Coiled-coil regions play a role in the function of the 
*Shigella flexneri* O-antigen chain length regulator

**Wzz<sub>pHS2</sub>**

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Regulation of the length of the O-antigen (Oag) chain attached to LPS in *Shigella flexneri* is important for virulence and is dependent on the inner-membrane protein Wzz. A lack of high-resolution structural data for Wzz has hampered efforts so far to correlate mutations affecting function of Wzz with structure and describe a mechanism for chain length regulation. Here we have used secondary structure prediction to show that the periplasmic domain of the Wzz<sub>pHS2</sub> protein has three regions of significant coiled-coil (CC) potential, two of which lie within an extended helical region. We describe here the first site-directed mutagenesis study to investigate the role of individual predicted CC regions (CCRs) in Wzz function and oligomerization. We found that CCRs 2 and 3 are necessary for wild-type Oag chain length regulation by Wzz<sub>pHS2</sub>. The in vivo cross-linking profile of mutants affected in the three CCRs was not altered, indicating that individually each CCR is not required for oligomerization. Interestingly, the CCR3 mutation resulted in a temperature-sensitive phenotype and an inhibitory effect on Oag polymerization. Analysis of Wzz<sub>pHS2</sub> and the mutant constructs in a *S. flexneri* degP mutant showed that DegP did not affect the function of wild-type Wzz<sub>pHS2</sub> but its presence influenced the phenotype of the Wzz<sub>pHS2</sub> CCR3 mutant. Additionally, the phenotype of the Wzz<sub>pHS2</sub> CCR3 mutant was suppressed by a *cis* mutation near the putative cytoplasmic C-terminus of Wzz<sub>pHS2</sub>.

**INTRODUCTION**

Biosynthesis of O-antigen (Oag) in *Shigella flexneri* occurs by a Wzy-dependent mechanism (Raetz & Whitfield, 2002; Valvano, 2003). Briefly, tetrasaccharide repeat units (RUs) linked to undecaprenol phosphate (Und-P) are assembled on the cytoplasmic face of the inner membrane (IM) and transferred to the periplasm via the putative flippase, Wzx, located in the IM. Polymerization of the RUs occurs in the periplasm and is dependent on the IM polymerase, Wzy. The process of polymerization involves the addition of the growing chain onto an Und-P-linked single RU such that the chain is increased by one RU at the reducing end. The resulting Oag chains are not random in length but are typically clustered around a certain number of Oag RUs determined by the presence of Wzz, also located in the IM. After polymerization, Oag chains are ligated to preformed lipid A-core by the IM ligase WaaL and transported to the outer membrane as LPS. In *Escherichia coli*, assembly of LPS in the outer membrane has recently been shown to require a protein complex involving Imp/RlpB (Wu et al., 2006) and Sperandeo et al. (2007) have also provided evidence that the essential genes *lptA* and *lptB* are involved. It is thought that components of the Oag biosynthesis machinery such as Wzz, Wzx and Wzy may interact to form a complex in the IM. At present, the mechanisms and putative protein–protein interactions underlying the biosynthesis of Oag are not well understood, in part due to the difficulties encountered with expression and purification of the integral membrane proteins involved.

*S. flexneri* 2a strains have a bimodal distribution of Oag consisting of short-type (S-type) and very-long-type (VL-type) Oag chains. To maintain a bimodal pattern *S. flexneri* 2a has two forms of Wzz encoded by two separate genes. The *wzzSF* gene is located on the chromosome and is responsible for S-type Oag chains of 11–17 RU (Morona et al., 1995) while the *wzzpHS2* gene is located on a small plasmid called pHS-2 and is responsible for VL-type Oag chains of approximately 90–100 RU (Stevenson et al., 1995). The VL-type Oag chains resulting from the presence of *wzzpHS2* are advantageous to the cell because they confer resistance to complement (Hong & Payne, 1997). A model

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**Abbreviations:** CC, coiled-coil; CCR, coiled-coil region; IM, inner membrane; Oag, O-antigen; PCP, polysaccharide copolymerase; RU, repeat unit; S-type, short-type; TM, transmembrane; ts, temperature sensitive; VL-type, very long type.

The GenBank accession number for the sequence reported in this paper is EU220028.
has been described by Morona et al. (2003) where in the absence of S-type Oag chains it is postulated that surface-expressed IcsA can be masked by VL-type Oag, leading to defective actin-based motility, and therefore a mixture of the two chain lengths is required for full virulence.

Wzz homologues are found in all bacteria that use a Wzy-dependent mechanism for Oag biosynthesis. Proteins related to the Wzz homologues are also involved in capsule and exopolysaccharide biosynthesis, for example Wzc in E. coli and ExoP in Rhizobium meliloti (Becker et al., 1995; Whitfield et al., 1997). To reflect the related function of these proteins they have been grouped together as polysaccharide copolymerases (PCPs) (Morona et al., 2000b), but within this group they are subdivided according to the polysaccharides they process and other structural features. Wzz homologues form a distinct subgroup of PCPs known as the PCP1 family (Morona et al., 2000b). While primary sequence identity between pairs in this group may be as low as 23%, they are similar in size, ranging from 36 to 41 kDa, and share a common topology. They have two transmembrane (TM) domains flanking a periplasmic domain (Morona et al., 1995) and a conserved proline-rich region, PX2PX4SPKX1X10GGMXGAG, overlapping the second TM region (Becker & Puhler, 1998; Becker et al., 1995; Daniels & Morona, 1999; Morona et al., 2000b). The periplasmic domain of Wzz proteins is predicted to be mostly \( \alpha \)-helical in structure (Bastin et al., 1993) and has significant coiled-coil (CC) potential (Morona et al., 2000b). The features described above are illustrated for Wzz pHS2 in Fig. 1.

Two models for Wzz activity have been proposed. Bastin et al. (1993) described Wzz as a timer of Wzy activity, switching it between polymerization and transfer of the chain to WaaL for ligation. Alternatively, Morona et al. (1995) proposed that Wzz is a molecular chaperone that determines the ratio of Wzy to WaaL, which in turn affects ligation kinetics. While the models are not mutually exclusive, \textit{in vivo} genetic evidence is now accumulating that supports the molecular chaperone model. For example, overexpression of Wzy can alter the modal distribution of Oag chains, and altered ratios of Wzz in bimodal strains can favour production of one modal length over another, indicating that the correct balance of these proteins is an important aspect of Oag chain length regulation (Carter et al., 2007; Daniels et al., 1998). Additionally, Marolda et al. (2006) have also shown that concomitant expression of

![Fig. 1. Features of Wzz pHS2 as shown in a schematic diagram of the ORF (a) and at the nucleotide sequence and amino acid sequence level (b). Panel (a) shows the relative positions of the TM and CC regions (boxes) in the Wzz pHS2 ORF. The proline/glycine-rich region (Pro/Gly) is underlined. Underneath the ORF is a representation of the constructs created in this study, with their names to the right. The positions of mutations made within the CCRs are indicated by crosses and the substitutions identified above each construct. The ‘v’ indicates the extra downstream mutation observed in pRMA2544. Panel (b) shows the nucleotide and amino acid sequence of Wzz pHS2 (EU220028). Residues in CCR1 (aa 136–162), CCR2 (aa 196–225) and CCR3 (aa 22–247) are underlined. Residues targeted for mutation within these regions are in bold type. Predicted secondary structural features are highlighted with italicized coloured text: red indicates \( \alpha \)-helices and blue indicates \( \beta \)- strand.]
either Wzy or Wzz can interfere with complementation of heterologous systems with Wzx. Any model also needs to take into account the interchangeability of Wzz proteins (Klee et al., 1997; Murray et al., 2006).

Specific functional domains have not been identified in Wzz so far, and it is apparent from in vivo genetic studies that individual residues throughout Wzz are important (Daniels & Morona, 1999; Franco et al., 1998; Klee et al., 1997). It should be noted, however, that creation of hybrid Wzz proteins has indicated that the preference for a certain modal length can be determined by the C-terminal half of the protein (Daniels & Morona, 1999).

Oligomerization of Wzz is believed to be important for its correct function. In vivo chemical cross-linking has demonstrated that Wzz358 can form oligomers and that mutants with altered oligomerization profiles were non-functional (Daniels & Morona, 1999). Oligomerization has since been shown by in vivo cross-linking for other Wzz proteins (Daniels et al., 2002; Guo et al., 2006).

Studies using purified Wzz proteins and related proteins have recently been published, allowing greater insight into structural aspects of Oag chain length regulation. For example cryo-electron microscopy has been used to provide evidence of a tetrameric form of the related PCP2 protein, Wzc, which is involved in group 1 capsule biosynthesis in E. coli (Collins et al., 2006). Recently the Wzz protein from E. coli O86:H2 has been purified and analysed by circular dichroism, size-exclusion chromato-graphy and small-angle X-ray scattering, showing that this protein is largely helical in nature and can exist as tetramers in solution (Guo et al., 2006; Tang et al., 2007).

The CC motif (Burkhard et al., 2001; Lupas, 1996a) is a widespread structural element that mediates protein–protein interactions. Evidence is beginning to emerge that the CC regions (CCRs) in Wzz contribute to oligomerization and function. For example, the number of CCRs in Wzz proteins is positively correlated with the length of Oag chain they control (Morona et al., 2000), and recently it was shown that a periplasmic fragment of E. coli O86:H2 Wzz with predicted CC potential was sufficient for interaction with full-length Wzz in a pull-down assay (Tang et al., 2007).

We hypothesize that CCRs are involved in the function of WzzpHS2 via facilitation of protein–protein interactions. In this study we performed site-directed mutagenesis on WzzpHS2, creating a panel of mutants with alterations to predicted CCRs, and determined their ability to complement a S. flexneri Wzz mutant strain. We found that CCRs 2 and 3 are necessary for function of WzzpHS2; however, they were not essential for oligomerization. Alteration to CCR3 led to a protein with a temperature-sensitive phenotype and an inhibitory effect on Oag polymerization. The negative effects of this mutant were dependent on the presence of the chaperone/protease DegP. We also isolated a cis suppressor of the mutation in CCR3 located very near the putative cytoplasmic C-terminus of WzzpHS2, leading to the hypothesis that this region may play a role in regulating Wzz activity.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study are described in Table 1. Bacteria were routinely grown in Luria–Bertani broth (Bacto tryptone 10 g l⁻¹, Bacto yeast extract 5 g l⁻¹, NaCl 5 g l⁻¹) with aeration. Growth temperatures are stated for each experiment in the Results and figure legends. Antibiotics were used at the following concentrations where appropriate: ampicillin, 100 μg ml⁻¹; kanamycin, 50 μg ml⁻¹; chloramphenicol, 25 μg ml⁻¹. Luria–Bertani agar was LB broth with 15 g Bacto agar 1⁻¹ added.

**DNA methods.** The plasmids used and constructed in this study are described in Table 1. PCR for construction of clones was performed with Phusion High Fidelity DNA polymerase (Finnzymes). Oligonucleotides were purchased from Generics. Restriction enzymes, T4 DNA ligase and Taq DNA polymerase for general PCR screening were purchased from New England Biolabs. Cloning into the pGEM-T Easy vector was performed using reagents supplied with the vector (Promega). All constructs created in this study were confirmed by restriction digestion and sequencing. Applied Biosystems BigDye Terminator version 3.1 sequencing reagent was used for sequencing reactions in our laboratory according to the protocol outlined by the Australian Genome Research Facility (AGRF) (St Lucia, QLD). Sequencing samples were processed and analysed at the AGRF. Eppendorf thermocyclers 24 or 96 were used for PCR and sequencing. Plasmids were introduced into S. flexneri by electroporation. Electrocompetent cells were prepared by washing exponential-phase cells in ice-cold, ultrapure water (MilliQ) twice, with final resuspension in ice-cold 10% (v/v) glycerol. Electroporation was performed using a Bio-Rad GenePulser. E. coli DH5α was used as host during construction of clones with the following exceptions: JM110 was used as host when BclI restriction was required and DH5αpir was used for construction based on pCVD442. Plasmid DNA was introduced into E. coli strains via electroporation (as described above for S. flexneri) or chemical transformation. Chemically competent cells were prepared by washing exponential-phase cells in ice-cold 0.1 M MgCl2 followed by incubation for 1 h on ice in 0.1 M CaCl2 and final resuspension in ice-cold 0.1 M CaCl2 with 10% (v/v) glycerol.

**Cloning of WzzpHS2 into pLITMUS29 and site-directed mutagenesis of CCRs using overlap-extension PCR.** The 1.352 kb NsiI fragment containing wzz358 (EU220028) was digested from pMN4, a Cm² derivative of pH5-2 (Hong & Payne, 1997) and ligated into the NsiI site of pLITMUS29, resulting in plasmid pRMA2352. The NsiI fragment was sequenced and found to be in the same direction as the lac promoter encoded by the vector. Overlap-extension PCR was used to create specific basepair changes in individual CCRs of WzzpHS2. Two primers were designed to anneal within the plasmid pLITMUS29 sequence such that they flanked the NsiI site of WzzpHS2, fragment in pRMA2352: 5'-GGATCCAGCAGTATCC-TGGAG-3' and 5'-GGGCTTTGACTAGAGGGAAG-3'. To introduce specific mutations into CCR1, CCR2 and CCR3, respectively, pairs of overlapping internal primers were designed to contain the specific nucleotide substitutions [CCR1 (A135G/L138G), 5'-GATATATGGGCTCAATGCTG--TTCGAGTATCC-3' and 5'-GCACTAGGAGCTTCTGACGAG-3'], CCR2 (12049/K207G), 5'-GATAGGAGATGGAGAATGCAAGGCTATAGGGT-3' and 5'-AACTTCTAGCTTAGAGT-3'], CCR3 (R227G/L320G), 5'-GATTAAAA-ATTTGAAACCCAGGGAGAAGCAGAT-3' and 5'-ATCTCAGCCATGGAT-3'. For each mutant, a first round of
PCR was performed with each flanking primer and a corresponding internal primer using pRMA2352 as template, leading to the generation of two overlapping fragments. The second round of PCR was performed using only the flanking primers and the two overlapping fragments as the template, resulting in a single fragment. This final PCR product was digested with NdeI and cloned into the NdeI site of pLITMUS29, giving rise to pRMA2606, pRMA2354 and pRMA2548 containing the CCR1, CCR2 and CCR3 mutations, respectively. Constructs were sequenced to confirm the incorporation of these mutations in pLITMUS29. The CCR1 mutant in pLITMUS29 was then subcloned into the expression host, pRMA2352.

### Construction of a *S. flexneri* degP mutant strain.

Primer pairs were designed and used with the *S. flexneri* 2a 2457T genome sequence (NC_004741) and the primers used by Purdy et al. (2002), with the following sequences: 5′-TACATTGTCGAGCGAAGC-3′ and 5′-CACCACATTGGTCTGA-3′. These primers were used to amplify a 2982 bp region encompassing degP from *S. flexneri* strain RMA2163. The PCR product was cloned into pGEM-T Easy, resulting in pRMA2784. The 1 kb chloramphenicol resistance (CmR) fragment from pRMA2784 was then subcloned into the *Bcl*I site of pRMA2770, giving rise to pRMA2787. The XmnI/Spfl insert from pRMA2787 containing the interrupted degP gene was then subcloned into corresponding sites in pCVD442, resulting in pRMA2798, pRMA2798 was conjugated into RMA2163, and sucrose/Cm/Km-resistant and Ap-sensitive colonies were selected, one of which was isolated and named RMA2804. The CmR cartridge was not removed using the FLP/FRT feature.

### Purification of His<sub>6</sub>-WzzpHS2 and production of polyclonal WzzpHS2 antibodies.

Plasmid pMN4 was used as a template for PCR to amplify the WzzpHS2 ORF using oligonucleotides 5′-GGGGTACCAAGAATCTGCTTGCTGAC-3′ and 5′-AGCAATTTACTAATTACACACAC-3′, which contain *Kpn*I and *Pst*I sites respectively (underlined). This fragment was cloned into the corresponding sites of the His-tag expression vector pQE-30, resulting in plasmid pRMA2274. N-terminally His<sub>6</sub>-tagged WzzpHS2 was purified from SG13009(pREP4)(pRMA2274) by the following method. The strain was grown overnight with selection. Two litres of broth was inoculated with 40 ml of the overnight culture and the culture was grown for 3 h with selection before induction by the addition of 0.5 mM IPTG. The induced culture was grown for a further 5 h before the cells were harvested by centrifugation. Harvested cells were kept frozen overnight at –20 °C, thawed the next day and washed in 50 mM Tris/HCl pH 7.5. Washed cells were resuspended in 10 mM HEPES, 1 mM MgCl₂ and passed through a French press twice. Unlysed cells and inclusion bodies were removed by low-speed centrifugation. The whole-membrane fraction was isolated from the supernatant by ultra-high-speed centrifugation.

### Table 1. Strains and plasmids used in this study

<table>
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<th>Strain or plasmid</th>
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<td>This study</td>
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*Ap<sup>R</sup>, ampicillin resistance; Km<sup>R</sup>, kanamycin resistance; Cm<sup>R</sup>, chloramphenicol resistance; Te<sup>R</sup>, tetracycline resistance; vir<sup>−</sup>, virulence plasmid negative.

†R. Morona & L. Van Den Bosch, unpublished data.

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Coiled-coil regions are important for function of WzzpHS2.
RESULTS

Overexpression, purification and production of anti-WzzpHS2 antibodies

In order to analyse WzzpHS2 using Western immunoblotting we overexpressed and purified WzzpHS2 and then used the purified protein to generate polyclonal antibodies. His6-WzzpHS2 was overexpressed from pRMA2274 in E. coli SG13009 and purified as described in Methods. The purification was followed by SDS-PAGE and Coomassie staining as shown in Fig. 2. A single band of approx 43 kDa was detected in the eluted fraction (Fig. 2, lane 4), which corresponded with the expected size of His6-WzzpHS2. The purified protein was used to generate rabbit polyclonal antiserum, which was absorbed and affinity-purified and is referred to as WzzpHS2 polyclonal antibodies herein.

Secondary structure prediction and mutagenesis of helical regions in WzzpHS2

Bastin et al. (1993) have predicted that the periplasmic domain of Wzz proteins from E. coli and Salmonella has at least two amphipathic α-helical regions, and this is supported by circular dichroism spectroscopy data indicating that E. coli Wzz is largely α-helical in structure (Guo et al., 2006). Analysis of the WzzpHS2 sequence (EU220028) with the PredictProtein server (Rost et al., 2004) also suggests that the periplasmic region is largely α-helical, with several small isolated areas of β-strand (Fig. 1b). Of note is a long continuous helical region predicted to lie between residues 178 and 246. This region may extend 102 Å (10.2 nm) into the periplasm if a rise of 1.5 Å is assumed for each residue in the helix, giving WzzpHS2 a long hairpin shape anchored in the cell membrane as predicted by Bastin et al. (1993) and now supported by analysis of purified Wzz by small-angle X-ray scattering (Tang et al., 2007). Long periplasmic helices of the same length are also predicted for other Wzz proteins, such as WzzFepE from Salmonella typhimurium, which confers V1-type Oag modal lengths, and also in those that confer...
significantly different modal lengths such as WzzSF and WzzST from *Shigella flexneri* and *Salmonella typhimurium*, respectively (data not shown).

The α-helical region in the Wzz group of proteins (PCP1) has the potential to form CCs (Morona *et al.*, 2000b). The structure of a classical CC (reviewed by Lupas, 1996a) is based on the presence of a heptad repeat in the amino acid sequence. Each position in the heptad is nominally labelled (a), (b), (c), (d), (e), (f), (g), where (a) and (d) are usually hydrophobic. In CC helices, residues in position (a) are found directly above each other on the same side of the helix every second turn, and the hydrophobic residues in position (d) also line up on the same side of the helix as they are found approximately one complete turn after position (a). CCs form when two or more helices interlock in an antiparallel or parallel fashion and are stabilized by hydrophobic core interactions between residues in the (a) and (d) positions. In addition to hydrophobic interactions, polar and ionic interactions between coils play a role and can influence orientation, oligomerization and stability (Burkhard *et al.*, 2001; Lupas, 1996a). We used the COILS program (Lupas, 1996b; Lupas *et al.*, 1991) to show that WzzpHS2 is predicted to have three CCRs, which are located between residues 135 and 162 (CCR1), residues 196 and 225 (CCR2), and residues 224 and 247 (CCR3). The residues within these regions have >50% probability of forming a CC in one or more reference windows of 28, 21 or 14 residues, shown graphically in Fig. 3(a). CCR2 and CCR3 were noted to overlap with the majority of the predicted extended α-helix. In addition to graphical output, COILS also designates the position of the sequential heptad repeats along the predicted CC sequence (data not shown), allowing identification of residues in key positions such as (a) and (d).

To investigate the role of the CCRs in function of WzzpHS2, two residues in the (a) and (d) positions in each CCR were targeted for mutation by overlap-extension PCR (Fig. 1b). They are as follows, with the CC heptad position in parentheses: Ala135 (a) and Leu138 (d) in CCR1 were changed to Gly in pRMA2606; Ile204 (a) and Lys207 (d) in CCR2 were changed to Gly in pRMA2354; and Arg227 (a) and Leu230 (d) in CCR3 were changed to Gly in pRMA2548. We changed the chosen residues to Gly since this is a known helix-breaker. Prediction of CC potential using the mutant amino acid sequences in the COILS program confirmed that the mutations resulted in a

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**Fig. 3.** Prediction of CCRs in WzzpHS2 using COILS (http://www.ch.embnet.org/software/COILS_form.html). Each panel shows the probability of a residue being in a CCR on the y-axis and residue number along the x-axis. The COILS program was used to predict CCRs in the sequence of wild-type WzzpHS2 (EU220028) (a), the CCR1 mutant (b), the CCR2 mutant (c), and the CCR3 mutant (d). Residues with a probability of >50% were designated part of CCRs. The locations of CCR1, 2 and 3 as predicted from the wild-type sequence are detailed in the text. The MTIDK matrix with weights on positions (a) and (d) was used. Each set of peaks is labelled above with its corresponding CCR designation.
reduction in probability of forming a CC for each individual region (Fig. 3b, c d). During the screening for the CCR3 mutant we isolated a clone with a downstream mutation in addition to the CCR3 mutations. This clone was named pRMA2544, and has an S366G substitution (see Fig. 1a for summary of mutant constructs). The mutant forms of WzzpHS2 expressed from pRMA2606, pRMA2354, pRMA2548 and pRMA2544 are herein referred to as WzzpHS2-CCR1, WzzpHS2-CCR2, WzzpHS2-CCR3, WzzpHS2-CCR3/S366G, respectively.

Role of predicted CCRs in LPS Oag chain length regulation

We then examined the effect on LPS Oag production of the mutant WzzpHS2 protein constructs in S. flexneri RMA2163. This strain is naturally deficient for WzzpHS2 since it does not possess the pHS-2 plasmid, and it also has a disrupted copy of chromosomal wzz (Table 1). Consequently, in the absence of both forms of wzz, the LPS Oag chains it produces have unregulated length. This can be seen in Fig. 4(a), lanes 1 and 7, where RMA2163 is carrying pLITMUS29. Wild-type WzzpHS2 resulted in LPS with VL-type Oag chains at both 30 °C and 37 °C (Fig. 4a, lanes 2 and 8). WzzpHS2-CCR1 had no significant impact on production of VL-type Oag chains (Fig. 4a, lanes 3 and 9). WzzpHS2-CCR2 caused a loss of VL-type Oag chain modality and the majority of the LPS Oag chains remained as a continuous unregulated ladder (Fig. 4a, lanes 4 and 10). WzzpHS2-CCR3 caused a loss of VL-type Oag chains and additionally inhibited production of all lengths of LPS Oag chains, especially noticeable at 37 °C (Fig. 4a, lane 5). A temperature-sensitive (ts) effect was also noted for WzzpHS2-CCR3, with production of VL-type Oag chains being partially restored and some production of shorter Oag chain lengths noted at 30 °C (Fig. 4a, lane 11). In addition to the ts effect, the function of WzzpHS2-CCR3 was restored when it carried the cis S366G mutation in WzzpHS2-CCR3/S366G, which allowed production of both VL-type and shorter Oag chains at both 37 °C and 30 °C (Fig. 4a, lanes 6 and 12). Immunoblotting with polyclonal WzzpHS2 antibodies was performed to monitor expression from the same strains, as shown in Fig. 4(b). A single reactive band of approximately 40 kDa, corresponding to the size of WzzpHS2, was detected for each construct.

The inhibitory effect of the WzzpHS2-CCR3 mutant was investigated further by coexpressing this mutant with WzzSF (wild-type) (Fig. 5) in S. flexneri in order to determine if WzzpHS2-CCR3 exhibited any negative dominant effects. We observed LPS with S-type Oag chains when WzzSF and WzzpHS2-CCR3 were coexpressed (Fig. 5, lanes 5 and 6), indicating that the inhibitory effect can be rescued by a relatively small amount of WzzSF expressed from a single copy on the chromosome and therefore that WzzpHS2-CCR3 does not exhibit negative dominance over WzzSF. We performed this experiment at 30 °C and 37 °C and while temperature had no effect on production of S-type Oag chains, the production of VL-type Oag chains by WzzpHS2-CCR3 was abolished at 37 °C, highlighting the Ts nature of this mutant. Protein expression from the WzzpHS2 constructs in the RMA2162 strain background was monitored by Western immunoblotting, and the presence of a band migrating at approximately 40 kDa was detected for all strains (Fig. 5b).

Analysis of CCR mutants in a degP::CmR background

The effect of temperature on WzzpHS2-CCR3 led us to hypothesize that the function of WzzpHS2 and/or mutant forms of WzzpHS2 was affected by the ts periplasmic chaperone/protease DegP (previously known as HtrA) (Lipinska et al., 1989; Strauch & Beckwith, 1988; Strauch et al., 1989). To test this we constructed a S. flexneri degP::cmR mutant based on RMA2163 (wzz::kmR), and designated it RMA2804. On L agar this strain produced colonies of smaller size than the parent strain at 42 °C and normal-sized colonies at 30 °C and 37 °C (data not shown). It also showed a growth defect in broth at 42 °C compared to the parent strain (data not shown), consistent with the phenotypes reported for S. flexneri and E. coli degP strains (Lipinska et al., 1989; Purdy et al., 2002; Strauch et al., 1989).

The wzzpHS2 constructs in pLITMUS29 were introduced into RMA2804 and the LPS profile was observed after growth of all strains at 30 °C (Fig. 6a). DegP did not affect LPS Oag production regulated by wild-type WzzpHS2, indicating that it is not essential for production of VL-type chains. Likewise, DegP had no effect on LPS Oag in the presence of the CCR1, CCR2 or CCR3/S366G WzzpHS2 mutants. Notably, we observed that the absence of DegP restored wild-type function to the WzzpHS2-CCR3 mutant (Fig. 6a, lane 10). DegP did not affect protein expression of the WzzpHS2 mutants as shown by Western immunoblotting (Fig. 6b).

Analysis of oligomerization of WzzpHS2 by in vivo cross-linking with formaldehyde

We next assessed the ability of WzzpHS2 and the mutant constructs to form oligomers, to determine if any of the LPS Oag phenotypes seen could be correlated with the oligomerization profile. Although oligomerization has not previously been demonstrated for WzzpHS2, we predicted it would form oligomers in a similar manner to WzzSF (Daniels & Morona, 1999). Cross-linking was performed in strains RMA2163 (wzz::kmR) and RMA2804 (wzz::kmR, degP::cmR); the strains were grown at 30 °C prior to cross-linking. Additionally, samples were solubilized at 60 °C (rather than 100 °C) prior to electrophoresis so as to preserve any cross-links. Western immunoblotting revealed a similar profile of cross-linked bands for all strains (Fig. 7). In cross-linked samples, species of approximately 80 kDa and 120 kDa were observed in addition to the monomer at
40 kDa. It was also noted that the species at 80 kDa appeared as a doublet and as many as three bands could be distinguished at or just below 120 kDa. WzzpHS2-CCR1 appeared to show a reduced amount of the 80 kDa and 120 kDa oligomers in the presence of DegP (Fig. 7, lanes 5 and 6); however, independently prepared samples showed no differences (Fig. 7, lanes 3 and 4). Very high molecular mass material that barely entered the separating gel was apparent in samples cross-linked with 1% formaldehyde (Fig. 7, lanes 5–14) but not detected when samples were prepared with 0.5% formaldehyde (Fig. 7, lanes 1–4). When samples were either solubilized at 100 °C or not treated with cross-linker only the monomer was detected (data not shown). In summary, we could not correlate the phenotypes of these strains to their ability to form oligomers under the conditions used.

Fig. 4. Analysis of LPS Oag chain length regulation by WzzpHS2 and WzzpHS2-CCR mutants in S. flexneri. pLITMUS29-wzzpHS2 constructs were introduced into strain RMA2163 (wzzSF::kmR) and grown at either 30 °C or 37 °C. (a) LPS samples were prepared, electrophoresed on an SDS-15% polyacrylamide gel and silver stained as described in Methods. Strains in each lane are as follows: 1 and 7, RMA2163(pLITMUS29); 2 and 8, RMA2163(pRMA2352); 3 and 9, RMA2163(pRMA2606); 4 and 10, RMA2163(pRMA2354); 5 and 11, RMA2163(pRMA2548); 6 and 12, RMA2163(pRMA2544). Migration of LPS with VL-type Oag is indicated by the bracket. The growth temperature of the strains is indicated above each gel. (b) Cells from exponential-phase cultures were harvested and resuspended in SDS-PAGE sample buffer. Samples were loaded onto an SDS 12% polyacrylamide gel; each lane corresponds to 4×10^8 bacterial cells. Protein was detected by Western immunoblotting using anti-WzzpHS2 antibodies. Migration positions of molecular mass standards are indicated on the left. Strains in each lane correspond to those directly above in (a).

≈ 40 kDa. It was also noted that the species at ≈ 80 kDa appeared as a doublet and as many as three bands could be distinguished at or just below 120 kDa. WzzpHS2-CCR1 appeared to show a reduced amount of the 80 kDa and 120 kDa oligomers in the presence of DegP (Fig. 7, lanes 5 and 6); however, independently prepared samples showed no differences (Fig. 7, lanes 3 and 4). Very high molecular mass material that barely entered the separating gel was apparent in samples cross-linked with 1% formaldehyde (Fig. 7, lanes 5–14) but not detected when samples were prepared with 0.5% formaldehyde (Fig. 7, lanes 1–4). When samples were either solubilized at 100 °C or not treated with cross-linker only the monomer was detected (data not shown). In summary, we could not correlate the phenotypes of these strains to their ability to form oligomers under the conditions used.
Fig. 5. Analysis of LPS Oag chain length regulation and protein expression in S. flexneri strain RMA2162 (WzzSF+) containing pLITMUS29-wzzpHS2 constructs after growth at 30 °C or 37 °C. (a) LPS samples were prepared, electrophoresed on an SDS-15% polyacrylamide gel and silver stained as described in Methods. Strains in each lane are as follows: 1, RMA2162(pLITMUS29); 2, RMA2162(pLITMUS29); 3, RMA2162(pRMA2352); 4, RMA2162(pRMA2352); 5, RMA2162(pRMA2548); 6, RMA2162(pRMA2548). Growth temperature (°C) is indicated above each lane. Migration of LPS with VL-type Oag is indicated by the bracket. (b) Cells from exponential-phase cultures were harvested and resuspended in SDS-PAGE sample buffer. Samples were loaded onto an SDS-12% polyacrylamide gel; each lane corresponds to 3 x 10^8 bacterial cells. Protein was detected by Western immunoblotting using anti-WzzpHS2 antibodies. Migration positions of molecular mass standards are indicated on the left. The constructs for each lane correspond to those directly above in (a).

Fig. 6. Analysis of the effect of DegP on LPS Oag chain length regulation by WzzpHS2 and WzzpHS2-CCR mutants in S. flexneri. pLITMUS29-wzzpHS2 constructs were introduced into RMA2804 (degP::cm^R, wzzSF::km^R) and RMA2163 (wzzSF::km^R) and the strains were grown at 30 °C. (a) LPS samples were prepared, electrophoresed on an SDS-15% polyacrylamide gel and silver stained as described in Methods. Strains in each lane are as follows: 1, RMA2163(pLITMUS29); 2, RMA2804(pLITMUS29); 3, RMA2163(pRMA2352); 4, RMA2804(pRMA2352); 5, RMA2163(pRMA2606); 6, RMA2804(pRMA2606); 7, RMA2163(pRMA2548); 8, RMA2804(pRMA2548); 9, RMA2163(pRMA2548); 10, RMA2804(pRMA2548); 11, RMA2163(pRMA2544); 12, RMA2804(pRMA2544). Migration of LPS with VL-type Oag is indicated by the bracket. (b) Cells from exponential-phase cultures were harvested and resuspended in SDS-PAGE sample buffer. Samples were loaded onto an SDS-12% polyacrylamide gel; each lane corresponds to 3 x 10^8 bacterial cells. Protein was detected by Western immunoblotting using anti-WzzpHS2 antibodies. Migration positions of molecular mass standards are indicated on the left. The constructs for each lane correspond to those directly above in (a).
DISCUSSION

In this study we have overexpressed and purified WzzpHS2 for the first time as a His6-tagged construct, and have used the purified protein to generate polyclonal antibodies. A source of purified WzzpHS2 will greatly facilitate future structural studies of this protein.

Secondary-structure analysis of WzzpHS2 revealed that it has three areas of CC potential within the periplasmic domain. This region is likely to form an extended region of \( \alpha \)-helices which may extend as far as 102 Å (10.2 nm) into the periplasm, possibly in a hairpin-like manner. Analysis of the secondary structures of several other Wzz proteins implies that this may be a common structural feature.

Construction of a panel of WzzpHS2 CCR mutants has allowed us to probe the relationship between function and predicted CC structural elements within this protein. Introduction of the mutants into a Wzz-negative strain was used to investigate WzzpHS2 activity in LPS synthesis. Mutations affecting CCR2 and 3 prevented complementation to wild-type phenotype without altering protein expression, indicating that these regions play a role in Oag chain regulation by WzzpHS2. The alterations we made to CCR1 were tolerated, with no change in function detected. CC motifs mediate subunit oligomerization and other protein–protein interactions; therefore we hypothesized that this may be the role of the CCRs in WzzpHS2. However, in our analysis of oligomerization of WzzpHS2 by \textit{in vivo} cross-linking we were not able to correlate the CCR2 and 3 mutant phenotypes with oligomerization, as discussed below.

\textit{In vivo} cross-linking was used to show oligomerization of WzzpHS2 for the first time, revealing several large complexes containing WzzpHS2. The bands appearing at \( \approx 80 \) kDa and \( \approx 120 \) kDa indicate that WzzpHS2 may be forming dimers and trimers. A very large complex of \( \approx 220 \) kDa was also noted, raising the possibility that WzzpHS2 can form oligomers containing six or more subunits. The appearance of doublets at \( \approx 80 \) kDa is consistent with results reported by Daniels & Morona (1999). They detected doublets migrating to a position equivalent to the size of the WzzSF dimer and attributed this to the incorporation of a small WzzSF-related protein detected on their gels. We did not detect a smaller WzzpHS2-related product; however, it is possible that it was not resolved by our electrophoresis conditions. Overall the cross-linking profile for WzzpHS2 is complex and may represent species containing other proteins and/or different conformational forms of WzzpHS2 with altered mobilities as suggested for WzzSF (Daniels & Morona, 1999). Furthermore, the results of \textit{in vivo}
vivo cross-linking in other species also demonstrated multiple cross-linked bands for each Wzz (Daniels et al., 2002; Guo et al., 2006). In vitro studies with purified Wzz protein have shown that Wzz forms tetramers and larger structures (Guo et al., 2006; Tang et al., 2007), supporting the in vivo results.

Analysis of the WzzpHS2 CCR mutants by in vivo cross-linking showed no reproducible differences from the wild-type. It is possible that individually disrupting CCR2 and 3 is sufficient to disrupt function but not sufficient to disrupt oligomerization due to compensating interactions mediated by the remaining intact CCRs. Another possibility is that the role of the CCRs is to mediate hetero-interactions – hence the lack of effect on homo-interactions.

We also approached WzzpHS2 in vivo protein–protein interactions using two different bacterial fusion systems, the LexA protein–protein interaction system (Daines & Silver, 2000; Dmitrova et al., 1998) and the bacterial adenylate cyclase two-hybrid system (Karimova et al., 1998), but we were unable to demonstrate any WzzpHS2 interactions (data not shown). This may indicate that the cytoplasmic termini from separate WzzpHS2 monomers are not in close contact with each other. Alternatively, steric hindrance resulting from tight interactions between the TM regions of WzzpHS2 may prevent formation of active fusions.

Although the phenotypes displayed by the CCR2 and CCR3 mutants could not be linked to oligomerization in this study, we did make some additional observations of the phenotype displayed by WzzpHS2-CCR3. This mutant showed a ts defect in its ability to regulate VL-type Oag chain production. At 30 °C, WzzpHS2-CCR3 could complement the wzz mutant S. flexneri strain to varying degrees; however, this complementation was absent at 37 °C. It is possible that the mutations in CCR3 cause ts conformational changes in the structure of WzzpHS2.

We also observed that WzzpHS2-CCR3, in addition to its negative effect on VL-type Oag, significantly reduced the number of Oag chains of all lengths. This was noted at both 37 °C and 30 °C and was an effect dependent on the presence of DegP, as discussed in the following paragraph. To explore whether WzzpHS2-CCR3 could disrupt Oag biosynthesis in the presence of a wild-type Wzz protein, we co-expressed Wzzsf (wild-type) with WzzpHS2-CCR3 and demonstrated that LPS with S-type Oag chains could be produced. This indicated that WzzpHS2-CCR3 did not have a negative dominant effect on Wzzsf-regulated Oag production. It remains unclear how WzzpHS2-CCR3 interferes with Oag production. The lack of a negative dominant effect on Wzzsf, however, suggests that Wzzsf and WzzpHS2 participate in separate Oag biosynthesis and/or transport complexes. Data are accumulating to suggest that different Wzz proteins compete for influence over chain length regulation (Bastin et al., 1993; Carter et al., 2007; Stevenson et al., 1995). In particular it has been noted that Wzzsf competes more efficiently than WzzpHS2 in S. flexneri (Carter et al., 2007; Stevenson et al., 1995), and our data showing that Wzzsf expressed from the single chromosomal wzzsf gene can rescue the negative effect of WzzpHS2-CCR3 expressed from a high-copy-number vector support this.

Because of the ts and inhibitory phenotype of WzzpHS2-CCR3, we hypothesized that the ts periplasmic chaperone/ protease, DegP, was involved in chain length regulation by WzzpHS2. DegP expression is known to be upregulated in response to extracytoplasmic stress, such as misfolded proteins, sensed by the σ^E and Cpx pathways (Danese et al., 1995; Lipinska et al., 1988). Furthermore, degP is essential in pathogenic bacteria and is upregulated upon entry into the host (Clausen et al., 2002). In S. flexneri, degP is essential for virulence and efficient actin-based motility (Purdy et al., 2002, 2007). As noted above, the inhibitory effect of WzzpHS2-CCR3 on production of all lengths of Oag was dependent on the presence of DegP. This may be due to a direct interaction with DegP in response to misfolding of WzzpHS2-CCR3, resulting in a sequestered form of WzzpHS2-CCR3 that interferes with the Oag biosynthesis machinery. It is also possible that the effect of DegP is due to an indirect influence on Oag production; however, data presented here and by others (Purdy et al., 2007) show that degP does not influence wild-type LPS Oag production in S. flexneri and therefore wider-ranging effects are less likely. It is interesting to note that overexpressed Wzz leads to activation of the CpxAR extracytoplasmic stress-response system and upregulation of degP in Yersinia enterocolitica (Bengoechea et al., 2002). It has been proposed that excess Wzz is disadvantageous and that the cell senses this via periplasmic contacts with the CpxAR system (Bengoechea et al., 2002). The possibility that this system also recognizes misfolded Wzz proteins and potentially leads to direct interactions with DegP requires further investigation.

DegP is a highly conserved cage-forming serine protease that can also act as a chaperone (Clausen et al., 2002). Chaperone activity is evidenced by the ability of DegP to refold MalS (Spiess et al., 1999) and prevent protein aggregation over a wide range of temperatures (Skórko-Glonek et al., 2007). Protease activity rapidly increases as growth temperature rises from 30 °C (Skórko-Glonek et al., 1995; Spiess et al., 1999). It should be noted, therefore, that all assays performed in our degP::cmR^R mutant were at 30 °C due to the inability of this mutant to survive and grow normally at high temperatures. Further assays with purified DegP could be performed at higher temperatures in order to explore potential protease activity on WzzpHS2 which may not have been evident at the lower temperatures used here.

During the construction of WzzpHS2-CCR3, a mutant was isolated containing an additional S366G mutation which was found to suppress the WzzpHS2-CCR3 phenotype. It is unclear how this mutation acts as a cis suppressor, especially since it is located in the fourth-to-last residue of WzzpHS2 and therefore is predicted to be in the cytoplasm. A possible explanation for this result is that
this residue is involved in activation of WzzpHS2 and that signals sensed in the C-terminus can be transferred across the IM to the periplasmic domain. We have unpublished data showing that fusion of GFP to the C-terminus of WzzSa results in an 85% reduction in Wzz activity (Daniels, 1999) and furthermore, studies of hybrid Wzz proteins also support the idea that the C-terminus is important for function (Daniels & Morona, 1999). While post-translational regulation of Wzz activity has not been demonstrated as yet, activity of the related PCP2 proteins, Wzc and CpsC/D, is affected by phosphorylation that is mediated via cytoplasmic domains or subunits of these proteins (Morona et al., 2000a; Vincent et al., 1999; Wugeditsch et al., 2001). It may be possible that a similar mechanism for regulating the activity of WzzpPHS2 and other Wzz proteins exists. A mechanism such as this could be part of a timing mechanism as proposed by Bastin et al. (1993).

Elucidation of the structure–function relationship in Wzz proteins is part of ongoing work in our laboratory. Further investigation of the periplasmic CCRs will hopefully bring insights into how members of the Wzz family regulate Oag chain length. In addition to CC potential, the enigmatic periplasmic region of Wzz has the potential to protrude deep into the periplasm, and the implications of this are also being investigated. The interplay between DegP and altered forms of Wzz is another intriguing avenue of investigation that we are pursuing.

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