The transcriptional activator NhaR is responsible for the osmotic induction of *osmC_{p1}*, a promoter of the stress-inducible gene *osmC* in *Escherichia coli*

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Two overlapping promoters, *osmC_{p1}* and *osmC_{p2}*, direct the transcription of the *osmC* gene of *Escherichia coli*. The proximal promoter, *osmC_{p2}*, is induced upon entry into stationary phase under the control of $E\sigma^s$, the RNA polymerase that uses the σ^s (RpoS) sigma factor. Transcription from the distal promoter, *osmC_{p1}*, is independent of σ^s . Previous analysis demonstrated that the osmolarity of the growth medium modulates expression of both promoters. The use of an *E. coli* genomic library showed that the cloned *nhaR* gene was able to stimulate transcription of an *osmC-lac* reporter fusion. NhaR is a positive regulator of the LysR family, previously identified as an activator of *nhaA*, a gene encoding a Na^+/H^+ antiporter involved in adaptation to Na^+ and alkaline pH in *E. coli* and other enteric bacteria. NhaR was shown to activate only the expression of *osmC_{p1}* and to be necessary for the induction of this promoter by LiCl, NaCl and sucrose. Therefore, activation by NhaR is responsible for the osmotic induction of *osmC_{p1}*. In contrast to its action on *nhaA*, NhaR activation of *osmC_{p1}* is independent of H-NS. Activation of *osmC_{p1}* by NhaR requires a site located just upstream of the atypical –35 region of the promoter.

Keywords: transcriptional regulation, osmoregulation, bacterial promoters, NhaR

INTRODUCTION

To cope with environmental stresses, non-sporulating enterobacteria such as *Escherichia coli* or *Salmonella typhimurium* undergo global programmed modifications of their gene-expression pattern, leading to increased resistance to a number of chemical and physical agents including heat, oxidative agents and hyperosmotic shock (McCann *et al.*, 1991; Kolter *et al.*, 1993; Hengge-Aronis, 1996b). There is an overlap between the stimulons responding to different stresses, and many genes are induced by several types of stressful conditions. In the present work, we investigated the regulation of the stress-inducible gene *osmC* in *E. coli*. This gene is highly conserved among Gram-positive and -negative bacteria (Volker *et al.*, 1998). It encodes a putative envelope protein of unknown function that is required for resistance to organic peroxides in *Xanthomonas campestris* and *E. coli* (Mongkolsuk *et al.*, 1998; Conter *et al.*, 2001) and also for long-term survival in stationary phase in *E. coli* (Conter *et al.*, 2001).

Transcription of *osmC* is induced at elevated osmolarity, by exposure to short-chain fatty acids and at the onset of stationary phase (Gutierrez & Devedjian, 1991; Gordia & Gutierrez, 1996; Arnold *et al.*, 2001). *osmC* is transcribed from two overlapping promoters *osmC_{p1}* and *osmC_{p2}* (Fig. 1). *osmC_{p2}* is mainly transcribed by $E\sigma^s$, the RNA polymerase that uses σ^s (RpoS), a sigma factor that controls numerous genes expressed in response to starvation and during the transition to stationary phase (Hengge-Aronis, 1996b). Through its action on *osmC_{p2}*, σ^s is responsible for the growth-phase regulation of *osmC* transcription (Gordia & Gutierrez, 1996). In contrast, transcription from *osmC_{p1}* is independent of σ^s . During growth in minimal medium, the leucine-responsive protein has been shown to repress the transcription of *osmC_{p1}* and to activate that of *osmC_{p2}*, and the nucleoid-associated protein H-NS represses transcription from both promoters (Bouvier *et al.*, 1998). In cultures grown aerobically at 37 °C in rich medium, *osmC_{p1}* is probably transcribed by $E\sigma^{70}$, albeit at a very low level, and the exact relevance of this promoter to

Fig. 1. Organization of the *osmC* promoter region. The -10 and -35 boxes of *osmC*_{p1} are underlined with a dashed line; a bent arrow in a dashed line indicates an *osmC*_{p1} transcription start. *osmC*_{p2} does not exhibit a consensus -35 box, and its -10 box is denoted by a solid underline. The mutations *osmC*_{p11} ('Cp11') and *osmC*_{p21} ('Cp21'), abolishing the activity of *osmC*_{p1} or *osmC*_{p2}, respectively, are indicated above the sequence. Bent arrows indicate the 5' limits of the DNA fragments carrying *osmC*_{p1} that were used for deletion analysis. 'C1E' to 'C13E' refer to the oligonucleotides used to amplify the DNA fragments by PCR.

overall expression of *osmC* is questionable. In this work, we used a genetic screen to identify new regulators of *osmC*. The data reported here demonstrate that the product of the gene *nhaR* specifically activates *osmC*_{p1} in response to a number of solutes. NhaR is a regulator of the LysR family, previously identified as a transcriptional activator of the gene *nhaA*, encoding the NhaA Na⁺/H⁺ antiporter, the main system responsible for adaptation to Na⁺ and alkaline pH (in the presence of Na⁺) in *E. coli* and other enteric bacteria (Rahav-Manor *et al.*, 1992; Padan & Schuldiner, 1994; Dover *et al.*, 1996; Carmel *et al.*, 1997). We show that NhaR is a major determinant of the osmotic regulation of *osmC*_{p1}.

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 the *E. coli* K-12 wild-type strain MG1655 (Bachmann, 1996).

Culture conditions and enzyme assays. Cells were grown aerobically at 37 °C in Luria Broth medium with 0.17 M NaCl (LB170), 0.4 M NaCl (LB400) or without NaCl (LB0). β -Galactosidase activities were assayed as described by Miller (1992).

Genetic procedures. Bacterial strains carrying *nhaR*::Kan or *bns205*::Tn10 mutations were constructed by P1vir transduction, as described by Silhavy *et al.* (1984), using strains OR100 and GM229 (Table 1) as donors, and selecting for resistance to kanamycin (40 μ g ml⁻¹) or tetracycline (10 μ g ml⁻¹), respectively. The NhaR phenotype was tested by growth on minimal medium A plates (Miller, 1992) containing melibiose as a carbon source and 100 mM LiCl. Mutants exhibit very poor growth in comparison with that of an isogenic wild-type strain (Rahav-Manor *et al.*, 1992). Strain CLG723 carries a Φ (*malP-lacZ*) transcriptional fusion in which an intact *lac* operon is fused to the first gene of the *malPQ* operon (Debarbouille *et al.*, 1978). Strains carrying *osmC*_p-*lac* fusions were constructed as follows. DNA fragments harbouring various portions of the *osmC*_p region were PCR-amplified with oligonucleotides introducing *Eco*RI sites at both ends (sense: OsmC1E, OsmC9E, OsmC10E and OsmC13E; antisense: OsmC3E; Table 2). To generate fusions with the wild-type promoter region (*osmC*_{p1}⁺ *osmC*_{p2}⁺), the template was plasmid pCG321 (Table 1). To generate fusions with only one functional promoter, derivatives of pCG321

carrying the same insert with mutations *osmC*_{p11} (*osmC*_{p1}⁻ *osmC*_{p2}⁺) or *osmC*_{p21} (*osmC*_{p1}⁺ *osmC*_{p2}⁻) were used as templates (Bouvier *et al.*, 1998; Fig. 1). The DNA fragments were cloned in the unique *Eco*RI site of the vector pOM41 (Vidal-Ingigliardi & Raibaud, 1985). After transformation of CLG723 with the resulting plasmids, the *osmC*_p region was inserted in front of the Φ (*malP-lacZ*) fusion, in place of the *malP* promoter, by homologous recombination, as described previously (Gutierrez & Devedjian, 1991).

Construction of an *E. coli* genomic library. Chromosomal DNA was extracted from MG1655 cells by using the DNeasy Tissue kit (Qiagen) according to the manufacturer's protocols, and was partially digested with *Sau*3A. DNA fragments were ligated with *Bam*HI-digested low-copy-number vector pJPB209 (Pichoff *et al.*, 1995) and introduced by electrotransformation into strain CLG684 carrying an *osmC*_p-*lac* transcriptional fusion under the control of the two *osmC* promoters. The transformed cells were plated on MacConkey-lactose agar plus spectinomycin (100 μ g ml⁻¹).

Methods used with nucleic acids. Isolation of plasmid DNA, digestion with restriction enzymes, ligation with T4 DNA ligase, and transformation were carried out by using standard methods (Silhavy *et al.*, 1984; Sambrook *et al.*, 1989). Amplification of DNA fragments with Hot *Tub* DNA polymerase (Amersham) was performed according to the manufacturer's protocol. DNA sequencing was done with the thermo-sequenase sequencing kit (USB), using oligonucleotide 209H or 209E 5'-end-labelled with [γ -³²P]ATP.

Construction of plasmids. During the screening for *osmC* activators, we isolated an *nhaR*⁺ clone carrying *nhaR* on a 3968 bp *Sau*3A chromosomal DNA fragment ligated to the vector pJPB209. An internal deletion between two unique restriction sites (*Bsp*EI in the chromosomal insert and *Xma*I in the linker of pJPB209) yielded the plasmid pNHAR, carrying 1647 bp of chromosomal DNA with only one intact ORF (*nhaR*, transcribed from its own promoter). Plasmid pAPT*nhaR* was obtained by PCR amplification of *nhaR*, using NHAR1 and NHAR2 oligonucleotides (Table 2) and pNHAR as the template, digestion of the resulting DNA fragment with *As*eI and *Bam*HI, and ligation with the 7007 bp *Bam*HI-*Nde*I fragment of the vector pAPT156 (Table 1). *nhaR* was then placed under the control of the *lacUV5* promoter.

Preparation of *E. coli* crude extracts. Bacterial cells were grown in LB170 medium to an OD₆₀₀ of 0.6 and induced with 500 μ M IPTG for 2 h. Cells were harvested by centrifugation and then washed in Tris-NaCl buffer (10 mM Tris/HCl,

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype	Reference/source
<i>E. coli</i> strains		
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>)U169 <i>deoC1 flbB5301 rpsL150</i> <i>relA1 ptsF25 rbsR</i>	Casadaban (1976)
RH90	MC4100 <i>rpoS-359::Tn10</i>	Hengge-Aronis <i>et al.</i> (1993)
GM229	MC4100 <i>hns-205::Tn10</i>	E. Bremer
OR100	<i>melBLid</i> Δ <i>lacZY nhaR1::kan</i>	Rahav-Manor <i>et al.</i> (1992)
MG1655	F ⁻ λ^- <i>rph-1</i>	Bachmann (1996)
CF6343	MG1655 Δ <i>lacIZ</i> (<i>Mlu</i> I)	M. Cashel
CLG723	CF6343 Φ (<i>malP-lac</i>)*	Laboratory collection
CLG684	CF6343 Φ [<i>osmC</i> _{p1} ⁺ _{p2} ⁺ (1E)- Φ (<i>malP-lac</i>)]†	This study
CLG685	CF6343 Φ [<i>osmC</i> _{p1} ⁺ _{p2} ⁺ (1E)- Φ (<i>malP-lac</i>)]‡	This study
CLG686	CF6343 Φ [<i>osmC</i> _{p1} ⁺ _{p2} ⁻ (1E)- Φ (<i>malP-lac</i>)]‡	This study
CLG737	CF6343 Φ [<i>osmC</i> _{p1} ⁺ _{p2} ⁻ (9E)- Φ (<i>malP-lac</i>)]§	This study
CLG740	CF6343 Φ [<i>osmC</i> _{p1} ⁺ _{p2} ⁻ (10E)- Φ (<i>malP-lac</i>)]§	This study
CLG743	CF6343 Φ [<i>osmC</i> _{p1} ⁺ _{p2} ⁻ (13E)- Φ (<i>malP-lac</i>)]§	This study
CLG689	CLG686 <i>nhaR1::kan</i>	This study
CLG692	CLG686 <i>hns-205::Tn10</i>	This study
CLG695	CLG686 <i>hns-205::Tn10 nhaR1::kan</i>	This study
CLG762	CLG686 <i>rpoS-359::Tn10</i>	This study
Plasmids		
pAPT110	p15A derivative carrying <i>lacUV5</i> promoter	Polard & Chandler (1995)
pAPT156	pAPT110 derivative carrying <i>orfA</i> of IS911	Ton-Hoang <i>et al.</i> (1997)
pCG302	pBR322 carrying a 1 kbp <i>osmC</i> ⁺ fragment	Gutierrez & Devedjian (1991)
pCG321	pOM41 carrying a 91 bp <i>osmC</i> _p ⁺ fragment	Bouvier <i>et al.</i> (1998)
pJPB209	pSC101-derived vector	Pichoff <i>et al.</i> (1995)
pOM41	Promoter recombination vector	Vidal-Ingigliardi & Raibaud (1985)

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

† In this transcriptional fusion, a 91 bp *osmC*_p⁺ DNA fragment directs the transcription of the *malP-lac* hybrid operon. 1E refers to the utilization of oligonucleotide OsmC1E to obtain the *osmC* promoter DNA fragment by PCR amplification.

‡ In these transcriptional fusions, the *osmC* promoter regions, mutated in the -10 boxes of *osmC*_{p1} or *osmC*_{p2}, are carried on a similar 91 bp DNA fragment.

§ In these transcriptional fusions, the *osmC* promoter region, mutated in the -10 box of *osmC*_{p2}, is carried on DNA fragments of the sizes indicated in Fig. 1; '9E', '10E' and '13E' refer to the oligonucleotides used for PCR amplification of the *osmC* promoter DNA fragments.

Table 2. DNA primers used in this study

Primer	Sequence
OsmC1	5'-TTCGCCGGATTTTATTTCGG-3'
OsmC1E	5'-GGGAATTCGCCGGATTTTATTTCGG-3'
OsmC3	5'-GTTGCTCTCCTGTGGGC-3'
OsmC3E	5'-GGGAATTCGTTGCTCTCCTGTGGGC-3'
OsmC7	5'-CCCGGTAATCTATTGTGGG-3'
OsmC9E	5'-GGGAATTCATTTTATTCGGAATATCCTGC-3'
OsmC10E	5'-GGGAATTCGGAATATCCTGCTTATCC-3'
OsmC13E	5'-GGGAATTCCTGCTTATCCTCGTG-3'
NhaR1	5'-GGGATTAATGAGCATGTCTCATATC-3'
NhaR2	5'-GGGGATCCTTAACGCACCGCTGGA-3'
NhaA1	5'-CGTCTCCAGAAAGTCGTGATACCATC-3'
NhaA2	5'-CCGTCAAAAACGCATCTACCGCTG-3'
209E	5'-CAGCGGTATCATCAAC-3'
209H	5'-TAATCGCAACATCCGC-3'

pH 8.0, 100 mM NaCl). They were then washed in buffer B (20 mM HEPES, pH 8.0, 1 mM EDTA, 150 mM NaCl, 7 mM β -mercaptoethanol, 10%, v/v, glycerol) and lysed by sonication. Lysates were centrifuged for 1 h at 12000 g at 4 °C, and each supernatant was mixed with an equal volume of saturated ammonium sulphate and incubated for 30 min at 4 °C. After centrifugation at 12000 g at 4 °C, pellets were resuspended in buffer B and adjusted to 1 μ g protein μ l⁻¹ after assay of the total protein content (protein assay kit; Bio-Rad).

Electrophoretic mobility shift experiments. The DNA probes carrying the *osmC* promoter region were obtained by PCR amplification using pCG302 (Table 1) as a template and primers OsmC7 plus OsmC3, OsmC1 plus OsmC3 or OsmC13 plus OsmC3 (Table 2). The probe carrying *nhaA*_p was amplified from the DNA of bacteriophage λ 6H3 from Kohara's collection (Kohara *et al.*, 1987) and the primers NhaA1 and NhaA2 (Table 2). PCR amplifications were performed in the presence of 20 μ Ci [α -³²P]ATP. A 10 ng sample of the labelled DNA fragment was incubated for 10 min at room temperature with crude extract (0.6–2 μ g protein) in buffer B and 1 μ g poly(dI-dC)/poly(dI-dC) (Pharmacia) competitor DNA. The binding mix was loaded onto 5% polyacrylamide gels in TBE at a voltage of 6 V cm⁻¹, and run at a voltage of 12 V cm⁻¹.

RESULTS

Identification of new regulators acting *in trans* on *osmC* promoters

Strain CLG684 (Table 1) is a Δ *lac* derivative of the *E. coli* wild-type strain MG1655 carrying a transcriptional fusion, Φ [*osmC*_p– Φ (*malP*–*lac*)], as a single copy at the *malA* locus on the chromosome. In this strain, β -galactosidase is expressed under the control of a 91 bp DNA fragment carrying the two promoters *osmC*_{p1} and *osmC*_{p2}. In the MG1655 genetic background, expression of the *osmC* promoters was in all respects identical to that which we had previously observed in the genetic background of strain MC4100 (Gordia & Gutierrez, 1996; Bouvier *et al.*, 1998). In particular, *osmC*_{p1} was expressed at a low level and in a σ ^S-independent manner. To look for new regulators of *osmC* transcription, CLG684 was transformed with a genomic library of MG1655 (see Methods) and the transformants were plated on MacConkey-lactose agar supplemented with spectinomycin. CLG684 gives pale red colonies on MacConkey-lactose agar. Clones exhibiting either a pink colour (reduced expression) or a darker red colour (increased expression) were isolated. Plasmids from these clones were purified and used to retransform CLG684. Out of approximately 75000 independent colonies, 20 plasmids that yielded a darker red colour, and one that yielded a pink colour, were obtained.

Analysis of the new regulators of the *osmC*_{p1} promoter

The next step of our genetic screening was to determine which of the two *osmC* promoters was sensitive to the presence of the plasmids. Strains CLG685 and CLG686 carry *osmC*–*lac* transcriptional fusions expressed under the control of the *osmC*_{p2} promoter or the *osmC*_{p1}

promoter, respectively (Table 1). After transformation of these two strains with each candidate plasmid and plating on MacConkey-lactose agar, we observed that seven plasmids were able to stimulate expression of *osmC*_{p1} but had no apparent effect on *osmC*_{p2}. Previous work had established that (in standard laboratory conditions) *osmC*_{p2} was 10-fold more active than *osmC*_{p1}, suggesting that *osmC*_{p1} plays a minor role in *osmC* expression (Gordia & Gutierrez, 1996; Bouvier *et al.*, 1998). Therefore, we decided to focus our attention on these putative activators of *osmC*_{p1}. By making use of oligonucleotides hybridizing with either side of the pJPB209 multi-site linker (209E and 209H, Table 2), we then sequenced the two ends of the inserts on each of the seven candidate plasmids and compared the sequences with the *E. coli* genome, using the Colibri genebank (<http://genolist.pasteur.fr/Colibri/index.html>). Two of these clones carried the gene *rcsB*, encoding the response regulator of the two-component system RcsB/RcsC, and the analysis of these clones will be reported elsewhere. The five other clones carried different but overlapping DNA fragments from the same chromosomal region. All had in common the gene *nhaR*, already identified as a transcriptional activator of the Na⁺/H⁺ antiporter NhaA (Rahav-Manor *et al.*, 1992).

In addition, the sequence data showed that the single plasmid giving pink colonies (and thus able to repress expression of the *osmC*–*lac* fusion) carried the gene *hns*, which is already known to have a negative effect on the expression of the *osmC* promoters (Gutierrez & Devedjian, 1991; Bouvier *et al.*, 1998).

The gene *nhaR* is sufficient to activate *osmC*_{p1} when present in multicopy

The five *nhaR*⁺ clones carried inserts ranging in size from 3968 bp to 4490 bp. To demonstrate that *nhaR* was responsible for the stimulation of *osmC*_{p1}, we made an internal deletion on the *nhaR*⁺ plasmid carrying the smaller insert (see Methods), yielding a derivative (pNHAR; Table 1) with *nhaR* as the sole intact ORF. Strains CLG685 (*osmC*_{p1}[–] *osmC*_{p2}⁺) and CLG686 (*osmC*_{p1}⁺ *osmC*_{p2}[–]) were transformed with pNHAR or with the empty vector pJPB209. Overnight cultures of the four resulting strains were diluted 1000-fold in LB170 medium and grown for 300 min (to an OD₆₀₀ of approximately 2) before assay of β -galactosidase. Derivatives of CLG685 exhibited activities of 160 and 166 Miller units, in the presence of pJPB209 and pNHAR, respectively, demonstrating that overexpression of *nhaR* has no effect on transcription from *osmC*_{p2}. In contrast, the presence of pNHAR resulted in a ninefold activation of *osmC*_{p1} (257 Miller units for CLG686/pNHAR versus 27 Miller units for CLG686/pJPB209).

Expression of *osmC*_{p1} and *osmC*_{p2} in *nhaR* mutants

We then checked the effect of the chromosomal copy of *nhaR* on expression of the *osmC* promoters. Strains CLG685 and CLG686 and their respective *nhaR*

Fig. 2. Effect of an *nhaR* mutation on transcription at the *osmC*_{p1} and *osmC*_{p2} promoters. Overnight cultures of the indicated strains were diluted 1000-fold into fresh prewarmed LB0 (open symbols) or LB400 (filled symbols). The OD₆₀₀ values of the cultures (dashed lines) and the β-galactosidase activities (solid lines) were monitored during growth. (a) Expression of *osmC*_{p2}: circles, CLG685 (*nhaR*⁺); triangles, CLG688 (*nhaR1::kan*). (b) Expression of *osmC*_{p1}: circles, CLG686 (*nhaR*⁺); triangles, CLG689 (*nhaR1::kan*). The experiment was repeated three times under the same conditions, and the results were identical. Data from one representative experiment are shown.

derivatives (CLG688 and CLG689) were grown in LB0 and LB400. Measurement of β-galactosidase activity in samples of these cultures indicated that, in the absence of NhaR, *osmC*_{p2} expression was unchanged (Fig. 2a) whereas *osmC*_{p1} expression was reduced in both media (Fig. 2b).

*osmC*_{p1} activation by NhaR is independent of H-NS and σ^S

Work on the effect of NhaR on NhaA expression had shown an effect of the nucleoid-associated protein H-NS on the action of NhaR (Dover *et al.*, 1996). Therefore, we investigated the expression of *osmC*_{p1} in CLG686 and CLG689 derivatives carrying an *hns* mutation (Fig. 3a, b). In the presence of NhaR, an *hns* mutation resulted in a slight increase in transcription from *osmC*_{p1}. In the absence of NhaR, *osmC*_{p1} activity was reduced, and no increase was observed in the *nhaR hns* double mutant. In addition, when introduced into derivatives of strain CLG686 carrying *hns* or *rpoS*

Fig. 3. Effects of *hns* and *nhaR* mutations on transcription from *osmC*_{p1}. Experiments were done as described in Fig. 2. (a) Cultures made in LB0. (b) Cultures made in LB400. Circles, CLG686 (*nhaR*⁺ *hns*⁺); triangles, CLG689 (*nhaR1::kan hns*⁺); diamonds, CLG692 (*nhaR*⁺ *hns205::Tn10*); squares, CLG695 (*nhaR1::kan hns205::Tn10*). Data from one of two experiments that gave identical results are shown.

mutations, the plasmid pNHAR activated *osmC*_{p1} to a level similar to that observed in the wild-type background (Table 3). Therefore, *trans*-activation of *osmC*_{p1} by overexpressed NhaR protein does not require σ^S or H-NS.

NhaR is responsible for *osmC*_{p1} induction by NaCl and LiCl

To characterize the physiological role of *osmC*_{p1} activation by NhaR in more detail, we investigated the effects of various osmolytes added to the growth medium (Fig. 4). In the *nhaR* wild-type strain CLG686 (Fig. 4, circles), addition of NaCl resulted in a twofold stimulation of β-galactosidase production. The most potent inducer appeared to be LiCl, which yielded a fourfold increase in induction. The non-ionic solute sucrose gave only moderate and temporary stimulation of transcription from *osmC*_{p1}. The effects of all these solutes were totally abolished in the absence of NhaR (Fig. 4, triangles). In similar experiments, expression of *lacZ*

Table 3. Deletion analysis of sequences necessary for activation of *osmC_{p1}* by NhaR

Strain*	Relevant genotype†	β -Galactosidase activity (Miller units)‡			
		pAPT110		pAPT $nhaR$	
		– IPTG	+ IPTG	– IPTG	+ IPTG
CLG686	$\Phi(osmC_{p1}(1E)-lac)$	18	22	55	475
CLG692	$\Phi(osmC_{p1}(1E)-lac)$ <i>hns::Tn10</i>	23	24	50	424
CLG762	$\Phi(osmC_{p1}(1E)-lac)$ <i>rpoS::Tn10</i>	10	11	69	523
CLG737	$\Phi(osmC_{p1}(9E)-lac)$	20	23	50	487
CLG740	$\Phi(osmC_{p1}(10E)-lac)$	13	11	69	723
CLG743	$\Phi(osmC_{p1}(13E)-lac)$	12	11	6	4

* Strains were transformed with the plasmid indicated.

† The extent of the DNA fragment carrying *osmC_{p1}* is as described in Fig. 1. All of the fragments carry the mutation *osmC_{p21}* (*osmC_{p1}*⁺ *osmC_{p2}*[–]).

‡ Cells bearing the *osmC_{p1}-lacZ* fusion with pAPT110 or pAPT $nhaR$ were grown in LB0 aerobically at 37 °C. Overnight cultures were diluted 1000-fold and grown for five generations. They were then diluted 40-fold in prewarmed medium with or without IPTG (500 μ M), and sampled for β -galactosidase assays after 2 h. Values are the means of the results from two independent experiments.

mediated by *osmC_{p1}* was independent of the presence of KCl and of the pH in the growth medium (data not shown).

The sequence required for activation by NhaR is immediately adjacent to the *osmC_{p1}* – 35 region

To identify the sequence required for the stimulation of *osmC_{p1}* by NhaR, we made use of a set of *osmC_{p1}-lac* fusion strains in which *osmC_{p1}* was carried on various DNA fragments. The *osmC_{p1}* fragments differed from each other by having different sequences 5' to the promoter (Fig. 1). These strains were transformed with pAPT $nhaR$, and the β -galactosidase activities were monitored with or without induction of *nhaR* expression. The results of this deletion analysis, shown in Table 3, indicated that 16 bp upstream from the *osmC_{p1}* – 35 box (DNA fragment obtained with oligonucleotide OsmC10E, in strain CLG740) were sufficient to obtain a 10-fold increase in *osmC_{p1}* expression upon induction of NhaR production. In contrast, stimulation was no longer observed with a promoter fragment leaving only 6 bp upstream of the –35 box (DNA fragment obtained with oligonucleotide OsmC13E, in CLG743), indicating that the sequences required for the activation by NhaR had been at least partially deleted in the latter construction.

Overexpression of NhaR produces an *osmC* promoter region binding activity in *E. coli* crude extracts

Crude extracts were prepared from strain CLG686 containing either the vector pAPT110 or the plasmid pAPT $nhaR$ and used for electrophoretic mobility shift experiments with DNA fragments carrying either the

nhaA or the *osmC* promoter region (see Methods). In agreement with previous observations (Rahav-Manor *et al.*, 1992), incubation with a crude extract containing overexpressed NhaR retarded the migration of a DNA probe carrying the *nhaA* promoter region (Fig. 5, compare lanes 2 and 3). The migration of a 91 bp *osmC_{p1}* DNA probe that was sufficient to produce an effect of NhaR *in vivo* (Table 3) was also retarded after incubation with a crude extract enriched in NhaR (Fig. 5, compare lanes 5–9 with lanes 10 and 11). No retardation was observed after incubation with a control crude extract that did not contain overexpressed NhaR, demonstrating that the retardation was not a non-specific effect of some protein present in the crude extract. A longer DNA probe extending further upstream of the *osmC_{p1}* promoter exhibited the same behaviour as the 91 bp DNA probe (Fig. 5, lanes 12–16). In contrast, a smaller DNA probe that did not carry enough sequence to be responsive to NhaR *in vivo* (Table 3) showed no retardation of its migration after incubation with the crude extract containing overexpressed NhaR (Fig. 5, lanes 17–19).

DISCUSSION

Several regulators have been shown to modulate the transcription of the stress-inducible gene *osmC* in *E. coli*. In the present work, we describe an additional positive regulator, NhaR, which specifically stimulates transcription of the σ^S -independent distal promoter *osmC_{p1}*. Until now, *osmC_{p1}* was considered as a very weak promoter. However, the data presented in this study show that under appropriate conditions, for instance when stimulated by NhaR, *osmC_{p1}* is as efficient as *osmC_{p2}* and can contribute significantly to

Fig. 4. Induction of *osmC_{p1}* by NaCl, LiCl or sucrose is NhaR-dependent. Strains CLG686 (*nhaR*⁺; circles) and CLG689 (*nhaR1::kan*; triangles) were grown in LB170 until an OD₆₀₀ value of 0.1 was reached (time 0). Then, portions of the cultures were subjected to an osmotic shock by the addition of (a) NaCl (0.3 M final concentration), (b) LiCl (0.3 M final concentration) or (c) sucrose (0.4 M final concentration). The β -galactosidase activity was monitored at intervals. Open symbols and dashed lines indicate untreated cultures; filled symbols and continuous lines indicate treated cultures. Data from one of two experiments that gave identical results are shown.

the expression of *osmC* under stress conditions during exponential phase.

Until now, the only gene that was known to be regulated by NhaR was *nhaA*, a gene encoding a Na⁺/H⁺ antiporter of *E. coli* (Rahav-Manor *et al.*, 1992; Dover *et al.*, 1996). This gene is necessary for adaptation to high salinity and alkaline pH in the presence of Na⁺, but is not essential (Goldberg *et al.*, 1987; Karpel *et al.*, 1988). Like *nhaA*, *osmC* is not essential in *E. coli* (Gutierrez & Devedjian, 1991). To date, the biochemical function of the OsmC protein remains unknown. However, we have shown that inactivation of *osmC* results in higher

sensitivity to organic peroxides and faster decay in viable cell counts of bacterial cultures during long-term stationary phase (Conter *et al.*, 2001). Therefore, it is clear that the physiological role of OsmC is to participate in the response of the cells to adverse conditions. We have shown previously that transcription of *osmC* is stimulated by elevated osmolarity, through the stimulation of its two promoters. It has been shown that osmotic shocks result in an accumulation of the σ^S sigma factor and that this can account for the osmotic induction of some σ^S -dependent genes (Hengge-Aronis *et al.*, 1993; Hengge-Aronis, 1996a). Since the transcription of *osmC_{p2}* is σ^S -dependent (Gordia & Gutierrez, 1996), the osmotic stimulation of this promoter may be mediated by σ^S . In contrast, transcription of *osmC_{p1}* is probably assured by the σ^{70} sigma factor, and the mechanism of its osmotic induction was not clear. The data presented here indicate that NhaR is responsible for the stimulation of *osmC_{p1}* by NaCl, LiCl, and, to a lesser extent, the non-ionic solute sucrose (Fig. 4). Therefore, NhaR is responsible for the osmotic induction of *osmC_{p1}*.

It has been demonstrated recently that *nhaA* is also transcribed from two promoters (Dover & Padan, 2001). A proximal, Na⁺- and NhaR-dependent promoter (P1), is the main promoter during exponential growth. A distal, Na⁺- and NhaR-independent, but σ^S -dependent, promoter (P2) becomes the major promoter upon entry into stationary phase. Therefore, it appears that *nhaA* and *osmC* exhibit similar complex regulatory patterns. Overall, having two different promoters inducible by different mechanisms provides a broader spectrum of conditions for the induction of *nhaA* and *osmC*. In early exponential phase, when the amount of σ^S in the cells is very low, the osmotic induction of *nhaA* and *osmC* is due to the NhaR-dependent activation of P1 and *osmC_{p1}*. At a later stage of growth, even in the absence of osmotic stress, the two genes are expressed under the control of σ^S , preparing the cells for eventually encountering stress during stationary phase. It must be noted that similar dual-promoter organization has been described for other stress-responsive genes. For instance, *proP*, a gene encoding a transporter for the osmo-protectant compound glycine betaine, is induced by both osmotic stress, through stimulation of a σ^S -independent promoter, and upon entry into stationary phase, through stimulation of a second, σ^S -dependent, promoter (Mellies *et al.*, 1995).

The molecular mechanism of activation of *nhaA_p* by NhaR has been studied extensively (Karpel *et al.*, 1991; Rahav-Manor *et al.*, 1992; Carmel *et al.*, 1997; Dover & Padan, 2001). It involves a Na⁺-dependent interaction between the activator and several binding sites near the promoter (Rahav-Manor *et al.*, 1992; Carmel *et al.*, 1997). The activation of *osmC_{p1}* by NhaR might also be direct, involving binding of NhaR near *osmC_{p1}*. Alternatively, it might be indirect, involving an unknown mechanism. Our band-shift experiments show that overexpression of NhaR results in the appearance of a promoter-binding activity in the crude extracts (Fig.

Fig. 5. Crude extracts containing over-produced NhaR retard the electrophoretic migration of DNA fragments carrying the *nhaA* or *osmC* promoters. Approximately 10 ng of radiolabelled DNA fragments carrying either the *nhaA* (lanes 1–3) or the *osmC* (lanes 4–19) promoter region were run on polyacrylamide gels, either alone (lanes 1, 4, 12 and 17) or after incubation with the indicated amounts (in μ g total protein) of crude extracts from strain CLG686 bearing pAPT110 or pAPT*nhaR*. The *osmC* promoter fragments were amplified with the OsmC7–OsmC3 (7–3), OsmC1–OsmC3 (1–3) or OsmC13–OsmC3 (13–3) primer pairs to give products of 138, 91 or 68 bp, respectively. The *nhaA* promoter fragment was 282 bp long.

5). This binding is sequence specific, since it is only observed with a DNA probe carrying enough sequence upstream from the promoter to confer an NhaR-dependent stimulation of transcription from *osmC_{p1}*. However, we do not know if NhaR is directly responsible for this binding. Comparison of the sequences of the NhaR-binding sites near *nhaA_p* shows that the recognition sequence is quite variable (Carmel *et al.*, 1997), and it is not possible to derive a clear consensus with which to scan the sequence near *osmC_{p1}*. We note also that the pattern observed in band-shift experiments with the *osmC_{p1}* DNA fragments is quite different from that obtained with the *nhaA_p* DNA (Fig. 5). The retarded bands migrate at almost the same rate as the naked *osmC_{p1}* DNA fragments, suggesting that the overall structure of the *osmC_{p1}*–NhaR complex is different from that of the *nhaA_p*–NhaR complex. In addition, we constructed and purified a variant of NhaR, carrying a six-histidine tag at its amino-terminal end. Although we could reproduce with this modified protein the binding to *nhaA_p* reported previously (Carmel *et al.*, 1997), this variant of NhaR was unable to bind the 91 bp *osmC_{p1}* DNA fragment (data not shown). In view of the weak binding of native NhaR, it is possible that minor structural alterations induced by the His₆ tag render binding by the tagged protein too weak to be observed in band-shift experiments. Additional experiments will be needed to resolve this question.

After leucine-responsive protein, H-NS and σ^S , NhaR and RcsB are the fourth and fifth regulators of *osmC* transcription to have been identified. The participation of so many factors in the regulation of *osmC* illustrates the notion of cooperation of global regulators in the

fine-tuning of stress-inducible genes (Bouvier *et al.*, 1998; Hengge-Aronis, 1999). Work on the relationships between these multiple factors is in progress in our laboratory.

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