O-antigen modal chain length in Shigella 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 WzzpHS-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 Δrfah 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 Δrfah 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 wzzpHS-2 also altered OAg chain-length distribution. Transcription of wzzB and wzzpHS-2 genes was regulated during bacterial growth but in an RfaH-independent manner. Overall, these findings indicate that expression of the wzy, wzzB and wzzpHS-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...
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 WzzpHS-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 wzzpHS-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, WzzpHS-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 wzzpHS-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 wzzpHS-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, wzzpHS-2 and wzy genes. DNA fragments containing the S. flexneri 2457T rfaH (GeneID:1080050), wzzB (GeneID:1077593), wzzpHS-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 wzzpHS-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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties</th>
<th>Source or reference</th>
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<tr>
<td><strong>S. flexneri 2a</strong></td>
<td>Wild-type</td>
<td>ISP†</td>
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<tr>
<td>2457T</td>
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<td>This study</td>
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<td>MSFI47</td>
<td>2457T ArsH</td>
<td>Koop et al. (1987)</td>
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<tr>
<td>MSFI07</td>
<td>2457T wzz&lt;sub&gt;2457T&lt;/sub&gt;Δ::aph, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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**Plasmids**

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<tr>
<th>Plasmid</th>
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<th>Source or reference</th>
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<tr>
<td>pFY1</td>
<td>Promoterless cloning vector, Amp&lt;sup&gt;R&lt;/sup&gt; lac Φ' lac replicon, lacZ&lt;sup&gt;+&lt;/sup&gt; Y&lt;sup&gt;+&lt;/sup&gt; A&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Koop et al. (1987)</td>
</tr>
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<td>pCB315</td>
<td>pFY1 containing a 315 bp fragment including the promoter region of rfaH of <em>S. flexneri</em> 2a 2457T</td>
<td>This study</td>
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<td>pCB326</td>
<td>pFY1 containing a 326 bp fragment including the promoter region of wzzB of <em>S. flexneri</em> 2a 2457T</td>
<td>This study</td>
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<tr>
<td>pCB280</td>
<td>pFY1 containing a 280 bp fragment including the promoter region of wzz&lt;sub&gt;2457T&lt;/sub&gt; of <em>S. flexneri</em> 2a 2457T</td>
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<td>pCB261</td>
<td>pFY1 containing a 261 bp fragment of an intragenic region of rfaH of <em>S. flexneri</em> 2a 2457T</td>
<td>This study</td>
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<td>pKD46</td>
<td>bla&lt;sub&gt; PBAD&lt;/sub&gt; gam bet eso pSC101 oriTS</td>
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<tr>
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<td>bla FRT aph FRT P51 P52 oriR6K</td>
<td>Datsenko &amp; Wanner (2000)</td>
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<td>pCP20</td>
<td>bla cat cI857 ΔP&lt;sub&gt;n&lt;/sub&gt; fbp pSC101 oriTS</td>
<td>Cherepanov &amp; Wackernagel (1995)</td>
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<td>wzz&lt;sub&gt;2457T&lt;/sub&gt; gene cloned into pGEM-T Easy</td>
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*†*Amp<sup>R</sup>, ampicillin resistant; Kan<sup>R</sup>, kanamycin-resistant.

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 pFY1 to generate plasmid pCB261, which was used as negative control.

**RNA extraction.** Bacterial cells grown to early exponential (OD<sub>600</sub> 0.1) and stationary phase (OD<sub>600</sub> 1.5) were incubated with lysozyme (1 mg ml<sup>-1</sup>) 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<sub>260</sub>/A<sub>280</sub>). A ratio A<sub>260</sub>/A<sub>280</sub> > 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<sub>580</sub> 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 Bacteriologicos) 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).
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 (OD600) 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.

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 DrfaH mutant. As shown in Fig. 3(a), MSF487 lacked LPS with VL-OAg chains at both exponential and stationary phases (lanes 2 and 3).
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 ΔrfaH strains. The results showed a drastic reduction (120-fold) of wzy expression in the ΔrfaH 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
phenomenon further, we transformed the DrfAH 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 DrfAH 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 wzzpHS-2 genes into the multicopy plasmid pGEM-T. The resulting plasmids, pJC139 (wzzB) and pJC144 (wzzpHS-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 wzzpHS-2 gene (2457T/pJC144) resulted in higher production of VL-OAg than the
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 wzzpHS-2 promoter regions to lacZ in plasmid pFZY1. The resulting plasmids, pCB326 and pCB280, respectively, were transformed into the wild-type and ΔrfaH strains and β-galactosidase activity was assayed during growth (Fig. 6). Expression of both wzzB and wzzpHS-2 genes increased during the growth cycle, reaching maximal activities at stationary phase. However, no differences in expression levels were observed in the ΔrfaH 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.

**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 ΔrfaH 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 ΔrfaH mutant was not attributable to a deficit in the expression of either wzzB or wzzpHS-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 ΔrfaH mutant compared to the wild-type.

Interestingly, complementation of the ΔrfaH 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 would...
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 WzzpHS-2 in determining the OAg modal distribution. In addition, our results showing that overexpression of WzzB completely shifts the OAg distribution from VL-OAg to S-OAg, but that overexpression of WzzpHS-2 cannot do the opposite as efficiently, support the hypothesis of Stevenson et al. (1995), who proposed that WzzpHS-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 ΔrfaH mutant is unable to produce VL-OAg even though RfaH has no impact on wzzpHS-2 expression. Second, overexpression of wzy in a ΔrfaH 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 wzzpHS-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 WzzfepE (Murray et al., 2003). It has been shown that while wzzB expression is tightly regulated in response to conditions of low Mg²⁺ plus Fe³⁺ through the PmrA/PmrB and RcsC/YojN/RcsB systems (Delgado et al., 2006), wzzfepE expression is regulated by conditions that stimulate swarming motility (Wang et al., 2004). Additional control of wzzfepE 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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