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Multistress Regulation in *Escherichia coli*: Expression of *osmB* Involves Two Independent Promoters Responding either to σ^S or to the RcsCDB His-Asp Phosphorelay

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Transcription of the *Escherichia coli osmB* gene is induced by several stress conditions. *osmB* is expressed from two promoters, *osmBp1* and *osmBp2*. The downstream promoter, *osmBp2*, is induced after osmotic shock or upon entry into stationary phase in a σ^S-dependent manner. The upstream promoter, *osmBp1*, is independent of σ^S and is activated by RcsB, the response regulator of the His-Asp phosphorelay signal transduction system RcsCDB. RcsB is responsible for the induction of *osmBp1* following treatment with chlorpromazine. Activation of *osmBp1* by RcsB requires a sequence upstream of its −35 element similar to the RcsB binding site consensus, suggesting a direct regulatory role. *osmB* appears as another example of a multistress-responsive gene whose transcription involves both a σ^S-dependent promoter and a second one independent of σ^S but controlled by stress-specific transcription factors.

To adapt to adverse conditions, bacterial cells induce specific families of genes that promote growth or survival in stressful environments. Many such genes can be induced by a variety of stresses through several transcriptional regulators acting on one or several stress-inducible promoters. In *Escherichia coli*, *osmB* is an example of a multistress-responsive gene, and we are investigating its regulation with the aim of understanding better the complex interplay of regulators resulting in multistress response. *osmB* encodes an outer membrane lipoprotein of an as yet unknown function (21). First identified as an osmotically inducible gene, *osmB* is also induced upon entry into stationary phase in a σ^S-dependent manner (13, 18, 20). RNA mapping experiments suggested that *osmB* is expressed under the control of two promoters. The downstream promoter, named *osmBp2*, was unambiguously characterized by deletion mapping and RNA analysis (20). In contrast, the proposed upstream promoter, *osmBp1*, contained no sequences similar to the canonical −10 and −35 elements upstream from the mRNA 5′ end determined by RNase protection experiments, and its identification remained uncertain (20).

Recently, transcriptome analysis revealed that *osmB* is a target of the RcsCDB His-Asp phosphorelay system (7, 16; our unpublished results). Initially found to regulate the synthesis of the capsular polysaccharide in *E. coli* (11), the response regulator RcsB was also shown to activate *osmCp1*, a σ^S-independent promoter of the multistress-responsive gene *osmC*. This activation occurs through the binding of RcsB to a site upstream from the −35 box of *osmCp1* (5, 25, 26) and is responsible for the induction of *osmCp1* upon exposure to the cationic amphipathic molecule chlorpromazine (3).

We investigated here the organization of the *osmB* promoter region and the mechanism of activation of *osmB* transcription by RcsCDB. We demonstrate that *osmB* is transcribed under the control of two independent promoters. The downstream promoter, *osmBp2*, is σ^S dependent and responsible for the response to the phase of growth and to osmotic shock, whereas the upstream promoter, *osmBp1*, is σ^S independent and the target of the response regulator RcsB.

Transcription of *osmB* is activated by RcsB but not by its cofactor, RcsA. To investigate the regulation of *osmB* transcription, we constructed a set of *osmB-lac* transcriptional fusions. DNA fragments amplified from chromosomal DNA with various oligonucleotide pairs (Fig. 1) were cloned in the vector pRS550, recombined on bacteriophage λRS45, and installed in single copy in the chromosome of the wild-type strain CF6343 (9) as described by Simons et al. (24). In strain SK1755, the *osmB-lac* fusion is driven by a 465-bp DNA fragment amplified with the oligonucleotides osmBup and osmBdown (Fig. 1). SK1755 was transformed with the plasmid pHRcsB (2) or pHRcsA (5), expressing *rcsB* or *rcsA*, respectively, under the control of *lacp*, and the effect of overproduction of RcsB or RcsA on *osmB* promoter activity was tested by monitoring β-galactosidase activity (22) in the absence or presence of IPTG (isopropyl-β-D-thiogalactopyranoside) (Fig. 2). In agreement with previous data from transcriptome analysis (7, 16), these experiments demonstrated that the overproduction of RcsB strongly induced transcription from the *osmB* promoter region. In contrast, the overproduction of RcsA, a cofactor of RcsB necessary for the full activation of the capsule synthesis *cps* genes (11, 27), had no effect on transcription from *osmBp*. Since it has been shown previously that in identical experimental conditions, RcsA accumulation is sufficient to repress tran-

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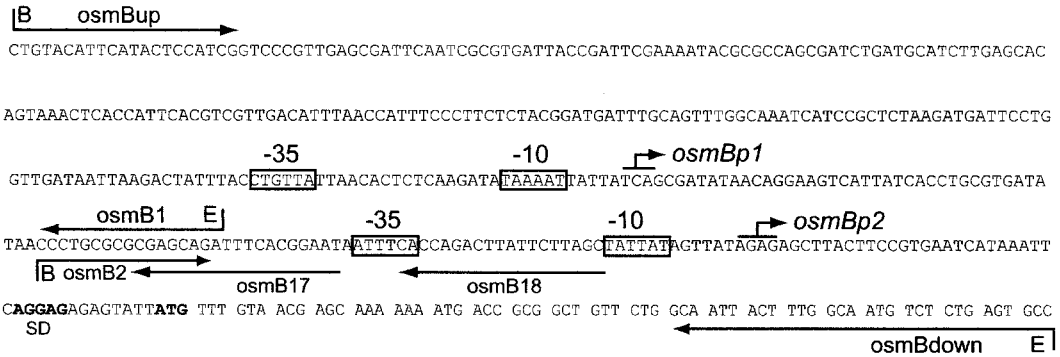

FIG. 1. Sequence of the *osmB* promoter region. Arrows indicate the oligonucleotides used for mRNA 5′-end mapping or to amplify the DNA fragments used to construct transcriptional *lac* fusions. B and E, BamHI and EcoRI restriction sites. The −35 and −10 elements of *osmBp1* and *osmBp2* are boxed. Broken arrows indicate the transcription starts. The Shine-Dalgarno sequence (SD) and translation start of *osmB* are shown in bold letters.

scription from the *flhD* promoter (9), we conclude that *osmB* belongs to the RcsA-independent subfamily of RcsB targets.

Expression of the proximal promoter *osmBp2* is controlled by σ^S but not by RcsB. Strain CLG250 contains an *osmB-lac* transcriptional fusion driven by a 176-bp DNA fragment amplified with the oligonucleotides osmB2 and osmBdown (Fig. 1). This DNA fragment carries the previously characterized *osmBp2* promoter (20). The introduction of an *rpoS*::Tn*10* mutation in this strain yielded strain CLG254. As shown in Fig. 3A, the pattern of β-galactosidase-specific activity was typical of promoters poorly expressed during exponential phase and induced upon entry into stationary phase in a σ^S-dependent manner (Fig. 3A). The overproduction of RcsB in strain CLG250 had no effect on the production of β-galactosidase, modifying neither the level of expression nor the stimulation at the end of exponential phase (Fig. 3B). Therefore, *osmBp2* appears to be an RcsB-independent promoter. We also tested the response of this promoter after an osmotic shock (Fig. 4). During exponential growth in a medium of low osmolarity (LB broth without NaCl [LB0N]), the addition of NaCl to a 0.4 M final concentration stimulated transcription from *osmBp2* in strain CLG250. In contrast, NaCl had no effect in the *rpoS* mutant strain CLG254, demonstrating that the osmotic shock induction of *osmBp2* is entirely dependent on σ^S. This behavior is in agreement with previous work that demonstrated that σ^S

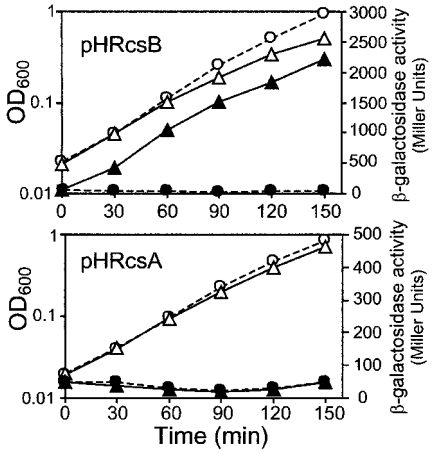

FIG. 2. *osmB* transcription is stimulated by RcsB but not by its cofactor, RcsA. Strain SK1755 (Φ[*osmBp1-osmBp2-lac*]), transformed with plasmid pHRcsB or pHRcsA, as indicated, was grown in LB broth, and the optical density at 600 nm (OD_{600}) (open symbols) and β-galactosidase activity (filled symbols) were followed during growth. At time zero, half of each culture was kept in LB broth (circles, dashed lines) and the other half (triangles, full lines) was treated with IPTG (0.5 mM) to induce the production of RcsB or RcsA.

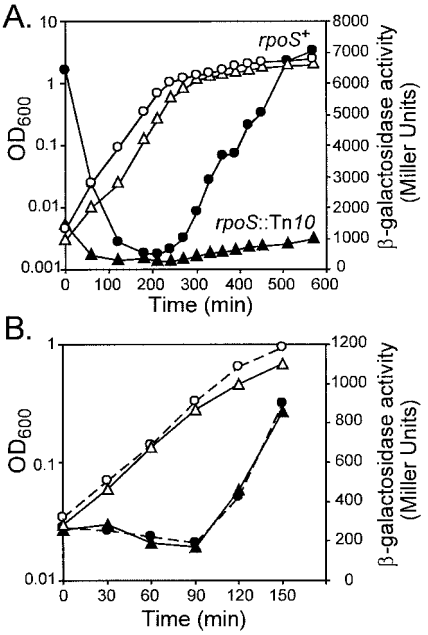

FIG. 3. *osmBp2* is induced upon entry into stationary phase in a σ^S-dependent manner but is not stimulated by overexpression of RcsB. (A) Strains CLG250 (Φ[*osmBp2-lac*]) (circles) and CLG254 (Φ[*osmBp2-lac*] *rpoS*::Tn*10*) (squares) were grown in LB0N, and OD_{600} (open symbols) and β-galactosidase activity (filled symbols) were monitored during growth. (B) CLG250 transformed with plasmid pHRcsB was grown in LB broth, and the OD_{600} (open symbols) and β-galactosidase activity (filled symbols) were monitored during growth. At time zero, half of the culture was kept in LB broth (circles, dashed lines) and the other half was treated with IPTG (0.5 mM) to induce the production of RcsB (triangles, full lines).

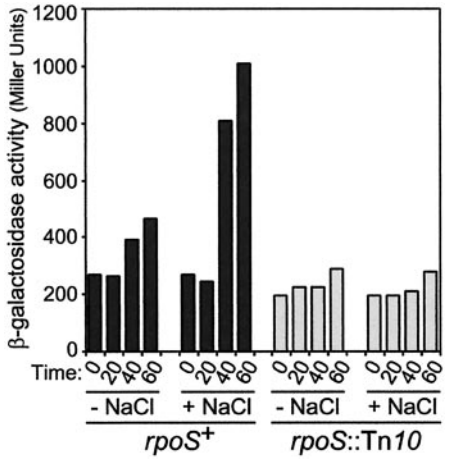

FIG. 4. σ^S is responsible for osmotic shock induction of *osmBp2*. Strains CLG250 (Φ[*osmBp2-lac*]) (dark gray bars) and CLG254 (Φ[*osmBp2-lac*] *rpoS*::Tn*10*) (light gray bars) were grown in LB0 until early exponential phase. At time zero, half of the culture was kept in LB0 (− NaCl) and the other half was treated with 0.4 M NaCl (+ NaCl), and β-galactosidase activity was monitored at 0, 20, 40, and 60 min.

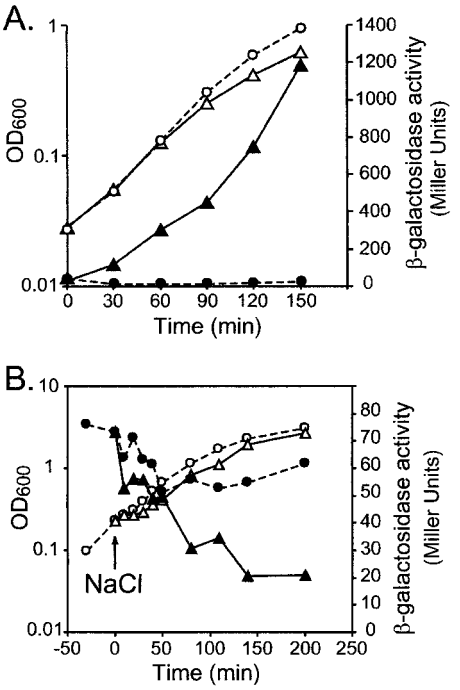

FIG. 5. *osmBp1* is stimulated by overexpression of RcsB but neither upon entry into stationary phase nor by osmotic shock. (A) Strain CLG249 (Φ[*osmBp1-lac*]) transformed with plasmid pHRcsB was grown in LB broth, and the OD_{600} (open symbols) and β-galactosidase activity (filled symbols) were monitored during growth. At time zero, half of the culture was kept in LB (circles, dashed lines) and the other half was treated with IPTG (0.5 mM) to induce the production of RcsB (triangles, full lines). (B) CLG249 was grown in LB0N, and the OD_{600} (open symbols) and β-galactosidase activity (filled symbols) were monitored during growth. At time zero, half of the culture was kept in LB0N (circles, dashed lines) and the other half (triangles, full lines) was treated with NaCl (0.4 M).

accumulates in the cells after an osmotic shock (23), leading to the osmotic induction of a number of its target genes (17, 19). It is also consistent with data obtained in vitro showing that transcription of *osmBp2* by $E\sigma^S$ is stimulated in the presence of high concentrations of K^+ glutamate, a compound known to accumulate in osmotically stressed cells (6).

Expression of the upstream promoter *osmBp1* is controlled by RcsB and independent of σ^S. Strain CLG249 contains an *osmB-lac* transcriptional fusion driven by a 306-bp DNA fragment amplified with the oligonucleotides osmBup and osmB1 (Fig. 1). During growth in LB broth, CLG249 expressed β-galactosidase at a low basal level, and this activity was strongly induced by the overproduction of RcsB from plasmid pHRcsB (Fig. 5A). In contrast, this activity was repressed after osmotic shock and showed no stimulation upon entry into stationary phase (Fig. 5B and data not shown). In addition, introduction of an *rpoS*::Tn*10* mutation in strain CLG249 had no effect on β-galactosidase production. Altogether, these data demonstrate that the 306-bp osmBup/osmB1 DNA fragment carries a σ^S-independent promoter that is stimulated by RcsB. This promoter must be responsible for the increase in *osmB* transcription following activation of the RcsCDB phosphorelay (Fig. 2) (7, 16).

The physiologically relevant signal sensed by the RcsCDB phosphorelay is complex and, as yet, not totally clear (10, 16). However, it has been shown that RcsB can induce transcription of some of its targets after treatment with the amphipathic molecule chlorpromazine (3). A culture of strain CLG249 was treated with 0.1 mM chlorpromazine during exponential growth in LB0N, and this resulted in 4.5-fold induction of β-galactosidase activity (from 57 to 257 units after 100 min of treatment). This stimulation was totally abolished in the *rcsB* mutant strain CLG258 (from 54 to 45 units after 100 min of treatment). Therefore, *osmBp1* is inducible by treatment with chlorpromazine and RcsB is necessary for this induction.

Identification of *osmBp1*. We performed primer extension experiments to investigate the *osmBp1* mRNA 5′ end. Strain SK1158 [MG1655 Δ*lacIZ*(MluI) *rcsB*::*tet*] (9) was transformed with the expression vector pIM10 (4) or its derivative, $pHRcsB_{D56E}$, which can overproduce $RcsB_{D56E}$, a constitutive form of RcsB (2, 12). The two resulting strains were grown in LB broth until early exponential phase, IPTG (0.5 mM) was added, and total RNAs were extracted after 10 min by the hot-phenol method. We chose to use the $RcsB_{D56E}$ variant because its overproduction resulted in a higher and earlier induction of a number of targets of the RcsCDB system than that of wild-type RcsB (our unpublished data). Ten micrograms of the total RNA was hybridized with 1 pmol of oligonucleotide osmB17 or osmB18, radiolabeled with [γ-^{32}P]dATP (3,000 Ci/mmol; Amersham) and T4 polynucleotide kinase (New England Biolabs), and primer extension experiments were performed as described previously (15), except that 25 units of AMV reverse transcriptase (FINNZYMES) was used for each reaction. The two oligonucleotide probes, osmB17 and osmB18, are complementary of the *osmB* mRNA specie starting from *osmBp1* but not of the mRNA starting from *osmBp2* (Fig. 1). As shown in Fig. 6, the experiments with both probes identified a single 5′-end region, centered 148 nucleotides upstream from the translation start of *osmB*. The amount

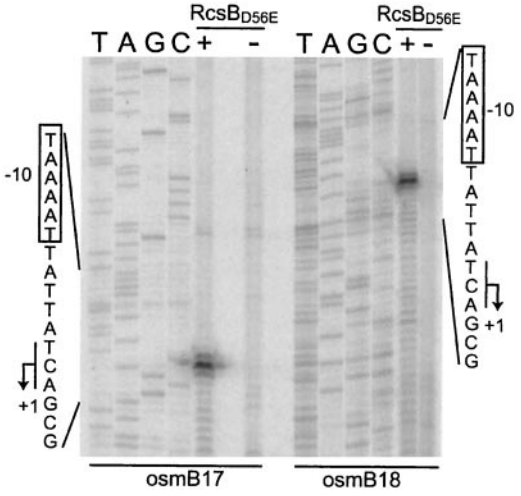

FIG. 6. Determination of *osmBp1* mRNA 5′ end. Total RNAs were extracted from a strain that was (+) or was not (−) overproducing $RcsB_{D56E}$, hybridized with ^{32}P-labeled osmB17 or osmB18 oligonucleotides, and treated with AMV reverse transcriptase before analysis on a denaturing polyacrylamide gel and autoradiography. The sequence ladders (lanes T, A, G, and C) were obtained by sequencing the *osmBp* region by using the same primers and the CircumVent sequencing kit (Biolabs) as described by the manufacturer.

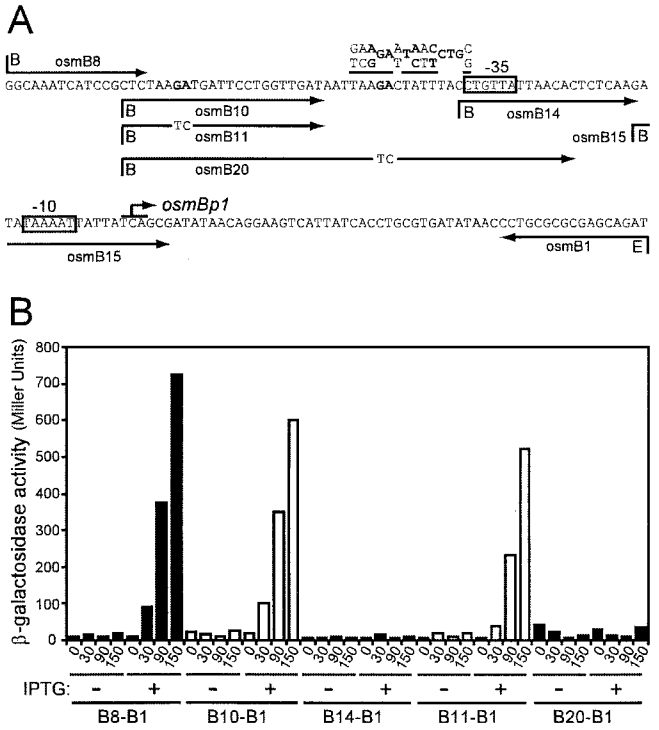

FIG. 7. Activation of *osmBp1* by RcsB necessitates a site upstream of the −35 element. (A) Arrows indicate the oligonucleotides used for the construction of Φ[*osmBp1-lac*] transcriptional fusions. osmB11 and osmB20 carry the GA-to-TC double mutations indicated. B and E, BamHI and EcoRI restriction sites. The consensus sequence of RcsB binding sites is shown above the *osmB* sequence, with the most-conserved positions in bold letters. (B) Strains carrying the indicated transcriptional fusions were transformed with plasmid pHRcsB and grown in LB broth. At time zero (OD_{600} of 0.1), cultures were not treated or were treated with IPTG (0.5 mM) to induce the production of RcsB, and β-galactosidase activity was assayed over time.

of this RNA was strongly dependent on the overproduction of $RcsB_{D56E}$. Just upstream from this 5′ end, a putative promoter that is composed of an almost-consensus −10 element and a more divergent −35 element (Fig. 1) separated by a 17-nucleotide spacer is found. We confirmed this location of *osmBp1* by deletion mapping. A DNA fragment amplified with the oligonucleotide pair osmB14/osmB1 (Fig. 7A) showed promoter activity similar to the basal activity exhibited by longer DNA fragments, whereas the DNA fragment obtained with osmB15/osmB1 (Fig. 7A) exhibited no promoter activity at all (Fig. 7B and data not shown). The *osmBp1* mRNA 5′ end identified here by primer extension analysis is located approximately 45-bp 5′ of that reported previously (20). This discrepancy may result from an artifact of the RNase protection experiments, and, because the nature of *osmBp1* as identified here is confirmed by both biochemical and genetic data, we propose to reassess this promoter.

Activation by RcsB requires a site upstream from the −35 element of *osmBp1*. To identify the sequence required for stimulation by RcsB, we constructed a set of *osmBp1-lac* fusion strains in which *osmBp1* was carried on DNA fragments with different 5′ ends upstream of the promoter (Fig. 7A). These strains were transformed with pHRcsB, and β-galactosidase activities were monitored with or without the induction of *rcsB* expression. The results of this deletion analysis (Fig. 7B) indicated that the 145-bp osmB8-osmB1 and 130-bp osmB10-osmB1 DNA fragments exhibited similar promoter activities and were sufficient to support activation after the overproduction of RscB. In contrast, the 95-bp osmB14-osmB1 DNA fragment carried a basal promoter activity that was insensitive to RcsB overproduction. A consensus has been proposed for the RcsB box (5), and several putative RcsB boxes could be recognized upstream from the −35 element of *osmBp1*. We then constructed *osmBp1-lac* transcriptional fusions expressed under the control of 130-bp DNA fragments similar to osmB10-osmB1 but carrying the GA-to-TC double mutations shown in Fig. 7A. The osmB11-osmB1 fragment remained fully responsive to RcsB overproduction. In contrast, the mutations introduced on fragment osmB20-osmB1 abolished the RcsB-dependent stimulation (Fig. 7B), demonstrating that the putative RcsB box located closest to the *osmBp1* −35 element is necessary for this stimulation. Altogether, these observations suggest a direct binding of RcsB at the identified box. However, the most-conserved positions in the RcsB boxes identified so far are GAnnnnnC (2, 9, 28), and we note that the putative RcsB box of *osmBp1* is lacking the C in its right half (Fig. 7). A search in the National Center for Biotechnology Information microbial genomes databases (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) revealed that OsmB is present and identical in all the *E. coli*, *Shigella flexneri* and *Salmonella* strains sequenced so far. Alignment of the promoter regions upstream of the *osmB* gene in some of these enterobacteria (8; EnteriX [http://globin.cse.psu.edu/enterix/]) shows a strong conservation of the sequence around the −10 element of *osmBp1* and downstream. In addition, in spite of a high degree of divergence within the spacer of *osmBp1*, a region upstream from the −35 element and encompassing the putative RcsB

box is also strongly conserved. Therefore, it is likely that *osmBp1* is functional and regulated similarly by the same regulators in the different enterobacterial species.

Architecture of multistress-responsive promoters. It is interesting to compare the organization of the *osmB* promoter region with that of other multistress-responsive genes. In particular, work on the *osmC* gene demonstrated that it is also a growth-phase and osmotically regulated gene transcribed under the control of two promoters: a downstream σ^S-dependent one and an upstream promoter independent of σ^S but regulated by auxiliary transcription factors (1, 5, 14, 25, 26). Thus, *osmB* and *osmC* respond to common stresses and exhibit promoter regions built on similar schemes. However, in spite of this great similarity, these two promoter regions use different ways to achieve the same type of regulatory response. For instance, the osmotic shock induction of *osmC* results from activation of its σ^S-independent *osmCp1* promoter by the NhaR activator, whereas its σ^S-dependent *osmCp2* promoter is not stimulated by osmotic shock (25, 26). In contrast, the osmotic induction of *osmB* occurs in a σ^S-dependent manner at the *osmBp2* promoter (Fig. 4), whereas the σ^S-independent *osmBp1* promoter is not activated by osmotic shock. At present, the molecular details explaining why the two σ^S-dependent promoters *osmBp2* and *osmCp2* respond differently to osmotic shock still await more investigations.

Finally, a general picture emerges from the comparison of genes such as *osmB* and *osmC*. Multistress-responsive promoters of this family are built by combining a patchwork of elements, based either on the general stress response sigma factor σ^S or on auxiliary regulators, each specific for a particular stress, that activate promoters recognized probably by the housekeeping sigma factor σ^{70}. These different elements cooperate to finely tune the transcription of each multistress-responsive gene, resulting in a precise level of expression in the different stress conditions.

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