

CpxR/OmpR Interplay Regulates Curli Gene Expression in Response to Osmolarity in *Escherichia coli*

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Curli fibers could be described as a virulence factor able to confer adherence properties to both abiotic and eukaryotic surfaces. The ability to adapt rapidly to changing environmental conditions through signal transduction pathways is crucial for the growth and pathogenicity of bacteria. OmpR was shown to activate *csgD* expression, resulting in curli production. The CpxR regulator was shown to negatively affect curli gene expression when binding to its recognition site that overlaps the *csgD* OmpR-binding site. This study was undertaken to clarify how the interplay between the two regulatory proteins, OmpR and CpxR, can affect the transcription of the curli gene in response to variation of the medium osmolarity. Band-shift assays with purified CpxR proteins indicate that CpxR binds to the *csgD* promoter region at multiple sites that are ideally positioned to explain the *csg* repression activity of CpxR. To understand the physiological meaning of this *in vitro* molecular phenomenon, we analyzed the effects of an osmolarity shift on the two-component pathway CpxA/CpxR. We establish here that the Cpx pathway is activated at both transcriptional and posttranscriptional levels in response to a high osmolarity medium and that CpxR represses *csgD* expression in high-salt-content medium, resulting in low curli production. However, *csgD* repression in response to high sucrose content is not mediated by CpxR but by the global regulatory protein H-NS. Therefore, multiple systems (EnvZ/OmpR, Cpx, Rcs, and H-NS) appear to be involved in sensing environmental osmolarity, leading to sophisticated regulation of the curli genes.

The ability of bacteria to recognize and adhere to specific surfaces is a fundamental aspect of microbial ecology and pathogenesis. Bacterial adhesins and fimbriae promote specific recognition and adhesion to diverse target molecules such as mammalian host tissue components or inorganic materials. Curli are highly adhesive proteinaceous fibers produced by *Escherichia coli* (37) and *Salmonella* spp. (15). Curli are involved in the colonization of inert surfaces and promote both initial adhesion and cell-cell interaction during biofilm development (3, 45, 57). Curli also mediate binding to a variety of host proteins (7, 37, 38, 54) and internalization of *E. coli* by eukaryotic cells (23). Production of these fibers has been shown to contribute to the symptoms seen during *E. coli* sepsis (8, 9, 27). Curli appear, therefore, as a virulence factor able to confer adherence properties to both abiotic and eukaryotic surfaces (29). The genes necessary for curli production are clustered in the *csgBA* and *csgDEFG* operons, which encode the curli subunits and regulate their transcription and transport, respectively. *csgD* encodes a key regulator of the FixJ family that positively regulates the production of curli and cellulose (11, 25, 49).

The ability to adapt rapidly to changing environmental conditions is crucial for the growth and pathogenicity of bacteria in their natural environments. Two-component systems that respond to particular stimuli by modifying the phosphorylated state of a cognate regulatory protein are the most prevalent

form of signal transduction mediating bacterial response to environmental signals. Three two-component systems (Cpx, EnvZ/OmpR, and Rcs) are implicated in the regulation of curli biogenesis in *E. coli* and contribute to the modification of the bacterial surface in response to any change in osmolarity, among others factors (43, 44, 55). The role of osmolarity in biofilm has also been reported in *Salmonella enterica* serotype Typhimurium (50), *Staphylococcus epidermidis* (31), and *Pseudomonas fluorescens* (39). Genetic data indicate that the RcsC sensor kinase negatively regulates *csgD* (21) and that EnvZ/OmpR (47, 57) and CpxA/CpxR (19) are implicated in the regulation of curli biogenesis. In electrophoretic mobility shift assays (EMSAs), both OmpR and CpxR response regulators have been shown to bind immediately upstream of the *csgD* promoter (44). OmpR was shown to bind to a consensus sequence located –49.5 bp upstream of the transcriptional start site of *csgD*, and this subsequently activates the *csgDEFG* expression that, in turn, increases the expression of *csgBA*. The CpxA/CpxR pathway was shown to negatively affect curli gene expression (19) by the binding of CpxR to its recognition site overlapping the OmpR-binding site (44). However, whether CpxR competes with OmpR for binding to the DNA is an open question. In addition to these three two-component systems, global regulators such as H-NS (1, 28, 36) and IHF (22) control the expression of the curli genes. Moreover, Crl, a potential thermosensor accumulating at 30°C, was recently shown to interact directly with σ^S , and this interaction promotes binding of the σ^S -holoenzyme (E σ^S) to the *csgBA* promoter (10). Curli regulators also include MlrA (12). Hence, nine regulators have been involved in the regulation of curli expression through a

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TABLE 1. *E. coli* K-12 strains and plasmids used in this study

<i>E. coli</i> strain or plasmid	Description ^a	Source or reference
<i>E. coli</i> strains		
MG1655 (PHL565)	λ^- F ⁻ <i>rph-1</i>	Lab collection
PHL1242	MG1655 Δ <i>cpxR::kanfrt</i>	This study
PHL1258	MG1655 Δ <i>cpxR</i> , flipase of the <i>kanfrt</i> cassette from PHL1242	This study
PHL1259	PHL1258 <i>csgD::uidA-kan</i>	This study
PHL1265	MG1655 <i>csgD::uidA-kan</i>	This study
PHL1089	PHL1265 <i>ompR331::Tn10</i>	44
PHL1311	PHL1265 <i>rscB::Tn10</i>	This study
PHL395	F ⁻ λ^- IN(<i>rrmD-rrmE</i>)1 <i>rph-1</i> Δ (<i>lacZ169</i>) Δ <i>hns-118</i> <i>zch-Tn10</i>	32
PHL1318	PHL1265 Δ <i>hns-118</i> <i>zch-Tn10</i>	This study
PHL1251	MG1655 <i>trp::(cpxR::lacZ-kan)</i>	This study
BL21(DE3)	F ⁻ <i>dem ompT hsdS</i> (r _B ⁻ m _B) <i>gal</i> λ (DE3)	Stratagene
PHL906	BL21(DE3) pCal-n-CpxR	44
PHL1227	BL21(DE3) pET20-OmpR	This study
TE2680	F ⁻ λ^- IN(<i>rrmD-rrmE</i>)1 <i>galK2</i> Δ (<i>lac</i>)X74 <i>rpsL galK2 recD1903::Tn10d-Tet</i> <i>trpDC700::putPA1303::[Kan^s-Cm^r-lac]</i>	2
Plasmids		
pET20-OmpR	pET-20b(+) with 760-bp fragment containing <i>ompR</i> ORF	This study
pCal-n-CpxR	pCal-n (Stratagene) with 700-bp fragment containing <i>cpxR</i> ORF	44
pCSG4	pUC19 with a 3.5-kb HindIII fragment containing intergenic region between <i>csgDEFG</i> and <i>csgBA</i>	36
pRS551	pBR322 with <i>lacZYA</i> genes; Amp ^r Kan ^r	53
pKOBEG	pSC101 <i>ts</i> , <i>araC</i> arabinose-inducible λ red γ β α operon (Cm ^r)	13
pKD4	Source of the excisable kanamycin-frt cassette	16
pCP20	<i>ts</i> (replicate at 30°C) plasmid bearing the <i>flp</i> recombinase gene; Cm ^r Amp ^r	14

^a Kan^s, Kanamycin sensitive; Amp^r, ampicillin resistant; Kan^r, kanamycin resistant; Cm^r, chlorophenicol resistant; ORF, open reading frame.

complex network of interactions between transcription factors and the *csg* regulatory region. This allows a fine-tuning of curli expression depending on environmental conditions such as osmolarity, temperature, or starvation.

The present study was undertaken to gain a better understanding of the molecular mechanisms mediating the repression of the *E. coli* curli genes in a high-osmolarity medium. We studied how the interplay between the two regulatory proteins *OmpR* and *CpxR* affect the transcription of the *csgD* gene. In order to understand the physiological meaning of this in vitro molecular phenomenon, we analyzed the effects of an osmolarity shift on the *CpxA/CpxR* pathway at three levels: (i) the *cpxR* expression level, as estimated from gene fusion; (ii) the *CpxR* synthesis level, as estimated by immunodetection; and (iii) the degree of *CpxR* phosphorylation, as estimated from two-dimensional gel electrophoresis combined with immunodetection. We establish here that the *Cpx* pathway is activated by high osmolarity and that *CpxR* binds to the *csgD* promoter at multiple sites. *CpxR* repressor activity is, however, restricted to selective environmental conditions, such as a high salt concentration. Other regulators, such as *H-NS* and *RcsB*, are involved in *csgD* repression in response to closely related stimuli. The participation of multiple and sophisticated regulatory systems in the control of *csg* genes suggests a role for curli in a broad spectrum of complex environments.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *E. coli* strains and plasmids used in the present study are listed in Table 1. The media used were Luria-Bertani broth and M63/2, a low-osmolarity minimal medium supplemented with glucose (0.2%). To obtain medium with high osmolarity, sucrose (8 and 20%) or NaCl (0.1 M) was added to M63/2-glucose. Kanamycin (50 μ g/ml), ampicillin (50 μ g/ml), tetracycline (10 μ g/ml), chloramphenicol (20 μ g/ml), and tryptophan (20

μ g/ml) were added into the medium when necessary. Congo red indicator plates were prepared as described by Hammar et al. (25); on these plates, curli-producing bacteria form red colonies, whereas non-curli-producing cells remain white.

Genetics methods. Phage P1 transductions were carried out as described by Miller (34). Transduction of the *cpxR::lacZ-kan* fusion was obtained by selection on kanamycin plates and verification of tryptophan auxotrophy. Transduction of *csgD::uidA-kan* was obtained by selection on kanamycin plates and verification of the white color of transductants on Congo red indicator plates.

Construction of the Δ *cpxR* mutant. The *cpxR* mutant of strain MG1655 was constructed by the use of the lambda-red recombination system as described in Chaverche et al. (13) and Derbise et al. (17) and detailed at <http://www.pasteur.fr/recherche/unites/Ggb/methodes.ang.html> with the use of the thermosensitive pKOBEG plasmid that carries the λ phage red γ β α operon under the control of the pBAD promoter. The *cpxR* gene (ATG to Stop) was first replaced by a kanamycin cassette flanked by two frt recombination sites amplified from the pKD4 plasmid (16). The Δ *cpxR::kanfrt* deletion was then transduced to MG1655, yielding the strain PHL1242. PHL1242 was then transformed with the thermosensitive pCP20 plasmid expressing the Flp flipase (14), thus leading to the excision of the kanamycin-frt cassette and leaving the natural *cpxR* promoter taking the control of the *cpxA* gene (strain PHL1258). The deletion of *cpxR* in both PHL1242 and PHL1258 was verified by PCR.

Construction of a *cpxR::lacZ* fusion. To obtain a *cpxR::lacZ* chromosomal fusion, a 718-bp DNA fragment corresponding to the *cpxR* promoter region and the beginning of the coding sequence, was amplified by PCR from MG1655 chromosomal DNA as the template and by using primers WB1 (5'-ATTAACA GGAGGGAATTTCGTGCCCGCCTG-3') and WB2 (5'-GCGCAGGATCCCG CGAATACGTG-3'), which contain, respectively, EcoRI and BamHI restriction sites (underscored sequences). The PCR fragment digested with EcoRI and BamHI was cloned into the corresponding sites of pRS551 (53) to give a transcriptional fusion to *lacZ*. Integration of the *cpxR::lacZ* fusion into the chromosome tryptophan locus was obtained as described by Elliott (20). Then, the chromosomal *trp::(cpxR::lacZ-kan)* fusion was transferred into a MG1655 strain by P1 transduction, resulting in the strain PHL1251.

Enzyme assays. β -Glucuronidase activity in toluene-treated samples was measured by spectrophotometrically monitoring the hydrolysis of *p*-nitrophenyl- β -*D*-glucuronide into *p*-nitrophenol at 405 nm (5). Specific activity was expressed as units per milligram of protein, where 1 U corresponds to 1 nmol of product liberated per mg of total protein. β -Galactosidase activity was measured by

monitoring the degradation of *o*-nitrophenyl- β -D-galactoside into *o*-nitrophenol, which absorbs at 420 nm (34). Specific activity was expressed as units per milligram of protein.

Overproduction and purification of OmpR-His₆ protein, preparation of OmpR antibodies. The *ompR* coding region was amplified by PCR from chromosomal DNA of the MG1655 strain by using the primers *ompRXbaI* (5'-GCTCTAGAGTTGCGAACCTTTGGGAGTAC-3') and *ompRXhoI* (5'-CCGCTCGAGTGTCTTAGAGCCGTCCGGTAC-3') carrying an XbaI and an XhoI site (underscored sequences), respectively. The 760-bp XbaI-XhoI fragment was then subcloned into the XbaI and XhoI unique sites of pET-20b(+). The resulting plasmid, pET20-OmpR, contains *ompR* under the control of the T7 promoter and with its own translational regulatory signals, and it expresses an OmpR protein with a His₆ motif at its C terminus. pET20-OmpR was introduced into strain BL21(DE3), yielding strain PHL1227. Three hours after IPTG (isopropyl- β -D-thiogalactopyranoside) induction, cells were harvested and suspended in 50 mM NaH₂PO₄ (pH 8), 300 mM NaCl, and 1 mM imidazole. Crude protein extracts were obtained by disrupting bacteria by sonication at 20 MHz or by vortexing bacteria with glass beads (Sigma). They were incubated overnight with nickel-nitrilotriacetic acid affinity resin. Washes and elution were performed as recommended by the manufacturer (Qiagen). The purification of the OmpR-His₆ protein yielded protein of 85% purity, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Antibodies to OmpR were obtained by immunization of a rabbit with purified OmpR-His₆.

Purification of the CBP-CpxR protein and preparation of CpxR antibodies. Construction of the calmodulin-binding protein (CBP)-CpxR fusion is described in a previous study (44). IPTG induction was carried out with a culture of strain PHL906. Crude protein extracts, obtained as described above, were incubated overnight with calmodulin affinity resin in CaCl₂ binding buffer. Washes and elution were performed as recommended by the manufacturer (Stratagene). The purification of the CBP-CpxR protein yielded protein of 90% purity, as judged by SDS-PAGE. Antibodies to CpxR were obtained by immunization of a rabbit with purified CBP-CpxR.

EMSA. The DNA probe D1D2, containing the *csdDEF* promoter region (-128 to +12), was obtained by PCR amplification from pCSG4 with primers D1 (5'-CCAAATGTACAAGCTTTCTATCATTTTC-3') and D2 (5'-GGATTACATCTGATTTCAATCTAGCC-3') (Fig. 3). The DNA probe was digested with HindIII and then ³²P labeled by using the Klenow fragment of DNA polymerase. Binding reactions were carried out in 20 μ l of 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol (DTT), 1 mM EDTA, 5% glycerol, 6 μ g of bovine serum albumin, 1 μ g of calf thymus DNA, and 20 mM acetyl phosphate as a phosphodonor molecule for OmpR and CpxR (30). After addition of the DNA probe (60,000 cpm), various amounts of purified OmpR-His₆ and/or CBP-CpxR proteins were added simultaneously. For the supershift assays (Fig. 4B), either 1 μ l of antiserum against OmpR or a nonspecific antiserum was added when necessary. The reaction mixtures were incubated for 30 min at 30°C prior to loading onto a 4% nondenaturing polyacrylamide gel (ratio of acrylamide to bisacrylamide, 80:1). Electrophoresis was carried out in 44 mM Tris-borate (pH 8)-0.5 mM EDTA. The ratio of the phosphorylated form of OmpR and CpxR in the binding reactions was determined by using bidimensional electrophoresis combined with immunodetection: 30% of each protein was phosphorylated under the conditions described here (data not shown).

Identification of OmpR in DNA-protein complexes after blotting of EMSA gels. The EMSA gel was electrotransferred onto a polyvinylidene difluoride membrane according to the method of Novak and Paradiso (35). Membranes were incubated with a 1:1,000 dilution of anti-OmpR antiserum. After extensive washing with Tris-buffered saline-0.1% Tween, the membrane was finally incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G. OmpR proteins were detected by chemiluminescence with an ECL Kit according to the manufacturer's instructions (Amersham).

Bidimensional electrophoresis. Proteins were extracted as follows. The MG1655 strain was grown in M63/2-glucose or M63/2-glucose-20% sucrose at 30°C for 48 h, and cells were washed and resuspended in Tris 50 mM (pH 8). Cocktails of protease inhibitors and phosphatase inhibitors (Sigma) were added prior to sonication at 20 MHz. The protein concentration of lysis supernatants was estimated by the Bradford assay and 50 μ g were precipitated with cold acetone at -20°C for at least 2 h. After centrifugation, the pellet was solubilized in 8 M urea-4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}-20 mM DTT-0.001% bromophenol blue-0.2% ampholytes 3-10. The passive rehydration of the 7-cm ReadyStrip IPG(5-8) (Bio-Rad) was performed in a Protean immunoelectrofocusing (IEF) cell for 15 h at 20°C. IEF itself was carried out at 50 V for 15 min, followed by a first linear gradient of 50 to 150 V for 30 min and a second linear gradient of 150 to 250 V for 15 min. The IEF was achieved when 4,000 V and 16,000 V · h were reached. Prior to the

second dimension, the IPG strips were equilibrated in 6 M urea-2.5% SDS-0.375 M Tris-HCl (pH 8.8)-30% glycerol-130 mM DTT for 15 min and then for 20 min in the same buffer with 135 mM iodoacetamide instead of DTT. SDS-PAGE was performed at 25 V for 30 min and was achieved at 200 V with 10% acrylamide gels.

Immunodetection and densitometric analysis. After SDS-PAGE, proteins were electrotransferred onto a semidry transfer cell (Bio-Rad) according to the manufacturer's protocol and were detected after immunoblotting with the anti-serum to CpxR and the second antibody, horseradish peroxidase-conjugated anti-rabbit immunoglobulin G. The protein bands were visualized by an enhanced chemiluminescence Western blotting kit (Amersham). The density of the protein bands or spots was quantified by using a densitometer and ImageMaster TotalLab software (Amersham).

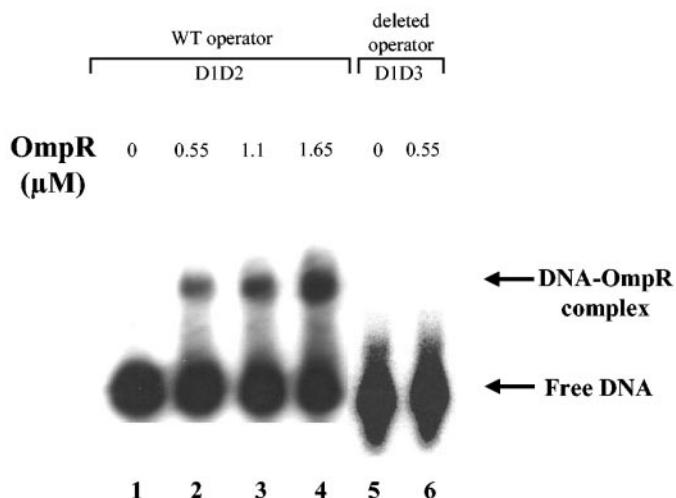
RESULTS

OmpR binds to a unique site at the *csd* promoter, whereas CpxR binds cooperatively to multiple sites. In order to understand how OmpR interacts with CpxR to affect regulation at the *csd* locus, we first assayed the binding of each purified regulator to a DNA fragment containing the transcriptional regulatory region of *csd*. The specificity of the detected complexes was previously demonstrated by a competition experiment with an excess of specific or nonspecific DNA competitor (44).

Increasing amounts of OmpR-His₆ were added to the DNA fragment encompassing the *csd* promoter and, unlike *S. enterica* serovar Typhimurium (22) but as previously shown in *E. coli* (44), only one complex was observed (Fig. 1A). According to the method described by Bading (4), the molecular weight of the protein participating in this protein-DNA complex was estimated to be 55 kDa, suggesting that the OmpR protein binds to the *csd* promoter as a dimer. Indeed, Harrison-McMonagle et al. have shown that OmpR acts as a dimer, with each monomer interacting with the DNA helix in the major groove (26). Two close OmpR-binding sites have been observed in serovar Typhimurium (22). The sequence of the first OmpR binding site (S1) was highly conserved in both species, as described in Fig. 1B. However, unlike the site in serovar Typhimurium, only a degenerated OmpR consensus was observed within the *E. coli* second site S2 (Fig. 1B). This discrepancy between the two regulatory sequences could explain why OmpR was able to form two complexes with the *csd* promoter region of serovar Typhimurium (22), whereas only one was observed with the homologous region of *E. coli* (Fig. 1A) (44). Indeed, no OmpR complex could be detected in *E. coli* when the first OmpR binding site S1 was deleted (Fig. 1A, lane 6).

Purified CBP-CpxR was assayed for its specific DNA binding to the *csd* promoter region. As opposed to results observed with OmpR, several protein-DNA complexes of decreasing mobility could be resolved with an increasing concentration of CpxR (Fig. 2A). This result suggests that CpxR interacts with several sites within the *csd* control region. Indeed, fuzzy unstable complexes could still be detected when the main CpxR-binding site was deleted (Fig. 2A, lane 9). Evaluation of mobility shift data from Fig. 2, lanes 4 to 7, yielded a calculated molecular sizes of 275, 330, 385, and 440 kDa, corresponding to the binding of five, six, seven, and eight CpxR dimers, respectively. However, the calculated molecular weight of the protein responsible for the shift in gel electrophoretic mobility can be overestimated if the protein exhibits DNA-bending properties (4). Using the consensus sequence defined for CpxR by Pogliano et al. (42) and recently refined by

A



B

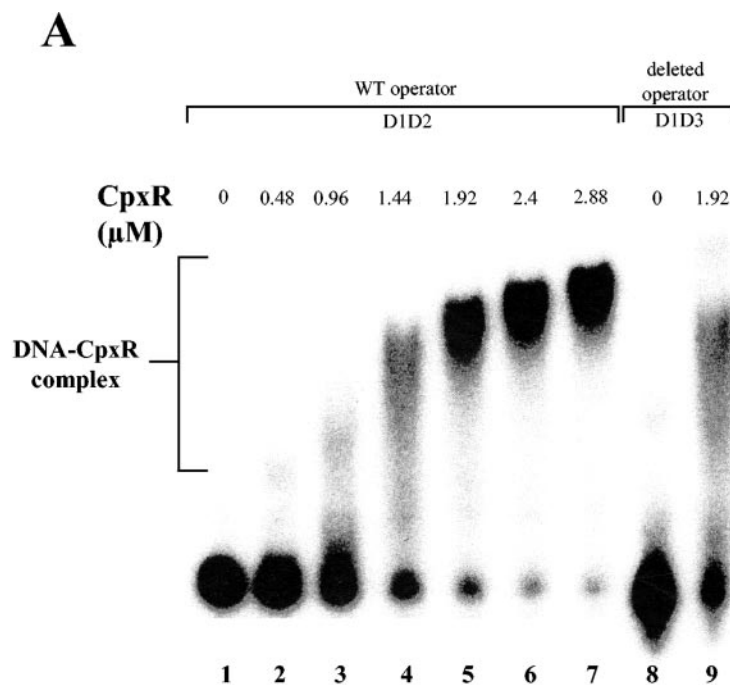
<u>OmpR consensus</u>	TTTntACA ⁺ TG _g TTT ⁺ aCA	<u>position</u>
<i>E. coli</i>	S1 [TTTGTACATTGATTTACA S2 [GAGCAATATAATTTTACA	-39 to -56 -59 to -76
<i>S. typhimurium</i>	S1 [TTTATATATAATTTTACA S2 [TTTGAACATTGGTTTACA	-41 to -58 -61 to -78

FIG. 1. (A) DNA-binding assay with purified OmpR-His₆ and the promoter region of *csgD*. ³²P-end-labeled wild-type *csgD* promoter (D1D2 probe, see Fig. 3) was incubated without protein (lane 1) or with 0.55 μM (lane 2), 1.1 μM (lane 3), or 1.65 μM (lane 4) OmpR-His₆. ³²P-end-labeled deleted *csgD* promoter (D1D3 probe, see Fig. 3) was incubated without protein (lane 5) or with 0.55 μM (lane 6) of OmpR-His₆. (B) Comparison of *E. coli* and *S. enterica* serotype Typhimurium OmpR binding sites. Site positions are relative to the transcriptional start site of *csgD* (*agfD*).

De Wulf et al. (18), we found six potential CpxR binding sites within the *csgD* promoter region (Fig. 2B). Figure 3 shows that two of them (sites 1 and 2) are positioned between the -35 box and the transcriptional start site, which is an ideal position to exert a repressor activity. Moreover, three other sites overlap the OmpR activator binding site (sites 3, 4, and 5). These sites could probably not recruit three dimers of CpxR at the same time, but they could certainly disturb OmpR binding and, consequently, *csgD* transcription activation. Interestingly, the intensity of the CpxR complexes formed did not increase gradually as a function of the protein concentration. Rather, a 1.5- to 2-fold increase in protein concentration was sufficient to switch from unstable complexes (Fig. 2A, lanes 3 and 4) to a large amount of stable complex formation (Fig. 2A, lanes 5 to 7). This suggests that multiple CpxR molecules were bound in a cooperative fashion, like the global repressor protein H-NS binding pattern (24). All of these data together indicate a significant regulatory potential of CpxR on *csgD* transcription.

OmpR and CpxR bind simultaneously at the *csgD* promoter. Since OmpR and CpxR share a common binding site (Fig. 3) and CpxR seems to bind preferentially in a cooperative fashion, we directly examined the possibility of a competitive binding of the two proteins at the *csgD* promoter. When increasing amounts of CpxR were simultaneously added to binding reac-

tions containing a constant quantity of OmpR, a major DNA-protein complex of slow mobility was observed. This complex comigrates with the DNA-CpxR complex (Fig. 4A, compare lanes 5 and 6 to lane 7). To determine whether OmpR is part of this slow mobility complex, antibodies raised to OmpR were added to the binding reactions. The addition of the anti-OmpR antibodies, but not of nonspecific antibodies, leads to a supershift of the OmpR containing-*csgD* promoter fragment (Fig. 4B, compare lanes 2, 7, and 8). The slow mobility complexes, observed when the two proteins are simultaneously added into the binding reactions (Fig. 4A, lanes 3 to 6), also upshift in the presence of anti-OmpR antibodies (Fig. 4B, lanes 3 to 6). These results indicate that, in addition to CpxR, the *csgD* promoter also binds OmpR. To confirm these results, anti-OmpR antibodies were used for the identification of the DNA-binding OmpR proteins after denaturing and Western blotting of the proteins of the EMSA gel onto a polyvinylidene difluoride membrane according to the method of Novak and Paradiso (35). Addition of a fourfold excess of unlabeled *csgD* promoter was required to increase the total amount of DNA-OmpR complexes and to allow for their immunodetection (Fig. 4C, compare lanes 2 and 4). This figure shows that the antibody-reactive band in the Western blot of the EMSA gel (Fig. 4C, lane 4) migrates at the same location as the slow



B

<u>CpxR consensus</u>	<u>GTAAA</u> _{nnnnn} <u>GTAA</u>	<u>score</u>	<u>position</u>
	N°		
	1	7/9	-5 to +9
	2	6/9	-14 to -27
	3	8/9	-55 to -42
	4	8/9	-45 to -58
	5	6/9	-59 to -46
	6	7/9	-107 to -94

FIG. 2. (A) DNA-binding assay with purified CBP-CpxR and the promoter region of *csgD*. ^{32}P -end-labeled wild-type *csgD* promoter (D1D2 probe) was incubated without protein (lane 1) or with 0.48 μM (lane 2), 0.96 μM (lane 3), 1.44 μM (lane 4), 1.92 μM (lane 5), 2.4 μM (lane 6), or 2.88 μM (lane 7) CBP-CpxR. ^{32}P -end-labeled deleted *csgD* promoter (D1D3 probe) was incubated without protein (lane 8) or with 1.92 μM (lane 9) CBP-CpxR. (B) Alignment of six putative CpxR binding sites. Scores are relative to the CpxR consensus defined by De Wulf (18). Site positions are relative to the transcriptional start site of *csgD*. CpxR binding sites are shown in Fig. 3.

mobility complex observed by autoradiography in the same conditions (Fig. 4A, lane 5). These *in vitro* experiments demonstrate that OmpR and CpxR bind simultaneously at the *csgD* promoter.

Response to high osmolarity: the Cpx activation pathway. Since growth in a high-osmolarity medium turns off curli transcription (44), it was tempting to suggest that CpxR represses the transcription of *csgD* in response to high osmolarity. Moreover, a previous study has shown that a two- to fivefold induction of the *cpxP::lacZ* fusion was observed when bacteria encounter high-osmolarity conditions, suggesting that the Cpx pathway is likely to be activated by high osmolarity (44). We further analyzed the effects of an osmolarity shift on the

Cpx pathway at three levels: (i) the *cpxR* expression level, as estimated from gene fusion; (ii) the CpxR synthesis level, as estimated by immunodetection; and (iii) the degree of CpxR phosphorylation, as estimated from two-dimensional gel electrophoresis combined with immunodetection. To determine whether osmolarity affects the transcription of the *cpxR* gene, a *cpxR::lacZ* fusion was constructed and introduced into the *trp* locus of MG1655 to obtain the merodiploid strain PHL1251. Figure 5A shows that transcription of the *cpxR* operon was positively affected at high osmolarity. We also examined the effects of osmolarity changes on the accumulation of CpxR by using an antiserum to CpxR. As shown in Fig. 5B, the cellular level of CpxR in the cells grown in high-osmolarity medium

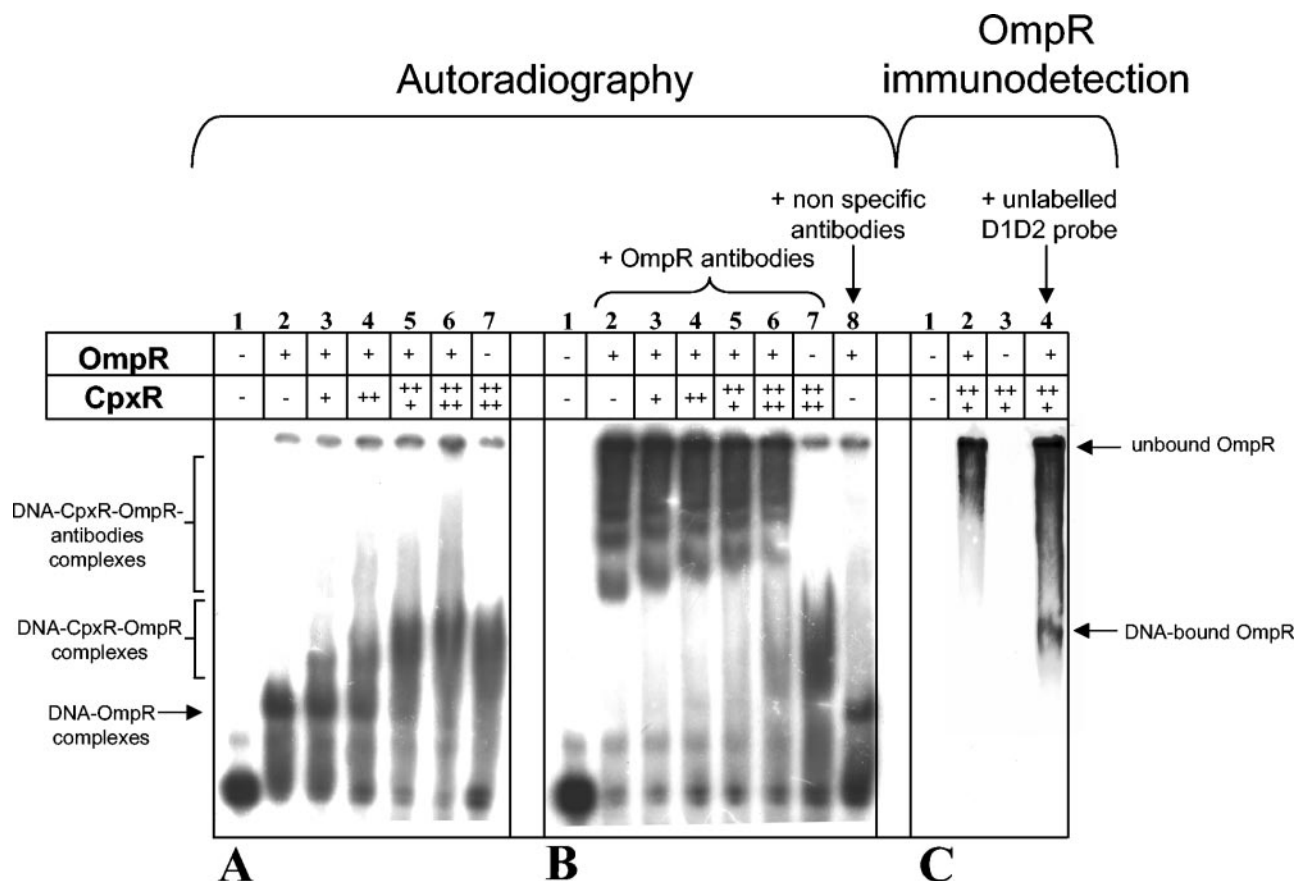


FIG. 4. CpxR and OmpR bind simultaneously at the promoter region of *csgD*. Band-shift assays were performed with pure CBP-CpxR, pure OmpR-His₆, and a ³²P-labeled *csgD* promoter fragment in the absence (A) or in the presence (B) of antibodies raised to OmpR. D1D2 probe without protein (lanes 1), and D1D2 probe with 1.65 μ M OmpR-His₆ (lanes 2) are as shown. Increasing amounts of CBP-CpxR were added in the binding reaction containing 1.65 μ M concentrations of OmpR-His₆. D1D2 probe with 1.65 μ M OmpR-His₆ and 0.48 μ M CBP-CpxR are shown in lanes 3; lanes 4 are the same as in lane 3 but with 0.96 μ M CBP-CpxR; lanes 5 are the same as for lanes 3 but with 1.92 μ M CBP-CpxR (lanes 5); lanes 6 are the same as for lanes 3 but with 2.88 μ M CBP-CpxR; lanes 7 show D1D2 probe with 2.88 μ M CBP-CpxR. A control lane contains D1D2 probe with 1.65 μ M OmpR-His₆ and nonspecific antibodies (panel B, lane 8). (C) Immunodetection of OmpR in DNA complexes after transfer of EMSA gel proteins onto a membrane as described in Materials and Methods. Lanes: 1, D1D2 probe without protein; 2, D1D2 probe with 1.65 μ M OmpR-His₆ and 1.92 μ M CBP-CpxR; 3, D1D2 probe with 1.92 μ M CBP-CpxR; 4, D1D2 probe with 1.65 μ M OmpR-His₆, 1.92 μ M CBP-CpxR, and a fourfold excess of unlabeled D1D2 probe.

not shown), and CpxR was previously shown to bind to a sequence centered at +10 relative to the transcriptional start site of *csgBA* operon (44). Hence, a total of 10 CpxR boxes could be identified in the *csg* intergenic region. This suggests that optimal *csg* repression depends on multiply bound CpxR molecules that interact in a complex way. From the *csgD* promoter sequence analysis (Fig. 3), we suggest that three different mechanisms of *csgD* repression are likely to occur: (i) CpxR sites 1 and 2 are in a good position to prevent RNA polymerase binding and consequently switch *csgD* expression off; (ii) binding of CpxR to sites 3, 4, or 5 is likely to disturb the activator OmpR binding to the *csgD* promoter (Fig. 3); and (iii) binding of CpxR to additional sites upstream *csgBA* promoter could affect both *csgB* and *csgD* transcription. Progressive occupancy of all of these CpxR sites could provide differential modulation of OmpR-mediated activation of *csgD*.

We have shown that OmpR is part of the stable complex formed when the two regulatory proteins OmpR and CpxR are present in binding reactions (Fig. 4). This result would rule out

the second mechanism (ii) and suggest that the first mechanism (i) alone could explain the CpxR-mediated repression of *csgD*. However, the presence of several nearly perfect CpxR consensus inside the OmpR box strongly suggests the existence of a competition between OmpR and CpxR. We propose that this configuration could allow for a gradual curli gene expression. Depending on the respective activation of the EnvZ/OmpR and Cpx pathway, a certain competition could occur and would moderately disturb OmpR-dependent *csgD* activation. When bacteria encounter environmental conditions that fully activate the Cpx pathway, CpxR-P should bind at sites 1 and 2 and allow an extremely efficient *csgD* repression even if OmpR is still bound to the *csgD* promoter (as is the case in our in vitro experiments in Fig. 4). We suggest that CpxR, via its specific binding sites arranged over an extended region of DNA, functions as a modulator of OmpR activation. The occurrence of such multiple repressor binding sites has been described for CytR in the *deoP2* promoter (41). The present study reveals the complexity of the control exerted on the *csgD* expression.

Growth of *E. coli* in a high osmolarity medium abolishes the

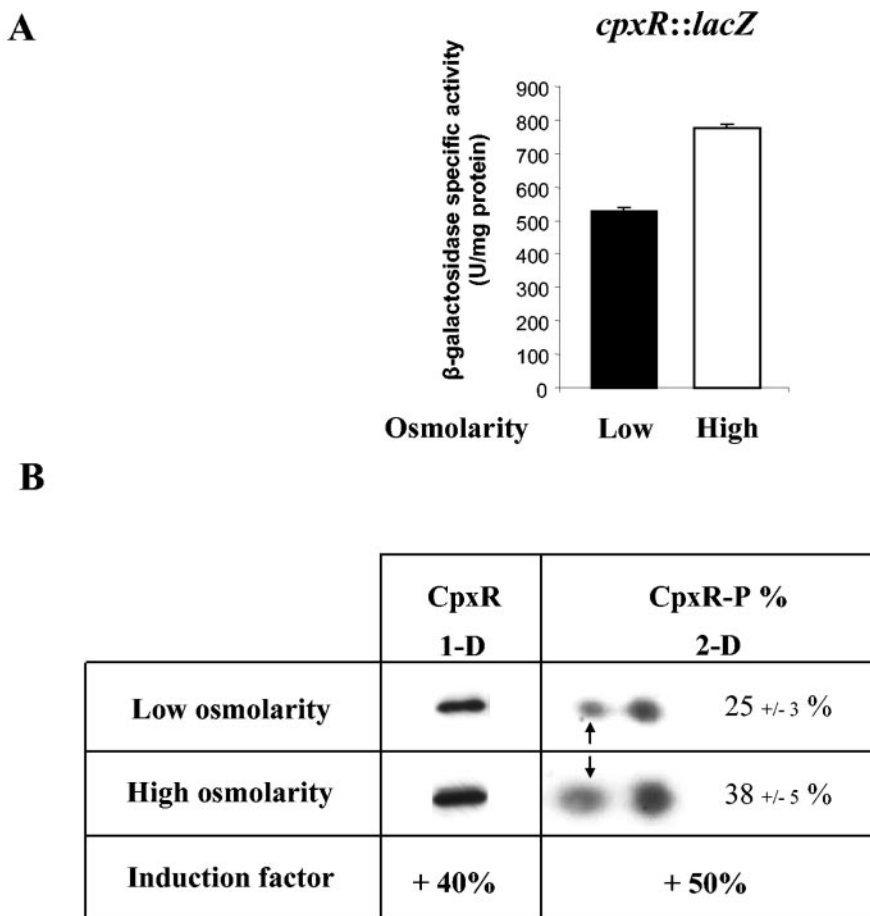


FIG. 5. Effect of high osmolarity on CpxR pathway induction. (A) Transcriptional activation. The β -galactosidase activity of the *cpxR::lacZ* merodiploid strain (PHL1251) was measured in stationary-phase cells grown in low-osmolarity (M63/2-glucose and tryptophan) or high-osmolarity (M63/2-glucose, tryptophan, and 20% sucrose) medium. The results are representative of five independent β -galactosidase assays. (B) Phosphorylated CpxR accumulation. Proteins extracted from cultures of MG1655 grown in low-osmolarity (M63/2-glucose) or high-osmolarity (M63/2-glucose and 20% sucrose) medium were analyzed by one-dimensional gel electrophoresis combined with immunodetection of CpxR. This revealed that the cellular level of CpxR is 40% higher in the cells grown in high-osmolarity conditions. This induction factor was calculated with data resulting from four independent experiments. To determine the percentage of phosphorylated CpxR form (CpxR-P), equivalent protein extracts were subjected to three independent two-dimensional gel electrophoresis analyses combined with immunodetection of CpxR as described in Materials and Methods. The position of phosphorylated CpxR is indicated by small arrows. The density of the protein spots was determined by a densitometer to calculate the percentage of CpxR-P. Analysis of purified CpxR phosphorylation by acetyl phosphate confirmed that the acidic form observed in our assays is the phosphorylated form of CpxR (data not shown). The experimental isoelectric point of CpxR is \sim 5.8.

expression of *csg* genes, resulting in low curli production and a planktonic mode of growth. We have shown here that the Cpx pathway is induced at both the transcriptional and the protein level in response to a high level of sucrose. These results indicate that, as previously suggested by using a target *cpxP-lacZ* fusion (44), the Cpx pathway responds to changes in osmolarity. With increasing salt or sucrose concentration, the activity of the *csgD* promoter decreases. However, when sucrose was used as an osmotic agent, CpxR had no effect on the *csgD::uidA* gene fusion expression (Fig. 6A). Although the Cpx pathway can sense both high ion and high sugar content, it seems that the CpxR repressor is only responsive to high salt. This suggests that very subtle regulatory interactions can take place between the *csgD* regulators. Interestingly, R mling and coworkers observed a slightly different situation in serovar Typhimurium. Whereas increasing the salt concentration led to a decrease in *csgD* promoter activity, no effect on *csgD* could

be observed when sucrose was used as an osmotic agent (48). Sequence divergences between the two species in the *csg* intergenic region are likely to account for these differences in the regulatory process (Fig. 1B).

We observed that a strong repression of *csgD* occurs in a high-sucrose medium independently of CpxR. In order to determine which proteins mediate this sucrose repression, mutation in two regulatory genes, *rceB* and *hns*, known to be involved in osmoregulation were introduced into the MG1655 *csgD::uidA* strain (2, 55). Recent genetic data indicate that the Rcs pathway negatively regulates *csgD* (21, 56). Indeed, we found that RcsB acts as a strong repressor of the *csgD* transcription in low-osmolarity medium. Nevertheless, the sucrose-mediated repression of the *csgD* operon was independent of RcsB (Fig. 6B). The *hns118* mutation was then introduced in the MG1655 *csgD::uidA* background. In the absence of H-NS, the *csgD* expression level in high-sucrose medium

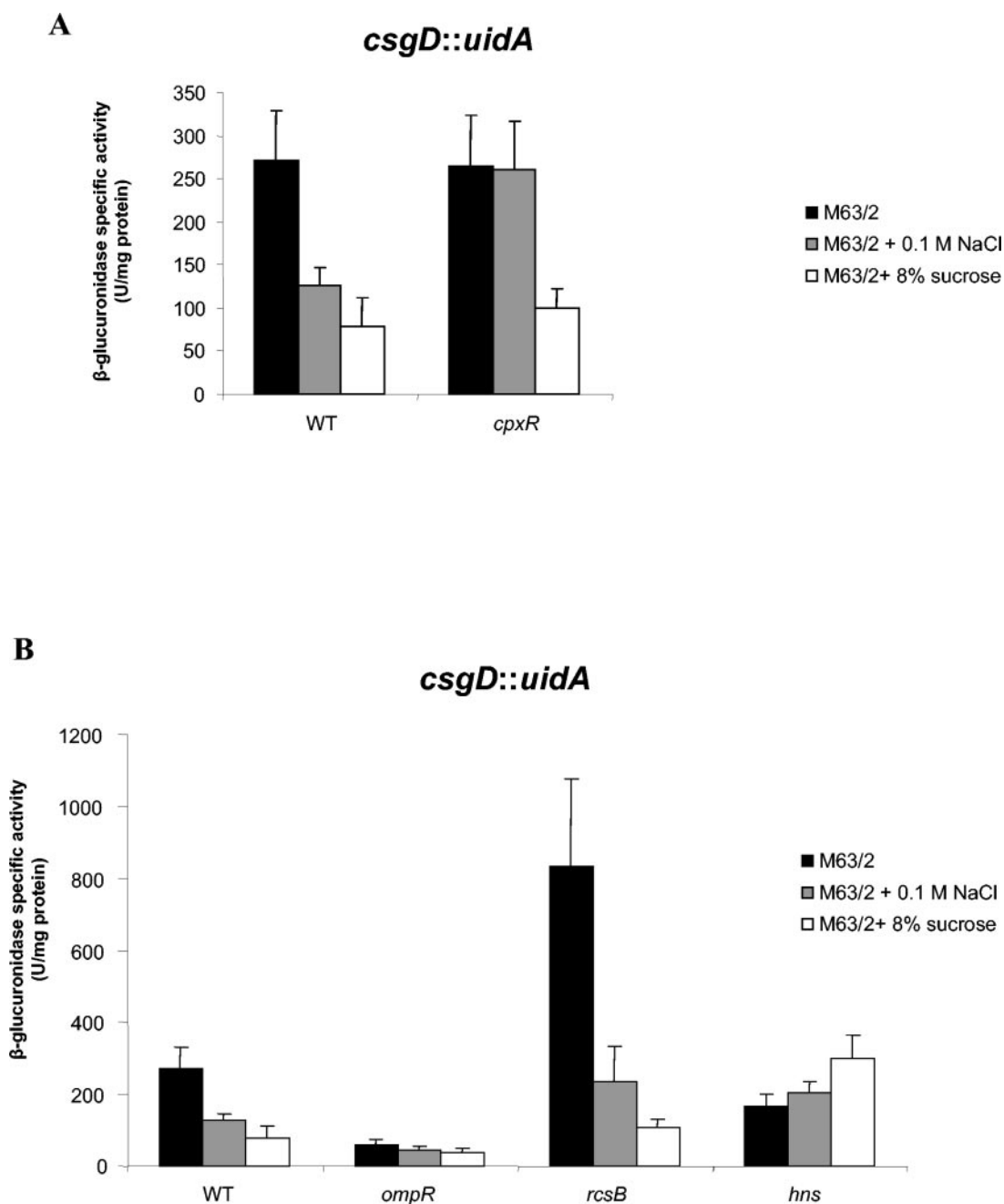


FIG. 6. (A) CpxR represses *csgD* transcription in high-salt-concentration conditions. The β -glucuronidase activities were determined for strains PHL1265 (MG1655 *csgD::uidA*) and PHL1259 (MG1655 Δ *cpxR csgD::uidA*). The results are means and standard deviations from three independent β -glucuronidase assays. (B) H-NS mediates *csgD* repression in high-sucrose medium. The β -glucuronidase activities were determined for strains PHL1265 (MG1655 *csgD::uidA*), PHL1089 (MG1655 *ompR331::Tn10 csgD::uidA*), PHL1311 (MG1655 *rcsB::Tn10 csgD::uidA*), and PHL1318 (MG1655 Δ *hns118 csgD::uidA*). The results are means and standard deviations from three independent β -glucuronidase assays. Cells were grown to early stationary phase at 30°C in M63/2–0.2% glucose supplemented or not with 0.1 M NaCl or 8% sucrose.

rises to the level observed in the wild-type strain grown in a low-osmolarity medium (Fig. 6B). This result shows that H-NS mediates the *csgD* repression in high-sucrose medium. H-NS is a histone-like nucleoid structuring protein that has no identified binding sequence but prefers an AT-rich DNA region with curved structures (52). Using rich medium and different genetic backgrounds, H-NS has previously been

shown to either activate or repress *csgD* transcription (22, 36). Although we show here that H-NS is the key repressor that switches off *csgD* transcription in a high-sucrose medium, our results also illustrate the fact that the role of H-NS in *csg* transcription is complex and depends on the strain background.

In response to osmolarity, a complex regulatory network

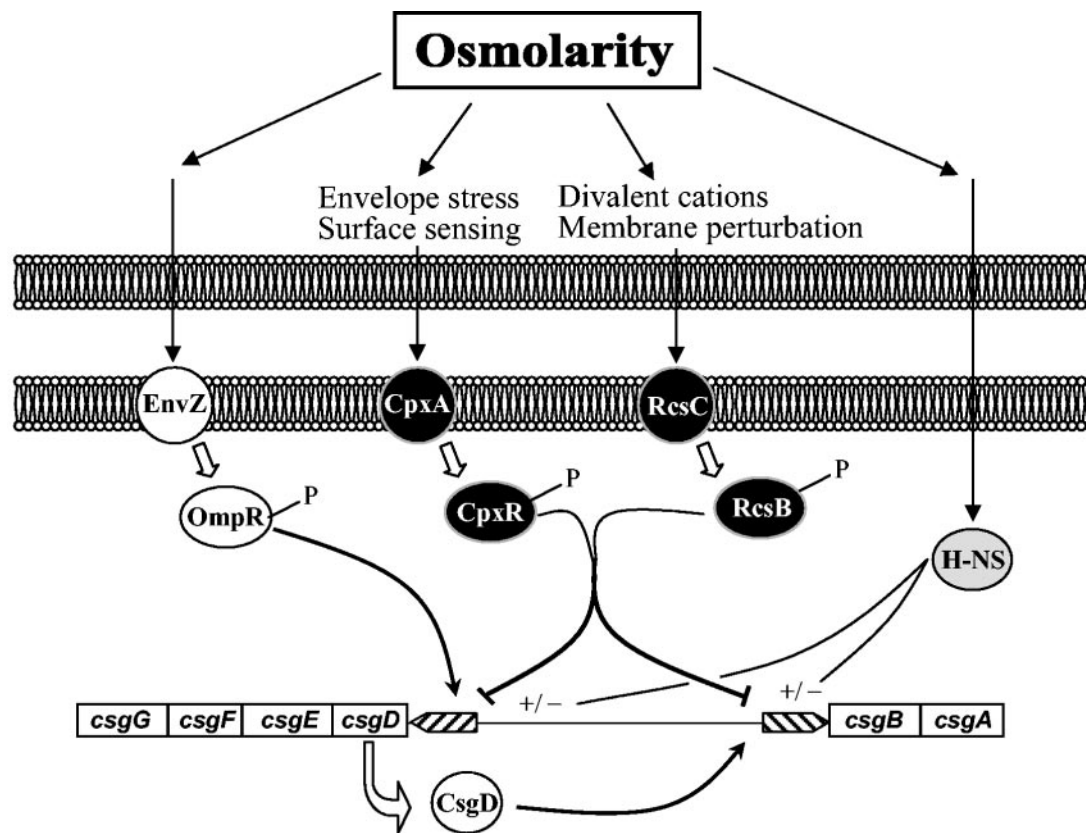


FIG. 7. Model of the regulatory network controlling curli production and biofilm formation in *E. coli*. Curli production at low osmolarity results from transcriptional activation of the *csgD* promoter by OmpR. When cells encounter high-salt medium, the Cpx pathway is activated. CpxR-P binds to the *csgD* promoter and consequently switches *csgD* expression off. The involvement of the regulatory systems EnvZ/OmpR, CpxA/CpxR, RcsC/RcsB, and H-NS in curli production is illustrated. Activation of all of these regulatory proteins is dependent on medium osmolarity. The different regulations are indicated by arrows for positive controls or via a line with a bar for negative controls. H-NS can exert either activation or repression, depending on the environmental conditions.

controls initial adhesion and biofilm formation via the *csgD* gene. Such complexity could be justified by the challenge faced by bacteria: switching from a planktonic mode of growth to biofilm communities. We propose a step-by-step model of biofilm development integrating osmolarity microenvironmental changes. In medium of low to moderate osmolarity, *csgD* is activated by OmpR, which results in high curli production by swimming cells (G. Jubelin and P. Stewart, unpublished observations). Cells with curli then bind easily to the inert surface when approaching it. We suggest that the key problem in curli synthesis is the repression of *csg* transcription through multiple repressor systems responding to closely related stimuli (Fig. 7), such as (i) surface sensing, envelope stress, and high salt content for CpxR; (ii) osmolarity, desiccation, and membrane perturbation for RcsB; and (iii) high sucrose content for H-NS. In response to immobilization to a surface, cells encounter conditions of higher osmolarity (33, 46) and gene expression is largely modified (6). Under these conditions, the Cpx pathway is activated, and accumulation of CpxR-P allows the cooperative binding of these proteins to the *csgD* promoter region, resulting in the repression of curli synthesis. It can be hypothesized that transient escape from neighboring cells can occur, for example, upon cell division completion. In this case, the “free” cells can sense the surrounding liquid medium, allowing

for OmpR-mediated activation of curli production. This may help to ensure the following anchoring to neighbor fixed cells through curli interactions, which will lead to new “stop” signals (contact and increasing osmolarity), turning the curli biogenesis back off.

The determination of the molecular consequences of complex “osmoswitches,” such as the one analyzed here, will require further investigations. It is likely however, owing to the wide range of external solute concentration encountered by enterobacteria within normal gastrointestinal tract environment or during gut or urinary tract infections (51), that the ability to respond to osmotic conditions play a role in enterobacterial colonization of specific niches.

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