cAMP receptor protein (CRP) positively regulates the *yihU*–*yshA* operon in *Salmonella enterica* serovar Typhi

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Salmonella enterica serovar Typhi (S. Typhi) is the aetiological agent of typhoid fever in humans. This bacterium is also able to persist in its host, causing a chronic disease by colonizing the spleen, liver and gallbladder, in the last of which the pathogen forms biofilms in order to survive the bile. Several genetic components, including the *yihU*–*yshA* genes, have been suggested to be involved in the survival of *Salmonella* in the gallbladder. In this work we describe how the *yihU*–*yshA* gene cluster forms a transcriptional unit regulated positively by the cAMP receptor global regulator CRP (cAMP receptor protein). The results obtained show that two CRP-binding sites on the regulatory region of the *yihU*–*yshA* operon are required to promote transcriptional activation. In this work we also demonstrate that the *yihU*–*yshA* transcriptional unit is carbon catabolite-repressed in *Salmonella*, indicating that it forms part of the CRP regulon in enteric bacteria.

INTRODUCTION

Salmonellae are aetiological agents of enteric human diseases, including typhoid fever and gastroenteritis, both of which are major public health threats (Pang *et al.*, 1995). Orally ingested bacteria that survive stomach acidity penetrate the intestinal mucosa via M cells and migrate to the spleen and liver via the lymph nodes, where they reside and replicate intracellularly within macrophages (Finlay, 1994; Zhang *et al.*, 2003). From the liver, *Salmonella* can be transported into the bile-rich gallbladder, forming biofilms, which provide a selective advantage by increasing its ability to persist under this environmental condition, causing a chronic or active infection (Crawford *et al.*, 2008; Fux *et al.*, 2005; Hazrah *et al.*, 2004; Parsek & Fuqua, 2004; Prouty *et al.*, 2002). The cluster *yihU*, *yihT*, *yihS*, *yihR*, *yihQ*, *yihP*, *yihO* and *yshA* (*yihU*–*yshA*) has been associated with biofilm formation in the gallbladder, since it is induced by bile, and mutants in the *yihO* and *yihP* (glucuronide transporters) genes result in a diminution of biofilm formation on surfaces coated with cholesterol, the main compound in gallstones (Crawford *et al.*, 2008). Moreover, this gene cluster is conserved throughout enteric bacteria, and can process and transport galacturonates as alternative sources of carbon (Blot *et al.*, 2002).

Interestingly, mutations of the *crp* gene cause defective intestinal colonization, i.e. they affect genes related to resistance to bile, motility, outer-membrane proteins (OMPs), sigma factor E (σ^E) and RpoS in *Salmonella* Choleraesuis, *Vibrio cholerae*, *Salmonella* Gallinarum and *Salmonella* Typhimurium (Chen *et al.*, 2010; Liang *et al.*, 2007; Rosu *et al.*, 2007).

The cAMP receptor protein (CRP) is one of the best-known global regulatory proteins in *Escherichia coli*; more than 260 CRP-binding sites have been identified to date (Xu & Su, 2009). The cAMP–CRP complex is involved in regulation of several catabolic functions, flagellum synthesis and toxin production (Botsford & Harman, 1992). CRP consists of a homodimer, which binds to cAMP and undergoes a conformational change that allows it to bind to the pseudo-palindromic consensus TGTGA-N₍₆₎-TCACA (Baichoo & Heyduk, 1999; Botsford & Harman, 1992; Busby *et al.*, 1994; Kolb *et al.*, 1983, 1993).

Hence, our interest was focused on studying the regulation of the *yihU*–*yshA* genes in *Salmonella enterica* serovar Typhi (S. Typhi) due to their putative role in virulence. In this work we report that the *yihU*–*yshA* gene cluster from S. Typhi is an operon with a transcriptional start site located 58 nt upstream of the *yihU* start codon. It is positively regulated by the global transcriptional factor CRP, which, in the presence of cAMP, interacts directly with the regulatory region of the *yihU*–*yshA* transcriptional unit, showing that the *yihU*–*yshA* operon belongs to the CRP regulon in *Salmonella*.

Abbreviations: CAT, chloramphenicol acetyltransferase; CRP, cAMP receptor protein; EMSA, electrophoretic mobility shift assay.

METHODS

Bacterial strains. The strains and plasmids used in this study are listed in Table 1.

Growth media and culture conditions. Bacterial strains were grown aerobically at 37 °C with vigorous shaking in Luria–Bertani (LB) medium (containing per litre: 10 g tryptone, 5 g yeast extract, 10 g NaCl), medium A (MA; containing per litre: 7 g nutrient broth, 1 g yeast extract, 2 g glycerol, 3.7 g K₂HPO₄, 1.3 g KH₂PO₄) (Kawaji *et al.*, 1979) and M9 minimal medium (with either 0.2 %, v/v, glycerol or 0.2 %, v/v, glucose as carbon source). Growth was monitored by measuring OD₆₀₀. When necessary, antibiotics were used at the following concentrations: ampicillin (Amp), 100 µg ml⁻¹; kanamycin (Km), 30 µg ml⁻¹; tetracycline (Tc), 12 µg ml⁻¹; spectinomycin (Sp), 50 µg ml⁻¹; streptomycin (Sm), 50 µg ml⁻¹.

DNA manipulation. All DNA manipulations were performed using standard genetic and molecular techniques (Sambrook *et al.*, 1989). Plasmid DNA was purified using the High Pure isolation kit (Roche). The oligonucleotides used for amplification by PCR and for primer

extension were synthesized by Integrated DNA Technologies (IDT) or at the Oligonucleotide Synthesis Facility of the Institute of Biotechnology, Universidad Nacional Autónoma de México (UNAM), and are listed in Table 2. Restriction enzymes, DNA and RNA polymerases, ligases, kinases and reverse transcriptase were provided by Invitrogen, New England Biolabs or USB, and used according to the manufacturers' instructions.

Construction of *S. Typhi* mutant strains In order to evaluate the functional role of CRP in the regulation of the *yihU-yshA* operon, a *crp* mutant in *S. Typhi* was obtained by substitution of the *crp* gene with the Km-resistance cassette (Datsenko & Wanner, 2000). The mutant strain was selected in LB/agar media supplemented with Km and glucose (0.8 %), as recommended by Fuentes *et al.* (2009). The mutation was confirmed by PCR and sequencing.

Construction of transcriptional fusions. Oligonucleotides (see Table 2) were designed in order to amplify the 5' intergenic regions of each one of the genetic components of the *yihU-yshA* cluster (Fig. 1). PCR fragments were double-digested with *Bam*HI/*Kpn*I or *Bam*HI/*Hind*III and ligated into the pKK232-8 Ω TcAp vector, which

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
STM 14028s	<i>S. Typhimurium</i> wild-type strain	Gil <i>et al.</i> (2007)
STY IMSS-1	<i>S. Typhi</i> 9.12, d, Vi serotype; Mexican reference clinical strain	Puente <i>et al.</i> (1987)
DH5 α	<i>E. coli</i> <i>fhuA2</i> Δ (<i>argF-lacZ</i>)U169 <i>phoA glnV44 ϕ80A(lacZ)</i> M15 <i>gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	Invitrogen
BL21(DE3)	<i>E. coli</i> <i>B dcmompThsdS</i> (r _B ⁻ m _B ⁻) <i>gal</i>	Invitrogen
BL21(DE3)- <i>crp</i>	<i>E. coli</i> BL21(DE3) carrying <i>crp</i> gene inserted into plasmid pET15b	This study
STY <hns99< h=""></hns99<>	<i>S. Typhi</i> IMSS-1 <i>hns99::Km^r</i> mariner	Flores-Valdez <i>et al.</i> (2003)
Plasmids		
pKK232-8 Ω Tc	Cloning vector for promoter analysis, Amp ^R , Tet ^R	
pYU-194	pKK232-8 derivative carrying the <i>yihU</i> promoter (nucleotides -194 to +251), Amp ^R	
pYU-145	pKK232-8 derivative carrying the <i>yihU</i> promoter (nucleotides -145 to +251), Amp ^R	
pYU-106	pKK232-8 derivative carrying the <i>yihU</i> promoter (nucleotides -106 to +251), Amp ^R	
pYU-86	pKK232-8 derivative carrying the <i>yihU</i> promoter (nucleotides -86 to +251), Amp ^R	
pYU-79	pKK232-8 derivative carrying the <i>yihU</i> promoter (nucleotides -79 to +251), Amp ^R	
pYU-66	pKK232-8 derivative carrying the <i>yihU</i> promoter (nucleotides -66 to +251), Amp ^R	
pYU-57	pKK232-8 derivative carrying the <i>yihU</i> promoter (nucleotides -57 to +251), Amp ^R	
pYU-19	pKK232-8 derivative carrying the <i>yihU</i> promoter (nucleotides -19 to +251), Amp ^R	
pYU-194-A	pKK232-8 derivative carrying the <i>yihU</i> promoter (nucleotides -194 to +251) with AGTGA→CCCCC substitution into positions -46 to -50, Amp ^R	
pYU-194-B	pKK232-8 derivative carrying the <i>yihU</i> promoter (nucleotides -194 to +251) with TTTC A→GGGGG substitution into positions -65 to -69, Amp ^R	
pYU-194-C	pKK232-8 derivative carrying the <i>yihU</i> promoter (nucleotides -194 to +251) with a AAAA deletion at positions -72 to -75, Amp ^R	
pYU-194-D	pKK232-8 derivative carrying the <i>yihU</i> promoter (nucleotides -194 to +251) with TGTGC→AAAAA substitution into positions -79 to -83, Amp ^R	
pYU	pKK232-8 derivative carrying the <i>yihU</i> promoter and structural gene, <i>yihT</i> promoter and structural gene, Amp ^R	
pYT	pKK232-8 derivative carrying the <i>yihT</i> promoter, Amp ^R	
pYS	pKK232-8 derivative carrying the <i>yihS</i> promoter and structural gene, Amp ^R	
pYR	pKK232-8 derivative carrying the <i>yihR</i> promoter and structural gene, Amp ^R	
pYQ	pKK232-8 derivative carrying the <i>yihQ</i> promoter and structural gene, Amp ^R	
pYP	pKK232-8 derivative carrying the <i>yihP</i> promoter and structural gene, Amp ^R	
pYO	pKK232-8 derivative carrying the <i>yihO</i> promoter and structural gene, Amp ^R	
pYA	pKK232-8 derivative carrying the <i>yshA</i> promoter and structural gene, Amp ^R	

Table 2. Oligonucleotides used in this study

All primers were sourced in this study.

Primer	Sequence (5'–3')
pYU-FOR	CGGGATCCAATCCGCTCTCCTTTGGCAT
pYT-FOR	CGGGATCCCTGATCAACAACTACATGAG
pYT-REV	GGGGTACCACGACGTTTTTGCCATCATT
pYS-FOR	CGGGATCCTATGTCACTCCAATGGTTTG
pYS-REV	GGGGTACCTTTTCGCGTTGATATCCAGGA
pYR-FOR	CGGGATCCGTCTGATGCTCCATCTCCAT
pYR-REV	GGGGTACCGTCGCAGCCGATGGAAAAAT
pYQ-FOR	CGGGATCCTATCCCTTTGCGTTGATATC
pYQ-REV	GGGGTACCGATATTCCGTAAAGAAGCAA
pYP-FOR	CGGGATCCGCGGAAACTATTGCCCACTT
pYP-REV	CCCAAGCTTAACCGTATGTTTCCGGGCCT
pYO-FOR	CGGGATCCAGAAGAAAGTCTATATCGGC
pYO-REV	CCCAAGCTTCGCGACAATTTGATTACCTG
pYA-FOR	CGGGATCCTTTTCTCTTGATATCGCCTTC
pYA-REV	CCCAAGCTTGAAGAAATACTTCGCCCCGA
pYU-194-FOR	CGGGATCCCCACATATTTACCCCTTCT
pYU-145-FOR	CGGGATCCATCCATCACGTAATACCTA
pYU-106-FOR	CGGGATCCGGTCATTTCCTCAAGCATTTT
pYU-86-FOR	CGGGATCCCGATGTGCTCATTTTAATTT
pYU-79-FOR	CGGGATCCCTCATTTTAATTTACGAAA
pYU-66-FOR	CGGGATCCCACGAAATGTTTAAAAAGTG
pYU-57-FOR	CGGGATCCTTTAAAAAGTGACGGCTGTC
pYU-19-FOR	CGGGATCCTACAAATACGATCACAACA
pYU + 251-REV	GGGGTACCGCATGGTGATAACAAATTCA
PextA	ACCCGATAAATGCTATAACT
PextB	ATCTGTCCCAACCCGATAAA
pYU-194-A-FOR	CACGAAATGTTTAAACCCCGGCTGTCAATTTTTTG
pYU-194-A-REV	CAAAAAATTGACAGCCGGGGGGTTTAAACATTTTCGTG
pYU-194-B-FOR	CGATGTGCTCATTTTAAGGGGGCGAAATGTTTAAAAAGTG
pYU-194-B-REV	CACTTTTTAAACATTTTCGCCCCCTTAAAAATGAGCACATCG
pYU-194-C-FOR	GCATTTTCGATGTGCTCAAAATTTACGAAATGTTTAAAAAGTGAC
pYU-194-C-REV	GTCATTTTTTAAACATTTTCGTGAAATTTGAGCACATCGAAAAATGC
pYU-194-D-FOR	CATTCTCAAGCATTTTCGAAAAACTCATTTTAATTTACG
pYU-194-D-REV	CGTGAATTTAAATGAGTTTTTCGAAATGCTTGAGGAATG
Δ crp-H1P1	AACAGAGGATAACCGCGCATGGTGCTTGGCAAACCGCAA- ACATGTAGGCTGGAGCTGCTTCG
Δ crp-H2P2	CGCGCCATTCTGACGGGATTAACGGGTGCCGTAGACGAC- GATCATATGAATATCCTCCTTAG
crp-F	AAAGCTATGCTAAAACAGACA
crp-R	TATCCAGACCTGCAATGTTG

contains a promoterless *cat* gene. To map the *yihU* regulatory region, different promoter fragments were generated by PCR, double-digested with *Bam*HI/*Kpn*I and then cloned into *Bam*HI/*Kpn*I-digested pKK232-8. Complete lists of the fusions obtained in this work are shown in Table 1. The numbering indicates the basepair coordinates for each fusion, upstream (negative) or downstream (positive) of the transcriptional start site (Fig. 5).

Chloramphenicol acetyltransferase (CAT) assays. CAT assays were performed as described previously (Gil *et al.*, 2009; Hernández-Lucas *et al.*, 2008). To prepare crude extracts, *S. Typhi* strains harbouring plasmids containing the transcriptional fusions described above were grown in MA, LB or M9 medium supplemented with ampicillin to OD₆₀₀ 0.6, 0.8, 1.0 and 1.4 (12 h). Cell samples were

collected by centrifugation (15 000 *g*) and then washed with 750 μ l TDTT buffer (50 mM Tris/HCl, pH 7.8, 30 mM DL-dithiothreitol). The bacterial pellet was resuspended in 600 μ l TDTT buffer and sonicated on ice until it was clear. Intact cells and debris were eliminated by centrifugation (15 000 *g*) for 15 min, and the supernatants were transferred to clean microcentrifuge tubes.

For the CAT assay, 5 μ l of each extract was added in duplicate to a 96-well ELISA plate, followed by 200 μ l of the reaction mixture containing 1 mM 5,58-dithio-bis (2-nitrobenzoic acid) (DNTB) (Boehringer Mannheim), 0.1 mM acetyl-CoA (Pharmacia Biotech)/0.1 mM chloramphenicol (Sigma) in 0.1 M Tris/HCl, pH 7.8. Changes in A_{410} were read at intervals of 5 s for 5 min, using a Ceres 900C scanning autoreader and microplate workstation and KC3

Fig. 1. Schematic representation of the *yihU-yshA* operon in *S. Typhi*. (a) PCR fragments of different lengths were amplified and ligated into the pKK232-8 Ω Tc promoterless *cat* vector. About 400 bp upstream of the ATG translational start codon of each of the *yihU-yshA* genes was cloned, to produce reporter plasmids pYU, pYT, pYS, pYR, pYQ, pYP, pYO and pYA. (b) CAT reporter activity levels for these plasmids were determined at OD₆₀₀ 0.4, 0.6, 0.8 and 1.0, and at 1.4 or 1.3 (12 h) in MA medium. Each bar represents the mean of three independent experiments performed in duplicate.

software (Bio-Tek Instruments) set in the kinetics mode. The activities were obtained by interpolation with a standard curve. Protein concentrations of the cell extracts were determined using a bicinchoninic acid (BCA) protein assay kit (Thermos). These values were used to calculate the CAT specific activity. BSA served as the protein standard. Each value represents the mean activity obtained from at least three independent experiments.

RNA isolation and primer extension analysis Total RNAs were isolated from samples of cultures grown in medium A at 37 °C and collected at 12 h. Samples (40 μg) of total RNA, isolated using a commercial kit (RNeasy, Qiagen), were denatured at 90 °C for 3 min and then slowly cooled to 42 °C. The RNA was annealed with [γ -³²P]ATP-labelled *yihU*-PExtA (annealing from position +25 to +6 with respect to the translational start site) or PExtB (annealing from position +35 to +16 with respect to the translational start site) (see Table 2). Primers were extended with AMV reverse transcriptase at 45 °C for 2 h, and the extended products were precipitated with 100 % (v/v) ethanol and analysed by electrophoresis in urea 8 % (v/v) polyacrylamide gels alongside sequencing ladders. Sequencing ladders were generated from the pYU-194 plasmid, which contained 194 and 252 bp upstream and downstream, respectively, of the *yihU* translational start codon.

Purification of His6-CRP protein. For CRP purification, the corresponding gene was cloned into the IPTG-inducible vector pET-15b (Novagen). The protein was purified as follows: 5 ml LB was inoculated with the corresponding strain and growth was at 37 °C overnight; afterwards, 100 μl of this preculture was inoculated into 500 ml LB medium up to OD₆₀₀ 0.6 and induced over 5 h with 1 mM IPTG. Samples were collected and centrifuged at 3000 g (Eppendorf 5702R centrifuge) for 30 min at 4 °C. The cell pellet was washed twice with binding buffer (0.5 M NaCl, 20 mM NaH₂PO₄, 5 mM

imidazole, pH 7.4). The cell suspension was disrupted using a sonic disruptor (Microson ultrasonic cell disruptor XL, Misonix). The suspension was centrifuged at 15 000 g for 10 min at 4 °C to collect cell debris. The supernatant was loaded onto a 1 ml nickel affinity chromatography column (HisTrap HP, GE Healthcare). The column was washed and equilibrated with binding buffer before extract loading, and washed with 10 volumes of binding buffer. The protein was eluted with increasing concentrations of imidazole (100, 250 and 500 mM). Fractions were monitored at 280 nm and resolved by SDS-PAGE. Finally, the purified protein was dialysed and stored at -20 °C in storage buffer (40 mM Tris, pH 7.4, 200 mM KCl, 20 %, v/v, glycerol). Protein concentrations were determined by BCA assays (Thermos).

Gel electrophoretic mobility shift assay (EMSA). Non-radioactive EMSAs were performed according to the protocol described by De la Cruz *et al.* (2007). The DNA fragments were obtained by PCR using the same primers used for transcriptional fusions (see Table 2, Fig. 5). Each probe (20 ng) was mixed with increasing concentrations of purified protein in the presence of 3 × CRP binding buffer [100 mM Tris, pH 8.0, 50 mM KCl, 5 % (v/v) glycerol, 250 μg BSA ml⁻¹, 100 μM cAMP, 1 mM DTT]. The mixture was incubated for 20 min at room temperature and then separated by electrophoresis in 6 % (v/v) native polyacrylamide gels in 0.5 × Tris/borate-EDTA buffer. The DNA-protein complexes were visualized by ethidium bromide staining.

Site-directed mutagenesis. In order to obtain mutated CRP-binding sites, mutagenic primers (see Table 2) were designed with PrimerX (<http://www.bioinformatics.org/primerx/index.htm>). Site-directed mutagenesis by overlap extension was performed as described by Sambrook *et al.* (1989), generating the mutated plasmids pYU-194-A, pYU-194-B, pYU-194-C and pYU-194-D (Fig. 6a).

RESULTS

Functional organization of the *yihU*–*yshA* genes of *S. Typhi*

In silico analyses to evaluate the transcriptional organization of the *yihU*–*yshA* cluster were performed using the FGENESB program (<http://www.softberry.com>), the ORF program (<http://bioinformatics.biol.rug.nl/>), and the EcoCyc (Keseler *et al.*, 2009) and Prodigic databases (Münch *et al.*, 2003). The results indicated that the *yihU*–*yshA* genes form a cluster of 9.6 kb, which is conserved throughout *E. coli*, *Salmonella* and *Shigella*, and that they have an operon organization (Fig. 1a).

In order to validate the *in silico* results, transcriptional fusions with the *cat* gene were obtained by amplifying the 5' intergenic regions of each gene (approximately 400 bp upstream and downstream of the theoretical ATG) and cloning them into the pKK232-8 vector (Fig. 1a). The results for the evaluated eight transcriptional fusions showed that only the first gene, *yihU*, possessed CAT activity ($541 \pm 33.7 \mu\text{mol min}^{-1} \text{mg}^{-1}$), indicating that in MA medium this is the only functional promoter in this gene cluster (Fig. 1b). These results support the notion that the functional genetic organization of *yihU*–*yshA* in *S. Typhi* is as an operon.

Identification of the *yihU* transcriptional start site by primer extension

Primer extension was performed to define the *yihU*–*yshA* promoter region in *S. Typhi*. Total RNA obtained from the wild-type *S. Typhi* strain harbouring a plasmid that contains the putative regulatory region of the *yihU* gene was annealed with two different oligonucleotides (PextA

and PextB). The results with the two primers showed that *yihU* possesses a transcriptional start site 58 nt upstream of the theoretical ATG of *yihU* (Fig. 2). The initiation site is associated with a -10 (5'-TACAAA-3') and a -35 box (5'-CTGTCA-3'), suggesting a sigma 70-dependent promoter that drives *yihU* transcription, such as that predicted with the Bacterial Promoter Prediction program.

CRP positively regulates the expression of the *yihU*–*yshA* operon

In order to identify global regulatory proteins involved in the genetic expression of the *yihU*–*yshA* operon, bioinformatics analyses of the 5' region of *yihU* were performed, and the results suggested that the *yihU*–*yshA* transcriptional unit is regulated by the global regulatory protein CRP. Virtual Footprinting (Münch *et al.*, 2005) and Bprom (<http://www.softberry.com>), programs that recognize and identify DNA patterns to analyse transcription factor binding sites in prokaryotes, were used and showed two putative CRP-binding sites: one near to the -35 box at -42.5 (5'-AGTGACGGCTGTCAAT-3'), located at nucleotides -50 to -35 ; and another further upstream at -75.5 (5'-TGTGCTCATTTTAATT-3'), located at nucleotides -83 to -68 . These sites will be referred to as CRP boxes I and II, respectively (Fig. 6a).

In order to determine whether CRP is involved in the genetic control of this operon, transcriptional fusions containing the full-length promoter region were transformed into the wild-type and into an isogenic *S. Typhi* *crp* mutant strain. The expression in the mutated strain showed an abolished activity as compared with the wild-type strain. This expression profile indicated that CRP is a positive regulator of the *yihU*–*yshA* operon (Fig. 3).

Fig. 2. Determination of the transcriptional start site of *yihU*. Primer extension assay using total RNA extracted from *S. Typhi*. The 5'-labelled (^{32}P) PextA and PextB primers (Table 2) located 25 and 35 bp downstream of the ATG translational codon, respectively, were used to obtain the respective cDNAs. The cDNAs were resolved in a sequencing gel together with a sequencing reaction performed with the primers, using as template a 445 bp fragment of the *yihU* promoter cloned into the pKK232-8 vector. The location of the translational start site is depicted in the nucleotide sequence at the bottom of the figure (italic and underlined type). The same result was obtained with both primers, but only the result with primer PextA is presented.

Fig. 3. Transcriptional analysis of the *yihU* promoter region in wild-type *S. Typhi* and *crp* mutant strains. The reporter plasmid pYU-194 was transformed into the *S. Typhi* IMSS-1 wild-type strain and the isogenic STY Δ*crp* mutant strain. The CAT reporter activity levels were determined at OD₆₀₀ 0.4, 0.6, 0.8 and 1.0, and at 1.4 or 1.3 (12 h) in MA medium. Bars represent the mean of three independent experiments performed in duplicate.

CRP binds to the *yihU* regulatory region

In order to determine whether the CRP protein directly regulates the genetic expression of *yihU-yshA*, EMSAs were performed with purified CRP and with the entire *yihU* regulatory region. The CRP protein was capable of binding to the 5' region of *yihU* in the presence of 100 μM cAMP, and a significant electrophoretic mobility shift occurred at 400 nM CRP (Fig. 4). An internal DNA coding fragment of *yihU* was used as negative control (YU-19). Moreover, no binding of CRP to the 5' region of *yihU* was observed in the absence of cAMP (Fig. 7a, b).

Two CRP boxes mediate *yihU* transcriptional activation

In order to determine the CRP-dependent sequences involved in the transcriptional activation of *yihU*, we constructed transcriptional fusions encompassing different lengths of the *yihU-yshA* promoter region (Fig. 5a). The fusions were transferred to the wild-type *S. Typhi* strain. The results (Fig. 5b) showed that the fusions from -194 to +251 (pYU-194), -106 to +251 (pYU-106) and -86 to +251 (pYU-86), had similar values of about $450 \pm 67.8 \mu\text{mol min}^{-1} \text{mg}^{-1}$. However, CAT reporter activities decreased for the fusions that contained DNA regions from -79 to +251 (pYU-79) and from -66 to +251 (pYU-66). Additionally, this activity was abolished in fusions that contain shorter regulatory regions starting at -57 to +251 (pYU-57). These data show that the sequence from -57 to -86 is relevant for the transcriptional activation of *yihU*. Interestingly, the fusions that include only CRP box I (pYU-66 and pYU-57) show 20 % and null activity as compared with the wild-type, respectively, whereas fusion pYU-79, which includes CRP box I and 12 bp of CRP box II, shows 40 % activity. Accordingly, only the pYU-86 fusion that harbours both CRP boxes completely shows a full activity commensurate

with the longest pYU-194 fusion. Together these data support the notion that the two identified CRP boxes are required for full transcriptional activity of *yihU* in *S. Typhi*.

To further delimit the specific nucleotides involved in the transcriptional activity mediated by CRP, substitutions and deletions were performed in both CRP boxes, using the

Fig. 4. Binding of the cAMP-CRP complex to the entire regulatory region of *yihU*. EMSAs of the purified CRP protein with the promoter region of the *yihU* gene. Different concentrations of CRP were incubated for 20 min at room temperature with 20 ng of the complete *yihU* regulatory region (PCR fragment YU-194) in the presence of 100 μM cAMP. As a negative control, we used 20 ng of an internal coding region of *yihU* that does not contain a CRP-binding site (PCR fragment YU-19). The gel was stained with ethidium bromide to visualize the free DNA as well as DNA-protein complexes (arrow).

Fig. 5. Expression profile of promoter fusions harbouring different lengths of the *yihU* regulatory region in *S. Typhi*. (a) Schematic representation of the *yihU* regulatory region showing the *yihU* +1 transcribed nucleotide. Different DNA fragments of 445, 396, 357, 337, 330, 317, 308 and 270 bp encompassing from -194, -145, -106, -86, -79, -66, -57 and -19, respectively, upstream of the transcriptional initiation site as well as 250 bp downstream of the mRNA initiation site were amplified by PCR and ligated into the pKK232-8 Ω Tc promoterless *cat* vector, generating reporter plasmids pYU-194, pYU-145, pYU-106, pYU-86, pYU-79, pYU-66, pYU-57 and pYU-19, respectively. (b) The CAT reporter activities of plasmids pYU-194, pYU-106, pYU-86, pYU-79, pYU-66, pYU-57 and pYU-19, harboured in *S. Typhi* wild-type strain IMSS-1, were determined at OD₆₀₀ 0.4, 0.6, 0.8, 1.0 and 1.4 (12 h) in MA medium. Each bar represents the mean of three independent experiments performed in duplicate.

longest pYU-194 plasmid as a template. The mutations (Fig. 6a) were done at -83 to -79 (pYU-194-D, TGTGC→AAAAA), -75 to -72 (pYU-194-C, ΔTTTT) and -69 to -65 (pYU-194-B, TTTCA→GGGGG): they were located at CRP box II. In addition, a substitution in CRP box I -50 to -46 (pYU-194-A, AGTGA→CCCCC) was done. Transcriptional activity of these constructs was measured in the wild-type *S. Typhi* strain.

The mutated fusions were compared with the longest wild-type pYU-194 fusion. The pYU-194-A substitution mutant resulted in no transcriptional activity (Fig. 6b), indicating that the mutated bases, -50 to -46 within CRP box I, are relevant for transcriptional activation. Moreover, a four T-deletion mutant (pYU-194-C), between positions -75 and -72, also showed essentially null activity; and the pYU-194-B substitution resulted in a decrease in activity of about 30%. Hence both mutations support the relevance of CRP box II. The substitutions in pYU-194-D did not affect transcription, indicating that this sequence is not a determinant of CRP activation. These data are in accordance with the transcriptional fusion data presented above, which reveal the role of both CRP boxes in the regulation of *yihU* (Fig. 6b).

Following the transcriptional mapping, a gel shift assay was performed with the PCR-amplified DNA fragments of plasmids pYU-194, pYU-86, pYU-66 and pYU-19 (Table 2). The full-length regulatory DNA fragment of -194 and fragment -86, which include the two CRP boxes, were fully shifted at 400 nM CRP (Fig. 7a), and fragment -66, which only contains CRP box I, was partially shifted (Fig. 7b). On the other hand, a PCR fragment, -19, devoid of the two CRP boxes, did not shift with the CRP protein (Fig. 7a, b). The mutated CRP box I, pYU-194-A (-50 to -46), did not shift as compared with the wild-type (Fig. 7c), in agreement with the corresponding null reporter activity shown above. The mutations in CRP box II, i.e. the pYU-194-B, pYU-194-C and pYU-194-D fragments, were shifted in a similar manner to the full-length wild-type fragment of pYU-194 (Fig. 7c). The results with the pYU-194-B and pYU-194-D fragments are in agreement with the moderate diminution (30%) or the null effect on the activity of the corresponding transcriptional fusions. Interestingly, the four-T deletion in pYU-194-C shifted to the same extent as the wild-type fragment, even though it was unable to promote transcriptional activity (Fig. 6b). CRP might be binding to this deletion to render a

Fig. 6. Mapping the CRP recognition sequences in the regulatory region of *yihU*. (a) Schematic representation of the substitutions and deletion obtained by site-directed mutagenesis in the complete regulatory region of *yihU*. The wild-type 5' regulatory region of *yihU*, with CRP boxes I and II, is shown. Bold, upper-case type indicates the mutated residues that result in altered reporter activity. The modified residues in pYU-194-A, pYU-194-B, pYU-194-C and pYU-194-D sequences are depicted. The transcriptional mRNA initiation site (+1) and the theoretical ATG are shown. (b) Expression profile of the wild-type and the mutagenized regulatory regions of *yihU* in *S. Typhi*. The reporter plasmid pYU-194 and mutagenized full-length regulatory regions (pYU-194-A, pYU-194-B, pYU-194-C and pYU-194-D) were transformed into the *S. Typhi* IMSS-1 wild-type strain. The CAT reporter activity levels were determined at OD₆₀₀ 0.4, 0.6, 0.8, 1.0 and 1.4 (12 h) in MA medium. Data represent the mean $\pm$ SD of three independent experiments performed in duplicate.

CRP–DNA conformation that does not promote transcriptional activation.

The *yihU-yshA* operon is carbon catabolite-repressed

Most bacteria can selectively use substrates from a mixture of different carbon sources. The genes that enable the use of secondary carbon sources are not expressed when glucose, or another preferred carbon source, is available (Brückner & Titgemeyer, 2002; Görke & Stülke, 2008). cAMP is a sensory signal in global gene control in *Salmonella*, responding to the availability of glucose, and hence affecting the expression of many catabolic operons (Botsford & Harman, 1992; Ullmann & Monod, 1968).

The largest pYU-194 transcriptional fusion was evaluated in MA medium using glycerol or glucose as carbon source. The addition of glucose had a suppressive effect on the expression of *yihU*, as compared with expression when growth was in glycerol (Fig. 8). Therefore, *yihU* is subject to carbon-dependent regulation that is consistent with the involvement of cAMP–CRP. In order to exclude the possibility that the content of the MA medium influences the results of the transcriptional assays, the same experiments were performed

in minimal medium with glucose or glycerol, with similar results (data not shown).

DISCUSSION

In *Salmonella*, the *yihU-yshA* cluster has been previously described, and it is hypothesized that these genes are involved in the processing and export of an exopolysaccharide to allow biofilm formation in gallstones. The literature indicates that these genes are overexpressed in the presence of bile when *Salmonella* colonizes the intestine and the gallbladder; therefore, this cluster could be relevant in the persistence of gallbladder stones (Crawford *et al.*, 2008; Darwin & Miller, 1999; Gibson *et al.*, 2006; Itoh *et al.*, 2008; Okuyama *et al.*, 2004; Saito *et al.*, 2009). On the basis of these data it is relevant to study several aspects of these genes, including their regulation.

The results of this work show that the *yihU-yshA* genes are organized in an operon, where only the first gene, *yihU*, contains a promoter (Fig. 1). As shown here, this operon is controlled by the global regulatory protein CRP (Fig. 3).

CRP controls the expression of over 200 transcriptional units, usually operons involved in the assimilation of

Fig. 7. CRP binds to different regions of the *yihU* promoter. EMSAs with different concentrations of CRP incubated with 20 ng DNA fragments encompassing different lengths of the 5' regulatory region of *yihU* (Fig. 5a): (a) YU-194, YU-86 and YU-19; (b) YU-194, YU-66 and YU-19. The PCR fragment YU-19 was used as a negative control as it lacks CRP regulatory sites. (c) EMSA of the CRP protein with the various mutations, as in Fig. 6(a), in the regulatory region of *yihU*. Different concentrations of CRP were incubated for 20 min at room temperature with 20 ng of either the full-length wild-type DNA fragment YU-194 or a mutagenized version. YU-19 was used as a negative control as it lacks the proposed CRP-binding sites. Experiments were made in the presence (+) or absence (-) of 100 μ M cAMP and the gels were stained with ethidium bromide to visualize the free DNA as well as the DNA-protein complexes (arrows).

carbon sources and in energy metabolism (Brückner & Titgemeyer, 2002; Görke & Stülke, 2008). Hence, we expected that if the cAMP-CRP complex positively regulated the *yihU-yshA* operon, its activity should

decrease in medium supplemented with glucose as the carbon source. In the absence of glucose, adenylate cyclase increases the intracellular amount of cAMP, the allosteric effector necessary for the binding of CRP to DNA to

Fig. 8. Glucose inhibits the transcriptional activity of the *yihU-yshA* operon. Expression profile of the pYU-194 full-length 5' regulatory region of *yihU* from *S. Typhi* wild-type strain IMSS-1 grown in MA medium supplemented with either glycerol or glucose. The CAT reporter activity levels were determined at OD₆₀₀ 0.4, 0.6, 0.8, 1.0 and 1.4 (12 h). Bars represent the mean of three independent experiments performed in duplicate.

activate transcriptionally the genes of the regulon (Brückner & Titgemeyer, 2002; Xu & Su, 2009). In agreement with this hypothesis, the activity levels of the full-length regulatory fusion pYU-194 decreased drastically when MA medium was supplemented with glucose instead of glycerol (Fig. 8).

CRP directly regulates by binding to the promoter region of *yihU* in the presence of cAMP (Fig. 4). The sequence of the CRP-binding site has been classically described as a pseudo-palindromic consensus, TGTGA-N₆-TCACA, and recently a slightly different CRP-binding site, TGCGA-N₆-TCGCA, has also been identified in *E. coli* and in other γ -proteobacteria (Busby & Ebright, 1999; Cameron & Redfield, 2006; Ivanov *et al.*, 1995; Kanack *et al.*, 2006; Kolb *et al.*, 1993; Pyles & Lee, 1996; Williams *et al.*, 1996; Xu & Su, 2009). The transcriptional activities of several deletions of the 5' end of the regulatory region (Fig. 5), and site-directed mutagenesis of the *yihU* promoter (Fig. 6), allowed the identification of the nucleotides within CRP boxes I (−50 to −46) and II (−72 to −75) that are essential for the transcription of the *yihU-yshA* operon. Accordingly, bioinformatics analyses have shown that the *yihU* regulatory region has two putative binding sites for CRP covering those bases, centred at positions −42.5 (5'-AGTGACGGCTGTCAAT-3'), located at nucleotides −50 to −35) and −75.5 (5'-TGTGCTCATTTTAATT-3', located at nucleotides −83 to −68).

The results obtained in this work indicate that it is necessary to have two CRP boxes for full *yihU* activation. To explain these data we hypothesize that initially CRP binds to CRP box I, near to the −35 box, recruiting and interacting with the RNA polymerase α -subunit, and that simultaneously, another CRP dimer binds upstream at the −83 to −68 region, stimulating transcription by direct contact with the RNA polymerase (Busby *et al.*, 1994). Hence, the C-terminal domains of the RNA polymerase α -subunits interact with both CRP dimers via activating region 1 of CRP (Belyaeva *et al.*, 1998).

This mechanism has been described for the tandem regulation of the *cdd* and *melR* promoters in *E. coli*; in both cases, a sequence close to the −35 and a second sequence located upstream are relevant for CRP transcriptional activation (Busby *et al.*, 1994; Savery *et al.*, 1996). Such a mechanism could be altered in the four-T deletion mutant pYU-194-C, which results in nearly null transcriptional activity (Fig. 6b). This may be the consequence of modifying the distance between the contact points of CRP with the RNA polymerase, or else of altering a binding site defined by the deletion.

This work opens the question of whether in the mammalian gallbladder environment or another abiotic system, a change in the available carbon source could allow CRP to mediate biofilm processing in *Salmonella* through this operon, promoting the persistence of the organism and its survival in different systems.

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