O-antigen modal chain length in S*higella flexneri* 2a is growth-regulated through RfaH-mediated transcriptional control of the *wzy* gene

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Shigella flexneri 2a 2457T produces lipopolysaccharide (LPS) with two O-antigen (OAg) chain lengths: a short (S-OAg) controlled by WzzB and a very long (VL-OAg) determined by Wzz_{pHS-2}. This study demonstrates that the synthesis and length distribution of the *S. flexneri* OAg are under growth-phase-dependent regulation. Quantitative electrophoretic analysis showed that the VL-OAg increased during growth while the S-OAg distribution remained constant. Increased production of VL-OAg correlated with the growth-phase-regulated expression of the transcription elongation factor RfaH, and was severely impaired in a Δ*rfaH* mutant, which synthesized only low-molecular-mass OAg molecules and a small amount of S-OAg. Real-time RT-PCR revealed a drastic reduction of *wzy* polymerase gene expression in the Δ*rfaH* mutant. Complementation of this mutant with the *wzy* gene cloned into a high-copy-number plasmid restored the bimodal OAg distribution, suggesting that cellular levels of Wzy influence not only OAg polymerization but also chain-length distribution. Accordingly, overexpression of *wzy* in the wild-type strain resulted in production of a large amount of high-molecular-mass OAg molecules. An increased dosage of either *wzzB* or wzz_{pHS-2} also altered OAg chain-length distribution. Transcription of *wzzB* and wzz_{pHS-2} genes was regulated during bacterial growth but in an RfaH-independent manner. Overall, these findings indicate that expression of the *wzy*, *wzzB* and wzz_{pHS-2} genes is finely regulated to determine an appropriate balance between the proteins responsible for polymerization and chain-length distribution of *S. flexneri* OAg.

INTRODUCTION

Shigella flexneri, the causative agent of shigellosis, is responsible for more than one million deaths per year, affecting particularly young children in developing countries. The infection manifests as a severe but self-limiting intestinal disease that results in inflammation and epithelial destruction (Jennison & Verma, 2004; Sansonetti, 2001). *S. flexneri* infection requires the expression of several virulence factors encoded in a 220 kb virulence plasmid. This plasmid includes two operons transcribed in opposite directions: the *ipa* operon encoding the invasion proteins (Ipa) and the *mxi/spa* genes encoding the type III secretion system for the translocation of Ipa proteins into the epithelial cells (Sansonetti & Egile, 1998).

In addition to invasion proteins, the lipopolysaccharide (LPS) plays a role in *Shigella* virulence (Morona *et al.*, 2003; Okada *et al.*, 1991; Sandlin *et al.*, 1995; Van den Bosch *et al.*, 1997). LPS, a major component of the outer membrane of Gram-negative bacteria, comprises three domains: the inner hydrophobic lipid A region, the oligosaccharide core and the outer O-polysaccharide chain or O antigen (OAg) that is exposed to the bacterial surface (Raetz & Whitfield, 2002; Valvano, 2003; Whitfield, 1995). Early reports showed that mutants of *S. flexneri* devoid of OAg or with defects in the core region were less virulent *in vivo*. Although these mutants invaded and replicated within non-polarized epithelial cells, they failed to spread to adjacent cells in a monolayer (Okada *et al.*, 1991; Sandlin *et al.*, 1995). The altered LPS structures in these

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Abbreviations: KDO, 2-keto-3-deoxyoctulosonic acid; OAg, O antigen; RU, repeated units; S-, short; VL-, very long.

mutants led to an incorrect localization and dysfunction of the IcsA protein (Sandlin *et al.*, 1995; Van den Bosch & Morona, 2003; Van den Bosch *et al.*, 1997). Also, adherence to and internalization into polarized intestinal epithelial cells are highly dependent on the length of the LPS, and require both the OAg and core regions (Kohler *et al.*, 2002). In addition to contributing to bacterial invasion, the OAg might, by itself, elicit inflammation and haemorrhage of the intestinal tissue (Zhong, 1999). Moreover, the OAg confers serum resistance by protecting the bacterium from the lytic action of complement (Hong & Payne, 1997).

LPS OAg synthesis is driven by complex biochemical mechanisms (Raetz & Whitfield, 2002; Valvano, 2003; Whitfield, 1995). In *S. flexneri*, OAg synthesis begins in the cytoplasmic face of the inner membrane with the addition of *N*-acetylglucosamine (GlcNAc) to the lipid carrier undecaprenyl phosphate. The additional sugars are added to the GlcNAc residue in a sequential manner to form a complete OAg unit that is translocated to the periplasmic side by the Wzx translocase. Then, the Wzy polymerase links the pre-formed OAg units, generating the OAg chain. The complete LPS is formed by the ligation of the OAg chain to pre-formed lipid A-core oligosaccharide by the WaaL ligase, which results in the release of undecaprenyl pyrophosphate. The Wzz protein is essential for generating a non-random OAg LPS structure, resulting in a preferred OAg chain length or modal distribution.

The LPS molecules of *S. flexneri* 2a have OAg with two preferred OAg chain lengths, a short (S-OAg) composed on average of 17 repeated units (RU) that is regulated by a chromosomally encoded WzzB protein (Morona *et al.*, 1995), and a very long LPS (VL-OAg) of about 90 RU. VL-OAg requires Wzz_{pHS-2}, which is encoded in plasmid pHS-2 (Stevenson *et al.*, 1995). The length distribution of the OAg modulates *S. flexneri* virulence, since mutants affected in wzz_{pHS-2} are more sensitive to serum killing and less virulent *in vivo*, while mutants in *wzzB* are defective in invasiveness and plaque formation (Hong & Payne, 1997; Morona *et al.*, 2003; Van den Bosch *et al.*, 1997).

Little is known about the regulation of OAg chain-length distribution. Hong & Payne (1997) reported that the expression of the very long chain length determinant, Wzz_{pHS-2}, is not regulated by a number of different environmental conditions such as iron concentration, temperature, pH and nutrients. More recently, Varela *et al.* (2001) showed that *S. flexneri* grown at 30 °C produced increased amounts of long chains relative to short chains, compared to bacteria grown at 37 °C. However, the mechanisms underlying this modulation were not investigated.

We previously demonstrated that production of OAg by *Salmonella* Typhi Ty2 varies during bacterial growth in direct relationship with the growth-regulated expression of the RfaH transcription elongation factor (Bittner *et al.*, 2002; Rojas *et al.*, 2001). RfaH controls the expression of OAg and core oligosaccharide biosynthesis genes (Bailey *et al.*, 1997; Pradel & Schnaitman, 1991; Wang *et al.*, 1998). Here, we demonstrate that the VL-OAg in *S. flexneri* increases significantly during growth while the S-OAg distribution remains relatively constant. VL-OAg production correlated with a growth-dependent regulation of the *rfaH* gene. Our results also indicate that RfaH is essential for expression of the *wzy* polymerase gene, but not for *wzzB* or wzz_{pHS-2}, suggesting that the cellular levels of Wzy are critical for VL-OAg production and normal OAg chain-length distribution in *S. flexneri* 2a.

METHODS

Bacterial strains, plasmids, media and growth conditions. Table 1 summarizes the properties of the bacterial strains and plasmids used in this study. Bacteria were grown aerobically in Luria–Bertani medium (LB) (10 g l^{-1} Bacto tryptone, 5 g l^{-1} Bacto yeast extract, 5 g l^{-1} NaCl). Solid medium contained 1.5 % (w/v) agar. Media were supplemented with 100 μg ampicillin ml^{-1} or 50 μg kanamycin ml^{-1} as appropriate.

Mutagenesis of the *rfaH* and wzz_{pHS-2} genes. Mutagenesis was performed by the method of Datsenko & Wanner (2000) to create chromosomal deletions by homologous recombination using PCR products. Primers were designed according to the DNA sequence information available for the *S. flexneri* 2a 2457T strain (Wei *et al.*, 2003) and plasmid pHS-2 (NC_002773). To disrupt the genes, *S. flexneri* 2a 2457T cells were first transformed with the temperature-sensitive plasmid pKD46, which expresses the λ Red recombinase system. These cells were transformed with PCR products that were generated using as template the pKD4 plasmid, which contains the FRT-flanked kanamycin-resistance gene (*aph*). The primers used carried 40 bases that were homologous to both edges of the gene targeted for disruption. The sequences of the oligonucleotide primers used in this study are available upon request. In the presence of the λ Red recombinase system, the integration of the amplicons resulted in the targeted replacement of the wild-type gene by the antibiotic-resistance cassette. The kanamycin-resistant transformants were replica-plated in the absence of antibiotic selection at 42 °C and finally assayed for ampicillin sensitivity to confirm the loss of pKD46. To obtain a non-polar deletion of the *rfaH* gene, the antibiotic-resistance gene was removed by transforming the gene replacement mutant with pCP20, which encodes the FLP recombinase (Cherepanov & Wackernagel, 1995). Transformants were plated on LB agar containing ampicillin and kanamycin at 37 °C. Individual colonies were replica-plated on LB agar, on LB agar containing ampicillin and on LB agar containing kanamycin. The plates were incubated at 42 °C. Transformants that had lost the resistance gene (*aph*) and plasmid pCP20 were selected as those colonies that were able to grow only on LB agar. Correct insertional gene replacement and the deletion of the antibiotic-resistance gene cassette were confirmed by PCR analysis.

Cloning of the *rfaH*, *wzzB*, wzz_{pHS-2} and *wzy* genes. DNA fragments containing the *S. flexneri* 2457T *rfaH* (GeneID:1080050), *wzzB* (GeneID:1077593), wzz_{pHS-2} (NC_002773) and *wzy* (GeneID:1078521) genes and their promoter regions were amplified by PCR. The amplicons were cloned into pGEM-T Easy as recommended by the supplier.

Construction of *lacZ* transcriptional fusions. The *rfaH*, *wzzB* and wzz_{pHS-2} promoter regions were amplified by PCR. The corresponding fragments were cloned into the pGEM-T Easy vector and then subcloned into plasmid pFZY1. This is a single-copy-number vector designed for the construction of transcriptional fusions to the *lac*

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties*	Source or reference
***S. flexneri* 2a**		
2457T	Wild-type	ISP†
MSF487	2457T Δ*rfaH*	This study
MSF107	2457T wzz_{pHS-2}::*aph*, Kan^R	This study
Plasmids		
pFZY1	Promoterless cloning vector, Amp^R F′*lac* replicon, $lacZ^+ Y^+ A^+$	Koop *et al.* (1987)
pCB315	pFZY1 containing a 315 bp fragment including the promoter region of *rfaH of S. flexneri* 2a 2457T	This study
pCB326	pFZY1 containing a 326 bp fragment including the promoter region of *wzzB* of *S. flexneri* 2a 2457T	This study
pCB280	pFZY1 containing a 280 bp fragment including the promoter region of wzz_{pHS-2} of *S. flexneri* 2a 2457T	This study
pCB261	pFZY1 containing a 261 bp fragment of an intragenic region of *rfaH* of *S. flexneri* 2a 2457T	This study
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 *ori*TS	Datsenko & Wanner (2000)
pKD4	*bla* FRT *aph* FRT PS1 PS2 *ori*R6K	Datsenko & Wanner (2000)
pCP20	*bla cat* cI857 λP_R *flp* pSC101 *ori*TS	Cherepanov & Wackernagel (1995)
pGEM-T Easy	TA cloning vector	Promega
pJC75	*rfaH* gene cloned into pGEM-T Easy	This study
pJC114	*wzy* gene cloned into pGEM-T Easy	This study
pJC139	*wzzB* gene cloned into pGEM-T Easy	This study
pJC144	wzz_{pHS-2} gene cloned into pGEM-T Easy	This study

*Amp^R, ampicillin resistant; Kan^R, kanamycin-resistant.
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operon (Koop *et al.*, 1987). The resulting plasmids, pCB315, pCB326 and pCB280, respectively, were transformed into *S. flexneri* 2457T. Also, a 261 bp PCR fragment of an intragenic region of the *rfaH* gene was cloned into pFZY1 to generate plasmid pCB261, which was used as negative control.

RNA extraction. Bacterial cells grown to early exponential (OD_{600} 0.1) and stationary phase (OD_{600} 1.5) were incubated with lysozyme (1 mg ml^{-1}) for 10 min at 4 °C, then total RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. Genomic DNA contamination from RNA samples was removed by treatment with Turbo DNase from Ambion. The integrity and purity of the RNA was assessed by denaturing agarose/formaldehyde gel electrophoresis and by nucleic acid/protein ratio (A_{260}/A_{280}). A ratio $A_{260}/A_{280} > 1.90$ was obtained for all samples.

Real-time quantitative RT-PCR. Expression of the *wzy* gene was examined by qRT-PCR. Five micrograms of total RNA was treated with 200 units of Superscript II Reverse Transcriptase (Invitrogen) by using gene-specific primers according to the manufacturer's recommendations. Quantitative PCR was performed using an Opticon 2 Thermal cycler PT (MJ Research) and SYBR Green technology (Platinum SYBR Green qPCR SuperMix-UDG, Invitrogen). Reaction mixtures containing no template and reaction mixtures containing DNase-treated RNA were included in each real-time PCR experiment to assess primer-dimers formation and residual chromosomal DNA, respectively. The identities of the amplicons resulting from the reactions were checked after amplification by melting curve analysis and amplicon DNA gel electrophoresis. The relative expression of *wzy* was normalized to the transcript levels of the *hisG* gene, whose expression remains constant throughout the bacterial growth (unpublished results), using the Relative Standard Curve method (Applied Biosystems). The statistical significance of differences in the data was determined using an unpaired Student's *t*-test.

LPS analysis. LPS was prepared as described elsewhere (Marolda *et al.*, 2006). Briefly, culture samples obtained at different times during growth were adjusted to OD_{600} 2.0 in a final volume of 1.5 ml LB. Cells were centrifuged and the pellets were suspended in 100 µl lysis buffer containing proteinase K, followed by hot phenol extraction and a subsequent extraction of the aqueous phase with ether. LPS was separated on 14 % (w/v) acrylamide gels using a Tricine-SDS buffer system (Lesse *et al.*, 1990) and visualized by silver staining (Marolda *et al.*, 2006). The concentration of LPS was determined by measuring 2-keto-3-deoxyoctulosonic acid (KDO) using the Purpald assay (Marolda *et al.*, 2006). Densitometry analysis was performed using the UN-SCANT-IT gel software (Silk Scientific). The ratio of the relative intensity of the lipid A-core band to the average intensity of the bands corresponding to the S-OAg and VL-OAg was calculated by quantifying the pixels in a narrow window across the centre of each lane. The densitometry analysis was calibrated by determining the ratio of the relative intensity of the lipid A-core to the average intensity of the O-antigen bands using a range of loading volumes of *S. flexneri* 2a 2457T LPS. The statistical significance of differences in the data was determined using the one-way ANOVA test and the Tukey post test.

Western blot analysis was performed as described by Marolda *et al.* (2006). Briefly, the gel was transferred to a PVDF membrane for 75 min at 250 mA and blocked for 90 min in 5 % (w/v) skim milk at room temperature. The membrane was incubated with a polyclonal rabbit antiserum against *S. flexneri* (Probac do Brasil, Produtos Bacteriológicos) as a primary antibody, and goat anti-rabbit HRP conjugated (Pierce) as a secondary antibody. Detection was performed using the SuperSignal West Pico chemiluminiscent substrate (Pierce).

***β*-Galactosidase assays.** Two hundred microlitres of an overnight culture in LB was inoculated into 100 ml of the same medium and grown in an orbital shaker. At different times during growth, a 1 ml sample was withdrawn to measure the bacterial growth (OD_{600}) and the β-galactosidase activity according to Miller (1972). Enzyme activities are expressed as Miller units. Each sample was analysed in triplicate in two independent experiments.

RESULTS

Bacterial growth phase affects O-antigen production in *S. flexneri*

To investigate OAg expression in *S. flexneri* 2a 2457T during growth, we analysed the LPS profiles in silver-stained polyacrylamide gels. Gel loadings were normalized by two parameters: (i) the bacterial density at the starting point of the LPS preparations and (ii) the concentration of KDO, a conserved sugar component in the lipid A-core region, as described in Methods. LPS analysis (Fig. 1a) revealed a differential growth-phase-dependent regulation of OAg synthesis. While the amount of VL-OAg increased throughout the bacterial growth, the S-OAg chains remained relatively constant. The densitometric quantification of the lanes in the gel showed a significant increase in the ratio of VL-OAg to the lipid A-core region in the samples grown to mid-exponential and stationary phases, compared to the sample grown to early exponential phase. In contrast, no significant differences in the amount of S-OAg relative to the lipid A-core during growth were observed (Fig. 1b). The differences in OAg expression were confirmed by Western blot analysis. As shown in Fig. 1(c), a higher amount of VL-OAg was detected at stationary phase (lane 2) compared to early exponential phase (lane 1). LPS obtained from a mutant lacking the VL-OAg (strain MSF107, Table 1) was also analysed (Fig. 1c, lane 3).

Production of VL-OAg correlates with *rfaH* transcription

The RfaH protein is a transcription elongation factor that positively regulates the LPS core and OAg genes in enterobacteria (Bailey *et al.*, 1997). To examine whether RfaH plays any role in growth-regulated OAg synthesis in *S. flexneri*, we constructed an *rfaH–lacZ* transcriptional fusion in a single-copy-number plasmid (pFZY1). The resulting plasmid, pCB315, was transformed into *S. flexneri* 2a 2457T. Plasmid pCB261, containing an intragenic region of the *rfaH* gene cloned in pFZY1, was also transformed as a negative control. The production of β-galactosidase by *S. flexneri*/pCB315 and *S. flexneri*/pCB261 was assayed at various stages of growth. Fig. 2 shows that β-galactosidase production driven by the *rfaH* promoter increases at late exponential phase, reaching maximal expression during stationary phase. Thus, the pattern of VL-OAg production reflects the differential *rfaH* expression during the bacterial growth cycle.

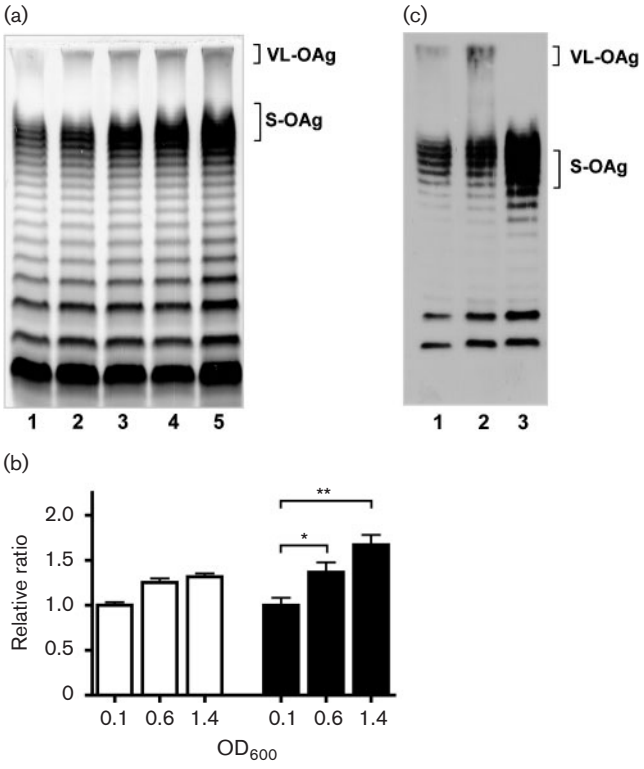

Fig. 1. Production of LPS in *S. flexneri* 2a 2457T during growth. (a) LPS profiles of cells grown in LB to the following OD_{600}: lane 1, 0.1; lane 2, 0.35; lane 3, 0.6; lane 4, 1.0; lane 5, 1.4. Samples from equal numbers of bacterial cells were loaded in each lane and analysed by Tricine/SDS-PAGE on a 14% (w/v) acrylamide gel followed by silver staining. (b) Densitometry analysis. Bars represent the levels of S-OAg (white bars) or VL-OAg (black bars) relative to the lipid A-core region at early exponential (OD_{600} 0.1), mid-exponential (OD_{600} 0.6) and stationary phase (OD_{600} 1.4). Values correspond to the number of pixels from the bands comprising each region relative to the pixels obtained at OD_{600} 0.1. Densitometry analysis was performed in four independent gels. Means ± SEM are shown. Asterisks indicate significant differences determined by the one-way ANOVA and Tukey post test (*$P<0.05$, **$P<0.001$). (c) Western blot analysis of LPS production by strains 2457T (wild-type; lanes 1 and 2) and MSF107 (2457T wzz_{pHS-2}::*aph*) (lane 3). Bacteria were grown to early exponential (OD_{600} 0.1, lane 1) and stationary phase (OD_{600} 1.4, lanes 2 and 3).

RfaH is required for O-antigen expression and normal chain-length distribution

To address the role of RfaH in OAg synthesis, we constructed strain MSF487, an isogenic derivative of *S. flexneri* 2a 2457T that carries a deletion of the *rfaH* gene, and monitored the production of LPS by SDS-PAGE. Analysis of the LPS profiles revealed that production of OAg is severely impaired in the Δ*rfaH* mutant. As shown in Fig. 3(a), MSF487 lacked LPS with VL-OAg chains at both exponential and stationary phases of growth (lanes 2 and

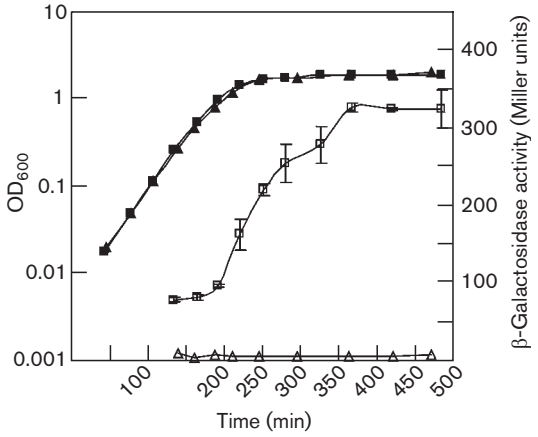

Fig. 2. Expression of *rfaH* in *S. flexneri* 2a 2457T during growth. Cells were transformed with plasmid pCB315 (*rfaH–lacZ*) or pCB261 (control). Growth was measured as OD_{600} for strains 2457T/pCB315 (■) and 2457T/pCB261 (▲). The expression of the *rfaH–lacZ* fusion for strains 2457T/pCB315 (□) and 2457T/pCB261 (△) was measured as β-galactosidase activity. Data are the means ± SD of triplicate assays in two independent experiments.

3). The absence of VL-OAg was confirmed in a gel loaded with a 10-fold excess of LPS sample (lane 4). Also, strain MSF487 produced a small amount of low-molecular-mass OAg chains (1–4 RU) and fewer S-OAg molecules migrating slightly faster than the wild-type S-OAg. In addition, greater amounts of unsubstituted core-lipid A molecules than in the wild-type strain were detected. When MSF487 was transformed with plasmid pJC75, harbouring the *rfaH* gene in a high-copy-number plasmid, the wild-type LPS phenotype was restored (Fig. 3b).

The defective LPS O-antigen in strain MSF487 was expected due to the presence of a conserved 8 bp motif, termed *ops* (operon polarity suppressor), in the 5′-untranslated region of the *S. flexneri wba* gene cluster (Bailey *et al.*, 1997) that encodes the majority of the genes required for OAg synthesis. RfaH recognizes RNA polymerase by interacting with the *ops* sequence, allowing transcript elongation in long operons (Artsimovitch & Landick, 2002). Hence, the RfaH function affects particularly transcription of distal genes. In *S. flexneri*, the *wzy* gene, which encodes the OAg polymerase, is the last gene in the *wba* operon (Morona *et al.*, 1994). Thus, we reasoned that absence of RfaH in strain MSF487 could result in low levels of transcription of *wzy* and impaired OAg polymerization. To investigate this hypothesis, we examined by qRT-PCR the transcript levels of *wzy* in the wild-type and Δ*rfaH* strains. The results showed a drastic reduction (120-fold) of *wzy* expression in the *rfaH* mutant compared to the wild-type ($P<0.0022$). When strain MSF487 was transformed with pJC75, a significant increase in the expression of *wzy* was observed ($P<0.0009$). The transcript levels of *wzy* in the complemented mutant were approximately 30 % of those obtained in the wild-type strain.

Overexpression of Wzy affects O-antigen chain-length distribution

The results described above indicated that the function of the *rfaH* gene is essential for *wzy* expression and normal polymerization of OAg in *S. flexneri* 2a. To investigate this

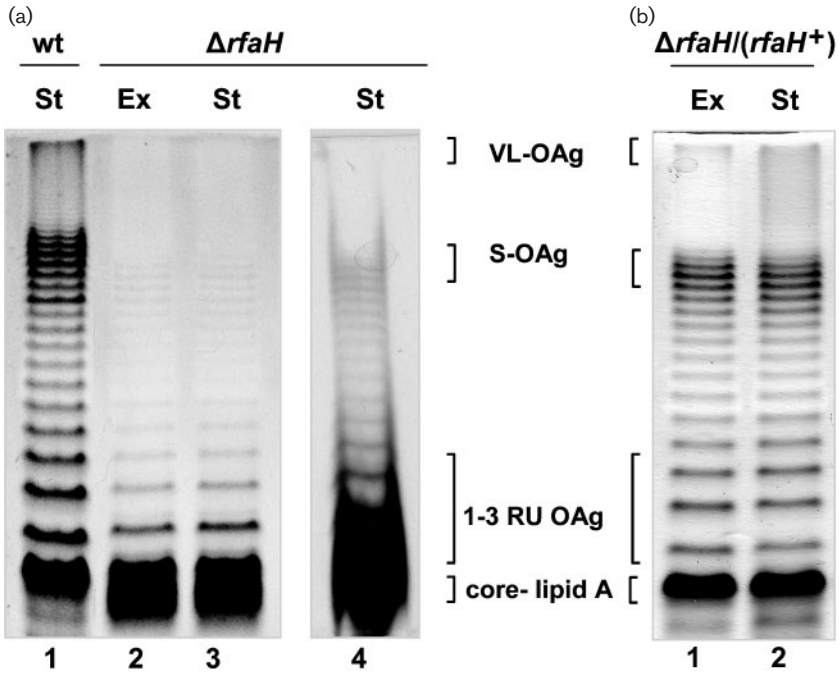

Fig. 3. Effect of the *rfaH* gene on LPS production by *S. flexneri* 2a during growth. LPS samples from equal numbers of bacterial cells grown to exponential (Ex) or to stationary (St) phase were loaded in each lane and analysed by Tricine/SDS-PAGE on a 14 % (w/v) acrylamide gel followed by silver staining. (a) Strains 2457T (wild-type, wt) and MSF487 (2457T Δ*rfaH*). Lane 4 was loaded with a 10-fold excess of MSF487 (2457T Δ*rfaH*) LPS. (b) Strain MSF487/pJC75 [2457T Δ*rfaH*/(*rfaH*$^+$)].

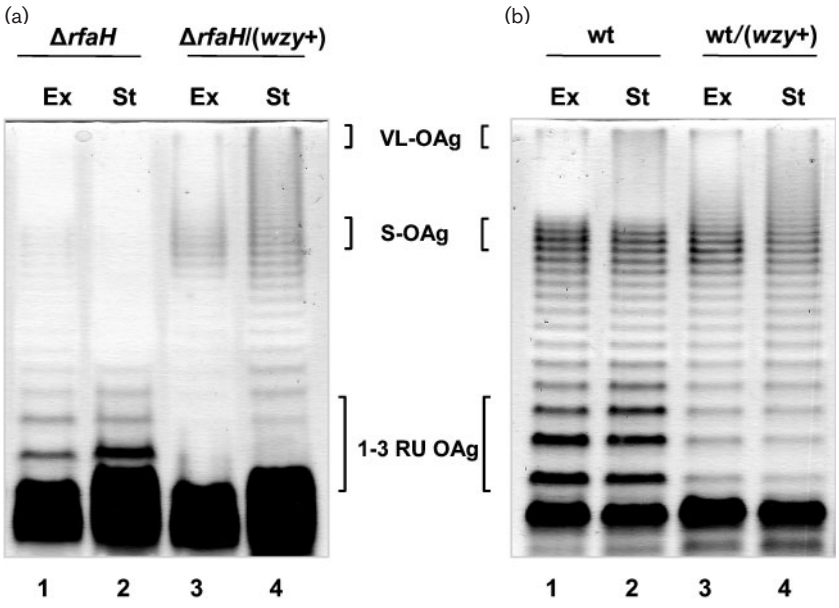

Fig. 4. Effect of overexpression of *wzy* on LPS production by *S. flexneri* 2a. Cells were transformed with plasmid pJC114 (wzy^+). LPS samples from equal numbers of bacterial cells grown to exponential (Ex) or to stationary (St) phase were loaded in each lane and analysed by Tricine/SDS-PAGE on a 14% (w/v) acrylamide gel followed by silver staining. (a) Strains MSF487 (2457T Δ*rfaH*) and MSF487/pJC114 [2457T Δ*rfaH*/(wzy^+)]. (b) Strains 2457T (wild-type, wt) and 2457T/pJC114 [2457T/(wzy^+)].

phenomenon further, we transformed the Δ*rfaH* mutant with plasmid pJC114, which carries the *wzy* gene in a multicopy plasmid. Analysis of the LPS patterns showed that both S-OAg and VL-OAg were produced at exponential and stationary phases of growth, while very small amounts of low-molecular-mass OAg molecules were detected (Fig. 4a, lanes 3 and 4). Thus, increased expression of *wzy* complements the defective LPS phenotype of the Δ*rfaH* mutant. However, close examination of the LPS profile exhibited by strain MSF487/pJC114 showed an altered LPS pattern compared to the wild-type: a higher amount of high-molecular-mass OAg molecules relative to low-molecular-mass OAg chains was observed. This result suggested that cellular levels of Wzy could influence not only OAg polymerization but also the chain-length distribution of OAg chains. To test this notion, we transformed the wild-type strain with pJC114 and analysed the LPS profiles during bacterial growth. As shown in Fig. 4(b), the LPS molecules had lower numbers of OAg repeat units of low molecular mass (1–3 RU) and fewer S-OAg chains than the wild-type LPS. In contrast, a large amount of high-molecular-mass OAg molecules were produced. These data indicate that levels of Wzy are important for VL-OAg expression.

Effect of overexpression of the chain-length determinants on O-antigen synthesis

To investigate the influence of an increased dosage of the genes that control chain-length distribution on OAg production during bacterial growth, we cloned the *wzzB* and wzz_{pHS-2} genes into the multicopy plasmid pGEM-T. The resulting plasmids, pJC139 (*wzzB*) and pJC144 (wzz_{pHS-2}), were transformed into the wild-type strain and LPS profiles were analysed (Fig. 5). *S. flexneri* 2457T/pJC139 produced greater numbers of S-OAg chains than the wild-type, but did not synthesize VL-OAg molecules. In this strain production of S-OAg was growth-phase-regulated, increasing at stationary phase. On the other hand, overexpression of the wzz_{pHS-2} gene (2457T/pJC144) resulted in higher production of VL-OAg than the

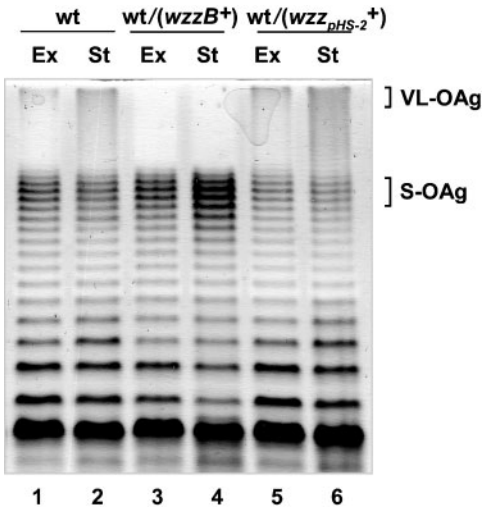

Fig. 5. Effect of overexpression of the O-antigen chain length regulators WzzB and Wzz_{pHS-2} on LPS production by *S. flexneri* 2a 2457T during growth. Cells were transformed with plasmid pJC139 ($wzzB^+$) or pJC144 ($wzz_{pHS-2}{}^+$). LPS samples from equal numbers of bacterial cells grown to exponential (Ex) or to stationary (St) phase were loaded in each lane and analysed by Tricine/SDS-PAGE on a 14% (w/v) acrylamide gel followed by silver staining. Strains are: 2457T (wild-type, wt), 2457T/pJC139 [(2457T/($wzzB^+$)] and 2457T/pJC144 [(2457T/($wzz_{pHS-2}{}^+$)].

wild-type, particularly during stationary phase. In contrast, this strain produced a small amount of S-OAg chains at both exponential and stationary phases of growth (Fig. 5). These results indicate that not only the levels of Wzy, but also those of the Wzz chain-length regulators are critical to determine the chain-length distribution of OAg in *S. flexneri* 2a. To investigate whether expression of the *wzz* genes is under growth-phase control, we constructed transcriptional fusions of the *wzzB* and wzz_{pHS-2} promoter regions to *lacZ* in plasmid pFZY1. The resulting plasmids, pCB326 and pCB280, respectively, were transformed into the wild-type and Δ*rfaH* strains and β-galactosidase activity was assayed during growth (Fig. 6). Expression of both *wzzB* and wzz_{pHS-2} genes increased during the growth cycle, reaching maximal activities at stationary phase. However, no differences in expression levels were observed in the Δ*rfaH* genetic background compared to those obtained in the wild-type strain. From these data, we conclude that the differential effect of growth phase on OAg chain-length distribution is not due to an *rfaH*-dependent regulation of either chain length determinant.

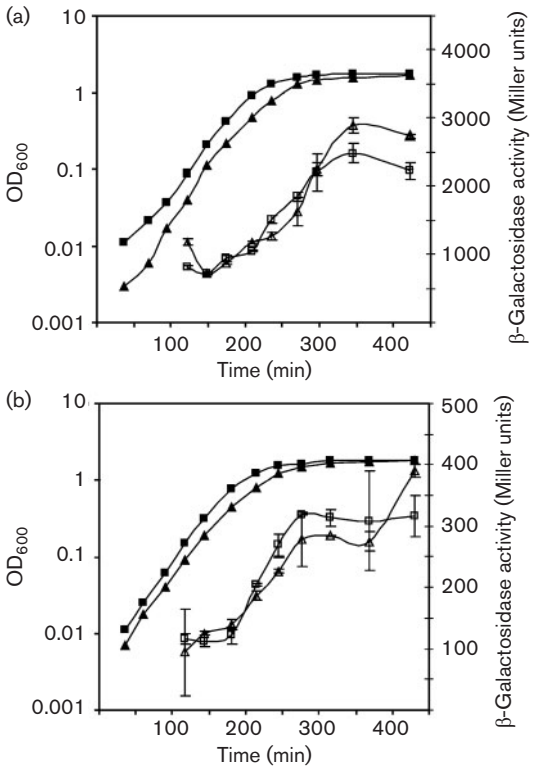

Fig. 6. Growth and expression of *wzzB–lacZ* and wzz_{pHS-2}–*lacZ* transcriptional fusions in *S. flexneri* 2a 2457T and Δ*rfaH* mutant. Cells were transformed with plasmids pCB326 (*wzzB-lacZ*) or pCB280 (wzz_{pHS-2}-*lacZ*). Growth was measured as OD_{600}. The expression of the *wzzB–lacZ* or wzz_{pHS-2}–*lacZ* transcriptional fusions was measured as β-galactosidase activity and expressed in Miller units. Data are the means ± SD of triplicate assays in two independent experiments. (a) Growth of strains 2457T/pCB326 (■, □) and MSF487/pCB326 (2457T Δ*rfaH*/pCB326) (▲, △). (b) Growth (■, ▲) and β-galactosidase activity (□, △) of strains 2457T/pCB280 (■, □) and MSF487/pCB280 (2457T Δ*rfaH*/pCB280) (▲, △).

DISCUSSION

Several studies have indicated a role for the LPS OAg in the pathogenesis of *S. flexneri* infection (Hong & Payne, 1997; Morona *et al.*, 2003; Sandlin *et al.*, 1995; Van den Bosch *et al.*, 1997). It has been reported that not only the presence of OAg side chains, but also the number and proper length distribution of OAg molecules are important for full virulence of *S. flexneri* 2a (Hong & Payne, 1997; Morona *et al.*, 2003). Yet, in contrast to the wealth of information available on the regulatory mechanisms that control expression of *Shigella* invasion proteins (Dorman & Porter, 1998; Sansonetti, 2001), little is known about environmental regulation of OAg synthesis and chain-length distribution.

In this study, we demonstrate a differential growth-phase regulation of OAg production in *S. flexneri* 2a. Our results showed that the production of VL-OAg correlates with the growth-dependent expression of the RfaH transcription elongation factor. LPS production was severely impaired in a Δ*rfaH* mutant which, while it was able to synthesize a small amount of low-molecular-mass OAg chains and a few S-OAg molecules, was totally devoid of VL-OAg. The LPS phenotype exhibited by the Δ*rfaH* mutant was not attributable to a deficit in the expression of either *wzzB* or wzz_{pHS-2} chain length regulators, but rather it was the result of diminished transcription of *wzy*. Quantitative RT-PCR results showed over 100-fold reduction in the transcript levels of *wzy* in the Δ*rfaH* mutant compared to the wild-type.

Interestingly, complementation of the Δ*rfaH* mutant with a high-copy-number plasmid carrying the *rfaH* gene could not restore wild-type levels of *wzy* transcription. Since RfaH controls the expression of an important number of membrane components (Bailey *et al.*, 1997) it is plausible that the deletion or overexpression of this regulator could generate a membrane stress response in order to maintain cellular homeostasis. Two recent studies support this notion. Nagy *et al.* (2006) showed that loss of RfaH not only had an impact on genes involved in LPS synthesis but also had an indirect and marked effect on a number of membrane components such as the flagellum/chemotaxis complex and type III secretion system. In addition, Bengoechea *et al.* (2002) demonstrated that overexpression of Wzz in *Yersinia enterocolitica* causes a membrane stress response that activates the CpxAR two-component signal transduction system, which in turn downregulates the expression of the OAg biosynthetic machinery.

The results discussed above suggested that an augmentation in RfaH levels increases Wzy expression and OAg polymerization during stationary phase. In support of this hypothesis, overexpression of *wzy* in the wild-type strain

results in an altered pattern of OAg synthesis displaced towards the production of high-molecular-mass chains. Our findings are in accordance with results obtained by Daniels *et al.* (1998) showing that complementation of a *wzy* mutant with the *wzy* gene cloned on a high-copy-number plasmid produces LPS with an increased OAg chain length.

The demonstration that overexpression of the chain length determinants can alter the OAg modal distribution further underlines the importance of a specific balance between Wzy, WzzB and Wzz_{pHS-2} in determining the OAg modal distribution, as proposed by Daniels *et al.* (1998). In addition, our results showing that overexpression of WzzB completely shifts the OAg distribution from VL-OAg to S-OAg, but that overexpression of Wzz_{pHS-2} can not do the opposite as efficiently, support the hypothesis of Stevenson *et al.* (1995), who proposed that Wzz_{pHS-2} does not compete efficiently with WzzB in influencing the OAg chain-length distribution in *S. flexneri*.

Altogether, our data suggest that regulation of Wzy levels is not only important for normal OAg polymerization but is also essential in defining the OAg modal distribution for the following reasons. First, a Δ*rfaH* mutant is unable to produce VL-OAg even though RfaH has no impact on wzz_{pHS-2} expression. Second, overexpression of *wzy* in a Δ*rfaH* background can restore the OAg bimodal distribution despite global OAg synthesis deficiency. Third, overexpression of *wzy* in the wild-type strain results in an altered pattern of OAg synthesis shifted towards the production of high-molecular-mass chains. And fourth, overexpression studies of the chain length regulators support the notion that there is a competition for the available pool of Wzy in order to determine a specific OAg modal distribution in *S. flexneri*.

The experiments with *lacZ* fusions demonstrated that transcription of both *wzzB* and wzz_{pHS-2} also increases upon entry into stationary phase, but in an RfaH-independent manner. Although at the moment we can only speculate how environmental conditions could modulate the OAg modal distribution in *Shigella*, several reports have begun to unravel a variety of mechanisms involved in OAg chain length regulation. *Salmonella* Typhimurium possesses two chain length regulators, WzzB and Wzz_{fepE} (Murray *et al.*, 2003). It has been shown that while *wzzB* expression is tightly regulated in response to conditions of low Mg^{2+} plus Fe^{3+} through the PmrA/PmrB and RcsC/YojN/RcsB systems (Delgado *et al.*, 2006), wzz_{fepE} expression is regulated by conditions that stimulate swarming motility (Wang *et al.*, 2004). Additional control of wzz_{fepE} expression by the flagellar master regulator FlhDC has been reported in *Escherichia coli* (Stafford *et al.*, 2005). In addition, genome-wide analysis of the DNA adenine methyltransferase (Dam) regulon in *E. coli* described *wzzB* as one of many genes found to be repressed by the presence of this regulator (Robbins-Manke *et al.*, 2005). Since Dam levels are downregulated in stationary phase (Seshasayee, 2007), the increase of *wzzB* transcription could be a result of diminished levels of Dam.

Unpublished results from our laboratories have implicated environmental signals such as oxygen availability and amino acid deprivation in the expression of *rfaH* and both chain-length regulators. These conditions are present in the stationary phase of growth and could therefore be responsible, at least in part, for the growth-phase regulation of these genes. How these and other environmental signals converge in order to regulate both LPS production and OAg modal distribution is under current investigation in our laboratories.

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