The OmpR Protein of *Escherichia coli* Binds to Sites in the *ompF* Promoter Region in a Hierarchical Manner Determined by its Degree of Phosphorylation*

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In *Escherichia coli* the *ompF* gene encodes a major outer membrane porin protein that is differentially regulated by the OmpR protein. OmpR acts as a positive as well as a negative regulator of *ompF* expression by binding to DNA sequences in the *ompF* promoter region. The DNA binding activity of OmpR is itself regulated by phosphorylation through the kinase protein EnvZ. Phosphorylation is believed to change the function of OmpR from an activator to a repressor molecule. By using purified OmpR and various regions of the *ompF* promoter we show that phosphorylation causes binding of OmpR to a DNA region between the −40 to −100 region of the *ompF* promoter previously shown to be important for *ompF* expression. As the amount of OmpR-phosphophorylates increases, a binding site located at a further upstream −360 to −380 region was occupied. This latter site has been reported to be important for *ompF* repression. Further experiments indicate that the −70 to −100 region is a high affinity site, while the −45 to −60 and −360 to −380 regions are low affinity sites. We also provide evidence that OmpR binding at the −360 to −380 region requires previous binding at downstream sequences, which is indicative of long range interactions between OmpR molecules. We interpret our results in terms of a model for *ompF* regulation involving hierarchical binding by phosphorylated OmpR and potential DNA looping.

In *Escherichia coli*, two major outer membrane porin proteins, OmpF and OmpC, are differentially regulated in response to changes in external medium osmolarity (1, 2). OmpF is preferentially produced under conditions of low osmolarity, while increases in osmolarity result in the loss of OmpF and the appearance of OmpC protein. Osmoregulation of porin proteins is controlled at the transcriptional level by the OmpR and EnvZ proteins (3–6). OmpR is a DNA-binding protein that interacts with the upstream promoter region of the *ompF* and *ompC* genes and regulates their expression (7–10). EnvZ is a transmembrane sensor that detects osmotic signals and modulates the function of OmpR through phosphorylation (11–16).

It is generally believed that at low osmolarity, EnvZ maintains relatively low levels of phosphorylated OmpR, thereby allowing significant expression of the *ompF* gene (17, 18). At high osmolarity, EnvZ is thought to change its signaling output and increase the relative amounts of OmpR-phosphate. High levels of OmpR-phosphate cause the *ompC* gene to become activated, while the *ompF* gene is repressed (7, 12, 18, 19). This model is supported by studies correlating phosphorylated OmpR levels with outer membrane porin production (12, 20). For example, pleiotropic envZ mutants such as *envZ473* and *envZ211* produce very high levels of phosphorylated OmpR and have an OmpF<sup>+</sup>, OmpC<sup>−</sup> outer membrane porin pattern (12, 18, 20). With regard to the *ompF* gene, several studies have suggested that OmpR converts from an activator to a repressor molecule when its phosphorylated levels are elevated (7, 18).

In *vitro* and *in vivo* footprinting studies have demonstrated that OmpR binds to at least two regulatory sites in the upstream region of *ompF* (8, 21) (see Fig. 8). One region is between −70 to −100 (relative to the transcriptional start site). The −70 to −100 region is essential for *ompF* activation and contains three tandemly arranged 10-bp elements (designated Fa, Fb, and Fc), which share sequence similarities with each other (see Figs. 8 and 9) (8, 21). Another regulatory site, termed the Cd box, is between −42 and −52 and is a 10-bp sequence unrelated to the −70 to −100 region (see Figs. 8 and 9). The OmpR<sub>472</sub> mutant protein is unable to bind to the −42 to −52 region (9, 21), and strains harboring the *ompR<sub>472</sub>* allele produce OmpF constitutively (4). Thus, the loss of binding at the −42 to −52 region correlates with the inability of OmpR<sub>472</sub> to repress *ompF* expression and implicates this site in negative regulation (21, 22). Another OmpR binding site located 360–380 bp upstream of the transcriptional start site has recently been identified (23). This region has been shown to be important for negative regulation of the *ompF* gene (24).

While the detailed mechanism of regulated *ompF* expression remains unclear, a correlation may exist between the extent of occupancy of binding sites in the *ompF* promoter and OmpR phosphorylation. That is, different levels of OmpR-phosphate may interact selectively with one region over another with the ultimate outcome affecting *ompF* expression. In this study we examined the phosphorylation requirement for OmpR-DNA interactions at the three binding sites in the *ompF* promoter. Our results indicate that OmpR binds sequentially to these sequences in a manner determined by the phosphorylated state of the protein. These results suggest a model of *ompF* regulation involving protein-protein interactions and DNA looping.

**EXPERIMENTAL PROCEDURES**

*Strains and Plasmid Construction—Plasmid pAB091 contains a 1.3-kilobase pair EcoRI-BgII insert from plasmid pMY222 (25) cloned into EcoRI-BamHI sites of phagemid pGEM3Z(f−) (Promega Corp., Madison, WI). This insert contains the 3′ portion of the *asnS* gene, a 0.49-

* The abbreviations used are: bp, base pair(s); FPLC, fast protein liquid chromatography.
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kilobase pair intervening sequence (ompF regulatory region) between ansS and the transcriptional start site for the ompF gene (see Fig. 9), and the 5′ portion of the ompF structural gene. The 5′ portion of the ompF gene was removed by exchanging SstI-SalI fragments between pAR091 and plasmid pGR241 (8). The resulting plasmid pAR092 contained a BamHI site at the 3′-region of the ompF regulatory region. Native occurring SstI, SalI, HaeII, AulI, or ScaI sites were used along with the common BamHI site of pAR091 to clone variable amounts of the ompF promoter into PRS414, a promoterless lacZ vector (26).

For DNase I footprinting a HaeII-HindIII fragment from plasmid pAR092 was treated with Klenow fragment and deoxyribonucleotides. This was digested with PstI and cloned into XbaI, PstI sites of pGEM3Zf(+), in which the XbaI site had been treated with Klenow fragment and deoxyribonucleotides. The resulting plasmid, pAR094, could be digested at flanking BamHI (adjacent to the HindIII site) or HindIII (adjacent to PstI) for radiolabeling with α-32PdGTP or α-32PdTTP, respectively. Labeling at the HindIII site allowed footprinting of the region between +2 to +100 region, while labeling at the BamHI site allowed footprinting of the region between −360 to −380. A 314-bp XbaI-BamHI region (−193 to +121) from plasmid pKI0033 (8) was used for most of the DNA mobility shift experiments.

Mutations were created in the ompF promoter in pKI0033 using a site-directed mutagenesis kit (Amersham Corp.). Three base pairs were altered in the C box (−42 to −51), which changed the sequence from ATTACATTGC to ATTGTCGTGC. This mutation was subcloned into plasmids pAR094. In another construction, a HindIII site was created in pKI0033 by making a T to G mutation at the −61 position. This clone, pAR001, was digested with XbaI and HindIII, which released a 130-bp ompF promoter fragment containing the region between −193 to −63 (F boxes alone). Digestion of pAR001 with the HindIII-BamHI fragment released a 184-bp ompF promoter fragment containing the region between −64 to −121 (Cd box).

E. coli strains AR134, AR135, and AR136, which express low copy numbers of plasmids with ColE1 replication origins, were created from MC4100, MH1471, and MH760, respectively. Each strain contains a pcnB80 allele, which was introduced by P1 transduction and selecting for a tightly linked Tn10 (27). The pcnB80 allele was introduced to minimize multicopy effects of the pBR322-based pAR094 vectors.

Binding of OmpR from Various Strains—Plasmids pAT428 (28) and pHY1430 (29) were used to express the wild-type and the transcriptional start site for the ompF gene. There is a 49-bp region (−193 to +121) from plasmid pKI0033 (8) was used for most of the DNA mobility shift experiments. A 314-bp XbaI-BamHI region (−193 to +121) from plasmid pKI0033 (8) was used for most of the DNA mobility shift experiments.

Footprinting—For DNA mobility shift experiments, various amounts of OmpR in the absence of ATP (lanes 1-4) and with ATP (lanes 5-8) represent DNA binding activities of OmpR following a 15-min incubation of EnvZ(C) with ATP in the absence of OmpR (lane 2), OmpR with ATP in the absence of EnvZ(C) (lanes 3 and 4), and EnvZ(C) with OmpR in the absence of ATP (lane 4). Protein-DNA complexes are indicated as a and b.

RESULTS

DNA Shift Experiments with Phosphorylated OmpR—DNA binding of phosphorylated and nonphosphorylated OmpR was examined using a 314-bp DNA fragment encompassing the region between −193 to +121 of the ompF promoter (XbaI/BamHI small fragment of pKI0033). We first phosphorylated EnvZ(C) at room temperature with ATP and then transferred phosphate from EnvZ(C) to OmpR on ice. The DNA binding activity of this newly phosphorylated EnvZ(C) was determined by mixing a portion of the reaction with the labeled promoter. The DNA binding reaction also contained an excess of EDTA to prevent any further phosphate transfer between EnvZ(C) and OmpR. Protein-DNA complexes were allowed to form, and complexes were resolved by nondenaturing electrophoresis. A representative autoradiograph of several experiments is shown in Fig. 1.

Under these experimental conditions, EnvZ(C) alone or low concentrations of purified (nonphosphorylated) OmpR hardly formed protein-DNA complexes (Fig. 1, lanes 2 and 3). However, brief incubation of OmpR with EnvZ(C) and ATP produced two protein-DNA complexes shown in Fig. 1 as “a”- and “b”-complexes (lanes 5–8). The a-complex migrated slower than the b-complex. As incubation of OmpR with EnvZ(C) and ATP was extended to increase the level of phospho-OmpR, more of the radiolabeled DNA appeared as the slower moving a-complex.

Fig. 1. DNA binding activities of pure OmpR following phosphorylation by EnvZ(C). EnvZ(C) was phosphorylated with ATP and placed on ice, and OmpR was added. At various times, aliquots representing approximately 25 ng of OmpR were mixed with approximately 5000 cpm of an end-labeled XbaI-BamHI ompF promoter fragment containing the region between −193 to +121 (Cd box). As incubation of OmpR with EnvZ(C) and ATP was extended, more of the radiolabeled DNA appeared as the slower moving a-complex.

50 mM EDTA, 50 mM KCl, 1 mM MgCl2, and 125 ng of poly[d(dC)] (Boehringer Mannheim). Complexes were allowed to form for 20 min at room temperature, and then samples were directly applied to a 5% nondenaturing polyacrylamide gel. The gel was dried and processed for autoradiography. For partially purified samples, 4–36 ng of OmpR protein was added directly to the DNA binding reaction.

For DNA mobility shift experiments, various amounts of OmpR were phosphorylated as above and added to DNA binding reactions containing approximately 50,000 cpm of labeled probe. Approximately 2 ng of a freshly prepared dilution of DNase I (0.5 μg/ml) in 10 mM MgCl2, was added, and complexes were digested for 1 min at room temperature. The reaction was terminated by the addition of stop buffer (3 mM sodium acetate, 2% SDS, 25 mM EDTA, 50 μg/ml RNAse A). Samples were extracted once with phenol:chloroform, ethanol-precipitated, with 70% ethanol, dissolved in sequencing dye, and applied to a 6% polyacrylamide sequencing gel containing 8 M urea.

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and eventually all of the DNA were present as the a-complex (lane 8). These results demonstrate that in vitro, DNA binding of OmpR to the ompF promoter proceeds through two complexes, both of which require phosphorylated OmpR.

Competition experiments were performed to study the OmpR binding sites involved with the a- and b-complexes (Fig. 2). In these experiments, protein-DNA complexes were initially formed, and then an excess of unlabeled DNA fragment was added. The unlabeled DNA represented the 314-bp DNA promoter (lanes 3 and 4), 130-bp DNA fragments containing the −70 to −100 region (lanes 5 and 6), a 184-bp fragment containing the −40 to −60 region (lanes 7 and 8), or a 180-bp DNA fragment from an unrelated gene (lane 9). Results in lanes 3–9 should be compared with phosphorylated OmpR mixed with the labeled promoter in the absence of any competitor DNA (lane 2). The labeled DNA in the absence of OmpR is shown in lane 1. OmpR-DNA complexes are shown as a and b.

The higher molecular weight of the a-complex is due to further OmpR interactions at the −40 to −60 region (see below). Nevertheless, at an 8-fold molar excess, this region caused a minor change in the ratios of a- to b-complexes but otherwise did not significantly compete either the a- or b-complex. These results may indicate that the −40 to −60 region is a low affinity site that requires previous occupancy of the high affinity −70 to −100 region before it can be bound. This aspect is developed in further studies (see below).

Analysis of Partially Pure Forms of OmpR—To correlate the a and b protein-DNA complexes with various porin phenotypes, we examined the DNA binding activities of partially purified OmpR from ompR472 and envZ473 strains that have OmpF/ompC and OmpF/ompC outer membrane porin phenotypes, respectively (5). OmpR was also prepared from procainetreated MC4100 cells to stimulate phosphorylation of wild-type OmpR by wild-type EnvZ. Procaine was used in place of high osmolarity to avoid the growth inhibitory effects of high osmolality and to limit the contribution of cross-talk pathways that are alternative routes of OmpR phosphorylation (30–32).

As OmpR-phosphate is usually lost during complete purification, we attempted a rapid but partial purification to preserve its in vivo phosphorylated state. This involved applying cell extracts to a DEAE-Fastflow column, which was attached to a FPLC chromatography system (Pharmacia). Column fractions eluting from the DEAE matrix between 70 and 80 mM NaCl were immediately frozen at −20°C as 35% glycerol mixtures. In general, we could partially purify OmpR within a few hours after preparing cell extracts with the preparations remaining active for DNA binding for at least a week.

DNA binding of the OmpR preparations was tested by mobility shift experiments using the 314-bp promoter fragment (Fig. 3). OmpR from untreated or procainetreated cells produced protein-DNA complexes identical to those obtained with in vitro phosphorylated OmpR (Fig. 3, lanes 1–6; compare with Fig. 1, lanes 4–8). OmpR from untreated or procainetreated cells produced protein-DNA complexes identical to those obtained with in vitro phosphorylated OmpR (Fig. 3, lanes 1–6; compare with Fig. 1, lanes 4–8). OmpR was partially purified from procainetreated (lanes 1–3) and untreated (lanes 4–6) MC4100 cells, M760 (ompR472) cells (lanes 7–9), and MH1471 (envZ473) cells (lanes 10–13). Samples representing approximately 8 ng (lanes 1, 4, 7, and 11), 16 ng (lanes 2, 5, 8, and 12), or 32 ng (lanes 3, 6, 9, and 13) of OmpR were tested for their relative DNA binding profile using the 314-bp XbaI-BamHI ompF promoter fragment. Lane 10 shows the DNA binding activity of approximately 4 ng of OmpR prepared from strain MH1471. Protein DNA complexes are designated as a, b, b', or c. The b'-complex appears to be a variant of the b-complex and was only observed for the OmpR472 protein.

Previous studies have shown that the OmpR472 mutant binds to the −70 to −100 region but does not bind to the −40 to −60 region (9, 21). Given these results, the appearance of the b'-complex by our OmpR472 preparation would be consistent with an interaction occurring at the −70 to −100 region. Due to technical problems we could not confirm this result by DNease I footprinting using OmpR472 in these preparations (although we verified this by footprinting studies using purified OmpR472 protein (Fig. 5)). Therefore we tested these preparations for DNA binding to an ompF promoter, which had several base changes in the −40 to −60 region.

As shown in Fig. 4, the wild-type OmpR preparation formed both the a- and b-complexes with the wild-type promoter but only produced the b-complex with the DNA having the dis-
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Fig. 4. DNA binding of partially purified forms of OmpR to an ompF promoter having mutations in the -42 to -52 region. Approximately 10 ng of partially purified OmpR from procaine-treated MC4100 cells (lanes 2 and 6), MH760 cells (lanes 3 and 7), and corresponding fractions from an ompR::Tn10 strain TK821 (lanes 4 and 8) were used to form complexes with the XbaI-BamHI ompF promoter fragment (lanes 1-4) or with an XbaI-BamHI ompF promoter fragment having mutations in the -42 to -51 region (lanes 5-8). Protein DNA complexes are designated as in Fig. 3. The wild-type and mutant promoters in the absence of any protein are shown in lanes 1 and 5, respectively.

ruptured C box region. The OmpR472 preparation formed the b' complex with either the wild-type or mutant promoter region. Therefore, these experiments, along with the competition results (Fig. 2), demonstrate that the b- and b'-complexes result from interactions at the -70 to -100 region because (a) they are not affected by mutations at the -42 to -52 region and (b) the complexes can be competed with DNA containing the -70 to -100 region. The a-complex can also be competed with DNA containing the -70 to -100 region, but its formation was dramatically affected by the C box mutant. Therefore we conclude that the a-complex arises from interactions at both the -70 to -100 and -40 to -60 regions.

Another protein-DNA complex designated as a "c-complex" was also present in the OmpR preparations (Fig. 3, lanes 1-9; Fig. 4, lanes 2, 3, 6, and 7). The c-band also appeared when complexes formed between in vitro phosphorylated OmpR and DNA were subjected to limited proteolysis (data not shown). Based on these latter results, it appears that our preparations either contained a proteolyzed form of OmpR still capable of DNA binding or were becoming degraded after complex formation.

DNase I Footprinting of the Upstream Region of the ompF Promoter—An OmpR binding site has recently been found 360 to 380 bp upstream to the ompF transcriptional start site (23). While it is believed to act as a repressor site to prevent ompF transcription, the involvement of phosphorylated OmpR is not understood. To address this question, we carried out DNase I footprinting experiments in which this upstream site was carried within a 470-bp HaeII-PstI ompF promoter fragment of pAR094. This DNA encompasses the region from -470 to +1 and thus contains the -40 to -60 and the -70 to -100 regions as well as the -360 to -380 region. For footprinting experiments, pure wild-type OmpR or OmpR472 protein were phosphorylated in vitro using wild-type EnvZ(C) to form the protein-DNA complexes.

Fig. 5A shows a DNase I footprint of the -40 to -100 region using the ompF promoter fragment labeled at the downstream HindIII site. A broad band of protection was seen in the -40 to -100 region with 150 ng of OmpR. Such protection has been reported previously (9, 21). DNase I footprinting using the same ompF promoter fragment labeled at the upstream BamHI site shows a similar increase in protection of the -360 to -380 regions with an increase in the amount of phosphorylated OmpR (Fig. 5B). Significant protection of this region required 300 ng of OmpR. Although this amount is not much greater than that needed for protection of the -40 to -100 region, panel B of Fig. 5 clearly shows that protection of the downstream sequences occurs before protection of the -360 to -380 region.

The mobility shift experiments described in the previous section showed that the mutant OmpR472 protein was unable to produce the slower migrating a-complex. This was further tested by DNase I footprinting of the ompF promoter using purified OmpR472 phosphorylated in vitro. As with wild-type OmpR, increasing levels of phosphorylated OmpR472 produced increased protection of the -60 to -100 region (Fig. 6A). However, protection of the -60 to -100 region required 400 ng of OmpR472, as compared with 150 ng of wild-type OmpR required for the same protection. In addition, OmpR472 was unable to protect the -40 to -50 region except at 1 µg of OmpR472.
Various amounts of OmpR472, phosphorylated in vitro, Maxam-Gilbert G (lane 1) and G+A (lane 2) reactions show the locations of the protected regions. Various amounts of OmpR472, phosphorylated in vitro as described under “Experimental Procedures,” were incubated with ATP in the absence of EnvZ(C). For wild-type OmpR, protection of the -40 to -50 region was observed at 150 ng of protein. Protection of the -360 to -380 region of ompF also required greater amounts of OmpR472 as compared with wild-type OmpR. Protection of this region occurred with 300 ng of wild-type OmpR. In contrast, 800 ng of OmpR472 was required for the same level of protection.

To determine whether the -360 to -380 region could be bound by OmpR in the absence of the downstream sequences, a fragment of the ompF promoter containing the sequence between -465 to -195 was used for DNase I footprinting as above. No protection was observed when as much as 1 μg of OmpR was used (data not shown). Furthermore, 4-point mutations were created at -363 to -366. DNase I footprints using this promoter and phosphorylated OmpR showed a loss of protection by OmpR to the entire -360 to -380 region (data not shown).

DNase I footprinting was also performed with a promoter containing the 3-point mutations in the Cd box (Fig. 7). Footprint of this mutant promoter showed that only the -58 to -100 region was protected by phosphorylated OmpR. Because the amount of OmpR needed for protection of this region was similar to that observed for the wild-type promoter, the loss of binding to the Cd box does not affect the affinity of OmpR for the -58 to -100 region. Similarly, the -360 to -380 region was also protected in the mutated promoter, although the protection was not as great as that seen in the wild-type ompF promoter. Thus, binding to the -360 to -380 region is also not dependent upon binding to the Cd box. From these results as well as those described above, OmpR binding to the -360 to -380 region is most likely to be dependent upon binding to the -60 to -100 sequence.

Within the -360 to -380 region, there are two 10-base pair

Fig. 6. DNase I footprinting of the downstream and upstream regions of the ompF promoter in the presence of OmpR472 (V203M) phosphorylated in vitro. Maxam-Gilbert G (lane 1) and G+A (lane 2) reactions show the locations of the protected regions. Various amounts of OmpR472, phosphorylated in vitro as described under “Experimental Procedures,” were incubated with ATP in the absence of EnvZ(C).

Fig. 7. DNase I footprinting of the downstream and upstream regions of the ompF promoter containing the mutated Cd sequence in the presence of wild-type OmpR phosphorylated in vitro. Various amounts of wild-type OmpR, phosphorylated in vitro as described under “Experimental Procedures,” were incubated with ATP in the absence of EnvZ(C).
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A

\[
\begin{array}{cccccc}
\text{A} & \text{B} & \text{C} & \text{D} & \text{E} & \text{F} \\
\text{Fe} & \text{Ff} & \text{Fc} & \text{C} & \text{Cd} & \text{Ce} \\
\text{TTTACATT} & \text{TTTACATT} & \text{TATGCCT} & \text{GAATTAC} & \text{TTTACATT} & \text{TTTACATT} \\
\text{TCGACGATT} & \text{TTACATCAG} & \text{AGACATCG} & \text{ATACTCT} & \text{TTTACATT} & \text{TTTACATT} \\
\end{array}
\]

B

| F box consensus: | T box consensus: |
|-----------------|-----------------|
| TTTACATT | TGAA-CAT-* |
| TTTACATT | TGAA-CAT-* |
| TTTACATT | TGAA-CAT-* |
| TTTACATT | TGAA-CAT-* |

**Fig. 8. Upstream sequences of the ompF and ompC promoters.** Panel A shows the locations of the F boxes and C boxes within the ompF and ompC promoters (9, 21). The numbers indicate the DNA region, taking +1 to be the mRNA start site. Panel B shows the homologies of the newly designated Fe and Ff boxes with the previously deduced F box consensus sequence (21). Bases different from the consensus sequence are shown with the lower case letters.

sequences (shown as Fe and Ff in Fig. 8) that have similarities to the sequences previously assigned as F boxes (see Fig. 8). Although we consider sequences of the F boxes to be high affinity binding sites, the low affinity of OmpR for the -360 to -380 region could possibly be due to the presence of only two 10-bp sequences rather than the 3 tandem sequences observed in the -70 to -100 region.

**Promoter Activity of ompF-lacZ Plasmids—**To correlate the in vitro DNA binding and DNaseI footprinting results, ompF-lacZ fusions were created and analyzed on MacConkey plates in various ompB penB80 strains. As shown in Fig. 9, ompF-lacZ plasmids produced phenotypes that depended on (a) the nature of the ompB allele contained in the strain and (b) the amount of ompF promoter DNA contained on plasmids. Three clones, containing between 0.5 and 1.12 kilobase pairs of DNA upstream to the ompF structural gene, were lacZ in an envZ473 strain but lacZ in both wild-type and ompR472 strains. These clones carry the repressor region between -360 and -380 in the ompF gene and thus must actively be preventing lacZ expression in the MH1471 strain. Clones that do not carry the upstream repressor site were lacZ in an MH1471 strain, indicating little repression of lacZ. Our results with plasmids are consistent with studies where ompF-lacZ fusions carried on bacteriophage were used to study ompF repression (18).

The ompF promoter mutant having base changes in the -42 to -52 region was also examined as an ompF-lacZ fusion. This construction still became lacZ in the envZ473 strain, AR135. This would indicate that the mutation does not disrupt either ompF expression or repression. To examine this further we compared β-galactosidase levels in liquid cultures for the AR134 strain transformed with plasmids pAR0102 (wt) and pAR0105 (mutated Cd). Both plasmids produced comparable β-galactosidase activities at low osmolarity (approximately 100 units/ml) and in both cases their levels were equally reduced at high osmolarity conditions (approximately 45 units/ml). These results suggest that the lack of DNA binding to the -42 to -52 region alone may not be sufficient to interfere with repression. In a related experiment, the 4-point mutations in the Ff box were also analyzed as an ompF-lacZ fusion (pAR0108). When AR135 cells were transformed with pAR0108, cells became pink due to a higher expression of the ompF-lacZ fusion. This would indicate that mutations in the Ff box partially disrupt negative regulation of ompF expression.

**DISCUSSION**

OmpR binds to three regions in the ompF promoter for regulated porin gene expression (9, 21). One of these, the -70 to -100 region, is essential for ompF activation, while the -42 to -52 and -360 to -380 regions represent binding sites from which OmpR facilitates negative regulation (9, 17, 21). It is not clear how OmpR controls ompF expression from these sites, but the DNA binding affinity for them is affected by the phosphorylation state of OmpR. In turn, the phospho-OmpR levels are well correlated with phenotypic changes in ompF expression.

In the present analysis, mobility shift assays were used to evaluate protein-DNA complexes formed by phosphorylated OmpR. Our results indicate that OmpR binding to the above target sites occurred in an ordered fashion that required increasing amounts of OmpR-phosphate. Occupancy occurred initially at the activator site between -70 to -100 and proceeded to the -42 to -52 region. DNaseI assays further showed that binding of the -42 to -100 region is followed by binding at the -360 to -380 region. Protein-DNA complexes formed by in vitro phosphorylated OmpR were very similar to those formed by in vivo phosphorylated forms of OmpR. This included the OmpR472 protein as well as OmpR prepared from a putative phosphatase-defective envZ mutant, envZ473.

Progressive binding of regulatory sequences by OmpR may be fundamentally important to the mechanism of ompF regulation. This idea is incorporated into the following model for ompF regulation. When OmpR-phosphate levels are low (such as at low osmolarity conditions) OmpR binds to the activator site at the -70 to -100 region to stimulate ompF expression. Elevation of OmpR-phosphate levels by high osmolarity conditions (12), ompC phosphatase mutants (20), or the presence of local anesthetics (32) facilitates occupancy at the -42 to -52 region with eventual binding at the -360 to -380 region. Filling of the -360 to -380 region then causes ompF repression. This mechanism fits well with the reported changes in phospho-OmpR levels measured in vivo.

OmpR binds to the -70 to -100 region in the absence of other DNA sequences (33). In contrast, binding at the -42 to -52 and
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-360 to -380 does not appear to occur without the -70 to -100 region (this study). We interpret these results as an indication of high and low affinity binding sites although it is not yet clear whether the high affinity site is defined more by its sequence or by the fact that there are three such binding sites juxtaposed to each other (8, 9, 21). In any event it appears that binding to the -70 to -100 region occurs before binding at other sites and thus may be a prerequisite for subsequent interactions on the DNA.

The -42 to -52 region has been correlated with negative regulation of ompF, because an OmpR472 protein fails to bind to this sequence and strains harboring this allele produce OmpR constitutively. While our studies confirm that OmpR472 does not bind the Cd box, we also show that OmpR472 binds poorly to the -360 to -380 sequence. Considering that the Cd mutant promoter was still capable of repressing ompF-lacZ expression, the Cd box is not absolutely required for ompF repression. On the other hand, sequences upstream of -100 have been shown to be essential for ompF repression, and point mutation of the Ff box results in a decrease in ompF-lacZ repression. Thus, the lack of repression by OmpR472 may be more readily explained on the basis of a lost interaction at the -360 to -380 sequence.

The observation that binding at the -360 to -380 region required downstream regulatory sequences suggests that OmpR molecules bound at this region are stabilized by OmpR molecules bound at other sites. Furthermore, binding to this low affinity site did not occur at the expense of the other low affinity -42 to -52 region. If the -70 to -100 region acts as a high affinity site then OmpR molecules bound to this region may be capable of multiple interactions with those bound at the low affinity regions. We envision that stabilization occurs through OmpR-OmpR interactions, because previous studies indicate that OmpR can form stable dimers upon phosphorylation (34). In this regard the ompF promoter has been shown to be inherently “bent” (35) and to have binding sites for integration host factor, which affects curvature of the ompF promoter (23, 36). OmpR-OmpR interactions might be greatly enhanced by DNA topology and factors that bring the bound OmpR molecules closer to each other. A putative DNA looping has been proposed to explain how the distantly located -360 to -380 region could regulate ompF expression. Evidence for long range interactions, together with the recognition of DNA bending at the ompF promoter, are consistent with this loop model.

DNA looping is important for regulated transcription of a number of prokaryotic systems and has been well characterized for the araCBAD operon (reviewed in Refs. 37 and 38). In this system, the araO2 site, 200 base pairs upstream of the promoter, is important for self-repression by the AraC protein. Helical twist experiments show that looping provides interactions between the araO2 site and the araI1 site, which is adjacent to the promoter to effect repression. Deletion of the araO1 and araI1 sites decreases the ability of AraC to bind to the araO2 site, indicating cooperative interaction. Such a model is certainly implicated by our studies, and experiments are currently underway to evaluate this mechanism for the regulation of ompF expression.

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FIG. 9. Phenotypic expression of ompF-lacZ fusions in ompB, pcnB strains. Various ompF-lacZ fusions having the same 3' BamHI sites were created. The clones differ with respect to the amount of additional ompF promoter sequence carried at the 5' end. Clones were used to transform AR134 (MC4100, pcnB80), AR135 (MH1471, pcnBBU), or AR136 (MH760, pcnB80) to ampicillin resistance. Transformants were grown in selective media overnight and then streaked on MacConkey ampicillin plates. After 12 h, colony color was judged as red (lac+), pink (lac+/-), or white (lac-). The line diagram represents a summary of relevant restriction enzyme sites between the 3' end of the asuS gene and ompF. In this diagram the ompF structural gene is replaced with the structural gene for β-galactosidase (lacZ). The OmpR DNA binding sites are shown. Relevant restriction sites are EcoRI (E), NruI (N), HaeII (H), Alul (A), SvaI (S), PstI (P), XbaI (X), and BamHI (B). The latter two sites were created by recombinant DNA methods.
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