Coiled-coil regions play a role in the function of the *Shigella flexneri* O-antigen chain length regulator Wzz$_{pHS2}$

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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$_{pHS2}$ 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$_{pHS2}$. 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$_{pHS2}$ and the mutant constructs in a *S. flexneri degP* mutant showed that DegP did not affect the function of wild-type Wzz$_{pHS2}$ but its presence influenced the phenotype of the Wzz$_{pHS2}$ CCR3 mutant. Additionally, the phenotype of the Wzz$_{pHS2}$ CCR3 mutant was suppressed by a *cis* mutation near the putative cytoplasmic C-terminus of Wzz$_{pHS2}$.
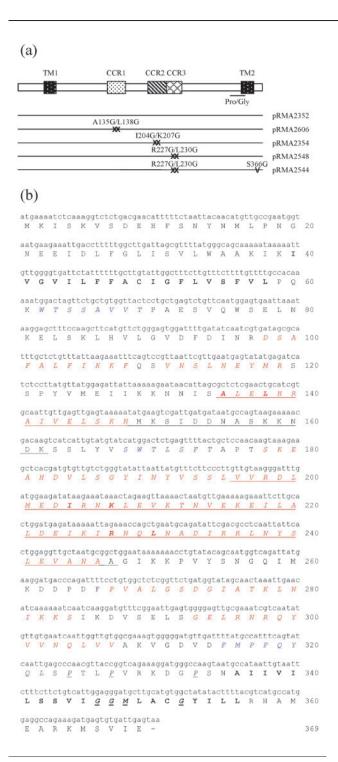
INTRODUCTION

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 *wzz$_{SF}$* gene is located on the chromosome and is responsible for S-type Oag chains of 11–17 RU (Morona *et al.*, 1995) while the *wzz$_{pHS2}$* 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 *wzz$_{pHS2}$* are advantageous to the cell because they confer resistance to complement (Hong & Payne, 1997). A model

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.

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 135–162), CCR2 (aa 196–225) and CCR3 (aa 22–247) are underlined. Residues targeted for mutation within these regions are in bold type. The predicted TM regions, TM1 (aa 40–58) and TM2 (aa 336–356) (Stevenson et al., 1995), are in black bold type. Residues identical to the consensus proline/glycine-rich motif (PX$_2$PX$_4$SPKX$_1$X$_{10}$GGMXGAG) (Becker & Puhler, 1998; Becker et al., 1995) are underlined and in italic type. Predicted secondary structural features are highlighted with italicized coloured text: red indicates α-helices and blue indicates β-strand.

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, PX$_2$PX$_4$SPKX$_1$X$_{10}$GGMXGAG, overlapping the second TM region (Becker & Pühler, 1998; Becker et al., 1995; Daniels & Morona, 1999; Morona et al., 2000b). The periplasmic domain of Wzz proteins is predicted to be mostly α-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, *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

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 Wzz$_{SF}$ 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 chromatography 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.*, 2000b), 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 Wzz$_{pHS2}$ via facilitation of protein–protein interactions. In this study we performed site-directed mutagenesis on Wzz$_{pHS2}$, 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 Wzz$_{pHS2}$; 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 Wzz$_{pHS2}$, 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 l^{-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 Geneworks. 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 *Bcl*I restriction was required and DH5αλpir 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 MgCl$_2$ followed by incubation for 1 h on ice in 0.1 M CaCl$_2$ and final resuspension in ice-cold 0.1 M CaCl$_2$ with 10 % (v/v) glycerol.

Cloning of Wzz$_{pHS2}$ into pLITMUS29 and site-directed mutagenesis of CCRs using overlap-extension PCR. The 1.352 kb *Nsi*I fragment containing *wzz$_{pHS2}$* (EU220028) was digested from pMN4, a Cm^R derivative of pHS-2 (Hong & Payne, 1997) and ligated into the *Nsi*I site of pLITMUS29, resulting in plasmid pRMA2352. The *Nsi*I 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 within individual CCRs of Wzz$_{pHS2}$. Two primers were designed to anneal within the pLITMUS29 sequence such that they flanked the *Nsi*I Wzz$_{pHS2}$ fragment in pRMA2352: 5′-GGATCCACGATATCC-TGCAG-3′ and 5′-AGGCCTTGACTAGAGGGAAG-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′-CATTAGC-GGTCTCGAGGGGCATCGTGC-3′ and 5′-GCACGATGCCCCTC-GAGACCGCTAATG-3′; CCR2 (I204G/K207G), 5′-GATGGAAGAT-GGAAGAAATGGACTAGAAGTT-3′ and 5′-AACTTCTAGTCCATT-TCTTCCATCTTCCATC-3′; CCR3 (R227G/L230G), 5′-GATAAAA-ATTGGAAACCAGGGGAATGCAGAT-3′ and 5′-ATCTGCATTCC-CCTGGTTTCCAATTTTTATC-3′]. For each mutant, a first round of

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Source or reference
Strains		
DH5α	*E. coli* general cloning host	Gibco-BRL
JM110	*E. coli* general cloning host, Dam$^-$	Laboratory collection
DH5αλpir	*E. coli* general cloning host, supports *pir*-dependent plasmids	Laboratory collection
SG13009(pREP4)	*E. coli* expression host, Km^R	Qiagen
RMA2163	*S. flexneri*, SFL1, Y serotype, $wzz_{SF}::km^R$, pHS-2$^-$, vir$^-$, Km^R	Laboratory collection†
RMA2804	RMA2163 $degP::cm^R$, Km^R Cm^R	This study
RMA2162	*S. flexneri*, SFL1, Y serotype, pHS-2$^-$, vir$^-$	Laboratory collection†
Plasmids		
pMN4	pHS-2 with insertion of Cm^R downstream of wzz_{pHS2}	Hong & Payne (1997)
pQE30	N-terminal 6 × His tag expression vector, Ap^R	Qiagen
pRMA2274	wzz_{pHS2} cloned in-frame with N-terminal 6 × His tag in pQE30	This study
pLITMUS29	High-copy-number cloning vector, blue/white colour screening, Ap^R	New England Biolabs
pRMA2352	wzz_{pHS2} wild-type subcloned into pLITMUS29	This study
pRMA2606	wzz_{pHS2} CCR1 mutant in pLITMUS29	This study
pRMA2354	wzz_{pHS2} CCR2 mutant in pLITMUS29	This study
pRMA2548	wzz_{pHS2} CCR3 mutant in pLITMUS29	This study
pRMA2544	wzz_{pHS2} CCR3/S366G mutant in pLITMUS29	This study
pGEM-T Easy	T/A cloning of PCR products, blue/white colour screening, Ap^R	Promega
pKD3	Template vector in lambda Red mutagenesis system, Cm^R Ap^R	Datsenko & Wanner (2000)
pRMA2770	*degP* PCR product cloned into pGEM-T Easy	This study
pRMA2784	cm^R cartridge amplified from pKD3 cloned into pGEM-T Easy	This study
pRMA2787	$degP::cm^R$ in pGEM-T Easy	This study
pCVD442	Positive selection suicide vector, *sacB*, *mob* RP4, *ori* R6K, Ap^R	Donnenberg & Kaper (1991)
pRMA2798	$degP::cm^R$ cloned into pCVD442	This study

*Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Cm^R, chloramphenicol resistance; Tc^R, tetracycline resistance; vir$^-$, virulence plasmid negative.

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

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 *Nsi*I and cloned into the *Nsi*I 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 the nucleotide changes. One construct was also isolated carrying a mutation in addition to the CCR3 mutation and was included in the study. The additional construct carries the substitutions R227G/L230G/S366G; it was named pRMA2544.

Construction of a *S. flexneri* degP mutant strain. Primers were designed, using the *S. flexneri* 2a 2457T genome sequence (NC_004741) and the primers used by Purdy *et al.* (2002), with the following sequences: 5′-TCATCATTTGCACGAAGC-3′ and 5′-CACCACCATTTCGGTTA-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 pRMA2770. A 1 kb chloramphenicol resistance (Cm^R) cartridge was amplified from pKD3 using oligonucleotides 5′-CGCGGATCCG-TGTAGGCTGGAGCTGCTTC-3′ and 5′-CGCGGATCCCCATAT-GAATATCCTCCTTA-3′ and cloned into pGEM-T Easy, giving pRMA2784. The 1 kb *Bam*HI Cm^R fragment from pRMA2784 was then subcloned into the *Bcl*I site of pRMA2770, giving rise to

pRMA2787. The *Xma*I/*Sph*I 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 Cm^R cartridge was not removed using the FLP/FRT feature.

Purification of His$_6$-Wzz$_{pHS2}$ and production of polyclonal Wzz$_{pHS2}$ antibodies. Plasmid pMN4 was used as a template for PCR to amplify the Wzz$_{pHS2}$ ORF using oligonucleotides 5′-GGGGTACCAAAATCTCAAAGGTCTCTGAC-3′ and 5′-AGCACT-GCAGTTTTACTCAATCACACTCATC-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$_6$-tagged Wzz$_{pHS2}$ 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 MgCl$_2$ 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

(85 000 **g**, 1 h, 4 °C, Beckman L8-80). The pellet containing the membranes was resuspended in 2 % (w/v) Sarkosyl and incubated at 37 °C with shaking for 30 min. The sample was centrifuged at ultra-high speed (as above) and the supernatant containing the soluble proteins was applied to 800 μl Ni-NTA slurry (Qiagen) previously equilibrated in buffer [1 % (v/v) Triton X-100, 20 mM Tris/HCl, 10 % (v/v) glycerol, 0.5 M NaCl, 100 mM NaH_2PO_4, pH 8.0]. Protein binding was allowed to proceed at room temperature for 1 h. The Ni-NTA was washed twice with wash buffer A [1 % (v/v) Triton X-100, 20 mM Tris/HCl, 10 % (v/v) glycerol, 0.5 M NaCl, 100 mM NaH_2PO_4 pH 8.0, 20 mM imidazole] and once with wash buffer B [1 % (v/v) Triton X-100, 20 mM Tris/HCl, 10 % (v/v) glycerol, 0.5 M NaCl, 100 mM NaH_2PO_4 pH 8.0, 30 mM imidazole]. His-tagged proteins were eluted by incubation in elution buffer (400 mM imidazole in equilibration buffer). The concentration of purified His_6-Wzz_{pHS2} was estimated to be 0.5 mg ml^{-1} using a BCA protein estimation kit (Pierce).

Specific polyclonal antibodies against His_6-Wzz_{pHS2} were obtained by immunizing a rabbit at several subcutaneous sites with purified protein emulsified in complete Freund's adjuvant. Serum was obtained, absorbed and affinity-purified against purified His_6-Wzz_{pHS2} (Daniels & Morona, 1999; Salamitou *et al.*, 1994).

LPS and protein analysis. Bacterial cultures were subcultured and grown to mid- to late exponential phase (as measured by OD_{600}). A standardized number of cells was harvested and used for preparation of whole-cell samples for Western immunoblotting and LPS profiling as described below. Whole-cell samples were solubilized in SDS-PAGE sample buffer (Lugtenberg *et al.*, 1975) at 100 °C for 5 min immediately prior to SDS-PAGE, except for formaldehyde cross-linked samples, which were heated to 60 °C for 5 min. Protein gels were either stained with Coomassie blue G250 or subjected to Western immunoblotting as follows: electrophoresed samples were transferred to nitrocellulose (GE/Osmonics), incubated with poly-clonal Wzz_{pHS2} antibodies at 1/100 dilution and detected using goat anti-rabbit horseradish-peroxidase-conjugated antibodies (KPL) and CPS-3 chemiluminescence substrate (Sigma). The molecular mass standard used was Benchmark protein ladder (Invitrogen). Small-scale LPS preparations were prepared and analysed as described previously (Murray *et al.*, 2003).

***In vivo* formaldehyde protein cross-linking.** Formaldehyde cross-linking was performed as described previously (Daniels & Morona, 1999; Prossnitz *et al.*, 1988) with the following modifications. Cells were harvested from exponential-phase cultures by centrifugation and washed once in ice-cold 10 mM potassium phosphate buffer, pH 6.8. Aliquots of 5×10^8 cells were pelleted by centrifugation and resuspended in 0.5 ml of the above buffer containing 0.5 % (v/v) or 1 % (v/v) formaldehyde (Sigma), or no formaldehyde, and incubated at 23 °C for 1 h. Samples were harvested by centrifugation, washed in ice-cold 10 mM potassium phosphate buffer, pH 6.8, and resuspended in 50 μl SDS-PAGE sample buffer (Lugtenberg *et al.*, 1975).

RESULTS

Overexpression, purification and production of anti-Wzz_{pHS2} antibodies

In order to analyse Wzz_{pHS2} using Western immunoblotting we overexpressed and purified Wzz_{pHS2} and then used the purified protein to generate polyclonal antibodies. His_6-Wzz_{pHS2} 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 His_6-Wzz_{pHS2}. The purified protein was used to generate rabbit polyclonal antiserum, which was absorbed and affinity-purified and is referred to as Wzz_{pHS2} polyclonal antibodies herein.

Secondary structure prediction and mutagenesis of helical regions in Wzz_{pHS2}

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 Wzz_{pHS2} 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 Wzz_{pHS2} 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 Wzz_{FepE} from *Salmonella typhimurium*, which confers VL-type Oag modal lengths, and also in those that confer
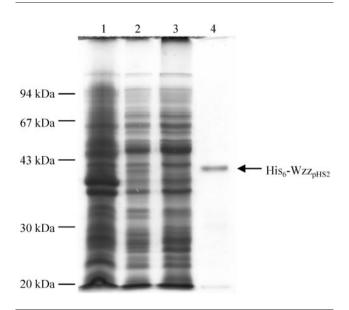

Fig. 2. Purification of His_6-Wzz_{pHS2}. Samples were taken during purification (refer to Methods for details), electrophoresed on an SDS-15 % polyacrylamide gel and stained with Coomassie G250. The lanes are samples of: 1, induced whole cells; 2, Sarkosyl-soluble supernatant extracted from the whole membranes; 3, flow-through fraction after binding to Ni-NTA resin; 4, elution fraction.

significantly different modal lengths such as Wzz$_{SF}$ and Wzz$_{ST}$ 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 Wzz$_{pHS2}$ 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 Wzz$_{pHS2}$, 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
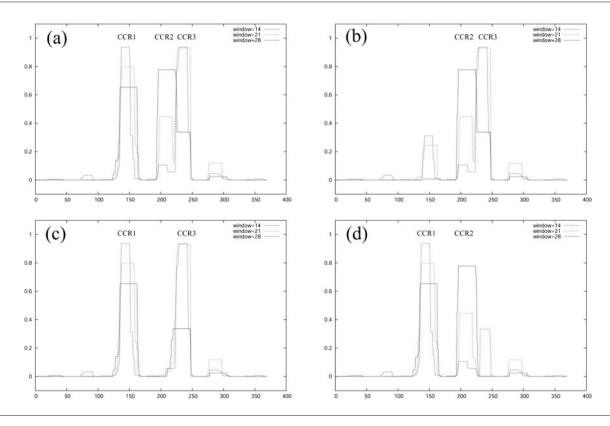

Fig. 3. Prediction of CCRs in Wzz$_{pHS2}$ using COILS (http://www.ch.embnet.org/software/COILS_form.html). Each panel shows the probability of a residue being in a CCR on the *y*-axis and residue number along the *x*-axis. The COILS program was used to predict CCRs in the sequence of wild-type Wzz$_{pHS2}$ (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 Wzz$_{pHS2}$ expressed from pRMA2606, pRMA2354, pRMA2548 and pRMA2544 are herein referred to as Wzz$_{pHS2}$-CCR1, Wzz$_{pHS2}$-CCR2, Wzz$_{pHS2}$-CCR3, Wzz$_{pHS2}$-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 Wzz$_{pHS2}$ protein constructs in *S. flexneri* RMA2163. This strain is naturally deficient for Wzz$_{pHS2}$ 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 Wzz$_{pHS2}$ resulted in LPS with VL-type Oag chains at both 30 °C and 37 °C (Fig. 4a, lanes 2 and 8). Wzz$_{pHS2}$-CCR1 had no significant impact on production of VL-type Oag chains (Fig. 4a, lanes 3 and 9). Wzz$_{pHS2}$-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). Wzz$_{pHS2}$-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 Wzz$_{pHS2}$-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 Wzz$_{pHS2}$-CCR3 was restored when it carried the *cis* S366G mutation in Wzz$_{pHS2}$-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 Wzz$_{pHS2}$ 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 Wzz$_{pHS2}$, was detected for each construct.

The inhibitory effect of the Wzz$_{pHS2}$-CCR3 mutant was investigated further by coexpressing this mutant with Wzz$_{SF}$ (wild-type) (Fig. 5) in *S. flexneri* in order to determine if Wzz$_{pHS2}$-CCR3 exhibited any negative dominant effects. We observed LPS with S-type Oag chains when Wzz$_{SF}$ and Wzz$_{pHS2}$-CCR3 were coexpressed (Fig. 5, lanes 5 and 6), indicating that the inhibitory effect can be rescued by a relatively small amount of Wzz$_{SF}$ expressed from a single copy on the chromosome and therefore that Wzz$_{pHS2}$-CCR3 does not exhibit negative dominance over Wzz$_{SF}$. 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 Wzz$_{pHS2}$-CCR3 was abolished at 37 °C, highlighting the ts nature of this mutant. Protein expression from the Wzz$_{pHS2}$ 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*::*Cm^R* background

The effect of temperature on Wzz$_{pHS2}$-CCR3 led us to hypothesize that the function of Wzz$_{pHS2}$ and/or mutant forms of Wzz$_{pHS2}$ was affected by the ts periplasmic chaperone/protease DegP (previously known as HtrA) (Lipińska *et al.*, 1989; Strauch & Beckwith, 1988; Strauch *et al.*, 1989). To test this we constructed a *S. flexneri degP*::*cm^R* mutant based on RMA2163 (*wzz*::*km^R*), 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 (Lipińska *et al.*, 1989; Purdy *et al.*, 2002; Strauch *et al.*, 1989).

The *wzz$_{pHS2}$* 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 Wzz$_{pHS2}$, 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 Wzz$_{pHS2}$ mutants. Notably, we observed that the absence of DegP restored wild-type function to the Wzz$_{pHS2}$-CCR3 mutant (Fig. 6a, lane 10). DegP did not affect protein expression of the Wzz$_{pHS2}$ mutants as shown by Western immunoblotting (Fig. 6b).

Analysis of oligomerization of Wzz$_{pHS2}$ by *in vivo* cross-linking with formaldehyde

We next assessed the ability of Wzz$_{pHS2}$ 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 Wzz$_{pHS2}$, we predicted it would form oligomers in a similar manner to Wzz$_{SF}$ (Daniels & Morona, 1999). Cross-linking was performed in strains RMA2163 (*wzz*::*km^R*) and RMA2804 (*wzz*::*km^R*, *degP*::*cm^R*); 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 ≈80 kDa and ≈120 kDa were observed in addition to the monomer at
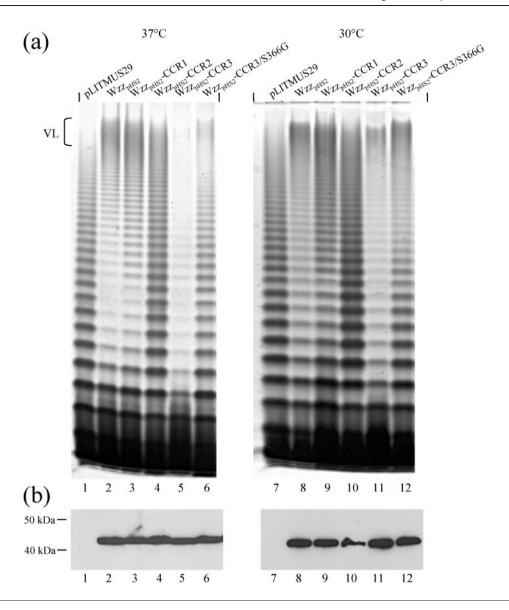
Fig. 4. Analysis of LPS Oag chain length regulation by Wzz$_{pHS2}$ and Wzz$_{pHS2}$-CCR mutants in *S. flexneri*. pLITMUS29-*wzz$_{pHS2}$* constructs were introduced into strain RMA2163 (*wzz$_{SF}$*::*km^R*) and grown at either 30 °C or 37 °C. (a) LPS samples were prepared, electrophoresed on an SDS-15 % 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-Wzz$_{pHS2}$ 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. Wzz$_{pHS2}$-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.
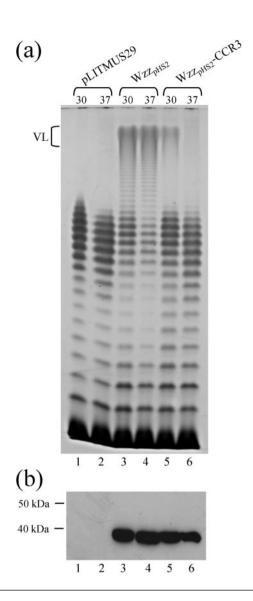
Fig. 5. Analysis of LPS Oag chain length regulation and protein expression in *S. flexneri* strain RMA2162 (Wzz_{SF}^+) containing pLITMUS29-wzz_{pHS2} constructs after growth at 30 °C or 37 °C. (a) LPS samples were prepared, electrophoresed on an SDS-15 % polyacrylamide gel and silver stained as described 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 3×10^8 bacterial cells. Protein was detected by Western immunoblotting using anti-Wzz_{pHS2} antibodies. Migration positions of molecular mass standards are indicated on the left. The constructs for each lane correspond to those directly above in (a).
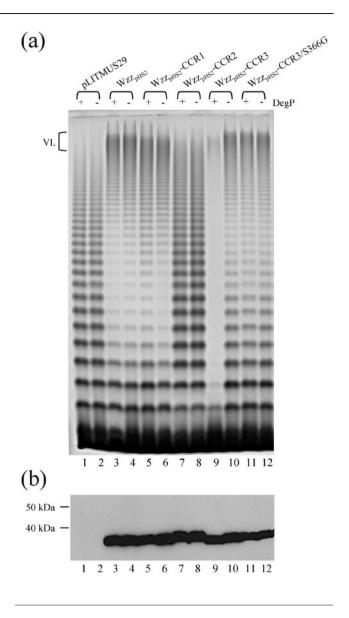

Fig. 6. Analysis of the effect of DegP on LPS Oag chain length regulation by Wzz_{pHS2} and Wzz_{pHS2}-CCR mutants in *S. flexneri*. pLITMUS29-wzz_{pHS2} constructs were introduced into RMA2804 ($degP::cm^R$, $wzz_{SF}::km^R$) and RMA2163 ($wzz_{SF}::km^R$) and the strains were grown at 30 °C. (a) LPS 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(pRMA2354); 8, RMA2804(pRMA2354); 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 3×10^8 bacterial cells. Protein was detected by Western immunoblotting using anti-Wzz_{pHS2} antibodies. Migration positions of molecular mass standards are indicated on the left side. The constructs for each lane correspond to those directly above in (a).
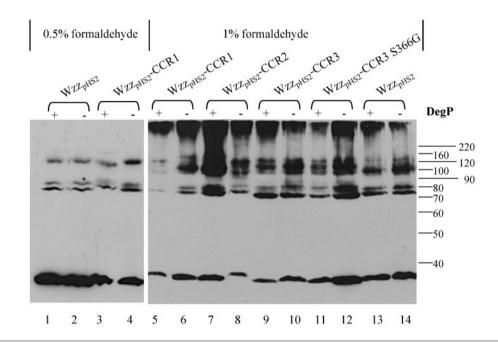
Fig. 7. Analysis of oligomerization of Wzz$_{pHS2}$ in *S. flexneri*. pLITMUS29-*wzz$_{pHS2}$* constructs were introduced into either RMA2163 (*wzz$_{SF}$*::*km^R*) or RMA2804 (*degP*::*cm^R*, *wzz$_{SF}$*::*km^R*). Cells were grown at 30 °C, washed in phosphate buffer and treated with either 1% or 0.5% formaldehyde as described in Methods. Whole-cell samples (3×10^8 cells) were electrophoresed on an SDS-12% polyacrylamide gel and immunoblotted with anti-Wzz$_{pHS2}$ polyclonal antibodies. Strains in each lane are as follows: 1, RMA2163(pRMA2352); 2, RMA2804(pRMA2352); 3, RMA2163(pRMA2606); 4, RMA2804(pRMA2606); 5, RMA2163(pRMA2606); 6, RMA2804(pRMA2606); 7, RMA2163(pRMA2354); 8, RMA2804(pRMA2354); 9, RMA2163 (pRMA2548); 10, RMA2804(pRMA2548); 11, RMA2163(pRMA2544); 12, RMA2804(pRMA2544); 13, RMA2163(pRMA2352); 14, RMA2804(pRMA2352). The migration positions and mass (kDa) of standards are indicated to the right.

DISCUSSION

In this study we have overexpressed and purified Wzz$_{pHS2}$ for the first time as a His$_6$-tagged construct, and have used the purified protein to generate polyclonal antibodies. A source of purified Wzz$_{pHS2}$ will greatly facilitate future structural studies of this protein.

Secondary-structure analysis of Wzz$_{pHS2}$ revealed that it has three areas of CC potential within the periplasmic domain. This region is likely to form an extended region of α-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 Wzz$_{pHS2}$ 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 Wzz$_{pHS2}$ 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 Wzz$_{pHS2}$. 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 Wzz$_{pHS2}$. However, in our analysis of oligomerization of Wzz$_{pHS2}$ by *in vivo* cross-linking we were not able to correlate the CCR2 and 3 mutant phenotypes with oligomerization, as discussed below.

In vivo cross-linking was used to show oligomerization of Wzz$_{pHS2}$ for the first time, revealing several large complexes containing Wzz$_{pHS2}$. The bands appearing at ≈80 kDa and ≈120 kDa indicate that Wzz$_{pHS2}$ may be forming dimers and trimers. A very large complex of >220 kDa was also noted, raising the possibility that Wzz$_{pHS2}$ can form oligomers containing six or more subunits. The appearance of doublets at ≈80 kDa is consistent with results reported by Daniels & Morona (1999). They detected doublets migrating to a position equivalent to the size of the Wzz$_{SF}$ dimer and attributed this to the incorporation of a small Wzz$_{SF}$-related protein detected on their gels. We did not detect a smaller Wzz$_{pHS2}$-related product; however, it is possible that it was not resolved by our electrophoresis conditions. Overall the cross-linking profile for Wzz$_{pHS2}$ is complex and may represent species containing other proteins and/or different conformational forms of Wzz$_{pHS2}$ with altered mobilities as suggested for Wzz$_{SF}$ (Daniels & Morona, 1999). Furthermore, the results of *in*

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 Wzz_{pHS2} 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 Wzz_{pHS2} *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 Wzz_{pHS2} interactions (data not shown). This may indicate that the cytoplasmic termini from separate Wzz_{pHS2} monomers are not in close contact with each other. Alternatively, steric hindrance resulting from tight interactions between the TM regions of Wzz_{pHS2} 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 Wzz_{pHS2}-CCR3. This mutant showed a ts defect in its ability to regulate VL-type Oag chain production. At 30 °C, Wzz_{pHS2}-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 Wzz_{pHS2}.

We also observed that Wzz_{pHS2}-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 Wzz_{pHS2}-CCR3 could disrupt Oag biosynthesis in the presence of a wild-type Wzz protein, we co-expressed Wzz_{SF} (wild-type) with Wzz_{pHS2}-CCR3 and demonstrated that LPS with S-type Oag chains could be produced. This indicated that Wzz_{pHS2}-CCR3 did not have a negative dominant effect on Wzz_{SF}-regulated Oag production. It remains unclear how Wzz_{pHS2}-CCR3 interferes with Oag production. The lack of a negative dominant effect on Wzz_{SF}, however, suggests that Wzz_{SF} and Wzz_{pHS2} 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 Wzz_{SF} competes more efficiently than Wzz_{pHS2} in *S. flexneri* (Carter *et al.*, 2007; Stevenson *et al.*, 1995),

and our data showing that Wzz_{SF} expressed from the single chromosomal wzz_{SF} gene can rescue the negative effect of Wzz_{pHS2}-CCR3 expressed from a high-copy-number vector support this.

Because of the ts and inhibitory phenotype of Wzz_{pHS2}-CCR3, we hypothesized that the ts periplasmic chaperone/protease, DegP, was involved in chain length regulation by Wzz_{pHS2}. 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; Lipińska *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 Wzz_{pHS2}-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 Wzz_{pHS2}-CCR3, resulting in a sequestered form of Wzz_{pHS2}-CCR3 that interacts negatively 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*::cm^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 Wzz_{pHS2} which may not have been evident at the lower temperatures used here.

During the construction of Wzz_{pHS2}-CCR3, a mutant was isolated containing an additional S366G mutation which was found to suppress the Wzz_{pHS2}-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 Wzz_{pHS2} and therefore is predicted to be in the cytoplasm. A possible explanation for this result is that

this residue is involved in activation of Wzz$_{pHS2}$ 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 Wzz$_{SF}$ 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 Wzz$_{pHS2}$ 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.

ACKNOWLEDGEMENTS

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