

The *rfaH* gene, which affects lipopolysaccharide synthesis in *Salmonella enterica* serovar Typhi, is differentially expressed during the bacterial growth phase

Gonzalo Rojas, Soledad Saldías, Mauricio Bittner, Mercedes Zaldívar, Inés Contreras *

Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, P.O. Box 174, Correo 22, Santiago, Chile

Abstract

We have cloned and sequenced the *rfaH* gene from *Salmonella enterica* serovar Typhi strain Ty2. The gene showed a high degree of similarity to the *rfaH* genes from *Escherichia coli* K-12 and *S. enterica* serovar Typhimurium. A *rfaH* mutant was constructed by site-directed mutagenesis. This mutant produced a rough lipopolysaccharide (LPS), with an incomplete core region. The defect in LPS expression that results from the *rfaH* mutation was corrected by a plasmid carrying the intact gene. The plasmid-borne *rfaH* gene also restored normal LPS synthesis in a *rfaH* mutant of *E. coli*. Reverse transcription-polymerase chain reaction analyses were performed to determine the effects of various environmental conditions on the expression of *rfaH*. The transcription of *rfaH* showed a growth-phase-dependent regulation, with maximal expression at the late exponential phase. Other environmental conditions, such as temperature or medium osmolarity, did not affect transcription of *rfaH*. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, plays an important role in the interaction between cells and environment, contributing to virulence in pathogenic bacteria. LPS is composed of three domains: the inner hydrophobic lipid A region, the oligosaccharide core, and the outer O-polysaccharide chain [1,2]. LPS biosynthesis has been extensively investigated, especially in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. Many of the genes involved in LPS synthesis have been sequenced and the functions of most of them are known [1,3–5]. However, the regulation of LPS expression is comparatively less understood.

Our interest has been in studying the genetics and reg-

ulation of LPS biosynthesis in *Salmonella enterica* serovar Typhi (*S. typhi*), a species that naturally infects only man causing the systemic disease typhoid fever. Because LPS is a major component of the bacterial surface, and mediates many of the interactions of *S. typhi* with its host [6], it is conceivable that its expression is regulated in response to changing environmental conditions encountered during infection. Studies conducted in other bacterial species have demonstrated that LPS biosynthesis is regulated in response to amino acid starvation [7], changes in the medium osmolarity [8] and growth temperature [9].

The transcription of genes involved in the synthesis and modification of the LPS core as well as O-polysaccharide is subject to positive regulation by the RfaH elongation factor [10–14]. This protein also regulates gene expression in operons encoding the synthesis, export and assembly of hemolysin toxin, capsular polysaccharides and the F pilus [15,16]. Recently, it has been shown that RfaH also regulates the expression of the hemin receptor protein ChuA [17]. Operons regulated by RfaH have a conserved 8-bp motif, termed *ops*, located in the 5'-non-coding region, that is required for RfaH-dependent regulation of gene

* Corresponding author. Tel.: +56 (2) 678 1658;

Fax: +56 (2) 222 7900.

E-mail address: inesccon@ciq.uchile.cl (I. Contreras).

expression. These sequences have been found in the leader regions of the O-polysaccharide and the Vi capsular polysaccharide in *S. typhi* [18].

In this study we describe the isolation of the *rfaH* gene from *S. typhi* and show that it is required for the normal expression of LPS. We also show that expression of *rfaH* is growth-phase-regulated at the transcriptional level.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and growth conditions

Bacterial strains used in this study were: *S. typhi* Ty2 (wild-type strain) obtained from the Instituto de Salud Pública, Santiago, Chile; *S. typhi* strain M8 (Ty2 *rfaH*::*cat*) constructed in this work; *E. coli* DH5 α (laboratory stock); *E. coli* strains CLM12 (*rfaH11 fadA*::Tn10), CLM13 (*fadA*::Tn10) [12], BW25113/pKD46 Δ (*araD-araB*)567 Δ *lacZ*4787 *lacI*p-4000(*lacI*^R) and BW25141 λ pir/pKD3 Δ (*araD-araB*)567 Δ *lacZ*4787 *lacI*p-4000(*lacI*^R) Δ (*phoB-phoR*)580 [19] provided by M.A. Valvano (University of Western Ontario, Canada).

Plasmid pBluescript/KSII was obtained from Stratagene. Plasmid pKZ17 is a derivative of pBR322 containing a 6.8-kb *Bam*HI fragment carrying the *E. coli rfaH* gene [12]. Plasmid pKHT19 is a pBluescript/KSII derivative containing a 723-bp fragment including the *rfaH* gene from *S. typhi* Ty2.

Bacteria were grown at 20 or 37°C in Luria–Bertani broth (LB, 10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract and 5 g l⁻¹ NaCl) supplemented with ampicillin (100 μ g ml⁻¹), chloramphenicol (20 μ g ml⁻¹) or tetracycline HCl (10 μ g ml⁻¹) as appropriate.

2.2. Isolation of the *rfaH* gene from *S. typhi*

DNA preparation, restriction endonuclease digestion and agarose gel electrophoresis were performed by standard procedures [20]. Polymerase chain reaction (PCR) amplification of the *S. typhi rfaH* gene was carried out in a Perkin Elmer 2400 GeneAmp PCR system (52°C annealing temperature and an elongation period of 90 s). Primers were designed from the DNA regions flanking the *S. typhi* sequence, as reported by the Sanger Centre [21], showing significant homology with the *rfaH* gene of *E. coli*. The primers were *rfaH*-1: 5'-gccagaaccttattgaggtcgatt-3' and *rfaH*-2: 5'-gtgatatttcctggcctcattgta-3'. The *Taq* polymerase-amplified product was separated on 1% agarose gels, purified using the Concert[™] system (Gibco BRL) and cloned into pBluescript/KSII after the addition of thymidine residues to the 3'-ends of the *EcoRV*-linearized vector. The recombinant plasmid was introduced in *E. coli* DH5 α cells by transformation via electroporation. Transformants were grown in LB medium for 90

min at 37°C. Recombinant clones were then selected on LB agar plates containing ampicillin.

2.3. Mutagenesis of the *rfaH* gene from *S. typhi*

Mutagenesis was performed according to the method described by Datsenko and Wanner [19] to disrupt specific chromosomal genes using PCR products. For this purpose, *S. typhi* Ty2 was transformed with plasmid pKD46, a temperature-sensitive, low copy-number plasmid that expresses the phage λ Red recombinase system from the arabinose-inducible *P*_{araB} promoter. The Red-mediated recombination is required to replace the targeted chromosomal sequence (*rfaH*) with an antibiotic resistance gene that is generated by PCR. *S. typhi* Ty2 cells carrying the Red helper plasmid were transformed by electroporation with a PCR product that was generated using plasmid pKD3 carrying a FRT-flanked *cat* gene as template and primers *rfaH*-A: 5'-agggaaacacctcgaagacaagcggttaagttg-cctgacaccatgatgaatctccttag-3' and *rfaH*-B: 5'-cagccctt-caaatgccttcctgatgatgacgctatcggtgtaggctggagctgcttcg-3'. These primers contained the FRT sequence (underlined) with 40- and 41-nucleotide extensions homologous to regions within the *rfaH* gene. Transformants were selected on LB agar plates containing chloramphenicol. After selection, mutants were purified non-selectively at 43°C and tested for ampicillin sensitivity to test for loss of the helper plasmid.

2.4. Complementation assays

Complementation of *S. typhi rfaH* mutant M8 strain was carried out by electrotransforming this strain with plasmid pKHT19 (carrying the *S. typhi* wild-type *rfaH* gene) or pKZ17 (carrying the *E. coli rfaH* gene). Transformants were selected on LB agar plates containing ampicillin and chloramphenicol. Complementations were also performed using the *E. coli rfaH* mutant CLM12 as the recipient strain. Transformants were selected on LB agar plates containing tetracycline and ampicillin.

2.5. LPS analysis

LPS gels of proteinase K-digested whole cell preparations were analyzed in a tricine–sodium dodecyl sulfate (SDS) buffer system [22]. Samples were prepared as described by Hitchcock and Brown [23]. Separating gels contained 14% acrylamide and 1.47% bisacrylamide, stacking gels were 3.85% acrylamide and 0.23% bisacrylamide. Gels were silver-stained by a modification of the procedure of Tsai and Frasch [24].

2.6. Reverse transcription (RT)-PCR

For expression analysis, each strain was grown to the selected OD₆₀₀ in 50 ml LB under the appropriate condi-

tions. RNA was extracted using the standard TRIzol[®] procedure. After DNase I treatment, RNA was reverse-transcribed using SuperScript II (200 U μl^{-1}) and anti-sense primers for *hisG*, *rfaH* and *katE* (HisG2, RfaH1 and KatE2, respectively). ssDNA was then amplified using the primers for genes: *hisG* (HisG1 and HisG2); *rfaH* (RfaH1 and RfaHint1); and *katE* (KatE1 and KatE2) (see legend to Fig. 3). The PCR products were analyzed by electrophoresis on 1.5% agarose gels.

3. Results and discussion

3.1. Isolation of the *S. typhi* *rfaH* gene

The JUMPstart sequence, a 39-bp element common to several polysaccharide gene clusters which contains the *ops* element, has been found in the non-coding region upstream of the O-polysaccharide gene clusters in different species of *Salmonella*, including *S. typhi* [18]. Because this sequence is essential for the RfaH-mediated regulation of transcription, we aimed to identify the *rfaH* gene in the *S. typhi* chromosome. We carried out PCR amplification using primers designed from the sequence reported for the *rfaH* gene of *E. coli* as described in Section 2. An amplification product of 723 bp was obtained (Fig. 1, lane 3), identical in size to PCR products obtained with *E. coli* and *S. enterica* serovar Typhimurium DNA as templates (not shown). The PCR product was cloned into pBluescript KSII+ to yield plasmid pKHT19, and the nucleotide sequence was determined. The sequence showed 100% identity with the corresponding sequence reported by the Sanger Centre [21]. Comparative analysis of the predicted sequence of the *S. typhi* RfaH protein with the data currently available [25] revealed 87% and 99% identity to the

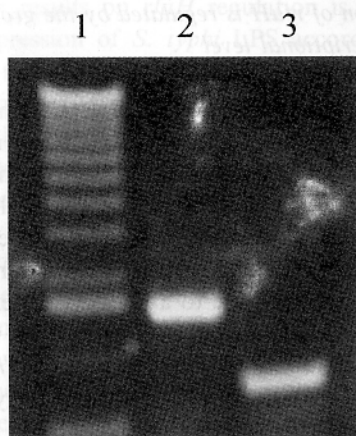


Fig. 1. PCR analysis of the *rfaH* gene from wild-type *S. typhi* Ty2 and one representative *rfaH::cat* mutant obtained by the PCR-mediated gene replacement method. Lane 1: marker 1-kb ladder; lane 2: 1500-bp fragment obtained with DNA from mutant M8 as template; lane 3: 723-bp fragment obtained with DNA from *S. typhi* Ty2 as template. Amplification primers were RfaH-1 and RfaH-2.

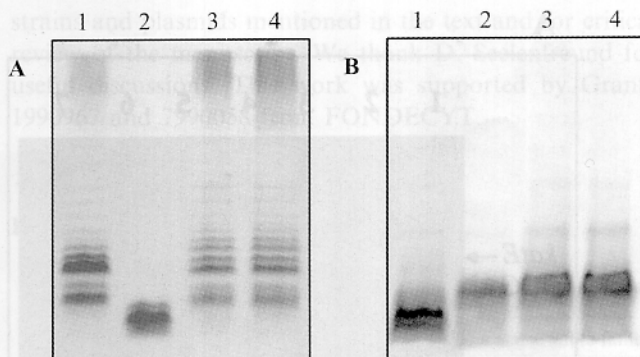


Fig. 2. Effect of the *rfaH* gene on LPS production by *S. typhi* (A) and *E. coli* (B). LPS was analyzed by tricine-SDS-PAGE. (A) Lane 1: Ty2 (wild-type); lane 2: M8 (*rfaH*⁻); lane 3: M8/pKZ17; lane 4: M8/pKHT19. (B) Lane 1: CLM12 (*rfaH*⁻); lane 2: CLM12/pKHT19; lane 3: CLM12/pKZ17, lane 4: CLM13 (*rfaH*⁺).

sequences of the RfaH proteins from *E. coli* K-12 and *S. enterica* serovar Typhimurium, respectively.

3.2. The *rfaH* gene product is required for normal expression of LPS

Site-directed mutagenesis of the chromosomal *rfaH* gene was carried out by a procedure based on the Red recombinase system [19] as described in Section 2. Eight chloramphenicol-resistant mutants were obtained, in which presumably the *cat* gene had interrupted the *rfaH* gene. The disruption of *rfaH* was confirmed by PCR analysis using primers *rfaH*-1 and *rfaH*-2. As shown in Fig. 1 (lane 2), an amplification product of 1500 bp was obtained with chromosomal DNA from one of such mutants (strain M8). The size of the PCR fragment indicated that the chromosomal sequence in the *rfaH* gene (approximately 200 bp) was replaced by the *cat* cassette (1100 bp) in this mutant. When the wild-type *S. typhi* Ty2 DNA was used as template, the amplification product of 723 bp was obtained (lane 3). Strain M8 was purified and LPS was analyzed by SDS-PAGE. Fig. 2A shows that the wild-type strain Ty2 produced a smooth-type profile with multiple high-molecular-mass bands, representing complete LPS (lane 1), while mutant M8 produced a rough LPS profile, in which

Table 1

Effect of growth temperature and osmolarity on expression of the *rfaH* gene^a

| Growth conditions | <i>rfaH</i> /hisG ^b |
|----------------------|--------------------------------|
| LB 85 mM NaCl, 20°C | 1.98 ± 0.41 |
| LB 85 mM NaCl, 37°C | 2.38 ± 0.37 |
| LB 50 mM NaCl, 37°C | 2.46 ± 0.35 |
| LB 170 mM NaCl, 37°C | 2.69 ± 0.22 |

^aBacteria were grown under the indicated conditions to an OD₆₀₀ = 0.4 and the mRNA levels were examined by RT-PCR.

^bmRNA levels of *rfaH* relative to levels of *hisG* mRNA. Data are the mean and standard deviation values from three independent assays.

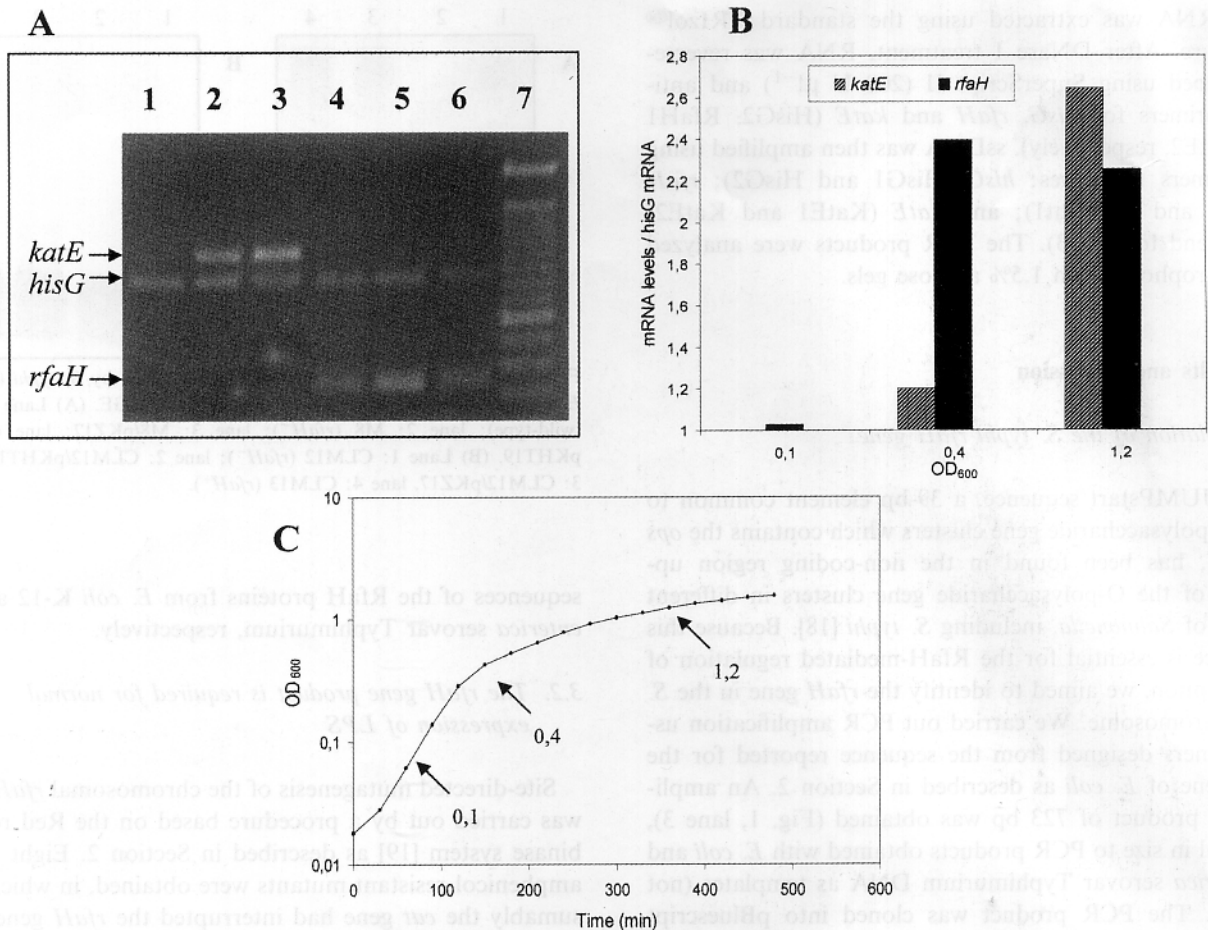


Fig. 3. Expression of *rfaH* mRNA during growth. (A) mRNA levels examined by RT-PCR in samples obtained at different stages of the growth curve. Lane 1: OD₆₀₀=0.1, primers for *katE* and *hisG*; lane 2: OD₆₀₀=0.4, primers for *katE* and *hisG*; lane 3: OD₆₀₀=1.2, primers for *katE* and *hisG*; lane 4: OD₆₀₀=0.1, primers for *rfaH* and *hisG*; lane 5: OD₆₀₀=0.4, primers for *rfaH* and *hisG*; lane 6: OD₆₀₀=1.2, primers for *rfaH* and *hisG*; lane 7: 100-bp ladder. (B) mRNA levels of *katE* and *rfaH* relative to levels of *hisG* mRNA. Each assay was repeated three times. Data from one representative experiment is shown. (C) Growth of *S. typhi* in LB at 37°C. Amplification primers were: HisG1: 5'-ccggatccagacaacaccgcttacgcatag-3' and HisG2: 5'-ccggatcctctccatggtttcccagaacaac-3'; RfaH1 5'-gccagaacctattttgagctgtatt-3' and RfaHint1 5'-tgccctccagcgtttatcatcag-3'; KatE1: 5'-gccctacgctgttgaagatttt-3' and KatE2: 5'-taccgtcacgctggaagtattgg-3'.

the ladder of bands representing the O-polysaccharide chains was absent (lane 2). In addition, strain M8 produced a fast migrating core region, indicative of a truncated core. When plasmid pKHT19, carrying the *S. typhi* *rfaH* gene, was introduced into the M8 strain, the wild-type phenotype was restored (lane 4). The same result was observed when strain M8 was transformed with plasmid pKZ17, which carries the *rfaH* gene from *E. coli* (lane 3).

Complementation was also accomplished by introducing plasmid pKHT19 into the *E. coli* CLM12 *rfaH* mutant strain. Fig. 2B shows that the *rfaH* gene from *S. typhi* complemented the defect in LPS synthesis of *E. coli* CLM12. Since *E. coli* K-12 does not express O-antigen, the effect of the *rfaH* mutation in strain CLM12 is manifested only as a truncated core region. The results demonstrate that the RfaH proteins from *E. coli* K-12 and *S. typhi* are functionally interchangeable, as was expected due to their high percentage of similarity.

3.3. Expression of *rfaH* is regulated by the growth phase at the transcriptional level

Although the regulatory effect of RfaH on the expression of the LPS core and O-polysaccharide genes has been well-documented, the environmental signals that modulate the expression of the *rfaH* gene are presently unknown. Initial attempts to investigate the *rfaH* expression at the level of transcription using Northern blots were unsuccessful. This was probably due to a low level of expression or high instability of the message. However, we were able to detect amplification of reverse transcribed *rfaH*-specific mRNA with RT-PCR, and used this strategy to test the effects of growth temperature and osmolarity on the transcription of *rfaH*. These conditions were selected because previous studies have shown them to be important for regulation of LPS expression in other bacteria [8,9]. The results (Table 1) showed no differences in the *rfaH*-specific

mRNA levels in cells grown at 20 or 37°C, and at different medium osmolarities ranging from 50 to 170 mM NaCl.

We also investigated whether *rfaH* expression changed during bacterial growth. As a control, we examined the levels of *katE* mRNA since this gene is known to be induced during stationary phase [26]. We also determined the mRNA levels of *hisG* as a loading control to normalize the results. We have previously demonstrated, using *lacZ* fusions, that expression of the genes from the *his* operon does not change in response to the bacterial growth phase (unpublished results). Fig. 3A shows the mRNA levels of *rfaH*, *katE* and *hisG* genes at different stages of growth as assessed by RT-PCR. The normalized data relative to *hisG* mRNA levels, demonstrate that the expression of *rfaH* is low in exponential phase but increases approximately two-fold (2.4-fold) when cells reach the late exponential phase, and remains high during the stationary phase (Fig. 3B). The control experiment with *katE* shows, as expected, high expression during the stationary phase (Fig. 3B). Our results demonstrate that the *rfaH* transcription is regulated in a growth-phase-dependent manner. Moreover, the comparison of *rfaH* and *katE* expression patterns during the bacterial growth suggests that *rfaH* may not be regulated by RpoS [27].

3.4. Concluding remarks

Marolda and Valvano [12] have suggested that the promoter of the *E. coli* O7-specific LPS gene cluster is constitutive in terms of transcription initiation and is regulated only at the level of mRNA elongation by RfaH. In *Salmonella*, the regulation of LPS core and O-polysaccharide gene clusters in response to changes in the environment has not been investigated. However, given the level of DNA sequence conservation as compared with *E. coli* [2], it is likely that the promoters of these clusters may be regulated in the same manner. The physiological relevance of our results on *rfaH* regulation is not apparent since the expression of *S. typhi* LPS according to SDS-PAGE does not change significantly when cells reach late exponential phase (unpublished). Since the RfaH protein is not a transcriptional activator but rather acts on the elongation of the mRNA, increasing levels of this protein would not necessarily result in an increased transcription of LPS biosynthetic genes. Therefore, we propose that the increased *rfaH* gene expression in late exponential and stationary phases may be required to ensure sufficient levels of the protein to adequately maintain the synthesis of LPS at these growth stages. Additional studies investigating changes in the amount of the RfaH protein under different growth phases are required to test this hypothesis.

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