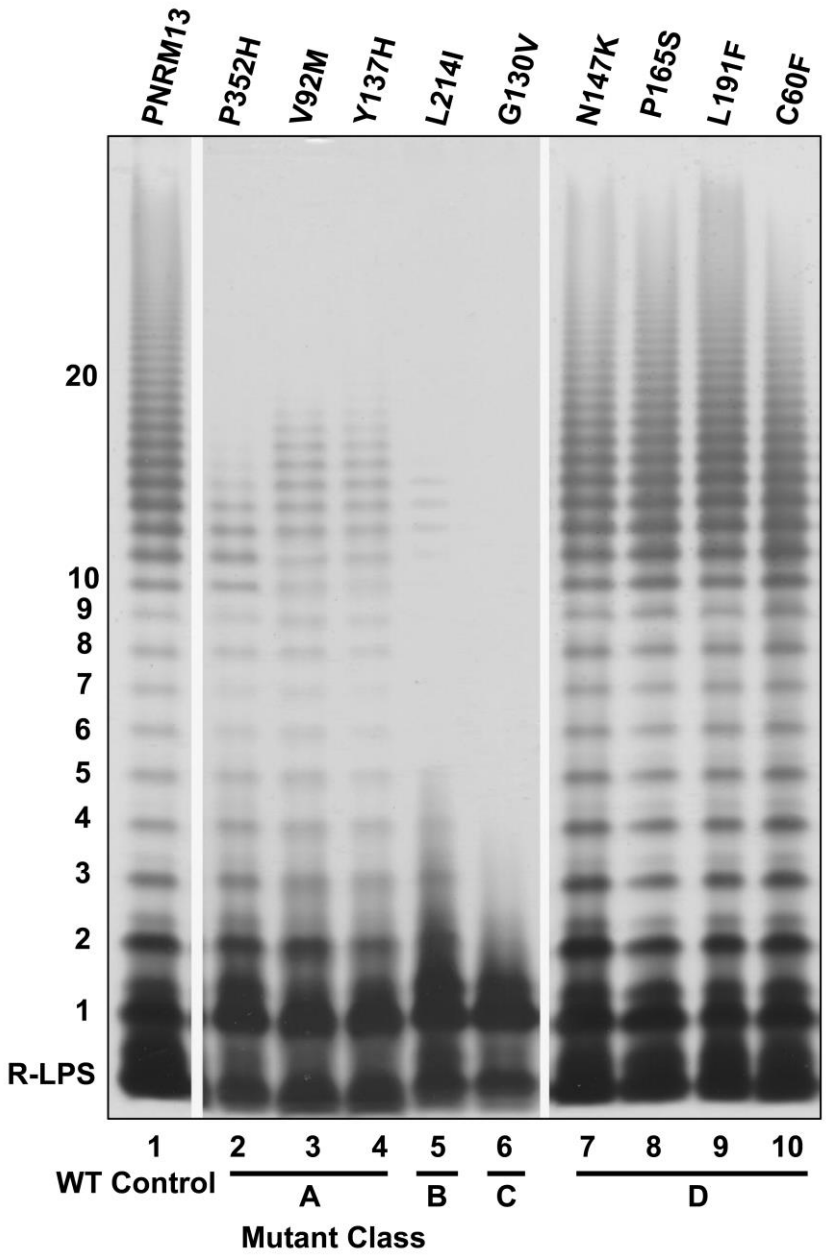

744

745 **Fig. 3. LPS phenotype conferred by different Wzy_{Sf} mutants expressed in PNRM6**
746 **(Δ*wzy*$_{Sf}$, [pAC/pBADT7-1]).**

747 Plasmid encoded mutated Wzy_{Sf} proteins were expressed in PNRM6. Strains were grown
748 and induced with arabinose as described in the Materials and Methods. LPS samples were
749 prepared, electrophoresed on a SDS-15% (w/v) PAGE gel, and silver stained (See

750 Materials and Methods). Strains were divided into various mutant Classes (A, B, C, and D)
751 based on their LPS phenotype.

752 Lane 1, the positive control strain PNRM13 (PNRM6 [pRMPN1]). Lanes 2-18 are the Δ*wzy*
753 strain (PNRM6) with plasmids encoding mutated Wzy_{Sf} protein. The Wzy_{Sf} mutants in each
754 lane are: 2. P352H (Class A), 3.V92M (Class A), 4. Y137H (Class A), 5. L214I (Class B), 6.
755 G130V (Class C), 7. N147K (Class D), 8. P165S (Class D), 9. L191F (Class D), 10. C60F
756 (Class D). Position of R-LPS is indicated and the numbers on the LHS indicate the Oag RU.

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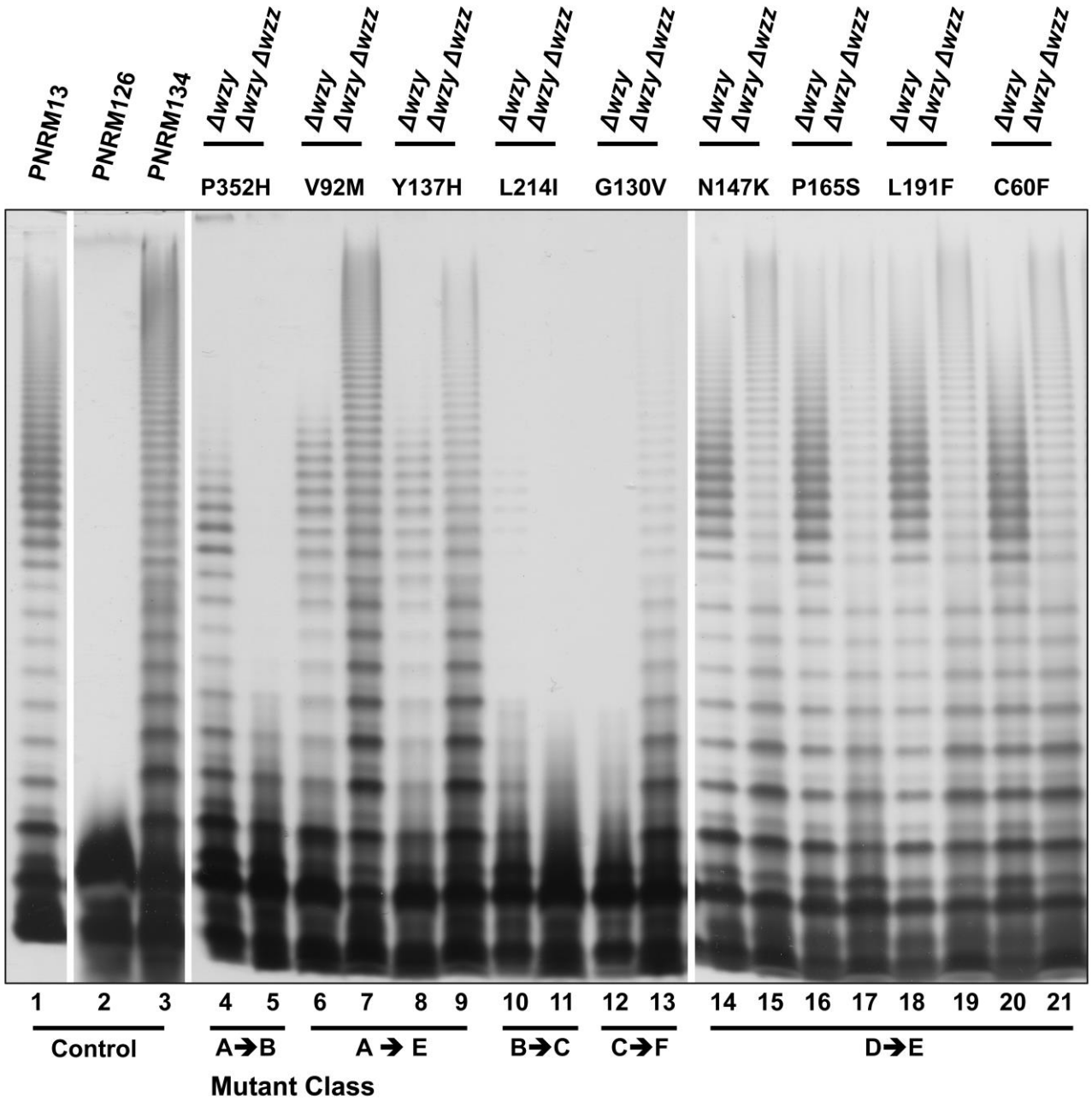

762

763 **Fig. 4. Comparison of the LPS phenotype conferred by the Wzy_{Sf} mutants expressed**

764 **in the ∆*wzy* and ∆*wzy* ∆*wzz* backgrounds.**

765 Plasmids encoding mutated Wzy_{Sf} proteins were expressed in PNRM126 (RMA4337

766 [pAC/pBADT7-1]) and PNRM6 (RMM109 [pAC/pBADT7-1]). Strains were grown and

767 induced as described in the Materials and Methods. LPS samples were electrophoresed on
768 a SDS-15% (w/v) PAGE gel and silver stained (See Materials and Methods).

769 Lanes 1-3 are: 1. PNRM13, and 2. PNRM126, and 3. PNRM134 (PNRM126 [pRMPN1]).
770 Lanes 4-36 are the *Δwzy* (PNRM6) or *Δwzy Δwzz* (PNRM126) strains with plasmids
771 encoding mutated Wzy_{Sf} protein. The Wzy_{SF} mutants in each lane are as follows: 4. P352H
772 (*Δwzy*), 5. P352H (*Δwzy Δwzz*), 6. V92M (*Δwzy*), 7. V92M (*Δwzy Δwzz*), 8. Y137H (*Δwzy*),
773 9. Y137H (*Δwzy Δwzz*), 10. L214I (*Δwzy*), 11. L214I (*Δwzy Δwzz*), 12. G130V (*Δwzy*), 13.
774 G130V (*Δwzy Δwzz*), 14.N147K (*Δwzy*), 15. N147K (*Δwzy Δwzz*), 16. P165S (*Δwzy*), 17.
775 P165S (*Δwzy Δwzz*), 18. L191F (*Δwzy*), 19. L191F (*Δwzy Δwzz*), 20. C60F (*Δwzy*), 21.
776 C60F (*Δwzy Δwzz*).

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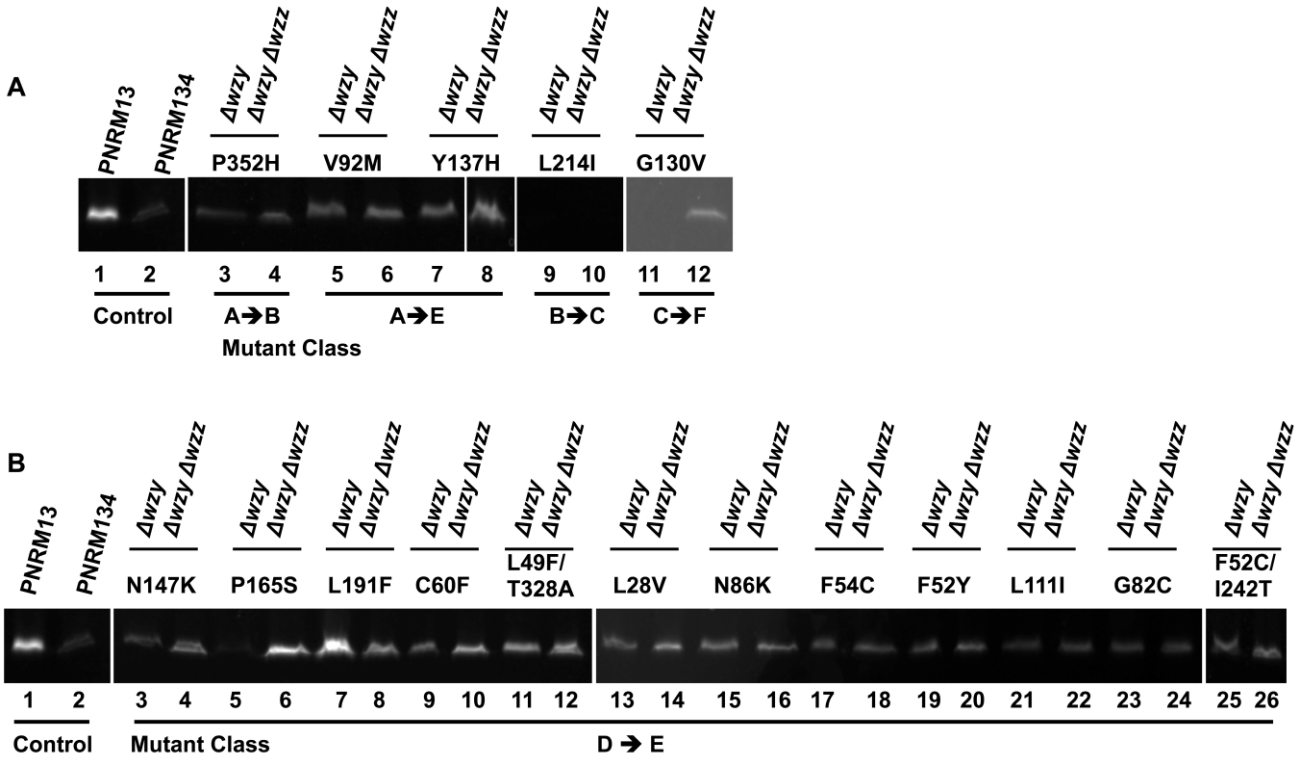

785

786 **Fig. 5. Protein expression level of the mutated Wzy_{Sf}-GFP compared to the positive**
787 **control.**

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

791 **A.** Strains in lanes 1-2 are as follows: 1. PNRM13 (PNRM6 [pRMPN1]), 2. PNRM134
792 (PNRM126 [pRMPN1]). Lanes 3-20 are the *Δwzy* or *Δwzy Δwzz* strains expressing mutated
793 Wzy_{Sf}-GFP. The Wzy_{Sf} mutants in each lane are as follows: 3. P352H (*Δwzy*), 4. P352H
794 (*Δwzy Δwzz*), 5. V92M (*Δwzy*), 6. V92M (*Δwzy Δwzz*), 7. Y137H (*Δwzy*), 8. Y137H (*Δwzy*
795 *Δwzz*), 9. L214I (*Δwzy*), 10. L214I (*Δwzy Δwzz*), 11. G130V (*Δwzy*), 12. G130V (*Δwzy*
796 *Δwzz*).

B. Strains in lanes 1-2 are as follows: 1. PNRM13, 2. PNRM134. Lanes 2-28 are the *Δwzy* or *Δwzy Δwzz* strains expressing mutated Wzy_{Sf}-GFP. The Wzy_{Sf} mutants in each lane are as follows: 3. N147K (*Δwzy*), 4. N147K (*Δwzy Δwzz*), 5. P165S (*Δwzy*), 6. P165S (*Δwzy Δwzz*), *7.* L191F (*Δwzy*), 8. L191F (*Δwzy Δwzz*), 9. C60F (*Δwzy*), 10. C60F (*Δwzy Δwzz*), 11. L49F/T328A (*Δwzy*), 12. L49F/T328A (*Δwzy/ Δwzz*), 13. L28V (*Δwzy*), 14. L28V (*Δwzy Δwzz*), 15. N86K (*Δwzy*), 16. N86K (*Δwzy Δwzz*), 17. F54C (*Δwzy*), 18. F54C (*Δwzy Δwzz*), 19. F52Y (*Δwzy*), 20. F52Y (*Δwzy Δwzz*), 21. L111I (*Δwzy*), 22. L111I (*Δwzy Δwzz*), 23. G82C (*Δwzy*), 24. G82C (*Δwzy Δwzz*), 25. F52C/I242T (*Δwzy*), 26. F52C/I242T (*Δwzy Δwzz*).