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 ($\sigma^E$) and RpoS in Salmonella Choleraesuis, Vibrio cholerae, Salmonella Gallinarium 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 pseudopalindromic 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.
**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), nutrient broth, minimum medium (with either 0.2 %, v/v, glycerol or 0.2 %, v/v, glucose as carbon source). Growth was monitored by measuring OD$_{600}$. 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 BamHI/KpnI or BamHI/ HindIII and ligated into the pKK232-8 QTcAmp vector, which is compatible with S. Typhi IMSS-1.
contains a promoterless cat gene. To map the yihU regulatory region, different promoter fragments were generated by PCR, double-digested with BamHI/KpnI and then cloned into BamHI/KpnI-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 OD 600 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 μl TDTT buffer (50 mM Tris/HCl, pH 7.8, 30 mM DL-dithiothreitol). The bacterial pellet was resuspended in 600 μl 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 μl of each extract was added in duplicate to a 96-well ELISA plate, followed by 200 μl of the reaction mixture containing 1 mM 5,5'-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 A410 were read at intervals of 5 s for 5 min, using a Ceres 900C scanning autoreader and microplate workstation and KC3

### Table 2. Oligonucleotides used in this study

All primers were sourced in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
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<tbody>
<tr>
<td>pYU-FOR</td>
<td>CGGGATCCAAATCCGTCTCCTTTTGCCAT</td>
</tr>
<tr>
<td>pYT-FOR</td>
<td>CGGGATCCCTGATCAACATGAG</td>
</tr>
<tr>
<td>pYT-REV</td>
<td>GGGGTACCACGACGTTTTGCCATCATTT</td>
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<tr>
<td>pYS-FOR</td>
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<tr>
<td>pYS-REV</td>
<td>CGGGATCCCTGAGAAAGTCTATATCGGC</td>
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<td>pYR-FOR</td>
<td>CGGGATCCGTCTGATGCTCCATCTCCAT</td>
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<tr>
<td>pYR-REV</td>
<td>GGGGTACCGTCGCAGCCGATGGAAAAAT</td>
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<tr>
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<tr>
<td>crp-R</td>
<td>TATCCAGAGCTTCTTTCG</td>
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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 grown 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 mM 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).

![Fig. 1. Schematic representation of the yihU–yshA operon in S. Typhi.](image-url)
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 Prodocic 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 ± 33.7 μmol min⁻¹ 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’-CTGTCGA-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’-TGTGCTATTGATTTTTA-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).
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 ± 67.8 μmol min 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. 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 Δcrp 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.

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).
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, TTTC→AGGGG); 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-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.

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, 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 ΩTc 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.
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
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. 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 μM cAMP and the gels were stained with ethidium bromide to visualize the free DNA as well as the DNA–protein complexes (arrows).]
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-N\(_6\)-TCACA, and recently a slightly different CRP-binding site, TGCGA-N\(_6\)-TCGCA, has also been identified in E. coli and in other \(\gamma\)-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′-AGTGACGGCTGGTCAAT-3′, located at nucleotides −50 to −35) and −75.5 (5′-TGTGCTCTTTTAATT-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 \(\alpha\)-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 \(\alpha\)-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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