O-antigen expression in *Salmonella enterica* serovar Typhi is regulated by nitrogen availability through RpoN-mediated transcriptional control of the *rfaH* gene

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The authors previously reported increased expression of the *Salmonella enterica* serovar Typhi (*S. typhi*) *rfaH* gene when the bacterial cells reach stationary phase. In this study, using a *lacZ* fusion to the *rfaH* promoter region, they demonstrate that growth-dependent regulation of *rfaH* expression occurs at the level of transcription initiation. It was also observed that production of the lipopolysaccharide (LPS) O-antigen by *S. typhi* Ty2 correlated with the differential expression of *rfaH* during bacterial growth. This was probably due to the increased cellular levels of RfaH, since expression of the distal gene in the O-antigen gene cluster of *S. typhi* Ty2, *wbaP*, was also increased during stationary growth, as demonstrated by RT-PCR analysis. Examination of the sequences upstream of the *rfaH* coding region revealed homologies to potential binding sites for the RcsB/RcsA dimer of the RcsC/YopJ/RcsB phosphorelay regulatory system and for the RpoN alternative sigma factor. The expression of the *rfaH* gene in *rpoN* and *rcsB* mutants of *S. typhi* Ty2 was measured. The results indicate that inactivation of *rpoN*, but not of *rcsB*, suppresses the growth-phase-dependent induction of *rfaH* expression. Furthermore, production of β -galactosidase mediated by the *rfaH-lacZ* fusion increased approximately fourfold when bacteria were grown in a nitrogen-limited medium. Nitrogen limitation was also shown to increase the expression of the O-antigen by the wild-type *S. typhi* Ty2, as demonstrated by a similar electrophoretic profile to that observed during the stationary phase of growth in rich media. It is therefore concluded that the relationship between LPS production and nitrogen limitation parallels the pattern of *rfaH* regulation under the control of RpoN and is consistent with the idea that RpoN modulates LPS formation via its effect on *rfaH* gene expression during bacterial growth.

Keywords: lipopolysaccharide, regulation, transcription, sigma factor

INTRODUCTION

Salmonella enterica serovar Typhi (herein abbreviated as *S. typhi*) causes typhoid fever in humans, a disease of great public health concern in many developing countries (Pang *et al.*, 1998). The detailed molecular mechanisms specifically involved in the pathogenesis of *S. typhi* are poorly understood due to the lack of an animal model. However, a great deal of information has been obtained from studies using *S. enterica* serovar Typhimurium, which infects a wide spectrum of animal

hosts and causes a typhoid-like disease in the mouse (Jones & Falkow, 1996). We are interested in the analysis of the biosynthesis and regulation of *S. typhi* surface polysaccharides, especially the lipopolysaccharide (LPS). This glycolipid surface molecule is an abundant component of the bacterial outer membrane and plays a role in pathogenesis by protecting micro-organisms from the lytic action of serum complement (Joiner, 1988). In *S. typhi*, LPS also interacts with intestinal epithelial cells during the initial stages of infection (Pier *et al.*, 1998; Lyczak *et al.*, 2001).

LPS has a tripartite structure that includes lipid A, a core oligosaccharide and the O-specific polysaccharide or O-antigen (Schnaitman & Klena, 1993; Raetz, 1996). The O-antigen is the most surface-exposed LPS component and displays enormous structural variability, resulting in a large variety of serotypes (Reeves, 1993). *S. typhi* also produces a group I exopolysaccharide known as the Vi antigen, which is made of a homopolymer of high molecular mass (Virlogeux *et al.*, 1996) and forms a capsular structure. The Vi antigen is found in virtually all clinical isolates from patients with acute typhoid infection. It protects *S. typhi* against complement-mediated lysis as well as phagocytosis (Kossack *et al.*, 1981).

The biosynthesis of exopolysaccharides is modulated by environmental factors through several regulatory systems (Virlogeux *et al.*, 1996; Arricau *et al.*, 1998; Whitfield & Roberts, 1999). One of these regulatory components is the RcsC/RcsB two-component system, which consists of a cytoplasmic sensor kinase (RcsC) and cytosolic response regulator (RcsB). More recently, a phosphorelay system involving the phosphotransmitter YojN was shown to be essential for transducing the signal from RcsC to RcsB in *Escherichia coli* (Takeda *et al.*, 2001). It is quite possible that the same mechanism operates in *S. typhi*, since a homologue of YojN is also present in this bacterium. In *E. coli*, stimuli like osmotic shock (Sledjeski & Gottesman, 1996) or growth at a low temperature (Whitfield & Roberts, 1999) result in the phosphorylation of RcsB, which associates with the proteolytically labile protein RcsA forming a heterodimer that acts as a positive transcriptional regulator (Stout & Gottesman, 1990). The activation of RcsC/YojN/RcsB-responsive promoters in *E. coli* and in other *Enterobacteriaceae* involves the recognition by the RcsB/RcsA dimer of a relatively conserved DNA sequence known as the 'RcsAB box' (Wehland & Bernhard, 2000). This box, consisting of 14 base pairs (TaAGaatATCctA), has been found in the upstream region of the promoter sequences of the colanic acid biosynthesis cluster of *E. coli* K-12, the K2 antigen cluster of *Klebsiella pneumoniae*, and the Vi antigen cluster of *S. typhi* (Wehland & Bernhard, 2000).

The synthesis of LPS in *S. typhi* involves a large number of genes, the majority of which are organized in various clusters located on separate regions of the bacterial chromosome. It is conceivable that LPS gene expression at all of these various sites must be coordinated to ensure that all necessary components are available at any given time. Yet the regulation of LPS synthesis is not well understood. In *E. coli* and *S. enterica* serovar Typhimurium, a covalent substitution of lipid A with 4-amino-4-deoxy-L-arabinose is regulated by the *pmrA* gene (Gunn & Miller, 1996), which encodes a transcription factor that is activated during growth under mildly acidic conditions, in a PhoP/PhoQ-dependent manner during Mg²⁺ limitation, or by exposure to Fe³⁺ ions (Guo *et al.*, 1997; Gunn *et al.*, 1998; Ernst *et al.*, 2001). This LPS modification reduces the net negative charge of the molecule, thus contributing to bacterial

resistance to cationic peptides and presumably enhancing intracellular survival within phagosomes. Another level of regulation involves the regulation of gene expression of the core biosynthetic cluster by the RfaH protein (Farewell *et al.*, 1991; Pradel & Schnaitman, 1991) and also by the heat-shock response (Karow *et al.*, 1991). RfaH is a homologue of the NusG factor that regulates gene expression of the haemolysin operon (Bailey *et al.*, 1992; Leeds & Welch, 1996, 1997), polysaccharide capsule genes (Stevens *et al.*, 1997), the F plasmid *tra* operon (Beutin & Achtman, 1979), and a gene involved in iron acquisition (Nagy *et al.*, 2001). RfaH regulation occurs during transcript elongation and depends on a 5'-proximal, transcribed nucleic acid sequence known as *ops* (for operon polarity suppressor; Nieto *et al.*, 1996; Bailey *et al.*, 1997) that induces transcriptional pausing *in vitro* (Artsimovitch & Landick, 2000) and *in vivo* (Leeds & Welch, 1997). It has been recently demonstrated that RfaH recognizes RNA-polymerase transcribing RfaH-regulated operons by interacting with the *ops* sequence in the exposed nontemplate DNA strand of *ops*-paused transcription complexes (Artsimovitch & Landick, 2002).

5'-proximally transcribed sequences containing *ops* elements exist in the O-polysaccharide gene clusters of many enteric bacteria (Hobbs & Reeves, 1994), suggesting that RfaH also plays a role in the regulation of the transcription elongation of O-antigen genes. In a previous study, Marolda & Valvano (1998) conducted a detailed analysis of the promoter region of the O7-specific genes in *E. coli*. Using single-copy-number fusions to a reporter gene, these authors did not observe any detectable regulation of the O7-specific promoter at the level of initiation of transcription, concluding that regulation only occurs at the level of mRNA elongation in an RfaH-dependent manner.

Therefore, modulation of the cellular levels of RfaH may contribute to coordinate expression of O-antigen and core LPS biosynthetic enzymes. Not much is known, however, about the regulation of the *rfaH* gene itself. In a previous study, we isolated the *rfaH* gene from the *S. typhi* strain Ty2 and confirmed that its function is essential for LPS expression (Rojas *et al.*, 2001). More importantly, we demonstrated that *rfaH* gene expression varies with the growth phase, with the highest expression during stationary phase (Rojas *et al.*, 2001). In this study, we provide evidence showing that regulation of *rfaH* depends, at least in part, on the activity of the RpoN alternative sigma factor and that differential *rfaH* expression influences a similar pattern of O-antigen production during the bacterial growth cycle.

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 in Luria-Bertani medium (LB: Bacto tryptone, 10 g l⁻¹; Bacto yeast extract, 5 g l⁻¹; NaCl, 5 g l⁻¹) or in minimal E medium (MgSO₄·7H₂O, 0.2 g l⁻¹; citric acid monohydrate, 2 g l⁻¹;

Table 1. *S. typhi* strains and plasmids used in this study

Strain or plasmid	Relevant properties*	Source or reference
Salmonella Typhi		
Ty2	<i>Salmonella enterica</i> serovar Typhi (<i>S. typhi</i>), wild-type	Institute of Public Health, Chile (ISP)
M8	Ty2 <i>rfaH</i> , Cam ^R	Rojas <i>et al.</i> (2001)
M161	Ty2 <i>rpoN</i> , Cam ^R	This study
M159	Ty2 <i>rcsB</i> , Kan ^R	This study
MEI120	Ty2 Φ (<i>bisD-lacZ</i>), Kan ^R	Lyczak <i>et al.</i> (2001)
Plasmids		
pGEM-T Easy	Cloning vector, Amp ^R	Promega
pSM334	pGEM-T Easy containing a 334 bp fragment of the <i>yigC-rfaH</i> intergenic region from <i>S. typhi</i> Ty2	This study
pKD46	pINT-ts derivative containing <i>araC-P_{araB}</i> and γ β exo DNA fragments	Datsenko & Wanner (2000)
pKD4	pANTS _{γ} derivative containing an FRT-flanked Kan ^R gene	Datsenko & Wanner (2000)
pKD3	pANTS _{γ} derivative containing an FRT-flanked Cam ^R gene	Datsenko & Wanner (2000)
pFZY1	Promoterless cloning vector, Amp ^R F' <i>lac</i> replicon, <i>lacZ</i> ⁺ Y ⁺ A ⁺	Koop <i>et al.</i> (1987)
pCE334	pFZY1 containing a 334 bp fragment of the <i>yigC-rfaH</i> intergenic region from <i>S. typhi</i> Ty2	This study
pKHT19	pBluescript/KSII derivative containing a 723 bp fragment including the <i>rfaH</i> gene from <i>S. typhi</i> Ty2	Rojas <i>et al.</i> (2001)

* Cam, chloramphenicol; Kan, kanamycin; Amp, ampicillin.

Table 2. Primers used in this study

Primer	Sequence
WrcsB1	CGG CGA ATT TGA AGA TTC CAC AGC ATT GAT CAA CAA CCT GTG TAG GCT GGA GCT GCT TCG
WrcsB2	TTA ATG CTG CGG TTG AGC TTC TTG GCG ATT TCG GTG ACC ATT CCG GGG ATC CGT GCA CC
WrpoN1	CAA CAG GCC ATC CGT CTG TTG CAG TTG TCT ACG CTG GAA CTG TGT AGG CTG GAG CTG CTT CG
WrpoN2	CAG TAA TTT CGA CGT TAT GTC CGG TGA TAT TGA GCT GCA TAT GAA TAT CCT CCT TAG
RfaH2	GTG ATA TTT GAT GGC GTC CAT TGT A
RfaHint2	AAG GCC TTC GTT TTC CGC GTA CCA TTT TT
WbaP591	CTC CCC GGG AAT GGA TAA TAT TGA TAA TAA G
WbaP592	TCG GAT ATC TTA ATA CGC ACC ATC TGC CC
HisG1	CCG GAT CCA GAC AAC ACC CGC TTA CGC ATA G
HisG2	CCG GAT CCT CTC CAT GGT TTC CCA GAA CAA C

K₂HPO₄·3H₂O, 13.1 g l⁻¹; NaNH₄HPO₄·4H₂O, 3.3 g l⁻¹) containing 0.2 % glucose as carbon source. Low-nitrogen E medium contained 1 % of the amount of nitrogen in E medium. Minimal media were supplemented with 50 µg cysteine ml⁻¹ and 50 µg tryptophan ml⁻¹. Media were supplemented with 100 µg ampicillin ml⁻¹, 20 µg chloramphenicol ml⁻¹ or 50 µg kanamycin ml⁻¹ as appropriate.

Construction of a *lacZ* transcriptional fusion to the *rfaH* gene.

The *S. typhi* Ty2 *rfaH* promoter region was amplified by PCR using the *rfaH2* and *rfaHint2* primers (Table 2), which were designed according to the DNA sequence information available for the *S. typhi* strain CT18 (Parkhill *et al.*, 2001). A 334 bp amplicon was cloned into the vector pGEM-T Easy, to yield plasmid pSM334. The 334 bp insert in pSM334 was then transferred into the plasmid pFZY1. This is a single-copy-

number vector designed for the construction of transcriptional fusions to the *lac* operon (Koop *et al.*, 1987). The resulting plasmid, pCE334, was transformed into *S. typhi* Ty2. The pFZY1 vector was also transformed as a negative control.

Mutagenesis of *S. typhi rcsB* and *rpoN* genes. Mutagenesis was performed according to the method described by Datsenko & Wanner (2000) to disrupt specific chromosomal genes using PCR products. For this purpose, *S. typhi* Ty2 was transformed with pKD46, a temperature-sensitive, low-copy-number plasmid that expresses the phage λ Red recombinase system under the control of the arabinose-inducible *P_{araB}* promoter. The Red-mediated recombination is required to replace the targeted chromosomal sequence with an antibiotic-resistance gene that is generated by PCR. *S. typhi* Ty2 cells

carrying pKD46 were transformed by electroporation with a PCR product that was generated using either plasmid pKD3 or pKD4 as templates. The plasmid pKD3 carries a chloramphenicol acetyltransferase gene flanked by FRT sites while pKD4 carries a gene encoding kanamycin resistance, also flanked by FRT sites. Primers WrpoN1 and WrpoN2 were used with the pKD3 template to obtain a product for disruption of *rpoN*. Primers WrcsB1 and WrcsB2 were used with the pKD4 template to obtain a product for disruption of *rscB*. The sequences of the primers are shown in Table 2. Transformants were plated on LB agar plates containing chloramphenicol, for the selection of *rpoN* mutants, or kanamycin, for the selection of *rscB* mutants. Transformants were replica plated in the absence of antibiotic selection at 43 °C and then assayed for ampicillin sensitivity, to confirm the loss of pKD46.

RT-PCR. For expression analysis, each strain was grown to the selected OD₆₀₀ in 50 ml LB. RNA was extracted using the standard TRIzol procedure. After DNase I treatment, RNA was reverse transcribed using SuperScript II (200 U µl⁻¹) and antisense primers for *hisG* and *wbaP* (HisG2 and WbaP592, respectively). Single-stranded DNA was then amplified using the primers for genes: *hisG* (HisG1 and HisG2) and *wbaP* (WbaP591 and WbaP592). The sequences of the primers are indicated in Table 2. The PCR products were analysed by electrophoresis on 1.5 % agarose gels.

LPS analysis. Culture samples were adjusted to OD₆₀₀ 2.0 in a final volume of 100 µl. Then, proteinase-K-digested whole-cell lysates were prepared as described by Hitchcock & Brown (1983) and LPS was separated on 14 % acrylamide gels using a Tricine/SDS buffer system (Lesse *et al.*, 1990). Gel loadings were normalized so that each sample represented the same number of cells. Each well was loaded with approximately 1 × 10⁸ c.f.u. Gels were silver stained by a modification of the procedure of Tsai & Frasch (1982). Densitometric analyses of the gels were performed using the UN-SCAN-IT gel software (Silk Scientific).

β-Galactosidase assays. Bacteria were grown overnight in LB or minimal E medium, subcultured and grown in 100 ml of the same medium on an orbital shaker. Every 30 min, a 2 ml sample was withdrawn to measure the bacterial growth (OD₆₀₀ and c.f.u. ml⁻¹) and the β-galactosidase activity according to Miller (1972). Enzyme activities (Miller units), normalized for cell density (OD₆₀₀), were calculated using the equation [(A₄₂₀ - 1.75A₅₅₀) × 1000]/[reaction time (min) × culture volume (ml) × OD₆₀₀]. Each sample was analysed in triplicate during at least three independent experiments.

RESULTS

Expression of *S. typhi* LPS during growth correlates with *rfaH* transcription

It is well documented that the RfaH elongation factor positively regulates the LPS core and O-polysaccharide genes. The environmental signals, however, that modulate the expression of the *rfaH* gene are largely unknown. In a previous study, we used RT-PCR analysis to test the effects of various growth conditions on the transcription of *rfaH* (Rojas *et al.*, 2001). We demonstrated that the transcription of *rfaH* in *S. typhi* Ty2 displays a growth-phase-dependent regulation, with maximal expression during late-exponential and stationary phases. In contrast, we found that environmental factors that are

known to be important for LPS regulation in other systems, such as growth temperature (Al-Hendy *et al.*, 1991) and osmolarity (Aguilar *et al.*, 1997), do not affect transcription of the *rfaH* gene in cells grown to late exponential phase (Rojas *et al.*, 2001). To determine whether the transcriptional regulation of the *rfaH* gene is exerted at the promoter level we constructed a transcriptional fusion of the *rfaH* promoter with a promoterless *lac* operon in a single-copy-number plasmid (pFZY1). The resulting plasmid, pCE334, was transformed into *S. typhi* Ty2. The pFZY1 vector was also transformed as a negative control. The production of β-galactosidase in strain Ty2/pCE334 was assayed during the various stages of growth in rich medium at 37 °C. The units of enzyme activity were corrected for the background levels determined in strain Ty2/pFZY1, which ranged between 0.5 and 5.0 Miller units during growth in LB medium. Fig. 1(a) shows that β-galactosidase production, driven by the *rfaH* promoter region, increases at the late exponential growth phase, reaching maximal expression during stationary phase. These results are consistent with the idea that growth-regulated expression of *rfaH* is exerted at the level of transcription. To rule out the possibility that the upregulation of the *rfaH* gene was a general growth-phase-dependent phenomenon, we assayed the production of β-galactosidase in *S. typhi* strain ME1120, which contains a *MudJ*(kan, *lac*) insertion in the *hisD* gene (Lyczak *et al.*, 2001). Fig. 1(a) shows that the *hisD-lacZ* expression does not change in response to the bacterial growth phase.

To examine whether the growth-regulated expression of *rfaH* has any biological significance in terms of LPS synthesis, we monitored the formation of *S. typhi* LPS during growth. Fig. 1(b) shows that the production of O-specific LPS varies during the bacterial growth. During the exponential phase (lanes 1 and 2), a very small amount of O-antigen is observed. As cells reach the late exponential phase, the formation of O-antigen increases (lanes 3, 4 and 5), and it is maximally expressed at the stationary phase (lanes 6 and 7). Densitometric analysis of the LPS gel in Fig. 1(b) revealed that the ratio of O-antigen to the lipid A-core region in the sample grown to OD₆₀₀ 1.239 (Fig. 1b, lane 7) was approximately twofold (183 %) higher than that of the sample grown to OD₆₀₀ 0.157 (Fig. 1b, lane 1). The pattern of O-antigen expression parallels the growth-regulated expression of *rfaH*, suggesting that the formation of O-antigen LPS by the bacterial cells during growth is modulated by the cellular levels of RfaH. In support of this notion, no changes in O-antigen expression during growth were observed when *S. typhi* Ty2 was transformed with the high-copy-number plasmid pKHT19, harbouring the *rfaH* gene (data not shown). Similar results were obtained when an *rfaH* null mutant was transformed with pKHT19.

The first step in the synthesis of the O-antigen in species of *Salmonella* involves the addition of galactose 1-phosphate onto undecaprenol-P to produce a galactose-P-P-undecaprenol intermediate that is strictly required

Fig. 1. Expression of *rfaH* and production of LPS in *S. typhi* Ty2/pCE334 at different points of the growth curve in LB at 37 °C. (a) Growth curve of *S. typhi* Ty2/pCE334 and expression of *rfaH-lacZ* transcriptional fusion. ○, Growth curve (OD₆₀₀); ●, β-galactosidase activity of the *rfaH-lacZ* fusion; □, growth of strain ME1120 (*hisD-lacZ* fusion) used as control; ■, β-galactosidase activity of the *hisD-lacZ* fusion. Data are the mean ± SD of three independent assays. (b) LPS profiles of cells grown to the following OD₆₀₀: lane 1, 0.157; lane 2, 0.353; lane 3, 0.462; lane 4, 0.572; lane 5, 0.775; lane 6, 1.004; lane 7, 1.239. All samples were adjusted to an OD₆₀₀ of 2.0 in a final volume of 100 μl for LPS preparation. LPS was analysed by Tricine/SDS-PAGE on a 14% acrylamide gel. Each well was loaded with approximately 1×10^8 c.f.u.

for the assembly of the O-antigen unit (Wang & Reeves, 1994; Wang *et al.*, 1996). This reaction is catalysed by the product of the *wbaP* gene, which is the terminal gene in the O-antigen cluster. Thus, the level of transcription of *wbaP* can be used to monitor whether RfaH modulates the expression of the entire *S. typhi* O-antigen gene cluster directly. We examined by RT-PCR the levels of *wbaP* mRNA in cells grown to mid-exponential phase and stationary phase. As a control, we also determined the mRNA levels of *hisG*. Fig. 2

Fig. 2. Expression of *wbaP* mRNA in *S. typhi* Ty2 at the exponential and stationary phases of growth. mRNA levels of *wbaP* and *hisG* were examined by RT-PCR in samples obtained at mid-exponential phase (OD₆₀₀ 0.2, lane 1) and stationary phase (OD₆₀₀ 1.0, lane 2). Arrows indicate RT-PCR products using *wbaP*- and *hisG*-specific primers. The PCR products were analysed by electrophoresis on a 1.5% agarose gel.

shows the mRNA levels of *wbaP* and *hisG* genes at different stages of growth as assessed by RT-PCR, in the wild-type Ty2 strain. The densitometric quantification of the lanes demonstrated that the expression of *wbaP* relative to the expression of *hisG* is low in mid-exponential phase but increases significantly (over 6.0-fold) when cells reach stationary phase (data not shown). The expression of *hisG* does not change in response to the bacterial growth phase (Fig. 1a and data not shown). The *wbaP*-specific mRNA levels in strain Ty2/pKHT19, which overexpresses the RfaH protein, remained unchanged during growth (data not shown). No *wbaP* transcription was detected in the *rfaH* null mutant. Taken together, our results indicate that the growth-phase regulation of O-antigen expression is associated with the growth-phase expression of RfaH. In contrast, the production of the lipid A-core region was not affected similarly, since a complete lipid A-core band was observed at all different stages of growth (Fig. 1b). This is not completely unexpected, since in a previous study, Marolda & Valvano (1998) have shown that RfaH-mediated regulation of the core operons in *E. coli* and *Salmonella* is less tight than the regulation exerted in the O-antigen operon. These differences in RfaH-mediated regulation may be due to structural differences that exist in the regions surrounding the *ops* elements in the O-antigen and core promoter regions (Marolda & Valvano, 1998).

Characterization of the *rfaH-yigC* promoter region

To investigate the factors influencing expression of the *rfaH* gene, the DNA sequence of the 334 bp insert in pCE334 was determined. The sequence was identical to that reported for the same region in *S. typhi* CT18 (www.sanger.ac.uk/Projects/S.typhi/blast_server.shtml). This fragment includes 128 bp from the *rfaH*

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gtgatatttcatggcgctccattgttagcctcttggtaagcgcatcattatcagcggttcacatcggtgatg 70
  S I E H R G N Y G R P L R M M
ctgtatttttggtaaatagcgtgaactctggcaaccaacgctaataccagatacggcttaaaggatgaag 140
  ↑ -10
tgtataaataacttcgcgcattGGCttttgctatGGCttgcgcCccgaacaagcgataagagtcattatgc 210
  -35 -10 M Q
aatcctggtatttactgtactgtcaaacgcgggcaacttcagcgtgctcaggaacacctcgaaagacaagc 280
  S W Y L L Y C K R G Q L Q R A Q E H L E R Q A
ggtaagttgctgacaccgatgatcaccctggaaaaatggtacgcgggaaacg 334
  V S C L T P M I T L E K M V R G K R

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Fig. 3. Sequence analysis of the *yigC-rfaH* intergenic promoter region using the Natural Network Promoter Prediction program. Arrows indicate the predicted transcription initiation sites for *rfaH* (C_{182}) and for *yigC* (T_{74}). Underlined sequences indicate the predicted -35 and -10 promoter regions for *yigC* (bases 84–105) and for *rfaH* (bases 148–176). Boxed segments indicate the putative RcsAB and RpoN boxes. Predicted translational start sites for *yigC* and *rfaH* are as labelled.

Fig. 4. Growth and expression of *rfaH-lacZ* transcriptional fusion in *S. typhi* *rpoN*/pCE334 and *rcsB*/pCE334 mutants. ○, □, Growth (OD_{600}) of *S. typhi* *rcsB*/pCE334 (○) and *S. typhi* *rpoN*/pCE334 (□); ●, ■, expression of *rfaH-lacZ* fusions in *S. typhi* *rcsB* (●) and *S. typhi* *rpoN* (■) mutants, measured as β -galactosidase activity. Data are the mean $\pm$ SD of three independent assays.

coding region and also 46 bp from the coding region of the upstream gene, *yigC*, which is transcribed in the opposite direction (Fig. 3). Therefore, the 334 bp fragment spans two divergently transcribed promoters.

The sequence of the intergenetic region was analysed with the Natural Network Promoter Prediction program (www.fruitfly.org/seq_tools/promoter.html) to predict potential promoter sites. The strongest predictions corresponded to positions 84 and 105 for the *yigC* promoter (score 0.84), and to positions 148 and 176 for the *rfaH* promoter (score 0.88). The program also predicted the location of the initiation of transcription at bases C_{182} and T_{74} for the *rfaH* and *yigC* transcripts, respectively (Fig. 3).

A closer examination of the sequences upstream of the *rfaH* promoter revealed homologies to potential binding sites of known regulatory proteins. The sequence 5'-TAAGCGCATCATTA-3' (Fig. 3) had similarities with

an RcsAB binding box, described in the upstream regions of promoter elements regulated by the RcsB/RcsA dimer (Wehland & Bernhard, 2000). Furthermore, sequences containing the conserved motifs 5'-TGCT-3' and 5'-TGGC-3' were also found (Fig. 3). These sequences resemble the -12 and -24 recognition regions for the alternative sigma factor RpoN, which is responsible for gene expression under nitrogen starvation (Shingler, 1996; Wang & Gralla, 1998).

RpoN regulates *rfaH* gene expression

To investigate whether RpoN or the RcsC/YojN/RcsB system could regulate the expression of *rfaH*, we constructed insertional mutations in the *rpoN* and *rcsB* genes of *S. typhi* Ty2 as described in Methods. The two mutant strains, named M161 (Ty2 *rpoN*) and M159 (Ty2 *rcsB*), were transformed with plasmid pCE334 and the β -galactosidase activity was measured during the bacterial growth. Fig. 4 shows that inactivation of *rpoN* abolished the growth-phase-dependent pattern of β -galactosidase production. In contrast, the *rcsB* mutant strain displayed a pattern of β -galactosidase production over the course of the growth experiment that was similar to that of the wild-type *S. typhi* Ty2 (Fig. 1a). These results suggest that the *rfaH* promoter activity can be modulated in an RpoN-dependent manner. To support this notion, the production of LPS by the mutant strain was also examined. Fig. 5(a) shows that the LPS profiles of the *rcsB* mutant remain identical to those of the wild-type strain (Fig. 1b). Densitometric analyses also revealed an approximately twofold increased proportion of O-antigen relative to lipid A-core in the samples obtained from cells grown to stationary phase (data not shown). In contrast, the proportion of O-antigen relative to lipid A-core was constant in the LPS profiles of the *rpoN* mutant irrespective of the growth stage (Fig. 5b).

Nitrogen limitation increases the transcription of the *rfaH* gene and the production of O-specific LPS

The results of the previous section demonstrate that expression of *rfaH* in *S. typhi* Ty2 is regulated, either directly or indirectly, by the alternative sigma factor RpoN. Several cellular functions are regulated by RpoN,

Fig. 5. Production of LPS in *S. typhi rcsB/pCE334* and *S. typhi rpoN/pCE334* mutants grown to different points of the growth curve in LB. (a) LPS profiles of *S. typhi rcsB/pCE334* grown to the following OD_{600} : lane 1, 0.100; lane 2, 0.186; lane 3, 0.445; lane 4, 0.668; lane 5, 1.021. (b) LPS profiles of *S. typhi rpoN/pCE334* grown to the following OD_{600} : lane 1, 0.104; lane 2, 0.256; lane 3, 0.401; lane 4, 0.676; lane 5, 1.181. All samples were adjusted to an OD_{600} of 2.0 in a final volume of 100 μ l for LPS

including utilization of various nitrogen and carbon sources, energy metabolism, chemotaxis, flagellation, and response to heat shock (reviewed by Buck *et al.*, 2000). But to our knowledge, a role for RpoN in regulation of LPS biosynthesis has not been described. Because of our results demonstrating that the growth-dependent regulation of the *rfaH* gene requires the RpoN function, we reasoned that *rfaH* expression would also be regulated by nitrogen limitation. To test this hypothesis, we grew *S. typhi/pCE334* in low-nitrogen minimal E medium and measured the β -galactosidase activity in the exponential phase of growth (OD_{600} 0.2). As shown in Table 3, expression of the *rfaH-lacZ* fusion increased nearly fourfold when bacteria were grown in the low-nitrogen medium. Additionally, we investigated the effect of nitrogen limitation on LPS production by the wild-type Ty2 strain grown to mid-exponential phase (OD_{600} 0.2). Fig. 6 (lane 2) shows that nitrogen limitation increased the expression of the O-antigen by the wild-type *S. typhi* Ty2, as demonstrated by a similar profile to that observed during the stationary phase of growth in rich medium (Fig. 1b). The relationship between LPS production and nitrogen limitation parallels the pattern of *rfaH* regulation under the control of RpoN and is consistent with the idea that RpoN modulates LPS formation via its effect on *rfaH* gene expression during bacterial growth.

DISCUSSION

Bacterial cells can sense host microenvironments and respond by coordinately regulating gene expression. Invasion of the intestinal epithelium and survival within macrophages are essential properties required for typhoidal pathogenesis. Therefore, *Salmonella* must sense the host environment, either at mucosal surfaces or within host cells, and respond by inducing or repressing gene expression. Essential to the adaptation of bacteria during host infection is the remodelling of the bacterial surface (Ernst *et al.*, 2001). This involves the modification of outer-membrane proteins and of LPS, the most abundant component of the bacterial envelope and a major pathogenic factor of *Salmonella* (Joiner, 1988; Rosenberger *et al.*, 2000; Ernst *et al.*, 2001). LPS is required for complement evasion (Joiner, 1988) as well as for invasion of intestinal epithelial cells (Pier *et al.*, 1998; Lyczak *et al.*, 2001) in the pathogenesis of *S. typhi*.

Evidence from other studies has demonstrated that the transcription of the LPS core and O-polysaccharide gene clusters is subject to positive regulation at the level of mRNA elongation by the RfaH protein (Farewell *et al.*, 1991; Pradel & Schnaitman, 1991; Bailey *et al.*, 1996; Marolda & Valvano, 1998; Wang *et al.*, 1998). RfaH promotes expression of operons encoding proteins

preparation. LPS was analysed by Tricine/SDS-PAGE on 14% acrylamide gels. Each well was loaded with approximately 1×10^8 c.f.u.

Table 3. Effect of nitrogen availability on the expression of *rfaH-lacZ* transcriptional fusion

Bacteria were grown in the indicated medium to OD₆₀₀ 0.2. Values are the mean $\pm$ SD of three independent assays. ND, Not determined because the *rpoN* mutant strain (M161/pCE334) does not grow in low-nitrogen E medium.

Strain	β -Galactosidase activity (Miller units)		
	LB	E medium	Low-nitrogen E medium
Ty2/pCE334	55.34 $\pm$ 4.58	65.02 $\pm$ 4.78	201.64 $\pm$ 45.17*
M161/pCE334	45.11 $\pm$ 7.89	60.23 $\pm$ 9.28	ND

* Significantly different from Ty2/pCE334 grown in LB ($P < 0.01$, ANOVA and Dunnett).

Fig. 6. Effect of nitrogen availability on LPS production by *S. typhi* Ty2 and *S. typhi rpoN*, analysed by Tricine/SDS-PAGE. Cultures were grown to mid-exponential phase. Samples were adjusted to an OD₆₀₀ of 2.0 in a final volume of 100 μ l for LPS preparation. LPS was analysed by Tricine/SDS-PAGE on 14% acrylamide gels. Each well was loaded with approximately 1×10^8 c.f.u. Lane 1, *S. typhi* Ty2 grown in E medium; lane 2, *S. typhi* Ty2 grown in low-nitrogen E medium; lane 3, *S. typhi rpoN* grown in E medium.

targeted to the cell surface or membrane (Bailey *et al.*, 1997) by promoting the efficient elongation of the mRNA (Artsimovitch & Landick, 2002). Based on a detailed deletion-fusion analysis of the *E. coli* O7 LPS promoter region, Marolda & Valvano (1998) have proposed a model involving premature termination of transcription relieved by the RfaH protein that operates to regulate the expression of O-specific polysaccharide genes. In this model, the site for premature transcription

termination is located within a relatively long untranslated 5' mRNA, and possibly depends on the formation of a hairpin. A similar 5' untranslated region is present in the case of the *Salmonella* O-antigen clusters. Therefore, it is reasonable to assume that a similar RfaH-dependent regulation of transcription elongation takes place in the *S. typhi* O-antigen gene cluster.

In this study, we have shown that the synthesis of the *S. typhi* O-antigen is regulated by RfaH in a growth-phase-dependent manner. Production of the O-antigen correlates with the differential expression of the *rfaH* gene during the bacterial growth, increasing at the late exponential phase and reaching maximal expression at the stationary phase. As has been shown in other bacteria (Marolda & Valvano, 1998; Wang *et al.*, 1998), we found that RfaH modulates the O-antigen genes by positively regulating gene transcription, as demonstrated by the increase in the *wbaP*-specific mRNA levels at the stationary phase of growth.

Previous work in our laboratory has shown that environmental conditions that are known to be important for LPS regulation in other systems, such as growth temperature (Al-Hendy *et al.*, 1991) and osmolarity (Aguilar *et al.*, 1997), do not affect transcription of the *rfaH* gene (Rojas *et al.*, 2001). In this study, we examined the sequences upstream of the *rfaH* promoter and found potential binding sites for the RcsB/RcsA dimer of the RcsC/YojN/RcsB phosphorelay system and for the RpoN alternative sigma factor. We therefore investigated the transcriptional activity of the *rfaH* gene under growth conditions that activate the RcsC/YojN/RcsB system or *rpoN*-mediated transcription. No effects were detected with osmotic shock or growth at low temperature, which are known to induce the expression of exopolysaccharides by activating the RcsC/RcsB system (Sledjeski & Gottesman, 1996; Whitfield & Roberts, 1999). Nor were changes in *rfaH* expression resulting from a mutation in the *rscB* gene found. In contrast, growth of *S. typhi* Ty2 in a nitrogen-limited medium induced *rfaH* gene transcription, and the

inactivation of the *rpoN* gene, which encodes the alternative sigma factor σ^{54} (σ^N), abolished the growth-phase-dependent induction of *rfaH* expression.

Most bacteria possess one copy of *rpoN*, which generally is constitutively expressed and not essential for survival and growth under favourable conditions (Buck *et al.*, 2000). RpoN is a specialized sigma factor that recognizes a subset of promoters in bacteria that control regulation of nitrogen metabolism as well as many other biological activities, transcribing genes with diverse physiological roles, including flagellation, chemotaxis, energy metabolism, RNA modification, electron transport, response to heat shock and expression of alternative sigma factors (Merrick, 1993; Buck *et al.*, 2000). RpoN-mediated transcription has also been associated with bacterial pathogenicity. Early reports showed that expression of pilin genes in *Pseudomonas aeruginosa* (Ishimoto & Lory, 1989) and in *Neisseria gonorrhoeae* (Klimpel *et al.*, 1989) required RpoN. More recently, Klose & Mekalanos (1998) reported that an *rpoN* null mutant of *Vibrio cholerae* was defective for colonization in an infant mouse model of cholera, and that this defect was distinct from the non-motile and glutamine synthetase phenotypes of the *rpoN* mutant (Klose & Mekalanos, 1998). Other authors have shown that a strain of *Proteus mirabilis* carrying a mutation in a gene which is highly homologous to ORF284 of the *rpoN* operon has a reduced ability to infect the urinary tract of CBA mice (Zhao *et al.*, 1999).

A role for RpoN in the expression of cell-surface polysaccharides has been demonstrated in the case of alginate production by *Pseudomonas aeruginosa* (Boucher *et al.*, 2000), but to our knowledge this is the first observation that RpoN plays a role in modulation of gene expression of O-antigen LPS genes. Our results suggest that RpoN acts directly or indirectly on *rfaH* gene expression to modulate O-antigen synthesis in an RfaH-mediated fashion. This regulation is manifested not only during the growth cycle but also under conditions of nitrogen limitation. Further studies are under way to characterize in detail the mechanism of RpoN action on the *rfaH* promoter region and the possible interrelation between stationary phase and nitrogen limitation in relation to O-antigen synthesis.

ACKNOWLEDGEMENTS

This investigation was supported by grants 1990967 and 7990058 from FONDECYT to I.C. and grant MOP10206 from the Canadian Institutes of Health Research to M.A.V.

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Received 22 May 2002; revised 7 August 2002; accepted 20 August 2002.