The Escherichia coli CpxA-CpxR Envelope Stress Response System Regulates Expression of the Porins OmpF and OmpC†

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We performed transposon mutagenesis of a two-color fluorescent reporter strain to identify new regulators of the porin genes ompF and ompC in Escherichia coli. Screening of colonies by fluorescence microscopy revealed numerous mutants that exhibited interesting patterns of porin expression. One mutant harbored an insertion in the gene encoding the histidine kinase CpxA, the sensor for a two-component signaling system that responds to envelope stress. The cpxA mutant exhibited increased transcription of ompC and a very strong decrease in transcription of ompF under conditions in which acetyl phosphate levels were high. Subsequent genetic analysis revealed that this phenotype is dependent on phosphorylation of the response regulator CpxR and that activation of CpxA in wild-type cells results in similar regulation of porin expression. Using DNase I footprinting, we demonstrated that CpxR binds upstream of both the ompF and ompC promoters. It thus appears that two distinct two-component systems, CpxA-CpxR and EnvZ-OmpR, converge at the porin promoters. Within the context of envelope stress, outer membrane beta-barrel proteins have generally been associated with the sigma E pathway. However, at least for the classical porins OmpF and OmpC, our results show that the Cpx envelope stress response system plays a role in regulating their expression.

The classical porins OmpF and OmpC are major constituents of the Escherichia coli outer membrane and account for approximately 2% of the total protein content of the cell (52). These proteins allow for the passive diffusion of solutes across the outer membrane. Many environmental factors have been identified that alter OmpF and OmpC expression, including osmolarity, temperature, pH, nutrient availability, and various toxins (23, 40, 54). The importance of this complex regulation for the cell is not understood, although it has been suggested that the differential regulation of the two porins may provide a means of balancing the competing needs of access to nutrients and protection from toxins (52).

The complex environmental regulation of the porins is implemented by a similarly complex regulatory network, whose components include the EnvZ-OmpR two-component system (reviewed in references 19, 34, and 63), the small RNAs MicF and MicG (9, 14), the sigma factors σ52 and σE (45, 55), the global regulator Lrp (21), and the histone-like protein IHF (30, 59). The EnvZ-OmpR system is a central element of this network, since phosphorylated OmpR (OmpR-P) is absolutely required for OmpF and OmpC expression. OmpR-P levels are modulated by the histidine kinase EnvZ in response to unknown stimuli. Low levels of OmpR-P activate transcription of ompF, while high levels repress ompF and activate ompC (22, 36, 61). In vitro studies revealed that OmpR binds to three sites (designated C1, C2, and C3) upstream of the ompC promoter (41, 66) and four sites (one distal site, F4, and three proximal sites, F1, F2, and F3) upstream of the ompF promoter (28, 29, 48, 66). However, despite this detailed characterization, the mechanism underlying the differential regulation of ompF and ompC by OmpR is not understood (25, 34).

Here we describe the results of a genetic screen and a subsequent analysis which indicated that a second two-component system, the CpxA-CpxR system, also regulates porin expression. The Cpx system responds to conditions associated with envelope stress, such as alkaline pH and overproduction of secreted proteins (10, 37, 64), and also to attachment of cells to surfaces (53). The previously characterized members of the Cpx regulon include proteins involved in the folding or degradation of misfolded proteins in the periplasm (18, 57) and in the assembly of structures on the cell surface (17, 26, 31, 39, 50, 51). Regulation and monitoring of porin status, however, have been associated with a separate envelope stress-responsive system controlled by σE (reviewed in references 1, 2, and 57). In particular, overexpression of OmpF or OmpC activates σE (45) but does not activate the Cpx pathway (12). We demonstrate here that both ompF and ompC porin genes are also members of the Cpx regulon. However, whereas activation of σE results in decreased expression of both ompF and ompC (60), activation of Cpx results in a strong decrease in ompF expression and an increase in ompC expression.

MATERIALS AND METHODS

Growth media. Minimal medium consisted of 60 mM K2HPO4, 33 mM KH2PO4, 7.6 mM (NH4)2SO4, 1.7 mM sodium citrate, and 1 mM MgSO4 (46) and was supplemented with 0.2% glucose or glycerol as indicated below. Luria-Bertani (LB) agar was obtained from Difco (Becton Dickinson, Sparks, MD). Medium A agar consisted of 7 g liter−1 glucose broth (Difco), 1 g liter−1 yeast extract (Difco), 0.2% glycerol, 20 mM K2HPO4, 10 mM KH2PO4, and 1.5% agar (Difco) (33). Ampicillin, when needed for plasmid maintenance, was added to a concentration of 50 μg/ml.

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of plasmid pKD46 by growth at 42°C, as described by Datsenko and Wanner.

The cat insertion in (the underlined regions are sequences flanking envZ in the E. coli genome). The PCR product was used to insert cat into envZ, as described above, and the resulting insertion was transduced into MDG147.

Strain MDG147 envZ:cat was then transformed with pCP20 to remove the cat gene (13) and then cured of the plasmid by growth at 42°C. This resulted in strain EPB91.

Fluorescence assay. Single colonies were grown to saturation in the appropriate medium at 37°C with aeration. Cultures were then diluted 1:1,000 into the same prewarmed medium and incubated further at 37°C with aeration. When these cultures reached an optical density at 600 nm (OD600) of 0.2, they were rapidly chilled in an ice-water slurry. Fluorescence was measured with a Quantamaster-4/2003 spectrophuorimeter (Photon Technology International, Lawrenceville, NJ). The excitation/emission wavelengths for cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) measurements were 434 nm/475 nm contrast.

Transposon mutagenesis and colony screening. For the transposon mutagenesis and colony screening procedures see the supplemental material.

Disruption of ackA-pta, cpxA, and envZ. To delete ackA-pta, a chloramphenicol resistance cassette was amplified from pKD3 (13) using primers 5'-GGTACTTCCATGCTGGATGTAAGTATCCGTTCGTTGAGCTGACCTGC TTC-3' and 5'-CCAGTGCGCCACGGGACAGGTCGTTAACCGGCTTGCCAT AATGGAATATCCTCCTTAG-3' (the underlined regions are sequences that flank the ackA-pta locus in the E. coli chromosome). The PCR product was inserted into the chromosome by Lambda Red-mediated allele replacement (13).

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Porin protein analysis. Two-milliliter cultures were grown to saturation in glucose minimal medium. They were then subcultured by 1:1,000 dilution into 50 ml of the same prewarmed medium and grown at 37°C with aeration. When the cultures reached an OD600 of ~0.2, they were rapidly chilled in an ice-water slurry. Fluorescence was measured with a QuantaMaster-4/2003 spectrophotometer (Photon Technology International, Lawrenceville, NJ). The excitation/emission wavelengths for cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) measurements were 434 nm/475 nm contrast.
TTTACGG-3' and 5'-GCTTTATGCTTTTTATTCACC-3', respectively. The F4 and ompC upstream regions were amplified by PCR with primer pairs 5'-CTAAATTTACTGCTTCCAAGG-3' and 5'-GCTTGAATTTATGCGATG-3', respectively. The products were cloned into TOPO cloning vector pCR4 by following the manufacturer's directions (Invitrogen). The resulting plasmids were used as templates to amplify DNA for the footprinting reactions. For generation of radiolabeled DNA, one primer of each pair listed above was labeled with 32P using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-32P]ATP (3,000 Ci/mmol; 5 mCi/ml; Perkin-Elmer, Shelton, CT). The PCR products were passaged through a QiAquick spin column per the manufacturer's instructions (QIAGEN) and subsequently purified on a 4% Tris-acetate-EDTA polyacrylamide gel. The desired fragments were removed from the gel, and the DNA was eluted into diffusion buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0, 0.1% SDS) for 1 h at 50°C. The eluted DNA was suspended in 4 volumes of QIAGEN buffer PB and passaged through a QiAquick spin column. The resulting DNA was denatured and reannealed with a gradient of 1°C per 45 s in the presence of 50 mM NaCl. CpxR was phosphorylated with phosphoramide as described previously (42), except that the reaction was carried out at 37°C. The binding reactions were performed in binding buffer (12% glycerol, 4 mM Tris-Cl, pH 7.6, 20 mM KCl, 2 mM EDTA, 1 mM dithiothreitol) with 1 μg of poly(dI-dC) (Roche, Indianapolis, IN) and 50,000 cpm of DNA for 20 min at 37°C. Then 125 ng of DNAse I (Roche) in 5 μl was added to each reaction mixture, and the reactions were stopped after 30 s with 380 mM sodium acetate, pH 5.2, 20 mM EDTA, and 2 μg glycerol (Roche). The reaction products were ethanol precipitated and resuspended in sequencing stop buffer (U.S. Biochemicals, Cleveland, OH). Then the reaction products were resolved in 4% Tris-acetate-EDTA polyacrylamide gel. The de-sired fragments were removed from the gel, and the DNA was eluted into sequencing buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 5.2, 20 mM EDTA, and 2 mM dithiothreitol) and verified by sequencing. This revealed insertions in 14 different genes (see the supplemental material).

To determine whether any of the isolated mutants showed altered porin expression under conditions other than growth on solid medium A agar, we measured the CFP and YFP fluorescence of liquid cultures in glucose minimal medium. Of the 14 mutants, 1 exhibited a particularly dramatic change in porin expression, with a roughly 17-fold decrease in ompF transcription and a 2-fold increase in ompC transcription (Fig. 2a). This mutant, strain EPB54-012, contained an insertion in cpxA, which encodes a histidine kinase. The insertion was just after bp 277 of the coding sequence for cpxA, which is the second gene in an operon with the gene for the cognate response regulator, cpxR. Colonies of the cpxA::kan mutant on medium A agar differed only subtly from the colonies with the original ring phenotype (see the supplemental material). However, due to the dramatic difference in porin transcription between the mutant and wild-type strains for growth in glucose minimal medium, we decided to further characterize this mutant.

Altered porin expression in the cpxA::kan mutant occurs under conditions associated with high levels of CpxR-P. To verify that the porin phenotype associated with EPB54-012 (cpxA::kan) was due to the loss of CpxA expression, we constructed an independent deletion of cpxA in MDG147, strain EPB134. This strain showed the same repression of ompF transcription and a 2-fold increase in ompC transcription (Fig. 2a). This mutant, strain EPB54-012, contained an insertion in cpxA, which encodes a histidine kinase. The insertion was just after bp 277 of the coding sequence for cpxA, which is the second gene in an operon with the gene for the cognate response regulator, cpxR. Colonies of the cpxA::kan mutant on medium A agar differed only subtly from the colonies with the original ring phenotype (see the supplemental material). However, due to the dramatic difference in porin transcription between the mutant and wild-type strains for growth in glucose minimal medium, we decided to further characterize this mutant.

RESULTS

Transposon mutagenesis identified CpxA as a regulator of porin expression. To monitor transcription of the porin genes ompF and ompC, we used the two-color fluorescent reporter strain MDG147, which contains chromosomal operon fusions of vhf to ompF and cfp to ompC (5). We observed that colonies of MDG147 growing on medium A agar plates initially showed uniform CFP and YFP fluorescence. However, as the colonies continued to grow, the fluorescence became nonuniform; the boundaries of the colonies showed an increased level of yellow fluorescence (increased ompF transcription) and a decreased level of cyan fluorescence (decreased ompC transcription) (Fig. 1). For colonies growing on LB agar, on the other hand, the CFP and YFP fluorescence was more uniform for both early and late growth of the colonies (data not shown). The characteristic pattern of fluorescence for colonies on medium A suggested that this would be a good starting point for genetic screening to search for additional regulators of porin expression.

Approximately 5,000 mini-Tn5 transposon insertions in MDG147 were selected on LB agar containing kanamycin and then spotted onto medium A agar without antibiotic selection. After at least 24 h of growth, spotted colonies were screened by fluorescence microscopy using a low-power (2.5×) objective. We observed a variety of colony fluorescence phenotypes, such as uniform fluorescence, concentric rings, and radial spokes, which were clearly distinct from the wild-type ring pattern shown in Fig. 1 (see figures in the supplemental material). These colonies were selected for further characterization. After transducing the transposon insertions back into MDG147 and verifying that the mutant phenotypes were stable, we determined the locations of the transposon insertions by DNA sequencing.
cpxA strains, conditions associated with high levels of acetyl phosphate, such as growth on glucose (43, 67, 68), apparently lead to high levels of CpxA (20, 21, 27, 28).

To test this hypothesis, we eliminated production of acetyl phosphate in our fluorescent reporter strains by deleting ackA and pta (43, 67, 68). Fluorescence measurements for cultures grown in glucose minimal medium revealed that, indeed, deletion of ackA-pta in a cpxA strain restored porin expression to wild-type levels (Fig. 2a). In addition, when the cpxA strain was grown in minimal medium with glycerol as the carbon source, conditions that result in low levels of acetyl phosphate (43, 67, 68), the porin expression was the same as that in the cpxA− strain (data not shown). When the same experiment was performed using minimal glycerol medium supplemented with 30 mM acetate, which results in high levels of acetyl phosphate (43, 67, 68), we observed elevated ompC transcription and greatly reduced ompF transcription for the cpxA strain (data not shown). Again, the porin levels returned to the wild-type levels when either cpxR or ackA-pta was deleted from the cpxA strain (data not shown).

It was possible that the results described above were some-how unique to the MG1655 strain background used to construct MG147. To verify that this was not the case, we also looked at the effect of cpx mutations in the fluorescent reporter strain MG131 (4), which was derived from MC4100. Due to strain differences, the CFP fluorescence was lower and the YFP fluorescence was higher in MDG131 than in MDG147. However, the relative changes in the CFP and YFP signals for the cpxA strains relative to the cpxA− strains in the MDG131 background were comparable to what we observed for MDG147 (Fig. 2).

To further test whether porin expression was mediated by CpxR-P, we used the allele cpxRD51A, which encodes a CpxR variant with an alanine at position 51 in place of aspartate (16). Strains with this mutation behave like cpxR null strains (16). A deletion of cpxA was constructed in a strain with the cpxRD51A mutation and grown on glucose minimal media. As shown in Fig. 3, porin expression in the cpxA cpxRD51A strain was comparable to porin expression in the wild-type strain.

Taken together, the results described above indicate that in the absence of CpxA and in the presence of a high concentration of acetyl phosphate, high levels of CpxR-P lead to strong repression of ompF and activation of ompC transcription.

Activation of CpxA decreasesompF expression and increasesompC expression. If the interpretation described above is correct, then activation of CpxA, which results in high levels of CpxR-P, should also lead to repression of ompF and activation of ompC. To test this hypothesis, we stimulated CpxA by overexpressing the periplasmic protein NlpE (64). Introduction of plasmid pLD404 (64), which constitutively overexpresses NlpE, into MDG147 resulted in a ~threefold increase in ompC transcription and an ~11-fold decrease in ompF transcription compared with the fluorescence levels for a strain containing an empty control vector (Fig. 4). Overexpression of NlpE in EPB62 (cpxR cpxA) did not affect ompF or ompC transcription (Fig. 4).

We also tested a constitutively active variant of CpxA, designated CpxA*, which lacks phosphatase activity (58). As shown in Fig. 5, the CpxA* strain exhibited a ~threefold increase in ompC transcription and a ~10-fold decrease in ompF transcription.
Strains were grown in glucose minimal medium with 50 μg/ml ampicillin to the mid-log phase (see Materials and Methods). The bars indicate the means from three independent gels, and the error bars indicate the standard deviations.

Transcription. Expression of CpxA* in the absence of CpxR resulted in no change in porin expression (Fig. 5).

**High CpxR-P decreases OmpF protein levels and increases OmpC protein levels.** To verify that the observed regulation of *ompF* and *ompC* transcription by the Cpx system results in regulation of porin protein levels, we examined the porin content in the cell envelope. Wild-type, *cpxA*, and *cpxRA* strains were grown in glucose minimal medium, and cell envelopes were analyzed by urea SDS-PAGE. The OmpC levels were ~2.2-fold higher in the *cpxA* strain than in the wild-type strain (Fig. 6). The OmpF levels in the *cpxA* strain were reduced to such an extent that they were below the detection level of the Coomassie blue-stained gel (Fig. 6). Disruption of both *cpxA* and *cpxR*, on the other hand, restored the outer membrane porin content to wild-type levels (Fig. 6). The results described above complement previous work in which a *cpxA* mutant was found to have reduced levels of OmpF in the membrane (44). In that study, the cause for this reduction in OmpF levels was not identified, but it was hypothesized to be due to an inability of CpxA− cells to properly incorporate OmpF into the outer membrane.

Repression of *ompF* by CpxR-P does not require MicF. Since OmpF expression is almost completely repressed in the presence of high CpxR-P levels, we wanted to determine whether CpxR-P could act through MicF, a small RNA that binds to and induces degradation of *ompF* mRNA (reviewed in reference 14). We therefore introduced a deletion of *micF* into wild-type and *cpxA* strains. The *micF* deletion that we used also eliminates *ompC*. (The *micF* gene lies upstream of and is transcribed in the opposite direction from *ompC*.) SDS-PAGE analysis of porin protein levels revealed that for cultures growing in glucose minimal medium, disruption of *cpxA* still resulted in reduced levels of OmpF in the Δ*micF* strain (Fig. 7). Thus, CpxR-P does not require MicF to repress OmpF expression.
CpxR-P represses ompF in the absence of EnvZ. One difficulty in separating the role of OmpR from the roles of other cellular components in regulating porin expression is that OmpR is required for transcription of ompF and ompC (63). Indeed, fluorescence analysis showed that a cpxA ompR double mutant failed to express either porin when it was grown in glucose minimal medium (data not shown), indicating that in the absence of OmpR, CpxR-P cannot act as a transcriptional activator of ompC or ompF.

OmpC expression increases with increasing OmpR-P levels, whereas OmpF is maximally expressed at intermediate levels of OmpR-P (61). It was thus possible that CpxR-P represses ompF and activates ompC by increasing OmpR-P levels. To test this possibility, we took advantage of the fact that in the envZ strain EPB91, phosphorylation of OmpR by acetyl phosphate leads to an OmpF−/− OmpC− phenotype (27; data not shown). Thus, in this background, a small increase in the OmpR-P level should result in an increase in ompF transcription. However, we found that for growth in glucose minimal medium, disruption of cpxA (EPB91) further reduced ompF transcription (Fig. 8). The level of transcription of ompC remained below the level of detection in the envZ cpxA strain (data not shown). Therefore, we concluded that the repression of ompF by CpxR-P does not require EnvZ or an increase in OmpR-P levels. Activation of ompC transcription by CpxR-P, on the other hand, is still dependent on increased levels of OmpR-P.

Inactivation of the Cpx system does not affect the osmoregulation of ompF and ompC transcription. High osmolarity has been shown to increase transcription of several Cpx-regulated genes and to increase the level of CpxR-P (32, 56). To determine whether the Cpx system plays a role in the osmoregulation of ompF and ompC, we compared the levels of YFP and CFP fluorescence of wild-type and cpxR−/− strain (data not shown). Therefore, we concluded that CpxR acts directly at the ompC and ompF promoters to regulate expression of these genes.

DISCUSSION

The Cpx and σE pathways respond to cell envelope stress. These two pathways are largely distinct, both in terms of their activating signals and in terms of the genes that they regulate. However, there is at least some overlap; the degP and skp genes are members of both the Cpx and σE regulons, and many other Cpx and σE-regulated genes have similar functional roles (1, 2, 18, 57). Based on the results presented here and recent work on σE-regulated genes (60), ompC and ompF are two addi-
tional members of the Cpx and σ^E^ regulons. However, porin regulation seems to highlight the differences rather than the similarities between the Cpx and σ^E^ systems. In particular, while σ^E^ downregulates expression of both porins (60), Cpx downregulates OmpF but upregulates OmpC.

Although the structure of OmpC has never been determined, permeability measurements suggest that it has a smaller pore than OmpF (52). In particular, toxins, such as bile salts and cephalosporins, pass through OmpF more readily than they pass through OmpC. Based on these observations, it has been suggested that reciprocal regulation of the two porins provides a means for *E. coli* to balance the need for nutrients and the need for protection from toxins (52). In environments such as freshwater supplies, which contain low levels of nutrients and toxins, OmpC would provide greater protection. In animal intestinal tracts, on the other hand, which are rich in nutrients and toxins, OmpC would provide greater protection. In light of our results, it is possible that in the natural environments for *E. coli*, toxins that induce the Cpx system are primarily permeable through OmpF. The Cpx regulation of porin expression would then be a simple way to provide protection from these compounds while avoiding the more drastic measure used by σ^E^ (60) of downregulating expression of both OmpF and OmpC.

A previous genome-wide analysis identified possible CpxR-P binding sites in the *ompC* and *ompF* regulatory regions. However, the consensus in the *ompF* promoter region was determined to be weak and was not characterized further (15). Our DNase I footprinting analysis provided evidence that CpxR-P acts directly at the *ompF* and *ompC* promoters. Repression of *ompF* by CpxR could result from prevention of either OmpR or RNA polymerase binding. Alternatively, CpxR could stimulate DNA loop formation and interaction with the upstream repressive site F4 (reviewed in reference 34). We identified a CpxR binding site >300 bp upstream of *ompC*. Although this site is at the terminus of *micF*, we found that a deletion of *micF* did not prevent CpxR-mediated repression of *ompF*. This site, however, may be important for *ompC* transcription. We found that high levels of CpxR-P failed to activate transcription of *ompC* in the absence of OmpR. This suggests that CpxR binding upstream of *ompC* acts in conjunction with OmpR binding to activate transcription.

Our results indicate that porin regulation by the CpxA-CpxR system and by the EnvZ-OmpR system converges at the porin promoters. Interestingly, this is not the first example of convergence of these two regulatory systems. Recent studies have shown that expression of curli fimbriae is also regulated by both CpxR and OmpR (17, 32). Other recent work with *Chlamydia* has identified interactions between OmpR and a CpxR homologue, ChxR (35). Unfortunately, without a better understanding of the stimulus for EnvZ and the mechanism of transcriptional control by OmpR and CpxR, it is difficult to determine the regulatory logic for the porin promoters. Two-component regulatory systems have generally been regarded as separate signaling cascades. However, increasingly, examples are emerging in which these systems are interconnected at various steps in their signaling pathways (for example, see references 6, 20, 24, 32, 47, 49, 65). Given the large regulons controlled by many of these systems, it seems likely that many

![DNase I footprinting of CpxR-P at *ompF* and *ompC*](http://jb.asm.org/). CpxR-P protects two distinct regions upstream of *ompF* (A and B) and of *ompC* (C and D). The concentration of CpxR-P (in nM) used in each lane is indicated at the bottom. The coordinates indicate positions relative to the *ompF* or *ompC* transcription start sites. Predicted CpxR binding sites (15) are indicated on the left by gray bars. Regions of protection are indicated by black bars and regions of higher affinity are indicated by striped bars on the right.
more examples of complex promoters regulated by multiple two-component systems will emerge.

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