NhaR and RcsB Independently Regulate the *osmCp1* Promoter of *Escherichia coli* at Overlapping Regulatory Sites

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Transcription of the *Escherichia coli osmC* gene is induced by several stress conditions. *osmC* is expressed from two overlapping promoters, *osmCp1* and *osmCp2*. The proximal promoter, *osmCp2*, is transcribed at the entry into the stationary phase by the σ^s sigma factor. The distal promoter, *osmCp1*, is activated by NhaR and RcsB. NhaR is a positive regulator of the LysR family and is known to be an activator of the *nhaA* gene encoding an Na$^+$/H$^+$ antiporter. RcsB is the response regulator of the RcsCDB His-Asp phosphorelay signal transduction system. Genetic data indicated that activation of *osmCp1* by both NhaR and RcsB requires the same short sequences upstream of the −35 region of the promoter. Accordingly, DNase I footprint analysis indicated that both activators protect an overlapping region close to the −35 box of the promoter and suggested that the regulatory effect is direct. Despite the overlap of the binding sites, each activator acts independent of the other and is specific for a particular stress. NhaR can stimulate *osmCp1* in response to an osmotic signal even in the absence of RcsB. RcsB is responsible for the induction of *osmCp1* by alteration of the cell envelope, even in the absence of NhaR. *osmCp1* as an example of multiple-stress-responsive promoter is discussed in light of a comparison of the NhaR and RcsB target regions in the *Enterobacteriaceae*.

When subjected to an environmental stress, bacterial cells induce specific families of genes that contribute to better survival under adverse conditions. Many of these genes are induced by several stresses through the action of a number of transcriptional regulators that bind to specific sites in the vicinity of stress-inducible promoters. In *Escherichia coli*, *osmC* is an example of a multiple-stress-responsive gene, and we are analyzing the regulation of this gene in order to better understand the complex interplay of regulators resulting in the multiple stress response. *osmC* belongs to a family of genes that are highly conserved in gram-positive and -negative bacteria that code for putative envelope proteins required for resistance to organic peroxides and long-term survival in the stationary phase (2, 7, 22, 34). In *E. coli*, *osmC* is induced by high osmolarity during the transition to the stationary phase and by the weak acids acetate and salicylate (1, 12, 15, 27). It is transcribed from two overlapping promoters, *osmCp1* and *osmCp2* (12, 15). The proximal promoter, *osmCp2*, is mainly recognized by σ^S and is responsible for growth phase regulation of the gene. In minimal medium, this promoter is also activated by the leucine-responsive protein and is repressed by the nucleoid-associated protein H-NS. Transcription from the distal promoter, *osmCp1*, is independent of σ^S and is repressed by both the leucine-responsive protein and H-NS in minimal medium (4). Recently, the following two positive regulators of *osmCp1* transcription have been identified: NhaR and RcsB (8, 33). NhaR is a positive regulator belonging to the LysR family and was previously identified as an activator of *nhaA*, a gene encoding an Na$^+$/H$^+$ antiporter (6, 9, 23, 28). NhaR is neces-

sary for the osmotic induction of *osmCp1* by NaCl, LiCl, and, to a lesser extent, the nonionic solute sucrose (33). RcsB is the response regulator of the RcsCDB His-Asp phosphorelay system, which was initially identified as a regulator of the synthesis of the capsular polysaccharide in *E. coli* (13). RcsB has been shown to be necessary for induction of *osmCp1* by the cationic amphipathic molecule chlorpromazine (CPZ) (7). Deletion analysis indicated that a short region upstream from the −35 box was sufficient for stimulation of *osmCp1* by overproduction of NhaR or RcsB (8, 33). RcsB directly activated *osmCp1* in in vitro transcription experiments and stimulated the binding of RNA polymerase to the promoter, but direct binding of RcsB to its DNA target has not been demonstrated so far (8). In this paper we show that RcsB and probably also NhaR are able to directly bind to overlapping sites next to *osmCp1*. We also show that the two activators act independent of each other to induce *osmCp1* in response to specific signals.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. All of the strains were derived from *E. coli* K-12 wild-type strain MG1655 (3).

Culture conditions and enzyme assay. Cells were grown aerobically at 37°C in Luria-Bertani broth with 0.17 M NaCl (5 g/liter) (LB170) or without NaCl. β-Galactosidase activities were assayed as described by Miller (20).

Genetic procedures. Strains carrying an *osmCp1-lac* or *osmCp2-lac* transcriptional fusion were constructed in strain CLG723, as described previously (33). The 138-bp *osmCp* DNA fragment controlling transcription of these fusions was obtained by PCR amplification by using the osmC7E sense primer (5′-GGGG GAATTCCCGGTAATCTATTGTGGG-3′) and the osmC3E antisense primer (5′-GGGAATTCGTTGCTCTCCTGTGGGC-3′) along with plasmid templates derived from pCG302 (Table 1) carrying the *osmCp11* mutation (*osmCp1 osmCp2*$^+$) that inactivates *osmCp1* or the *osmCp21* mutation (*osmCp1*$^+$ *osmCp2*) that inactivates *osmCp2* (12) (see Fig. 2). Bacterial strains carrying *nhaR*::*kan* and strains carrying *rcsB*::*tet* were constructed by P1vir transduction as described by Silhavy et al. (31) by using OR100 and MZ60 as donors and

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Reference or source
E. coli strains		
OR100	*melB*Lid Δ*lacZY nhaR1::kan*	28
MZ60	φ(*cps-lacZ*) *rcsB::tet*	36
CF6343	MG1655 Δ*lacIZ*(*Mlu*I)	M. Cashel
CLG723	CF6343 φ(*malP-lac*)[a]	Laboratory collection
CLG771	CF6343 φ[*osmCp1⁻p2⁺*(7E)-φ(*malP-lac*)][b]	This study
CLG772	CF6343 φ[*osmCp1⁺p2⁻*(7E)-φ(*malP-lac*)][c]	This study
CLG806	CLG772 *nhaR1::kan*	This study
CLG805	CLG772 *resB::tet*	This study
CLG809	CLG772 *nhaR1::kan rcsB::tet*	This study
Plasmids		
pCG302	pBR322 carrying a 1-kbp *osmC⁺* fragment	15
pAPT110	p15A derivative carrying *lacUV5* promoter, Kanʳ/Spʳ[d]	26
pAPT*nhaR*	pAPT110 derivative carrying *nhaR*	33
pAPT-6His-*nhaR*	pAPT110 derivative carrying *nhaR* with an N-terminal six-histidine tag	33
pHRcsB	pFAB1, pAPT110 derivative carrying *rcsB* with an N-terminal six-histidine tag	5
pHRcsB$_{D56E}$	pAPT110 derivative carrying *rcsB$_{D56E}$* with an N-terminal six-histidine tag	8

[a] The notation indicates a transcriptional fusion between the intact *lacZ* and *lacY* genes and the *malP* gene. In CLG723, this fusion is transcribed under control of the *malP* promoter.

[b] In this transcriptional fusion, the *malP-lac* hybrid operon is transcribed under control of a 138-bp DNA fragment carrying the *osmC* promoter region with the *osmCp11* mutation, which inactivates *osmCp1* (12). 7E refers to utilization of oligonucleotide OsmC7E (see Materials and Methods) to obtain the *osmC* promoter DNA fragment by PCR amplification.

[c] In this fusion, the transcription is directed by the same DNA fragment, but it carries the *osmCp21* mutation, which inactivates *osmCp2* (12).

[d] Kanʳ, kanamycin resistant; Spʳ, spectinomycin resistant.

selecting for resistance to kanamycin (40 μg/ml) and tetracycline (10 μg/ml), respectively.

Preparation of *E. coli* crude extracts. Bacterial cells transformed with the vector pAPT110 or derivatives of this vector (pAPT*nhaR*, pAPT-6His-*nhaR*, or pHRcsB) were grown at 37°C in LB170 until the optical density at 600 nm was 0.5, and then they were induced with 500 μM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. Cells were harvested by centrifugation, and the pellets were washed in Tris-NaCl buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl) and stored at −70°C. The cells were then washed in B buffer (20 mM HEPES [pH 8.0], 1 mM EDTA, 150 mM NaCl, 7 mM β-mercaptoethanol, 10% [vol/vol] glycerol) and lysed by sonication. The lysates were centrifuged for 1 h at 12,000 × *g* at 4°C, and the supernatants were each mixed with an equal volume of saturated ammonium sulfate and incubated for 30 min at 4°C. After centrifugation at 12,000 × *g* at 4°C, the pellets were resuspended in B buffer, and the protein concentrations were adjusted to 1 μg/μl after the total protein contents were assayed with a Bradford protein assay kit (Bio-Rad).

Purification of His₆-RcsB and His₆-RcsB$_{D56E}$ proteins. The six-histidine-tagged RcsB and RcsB$_{D56E}$ proteins were purified as described by Carballes et al. (5).

DNase I footprint analysis. DNase I footprint experiments were performed as described previously (10, 21), with the following modifications. The 188-bp *osmC* DNA fragment used for footprint analysis was generated by PCR with primers OsmC7 (5′-CCGGTAATCTATTGTGGG-3′) and OsmC14 (5′-GTTTGATAT CGCCTTCCC-3′) and contained 49 bp of the *osmC* coding sequence, as well as 139 bp upstream of the *osmC* translation start. For DNase I footprint experiments the ³²P-end-labeled probe (5 × 10⁴ cpm) was incubated with crude extract or purified protein as indicated below in a 16-μl solution containing 20 mM HEPES (pH 8), 1 mM EDTA, 150 mM NaCl, 7 mM β-mercaptoethanol, 10% (vol/vol) glycerol, and 1 μg of poly(dI-dC)/poly(dI-dC) (Pharmacia). After 5 min of incubation, DNase I (Appligene) was added at a final concentration of 1.25 μg/ml. The digestion was stopped after 3 min by adding 4 μl of a solution containing 1.5 M COONH₄, 0.25 M EDTA, and 125 μl of tRNA per ml. After ethanol precipitation, chloroform protein extraction, and another ethanol precipitation, the pellet was resuspended in 4 μl of loading buffer. Digests and their corresponding sequences (obtained with the same labeled primer) were analyzed on 6% denaturing polyacrylamide gels.

Comparison of protein and DNA sequences. Sequence analysis was performed by using the facilities of the National Center for Biotechnology Information microbial genome database (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes /bact.html) and of the Penn State Computational Molecular Biology Group (http://globin.cse.psu.edu/enterix/) (11).

RESULTS

RcsB and NhaR have overlapping binding sequences. In order to demonstrate that RcsB and NhaR directly bind to the *osmCp1* region and to identify their binding sites, we performed DNase I footprint experiments. To do this, we used a 188-bp DNA fragment that contained the upstream region of *osmCp1* and was sufficient for activation by RcsB and NhaR (8, 33).

Neither purified wild-type RcsB (Fig. 1A, lane 2) nor crude extract enriched in RcsB (Fig. 1C, lane 3) resulted in protection against cleavage by DNase I. We then used purified RcsB$_{D56E}$, a mutant form of the protein in which the conserved aspartate residue at position 56 was replaced by glutamate. This mutation makes the protein more active and is thought to mimic the phosphorylated state of the response regulator (5, 14). RcsB$_{D56E}$ gave a footprint on both DNA strands of the *osmCp1* region. The protected sequences extended from position −52 to position −40 on the template strand (Fig. 1A, lanes 3 and 4) and from position −52 to position −37 on the nontemplate strand (Fig. 1C, lane 4). Two sites on the template strand, at positions −48 and −16, appeared to be hypersensitive to DNase I (Fig. 1A). The protected region is in agreement with the location of the RcsB box proposed previously from genetic data (5, 8). The footprint was obtained with 10 μM RcsB$_{D56E}$ (Fig. 1A, lane 4), a concentration that is not high enough to observe an effect with wild-type RcsB (Fig. 1A, lane 2).

When the *osmCp1* DNA fragment was incubated with a crude extract from a wild-type strain, no protection against limited DNase I digestion was observed (Fig. 1B, compare lanes 1 and 3). In contrast, when the crude extract was prepared from a strain overexpressing NhaR, a footprint was obtained on both DNA strands (Fig. 1B and C), suggesting that
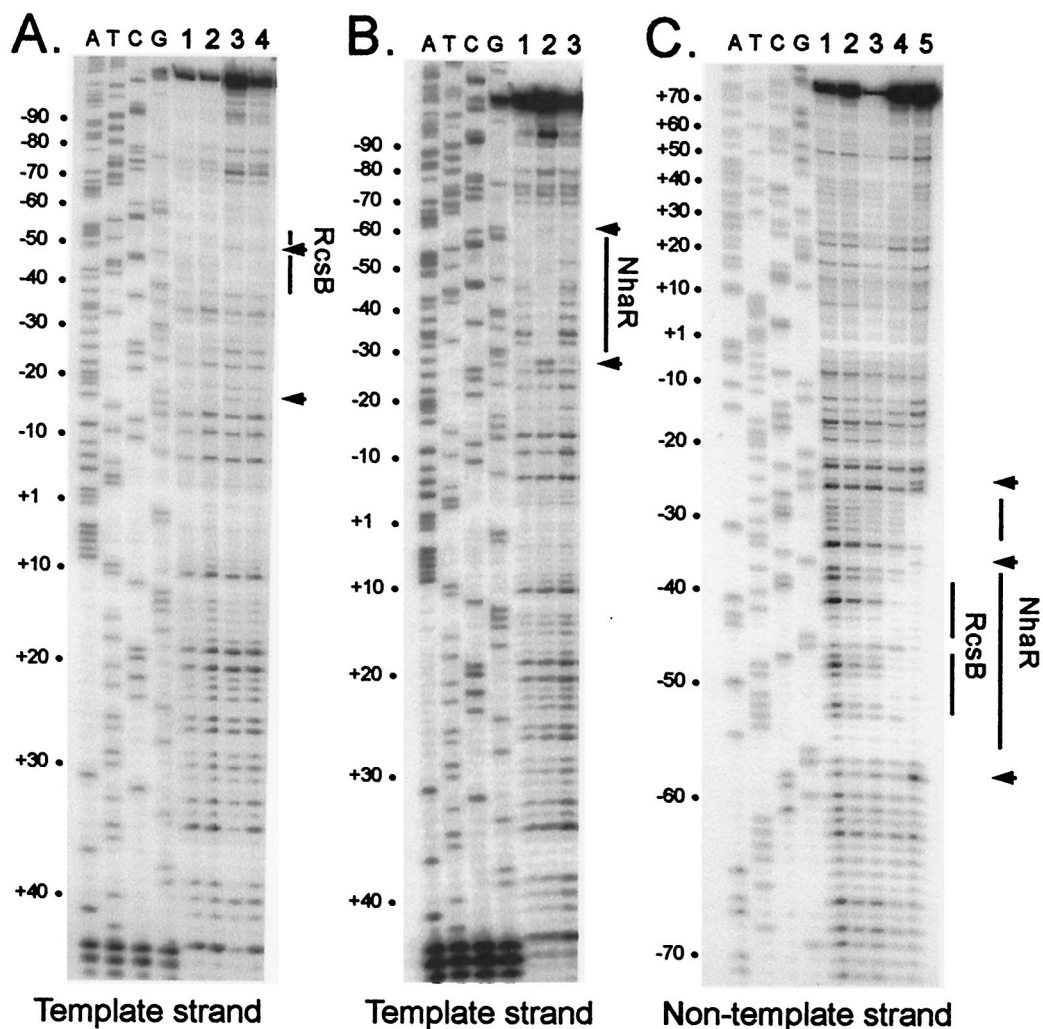
FIG. 1. DNase I protection footprint of NhaR and RcsB in the *osmCp1* region. A 188-bp DNA fragment encompassing the *osmC* promoter region, end labeled with ^{32}P on the strand indicated at the bottom, was incubated with purified RcsB or RcsB$_{D56E}$ protein or with crude extracts enriched or not enriched in RcsB or NhaR protein, digested with DNase I, and analyzed on denaturing polyacrylamide gels. The results of autoradiography of the dried gels are shown. (A) Lane 1, DNA probe alone; lane 2, DNA probe incubated with purified RcsB protein (10 μM); lanes 3 and 4, DNA probe incubated with 100 μM (lane 3) or 10 μM (lane 4) purified RcsB$_{D56E}$ protein. (B) Lane 1, DNA probe alone; lanes 2 and 3, DNA probe incubated with crude extract (2 μg of protein) of strain CLG772 transformed with pAPT*nhaR* (lane 2) or pAPT110 (lane 3). (C) Lane 1, DNA probe alone; lane 2, DNA probe incubated with crude extract (2 μg of protein) of strain CLG772 transformed with pAPT110; lane 3, DNA probe incubated with crude extract (2 μg of protein) of strain CLG772 transformed with pHRcsB; lane 4, DNA probe incubated with 100 μM purified RcsB$_{D56E}$ protein; lane 5, DNA probe incubated with crude extract (2 μg of protein) of strain CLG772 transformed with pAPT*nhaR*. The vertical lines and arrowheads indicate the positions protected from and hypersensitive to cleavage by DNase I, respectively.

NhaR directly binds to the *osmCp1* regulatory region. This footprint extended from position −61 to position −27 on the template strand and from position −57 to position −24 on the nontemplate strand. The protected region was limited at both sides by sites where there was enhanced DNase I cleavage. This region included not only the −35 box of *osmCp1* but also the RcsB binding site (Fig. 2). However, crude extracts prepared from a strain carrying an *rcsB::tet* mutation and containing plasmid pAPT*nhaR* gave the same footprint (data not shown), indicating that the binding of NhaR does not require RcsB.

NhaR and RcsB can independently activate the *osmCp1* promoter. Because RcsB and NhaR act through the same region upstream of *osmCp1*, we investigated putative functional interference between these two activators in regulating *osmCp1*.

RcsB or NhaR was overproduced in strains with mutations either in *nhaR* (CLG806) or in *rcsB* (CLG805), and the expression of a chromosomal *osmCp1-lac* fusion was monitored (Fig. 3). The 13-fold activation of *osmCp1* by overproduction of NhaR was the same in the presence or in the absence of RcsB, indicating that the activity of NhaR is independent of RcsB, in agreement with the footprint data.

As shown in Fig. 3B, transcription from *osmCp1* was stimulated by overproduction of RcsB both in wild-type and *nhaR* mutant backgrounds, indicating that RcsB can activate transcription of *osmCp1* independent of NhaR. However, we observed approximately twofold-higher expression when RcsB was overproduced in the absence of *nhaR*. Therefore, NhaR had a negative effect on activation by RcsB, presumably
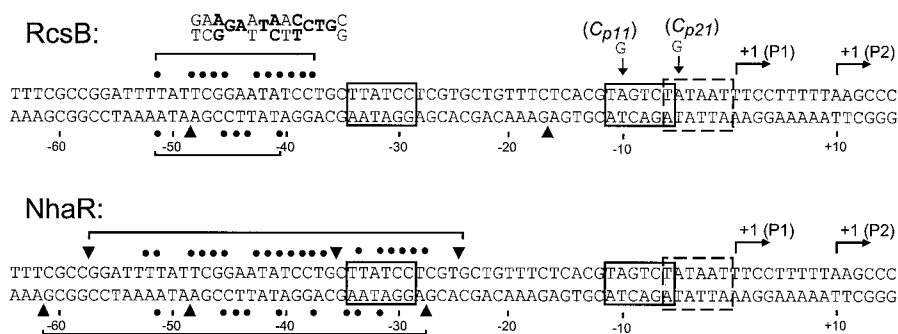
FIG. 2. Sequences of the RcsB and NhaR binding sites upstream of *osmCp1*. The sequence of the *osmC* promoter region is shown. Solid boxes indicate the −35 and −10 hexanucleotides of *osmCp1*. The dashed box indicates the −10 region of the proximal promoter *osmCp2*. Arrows indicate the transcription start sites. The *osmCp11* and *osmCp21* mutations, eliminating activity of *osmCp1* and *osmCp2*, respectively, are indicated. The dots and arrowheads indicate the sites protected from and hypersensitive to cleavage by DNase I, respectively. The consensus sequence for RcsA-independent RcsB sites is shown above the RcsB sequence.

through competition for the same binding site. We also observed that the basal expression was much reduced in an *nhaR* mutant background, indicating that NhaR is the major regulator of *osmCp1* during growth in Luria broth. The combination of a reduced basal level and higher induced expression
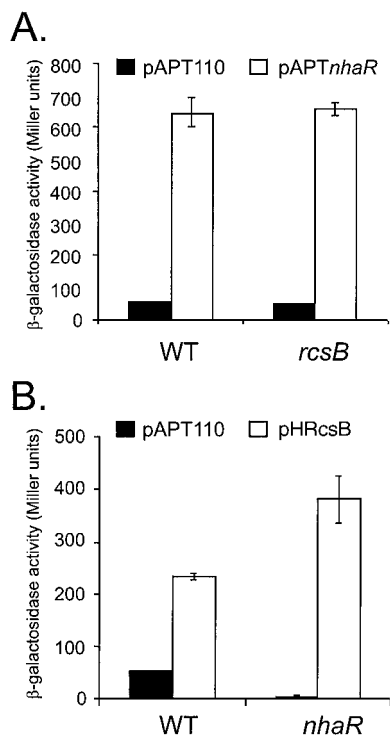

FIG. 3. RcsB and NhaR activate an *osmCp1-lacZ* transcriptional fusion independent of each other. Strain CLG772 (WT), carrying an *osmCp1-lac* fusion, and derivatives of this strain with mutations in *rcsB* (CLG805) (A) and *nhaR* (CLG806) (B) were transformed with plasmid pAPT110 (vector) (A and B), pAPT*nhaR* (A), or pHRcsB (B). Overnight cultures of these strains in LB170 containing spectinomycin were diluted 1,000-fold and grown for five generations. They were then diluted 40-fold in prewarmed medium with IPTG (final concentration, 500 μM), and samples were used for β-galactosidase assays after 2 h. The values are the means of the results of three independent experiments.

resulted in an induction rate that increased from 5-fold in the wild type to 100-fold in an *nhaR* mutant background (Fig. 3B). In control experiments with the isogenic strain CLG771, an *osmCp2-lac* transcriptional fusion was not affected by overproduction of NhaR or RcsB (data not shown).

NhaR and RcsB are specifically required for induction of *osmCp1* by different stress conditions. In agreement with previous results (33), we observed that NaCl and LiCl induced transcription from *osmCp1* and that this induction required *nhaR* (Table 2). In contrast, the response to NaCl or LiCl was independent of RcsB, because it was identical in *rcsB*⁺ and *rcsB* mutant backgrounds (compare CLG772 and CLG805 in Table 2). The cationic amphipathic molecule CPZ also induces *osmCp1*, and this activation requires a wild-type *rcsB* allele (7). In agreement, we observed that induction of *osmCp1* by CPZ was essentially eliminated in the absence of RcsB. In contrast, induction by CPZ was still observed in an *nhaR* mutant background, indicating that the RcsB-dependent response to CPZ does not require NhaR (Table 2). Altogether, these data demonstrate that NhaR and RcsB are able to transduce different regulatory signals to *osmCp1* independent of each other. We noted, however, that the activation by CPZ was increased in an *nhaR* background (92 versus 140 U), indicating that NhaR partially hampers the complete activation of *osmCp1* by RcsB.

Organization of RcsB and NhaR target regions in some members of the *Enterobacteriaceae*. *E. coli* strains EDL933 (O157:H7, enterohemorrhagic) (25) and CFT073 (O6:H1, uropathogenic) (35) both have an *osmC* gene, which encodes a protein that is the same length (143 amino acids) as and is almost identical to the protein of K-12 strain MG1655 (142 of 143 positions are identical). The *osmC* gene of *Shigella flexneri* serotype 2a (17) is identical to the *osmC* gene of MG1655. *Salmonella enterica* serovar Typhimurium LT2 (19) and *S. enterica* serovar Typhi CT18 (24) have an identical *osmC* gene, which encodes a protein with 92% identity to the protein of MG1655 (132 of 143 amino acids are identical). Homologues of NhaR and of the RcsCDB system are also found in all these strains. They exhibit between 85 and 100% identity with the proteins of *E. coli* MG1655, suggesting that NhaR and RcsB function similarly, if not identically, in these phylogenetically closely related bacteria. In order to see whether regulation of

TABLE 2. Effects of *nhaR* and *rcsB* mutations on osmotic and CPZ-induced stress responses of *osmCp1*

Strain (relevant genotype)	β-Galactosidase activity (Miller units)[a]			
	No addition	NaCl (0.3 M)	LiCl (0.3 M)	CPZ (0.1 mM)
CLG772	18 ± 2	70 ± 9	77 ± 6	92 ± 10
CLG805 (*rcsB::tet*)	13 ± 2	68 ± 5	62 ± 4	21 ± 1
CLG806 (*nhaR1::kan*)	14 ± 1	14 ± 2	15 ± 1	140 ± 15
CLG809 (*rcsB::tet nhaR1::kan*)	5 ± 1	7 ± 1	5 ± 1	5 ± 1

[a] Cultures of the strains were grown aerobically in Luria-Bertani broth without NaCl at 37°C. At an optical density at 600 nm of 0.1, NaCl, LiCl, or CPZ was added, and the β-galactosidase activity was assayed after 2 h. The values are the means of the results of two independent experiments for CLG809 and three independent experiments for the other strains.

osmC by NhaR and RcsB was also conserved, we compared the *osmC* promoter regions in these different bacteria (Fig. 4). The three *E. coli* strains and the *S. flexneri* serotype 2a strain had very similar sequences in the *osmC* promoter region, which was indicative of both the conservation of functional *osmCp1* and *osmCp2* promoters and the conservation of their regulation by NhaR, RcsB, and σ[S]. In contrast, in the two *Salmonella* strains, despite the conservation of very similar *osmC* genes, the DNA sequence is completely divergent upstream of the *osmCp2* −10 box (Fig. 4), suggesting that *osmC* regulation in these organisms might be different from that in *E. coli* and *S. flexneri*.

The *nhaA* promoter is the other known target of NhaR. All the *Escherichia*, *Shigella*, and *Salmonella* strains have closely related *nhaA* and *nhaR* genes. A comparison of the *nhaA* regulatory region (http://globin.cse.psu.edu/enterix; *Menteric* server) revealed very good conservation of the promoter and of the NhaR binding sites (data not shown). Similarly, comparison of the known RcsB targets at the *wza* (*cps*), *ftsA*1p, and *rprAp* promoters revealed strong conservation of the putative RcsB binding sites in the *Escherichia*, *Shigella*, and *Salmonella* strains (Fig. 5).

DISCUSSION

Previous work showed that *osmCp1*, one of the two overlapping promoters of the *osmC* gene, could be activated by the transcriptional regulators RcsB (8) and NhaR (33). Deletion analysis showed that activation by these two regulators required the same 16-bp DNA sequence upstream of the −35 box of *osmCp1*, raising the question of possible interference between RcsB and NhaR or even an indirect effect of one regulator through the other. The biochemical and genetic data reported here demonstrate that RcsB and NhaR act independent of each other, probably through direct binding to overlapping sites close to the *osmCp1* −35 box.

We previously showed with gel shift assays that RcsB$_{D56E}$, a constitutively active mutant form of RcsB, potentiated the binding of RNA polymerase to *osmCp1*, but binding of the activator alone to the RcsB box could not be demonstrated (8). The footprint analysis described here demonstrated that RcsB$_{D56E}$ binds on its own to its target DNA (Fig. 1), and to our knowledge, this is the first time that a footprint has been described with RcsB. It is not clear why binding of RcsB$_{D56E}$ to its target site is not seen in band shift experiments. Possibly, the affinity of binding is too low to obtain a stable complex during the electrophoretic migration. The consensus sequence derived from the comparison of several RcsB target regions was KMRGAWTMWYCTGS (W = A or T; K = G or T; M = A or C; R = A or G; W = A or T; Y = C or T; S = C or G), and this sequence allowed prediction of an RcsB box upstream from *osmCp1* (8). The binding site defined here by the DNase I footprint analysis confirms the predicted site; it is centered on position −41/−42, and the protection extends roughly over one turn of the DNA double helix (Fig. 2). We noted that the pattern of protection against DNase I is asymmetric, in agreement with the probable binding of RcsB as a dimer, with each monomer protecting one-half of the site.

A crude extract of wild-type *E. coli* does not contain enough of any protein to protect the *osmCp1* region against cleavage by DNase I in our experimental conditions (Fig. 1). In contrast, an extract enriched in NhaR protects a site that encompasses the −35 box of *osmCp1* and includes the region required for regulation by NhaR (33). Although the protected site includes the region protected by RcsB, RcsB is not necessary for the protection observed with extracts enriched in NhaR, and it is
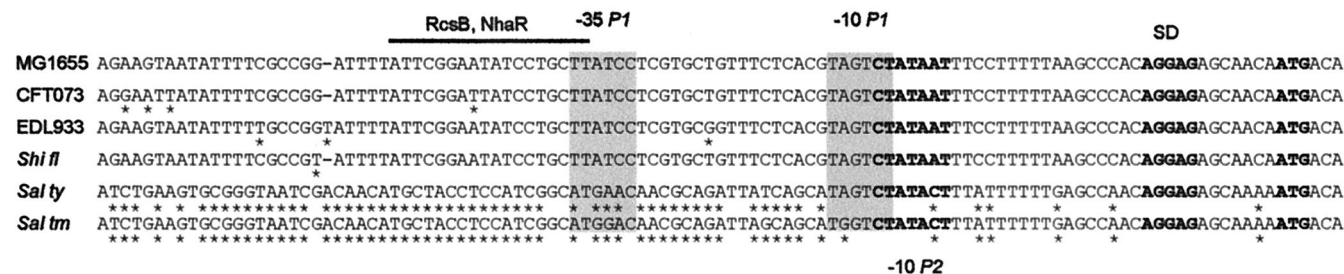

FIG. 4. Alignment of the DNA sequences of the *osmCp* regions of different members of the *Enterobacteriaceae*. MG1655, *E. coli* K-12 strain; CFT073, uropathogenic *E. coli* O6:H1 strain; EDL933, enterohemorrhagic *E. coli* O157:H7 strain; *Shi fl*, *S. flexneri* serotype 2a; *Sal thy*, *S.enterica* serovar Typhi CT18; *Sal tm*, *S.enterica* serovar Typhimurium LT2. The stars indicate differences compared with the MG1655 sequence. SD, Shine-Dalgarno sequence.
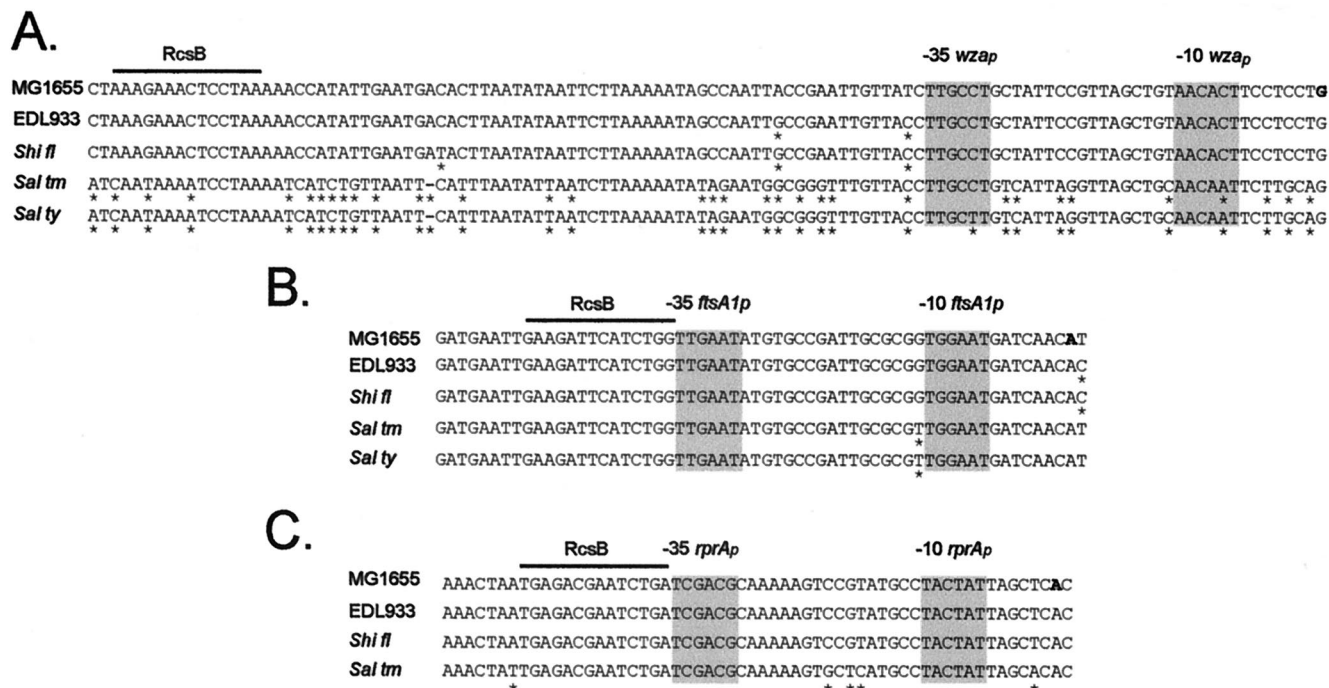
FIG. 5. Alignment of putative RcsB target sites of different members of the *Enterobacteriaceae*: regulatory regions of the *wza* (A), *ftsA*1p (B), and *rprA* (C) genes. MG1655, *E. coli* K-12 strain; EDL933, enterohemorrhagic *E. coli* O157:H7 strain; *Shi fl*, *S. flexneri* serotype 2a; *Sal thy*, *S.enterica* serovar Typhi CT18; *Sal tm*, *S.enterica* serovar Typhimurium LT2. The stars indicate differences compared with the MG1655 sequence. The −10 and −35 regions of the promoters (shaded background) and the transcription start sites (boldface type) have been described previously (5, 18, 32).

likely due to direct binding of NhaR upstream from *osmCp1*. NhaR belongs to the LysR family, a group of transcription activators characterized by unusually large binding sites (16, 28, 30). Upstream from the *nhaA* promoter, NhaR protects approximately 90 bp, spanning a compound site composed of three core binding sites separated by positions hypersensitive to DNase I (6). Because almost all the positions in the core binding sites vary from one site to another, no consensus sequence could be defined (6). The site protected at *osmCp1* is smaller than that protected at *nhaAp*, encompassing approximately 30 bp with positions hypersensitive to DNase I on both sides (Fig. 2). The NhaR binding site at *osmCp1* is composed of a single core binding site and thus appears to be simpler than the binding site at *nhaAp*. It is therefore likely that the affinity of NhaR for *osmCp1* is weaker than that for *nhaAp*, explaining why the same His-tagged NhaR variant is able to bind to *nhaAp* but not to a DNA fragment carrying *osmCp1* (6, 8).

osmC is a multiple-stress-responsive gene. Here, we established that induction of this gene by osmotic stress resulting from NaCl or LiCl addition and induction by membrane alteration due to CPZ are accounted for by independent pathways, involving different transcriptional regulators. Activated promoters often result from the association of intrinsically weak promoters and regulatory modules that allow the binding of specific activators that help RNA polymerase initiate transcription (29). During evolution, acquisition of a new module near a promoter should confer the ability to respond to a new specific stress by recruiting a dedicated regulator. However, most activators must be precisely located with respect to RNA polymerase, and this results in a strong constraint on the position of the binding site. In *osmCp1*, the same short sequence appears to be a bifunctional module that fulfills the requirements for accommodating two different transcriptional activators. Combined with utilization of overlapping alternative promoters, this makes the *osmC* regulatory region in *E. coli* K-12 unusually compact and complex. The three *E. coli* strains and the *S. flexneri* strain sequenced have almost identical *osmC* regulatory regions, suggesting that the patterns of regulation of *osmC* must be similar in all of these strains. In contrast, the *Salmonella* strains have a very different region (Fig. 4). Analysis of the sequence in *Salmonella* indicates that there is probably a functional, presumably σ^S-dependent homologue of *osmCp2*. In contrast, if there is a functional homologue of *osmCp1*, it must be located at a different position, because several sequence changes affect positions corresponding to the −10 or −35 regions of *osmCp1* and decrease the similarity to the consensus sequence for σ^70-dependent promoters. Experimental work is needed to determine the pattern of regulation of *osmC* in *Salmonella*, but the analysis of the promoter region suggests that it might be different from that in *E. coli*. We note that the difference in organization of the target promoters of NhaR or RcsB in *E. coli* and *Salmonella* is not a general phenomenon. For instance, comparison of the *nhaAp* regions revealed very good conservation up to the 5′ limit of the three NhaR binding sites, followed by complete divergence between the *Escherichia-Shigella* group and the *Salmonella* strains. Known targets for RcsB also show very good conservation of

the RcsB binding sites (Fig. 5), suggesting that again, the regulatory patterns and mechanisms are highly conserved in members of the *Enterobacteriaceae*. Finally, the very different organizations of the *osmC* promoter region suggest that acquisition of the *osmCp1* promoter and its regulatory cassettes was recent and occurred after separation of the genera *Escherichia* and *Salmonella*.

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