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Mutations in a Central Highly Conserved Non-DNA-Binding Region of OmpR, an *Escherichia coli* Transcriptional Activator, Influence Its DNA-Binding Ability

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OmpR is a transcriptional activator for the expression of outer membrane porin genes *ompF* and *ompC* in *Escherichia coli*. Its C-terminal half has been identified as the DNA-binding domain (K. Tsung, R. Brissette, and M. Inouye, J. Biol. Chem. 264:10104–10109, 1989). Recent studies have indicated that the N-terminal non-DNA-binding domain of OmpR is involved in modulating OmpR function through interaction with the EnvZ protein, a kinase and phosphatase for OmpR. We isolated and characterized two mutations, G94D and E111K, in the N-terminal domain of OmpR and one mutation, R182C, in the DNA-binding domain of OmpR. All three mutations abolished the ability of OmpR to bind to the *ompF* and *ompC* promoters in vivo, thus giving an OmpF$^-$ OmpC$^-$ phenotype. The decreased DNA-binding ability of the mutant OmpRs was not due to diminished phosphorylation of their N termini, since all the mutant OmpRs were found to be normally phosphorylated by EnvZ in vitro. The mutant OmpRs produced from multicopy plasmids were also found to inhibit completely the production of OmpF and OmpC in wild-type cells, and the complete inhibition depended on the function of EnvZ which was produced in *cis* or in *trans* from plasmids. The relationship of the possible alterations in OmpR by the mutations with the observed diminished binding ability is discussed.

The major outer membrane proteins of *Escherichia coli*, OmpF and OmpC, are reciprocally produced in response to the osmolarities of the culture media (29). The OmpF protein is produced at low osmolarity, while the OmpC protein is produced to a greater extent in high osmolarity. Mutational studies have identified a separate locus, *ompB*, which was found to alter osmoregulation patterns and affect the expression at the transcriptional level of both *ompF* and *ompC* (11). The *ompB* locus encodes two proteins, OmpR and EnvZ. OmpR is a cytoplasmic protein which functions as a transcriptional activator by binding to the promoter regions of both the *ompF* and the *ompC* genes (16, 20). The OmpR sequence responsible for DNA binding has been localized to the C-terminal portion of the protein and consists of 117 amino acid residues (27). The EnvZ protein, on the other hand, is an inner transmembrane protein with a topology similar to that of the chemotactic sensory receptors (8). In the current model for osmoregulation, EnvZ is an osmosensor which relays signals to OmpR through covalent modification. OmpR, in turn, functions as a transcriptional activator for both *ompF* and *ompC*. The EnvZ-OmpR protein pair is a member of a family of homologous sensory and regulatory proteins in bacteria (for a review, see reference 24). The EnvZ-like sensory proteins share sequence homologies at their C-terminal regions, while the OmpR-like regulatory proteins share sequence homologies at their N-terminal portions. It has been demonstrated that members of the sensory protein class are able to undergo autophosphorylation and then transfer the phosphate group to the corresponding regulator proteins. The protein pairs CheA-CheY, EnvZ-OmpR, and NtrB-NtrC have been shown in vitro to undergo a phosphotransfer reaction (25). In the case of the CheA-CheY protein pair, CheA undergoes autophosphorylation at His-48 (13) and in turn transfers the phosphate to CheY at Asp-57 (22). When OmpR was phosphorylated by EnvZ, the protein showed an elevated ability to activate transcription from the *ompF* and *ompC* promoters in vitro (1, 14). This enhancement of transcription correlates to an increased binding affinity of phosphorylated OmpR to the *ompF* and *ompC* promoters (3).

Until recently, mutations in OmpR that abolish or reduce OmpR binding to the *ompF* and/or *ompC* promoters are either in the C-terminal DNA-binding domain of OmpR (17) or, as we recently reported (4), at the putative phosphorylation center formed by Asp-11, Asp-12, and Asp-55 in the N-terminal domain of OmpR. In this study we have isolated and characterized three additional mutations in OmpR. Two of the mutations, G94D and E111K, are in the N-terminal domain of OmpR in a region highly conserved among the class of regulator proteins. The third mutation, R182C, is in the C-terminal DNA-binding domain of OmpR. All three mutations resulted in a loss of the ability of OmpR to activate the expression of the *ompF* and *ompC* genes in vivo, and this loss of activation by OmpR correlated with a loss of the mutant OmpRs in the ability to bind to the *ompF* and *ompC* promoters, as revealed by in vivo DNA footprinting. The mutant OmpRs, however, were able to undergo phosphorylation by EnvZ, in a similar manner as wild-type OmpR. Furthermore, these mutant OmpRs, produced from multicopy plasmids, were found to inhibit the expression of the *ompF* and *ompC* in cells producing wild-type OmpR from the chromosome. The results indicate that all three mutations probably resulted in defective C-terminal DNA-binding domains. The G94D and E111K substitutions in the N terminus may elicit a structural change in the C-terminal domain of OmpR. The R182C amino acid substitution, on the other hand, is likely to exert its inactivation effect directly at the site of the mutation.

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TABLE 1. Bacterial strains used

Strain	Genotype	Phenotype	Reference
MC4100	F⁻ Δ(*lac*)*U169 araD139 rpsL relA thiA flbB*	OmpF⁺ OmpC⁺	6
SG480Δ76	MC4100 Δ(*ompB bioH gntM malPQ malT*)	OmpF⁻ OmpC⁻	10
MH513	MC4100 *ara*⁺ Φ(*ompF-lacZ*⁺)*16–13*	OmpF⁻ OmpC⁺	12
MH760	MC4100 *ompB472*	OmpF⁺ OmpC⁻	12

MATERIALS AND METHODS

Strains and plasmids. Plasmids carrying mutations in the *ompB* locus are derived from plasmid pKL428. Plasmid pKL428 was constructed by inserting a 3-kb *Sma*I-*Hin*dIII fragment containing the *ompB* locus into the *Nru*I and *Hin*dIII sites of plasmid pACYC184 (21). Plasmids containing the *ompF* and *ompC* promoters used in in vivo DNA footprinting were previously described (27). Plasmid pKL0533 carrying an *ompF-lacZ* gene fusion has been described (27). The plasmid pRB003, which encodes a Tar-EnvZ fusion protein, Taz-1, has been described (28). *E. coli* strains used in this study are listed in Table 1.

Selection of OmpR mutations by in vitro mutagenesis. Hydroxylamine mutagenesis was carried out on cesium chloride-purified plasmid pKL428 containing the wild-type *ompB* locus essentially as described previously (5). Mutagenized plasmid DNA was then transformed into strain MH760 (*ompR2* OmpF⁺ OmpC⁻) which also harbored a compatible plasmid, pKL0533, carrying an *ompF-lacZ* gene fusion and the cells were plated on MacConkey plates to screen Lac⁻ cells. Plasmid DNA from the Lac⁻ colonies was isolated and transformed again to strain MH760 carrying plasmid pKL0533. In this way, three negative mutants carried on plasmids were isolated. Subsequent swapping of DNA fragments between the wild-type *ompB* plasmid, pKL428, and the mutant *ompB* plasmids revealed that the three mutations were located in the *ompR* gene. DNA sequencing by using the dideoxy method was carried out for the entire *ompR* gene in the three mutants (23).

Other methods. Analysis of outer membrane proteins (7), in vivo footprinting on plasmid DNA (27), and in vitro phosphorylation of OmpR (4) were performed as previously described.

RESULTS

Screening of OmpR mutations by in vitro chemical mutagenesis. Our initial intention for this study was to isolate mutant OmpR proteins which were capable of binding DNA but incapable of activating transcription. For this, plasmid pKL428 containing the *ompB* wild-type sequence (*ompR*⁺ *envZ*⁺) was treated with hydroxylamine in vitro (see Materials and Methods) and transformed to the *ompR2* mutant strain, MH760, harboring a pKL428 compatible plasmid, pKL0533, which carries an *ompF-lacZ* gene fusion. The *ompR2* strain produces a mutant OmpR which binds only to the *ompF* promoter weakly and thus has an OmpF⁺ OmpC⁻ phenotype. Dominant negative mutant OmpRs produced from the mutagenized plasmid could turn the Lac⁺ phenotype to Lac⁻. The transformants were selected on MacConkey plates. Three dominant negative OmpR mutations were thus selected. The regions of the mutations in the *ompR* gene were first identified by exchanging fragments with the wild-type plasmid and finally confirmed by DNA sequencing. Figure 1 is an illustration of the OmpR protein and shows the location of the three mutations isolated in this study. One mutation, located in the DNA-binding domain of OmpR, was found to be a C-to-T change at base position 544 (7), and as a result the amino acid at position 182 is changed from Arg to Cys. The other two mutations were found in the N-terminal half of OmpR. The first mutation is a G-to-A change in the DNA sequence at position 281, and as a result amino acid 94 is changed from Gly to Asp. The second mutation, on the other hand, is caused by a G-to-A change at position 331 and results in a Glu-to-Lys change at amino acid position 111.

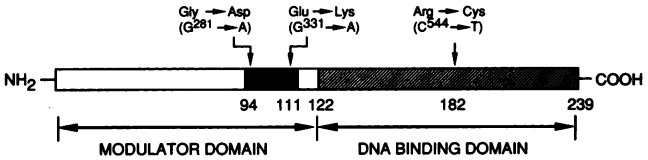

FIG. 1. Illustration of the domain structure of the OmpR protein showing the location of the amino acid substitutions of the mutant OmpRs. The hatched bar represents the DNA-binding domain previously identified (27). The solid bar represents the presumed modulator domain. The darkened area represents the central highly conserved sequence among the family of OmpR-like regulator proteins (25). The specific base substitution and the corresponding amino acid substitution is shown for each mutant.

The phenotype of the mutant OmpRs. The plasmids encoding the mutant OmpRs, R182C, G94D, and E111K and the parent wild-type plasmid, pKL428, as well as the vector control plasmid, pACYC184 were transformed into the *ompB* deletion strain, SG480Δ76 (Δ*ompR* Δ*envZ*), which has an OmpF⁻ OmpC⁻ phenotype. Outer membrane proteins were prepared from transformants grown in L-broth medium supplemented with chloramphenicol. The outer membrane proteins were isolated and analyzed on an urea-sodium dodecyl sulfate (SDS)-polyacrylamide gel (Fig. 2). In cells harboring the vector pACYC184, no production of OmpF and OmpC was observed. The wild-type *ompB* plasmid, pKL428, complemented the OmpF⁻ OmpC⁻ phenotype as expected (Fig. 2, lane OmpR). The three mutant OmpRs, however, were not able to support expression of OmpF and OmpC. Because the three mutant OmpRs were originally identified in cells encoding another mutant OmpR [MH760 (*ompR2* OmpF⁺ OmpC⁻)], we next examined whether the

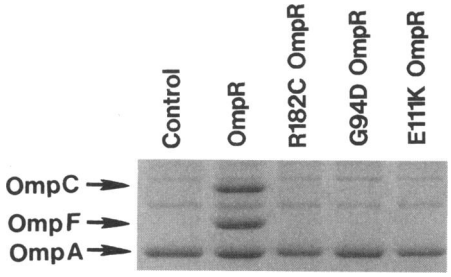

FIG. 2. Outer membrane protein profiles of an *ompB* deletion strain harboring various plasmids. *E. coli* SG480Δ76, transformed with various *ompB* plasmids, was grown in L-broth medium. The outer membrane porin proteins were isolated and analyzed on an urea-SDS-polyacrylamide gel and stained with Coomassie blue. Positions of the porin proteins are indicated. In the control lane, cells harbored the vector pACYC184.

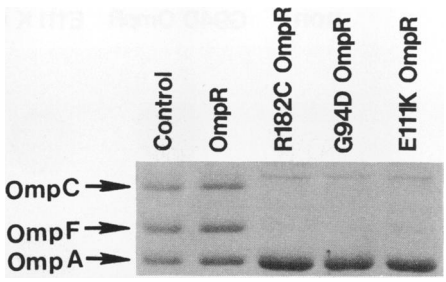

FIG. 3. Outer membrane proteins profiles of the wild-type *ompB* strain (MC4100) harboring various *ompB* plasmids. Gel conditions are the same as described in the legend to Fig. 2.

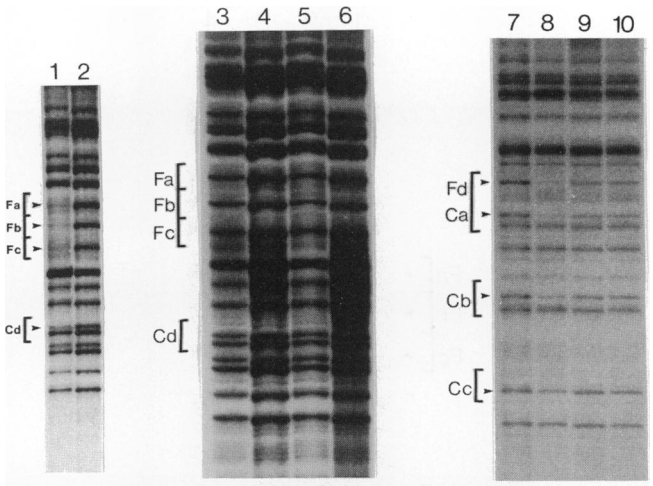

FIG. 4. In vivo methylation protection patterns of the *ompF* and *ompC* promoter regions in an *ompB* deletion strain. *E. coli* SG480Δ76 cells carrying either a plasmid containing the *ompF* (lanes 1 to 6) or *ompC* (lanes 7 to 10) promoter regions were grown in L-broth medium. Footprinting was carried out as described in Materials and Methods. Cells were doubly transformed with pACYC184 (lanes 2, 3, and 7) and with plasmids encoding wild-type OmpR and EnvZ (lanes 1 and 8), R182C OmpR and EnvZ (lane 4), G94D OmpR and EnvZ (lanes 5 and 9), and E111K OmpR and EnvZ (lanes 6 and 10). The arrows indicate the sites protected from methylation. Open brackets indicate the DNA sequence motifs recognized by OmpR in vivo as previously characterized (27).

three mutants would generate a dominant negative phenotype in cells encoding wild-type OmpR. As shown in Fig. 3, when the wild-type *ompB* plasmid (lane OmpR) was transformed into wild-type cells, production of OmpF and OmpC was identical to that in the case of the control plasmid, pACYC184. The mutant OmpR proteins, on the other hand, completely inhibited production of OmpF and OmpC in the wild-type strain.

DNA-binding properties of the mutant OmpRs. Using an in vivo DNA footprinting technique, we have shown in a previous study that wild-type OmpR binds to the promoter regions of *ompF* and *ompC* genes in vivo (27) and that the 117-amino-acid C-terminal domain of OmpR binds to the *ompF* and *ompC* promoters. In the present study we applied this in vivo DNA footprinting assay to examine the DNA-binding properties of the mutant OmpRs. Because the R182C OmpR mutation was located in the DNA-binding domain (Fig. 1), it was predicted that this mutant OmpR would not be able to bind to DNA, thus giving the $OmpF^-$ $OmpC^-$ phenotype. However, we had expected that the G94D and E111K OmpR mutants would still bind DNA normally, and the failure of these two mutant OmpRs to activate porin expression could have been caused by the loss of their activation function. The *ompB* plasmids containing the mutant *ompR* genes were transformed into the *ompB* deletion strain (SG480Δ76) containing compatible plasmids carrying either the *ompF* or the *ompC* promoter sequence (27). The double transformants were grown in L broth and treated with dimethyl sulfate. The respective promoter fragments were isolated, 5′ end labeled with [γ-^{32}P]ATP, cleaved at the methylation sites, and analyzed on DNA sequencing gels. In Fig. 4, lanes 1 to 6 and lanes 7 to 10 are the methylation protection patterns resulting from the *ompF* and *ompC* promoter regions, respectively. Wild-type OmpR protected the residues in the promoter regions as described previously (Fig. 4, motifs Fa, Fb, Fc, and Cd in lane 1 and motifs Fd, Ca, Cb, and Cc in lane 8) (27). As expected, the R182C OmpR lost its ability to bind to both the *ompF* (Fig. 4, lane 4) and *ompC* (data not shown) promoters. Contrary to our prediction in the case of the G94D and E111K OmpRs, both also showed a dramatic reduction in binding affinity for the *ompC* promoter (Fig. 4, lanes 9 and 10, respectively) and a total loss of binding to the *ompF* promoter (Fig. 4, lanes 5 and 6, respectively). Because the two mutations in the G94D and E111K OmpRs are in a region outside of the previously identified DNA-binding domain (27) (Fig. 1), these results indicate that the mutations indirectly affected the DNA-binding activity. The loss of the DNA-binding function for the G94D and E111K OmpRs could explain their null phenotype ($OmpF^-$ $OmpC^-$) in the *ompB* deletion strain (Fig. 2), but it raised the question concerning the mechanism by which these mutants exert the dominant negative phenotype in *ompB* wild-type cells (Fig. 3). To investigate this question further, the DNA footprinting experiment was also carried out with wild-type cells ($ompB^+$) harboring wild-type *ompB* or either of the *ompB* mutants together with the *ompF-lacZ* fusion plasmid (pKL0533) (Fig. 5). In cells harboring the vector plasmid, pACYC184 (Fig. 5, lane 1), protection by the wild-type OmpR produced from the chromosome was observed. The same pattern of methylation protection was observed when the cells carried the wild-type *ompB* plasmid, pKL428 (Fig. 5, lane 2). With the wild-type cells producing the mutant R182C (Fig. 5, lane 3) and E111K OmpRs (Fig. 5, lane 4), however, little, if any, protection of the *ompF* promoter was observed.

Requirement of EnvZ function for the maximum inhibitory effect of the mutant OmpRs. During the course of experiments described above, we found that when the *envZ* gene was removed from the mutant plasmid, the dominant negative effect of the mutant *ompR* gene became less severe. This phenomenon was more clearly demonstrated when the mutant *ompR* genes, encoding the G94D and E111K OmpRs, were introduced into a wild-type cell containing an *ompF-lacZ* fusion on the chromosome (MH513). Figure 6 shows the EnvZ effect on the N-terminal mutant OmpR inhibition of porin expression in wild-type (MH513) cells. MH513 cells without the mutant OmpRs gave a Lac^+ phenotype as monitored on MacConkey indicator plates (Fig. 6, dark spot none/none). With plasmids containing only the mutant *ompR* genes, the cells also gave a Lac^+ phenotype (Fig. 6, dark spots none/G94D OmpR and none/E111K OmpR) even though the expression levels of *ompF-lacZ* in these cells were only less than 20% of the wild-type level (data not shown). On the other hand, when the wild-type *envZ* gene

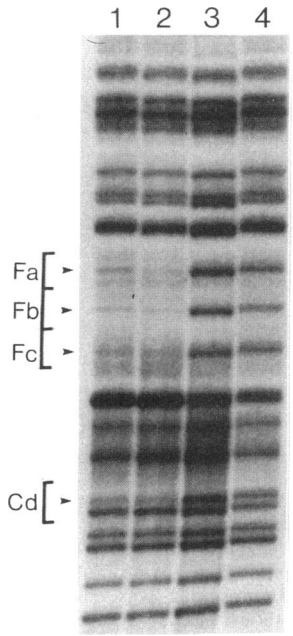

FIG. 5. In vivo methylation protection pattern of the *ompF* promoter region in the wild-type *ompB* strain. *E. coli* MC4100 cells carrying a plasmid containing the *ompF* promoter region were grown in L-broth medium. Footprinting was carried out as described in Materials and Methods. Cells were doubly transformed with pACYC184 (lane 1) and with plasmids encoding wild-type OmpR and EnvZ (lane 2), R182C OmpR and EnvZ (lane 3), and E111K OmpR and EnvZ (lane 4).

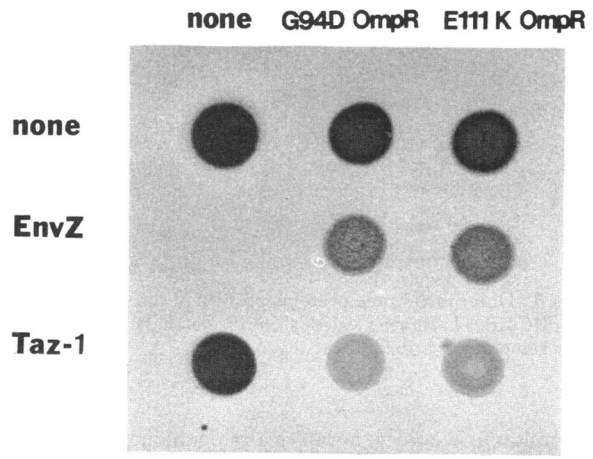

FIG. 6. In vivo analysis of *ompF* promoter activity in wild-type *ompB* cells. *E. coli* MH513 (*ompR*$^+$ *envZ*$^+$), which carries an *ompF-lacZ* chromosomal fusion, was transformed with various plasmids and grown in L-broth medium to log phase, and then aliquots of the culture were spotted on MacConkey indicator plates as shown. Cells transformed with pBR322 and pACYC184 (none/none) and cells harboring plasmids producing only the mutant OmpRs (none/G94D OmpR and none/E111K OmpR), both mutant OmpRs and wild-type EnvZ from the same plasmid (EnvZ/G94D OmpR and EnvZ/E111K OmpR), the Tar-EnvZ hybrid protein only (Taz-1/none), and mutant OmpRs and the Tar-EnvZ hybrid protein from different plasmids (Taz-1/G94D OmpR and Taz-1/E111K OmpR) are indicated.

was placed downstream of the mutant *ompR* genes as in the natural arrangement, the mutant plasmids caused a Lac$^-$ phenotype in wild-type cells (Fig. 6, light spots EnvZ/G94D OmpR and EnvZ/E111K OmpR). This complete inhibition could also be achieved by the C-terminal portion of EnvZ provided by the Tar-EnvZ hybrid protein produced in *trans* from another plasmid (Fig. 6, light spots Taz-1/G94D OmpR and Taz-1/E111K OmpR). As a control, the Tar-EnvZ hybrid (Taz-1) alone did not cause any inhibition (Fig. 6, dark spot Taz-1/none). Taz-1 has recently been shown to be able to complement an *envZ* deletion mutant (28). The EnvZ sequence in this hybrid protein is derived from the C-terminal EnvZ sequence consisting of 229 amino acid residues. This region is known to modify OmpR through a phosphate transfer reaction (2, 9, 14). Under the same conditions, the R182C OmpR showed a result similar to that shown here with the mutants G94D and E111K OmpRs (data not shown).

In vitro phosphorylation of the wild-type and mutant OmpRs by EnvZ. Recent in vitro studies have shown that EnvZ autophosphorylates and then transfers the phosphate group to OmpR (2, 9, 14). In turn, phosphorylated OmpR is able to enhance transcriptional activity from the *ompF* and *ompC* promoters (1, 14), most likely because of the increased binding ability of phosphorylated OmpR to the promoters (3). The result shown in Fig. 6 suggests that the mutant OmpRs are still able to interact with EnvZ. To verify this, we carried out an in vitro phosphorylation experiment as shown in Fig. 7. Membrane proteins containing EnvZ were isolated, labeled with [γ-^{32}P]ATP in vitro, and subsequently used to phosphorylate equal amounts of OmpRs contained in cell extracts prepared from *ompB* deletion (SG480Δ76) cells carrying various *ompB* plasmids. The R182C and E111K OmpRs (Fig. 7, lanes 4 and 6, respectively) were phosphorylated by EnvZ to the same extent as wild-type OmpR (Fig. 7, lane 3). On the other hand, G94D OmpR seemed less phosphorylated than the other OmpRs. It is not clear whether this is due to less phosphorylation or to a faster dephosphorylation of this mutant protein. It should be pointed out that the slightly slower mobility of the G94D OmpR has been confirmed by an antibody-blotting (Western immunoblot) assay (data not shown).

DISCUSSION

In this study we isolated three dominant negative *ompR* mutations which showed an OmpF$^-$ OmpC$^-$ phenotype.

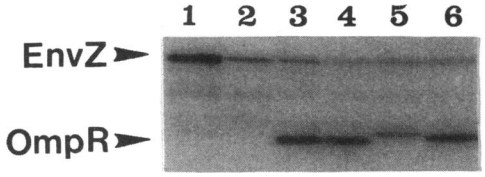

FIG. 7. In vitro phosphorylation of the mutant OmpRs. Shown is an autoradiogram after SDS-polyacrylamide gel electrophoresis of the in vitro phosphorylation experiment involving transfer of phosphate from the EnvZ-containing membrane fraction to OmpR-containing soluble cell extracts. A 7-μl volume of the phosphorylated EnvZ-containing membrane fraction and 3 μl of the soluble cell extract were mixed, and the mixture was incubated at 25°C for 2 min. The entire reaction mixture was applied to each lane. Soluble extracts, prepared from *ompB* deletion cells (*E. coli* SG480Δ76) harboring plasmid pACYC184 (lane 2) or plasmids producing wild-type OmpR (lane 3), R182C OmpR (lane 4), G94D OmpR (lane 5), and E111K OmpR (lane 6) or an equal volume of phosphate buffer (20 mM, pH 7.0) (lane 1) was added to ^{32}P-labeled EnvZ-containing membranes.

One of the mutant proteins, R182C OmpR, contained an amino acid substitution in the DNA-binding domain of OmpR. The other two mutant proteins, G94D and E111K OmpR, have amino acid substitutions in the N-terminal (non-DNA-binding) domain of OmpR. Examination of the DNA-binding characteristics of all three mutants revealed that they were incapable of binding to the OmpR-binding motifs previously determined for the upstream regions of the *ompF* and *ompC* promoters (27). Despite the lack of DNA-binding ability in these three mutant OmpRs, they all exerted a complete inhibition of OmpF and OmpC production in *ompB* wild-type cells, and this inhibition is EnvZ dependent.

OmpR mutations which affect DNA binding are usually found in the C-terminal DNA-binding domain (17), although early studies indicated that the N-terminal domain of OmpR also played a role in DNA binding. It was found that the DNA-binding affinity seemed lower for truncated C-terminal OmpRs lacking the N-terminal domain both in vivo (27) and in vitro (26). Recently, we characterized one class of N-terminal OmpR mutations which also affect DNA binding. This class of N-terminal OmpR mutations consists of amino acid substitution mutations at the Asp residues (D11, D12, and D55) which are considered to form an acidic pocket required for OmpR phosphorylation (4). Since phosphorylation of OmpR was shown to increase its DNA-binding activity (3), mutations at the phosphorylation site of OmpR were expected and were found to affect DNA binding. Phosphorylation of the N-terminal domain of OmpR probably promotes OmpR-OmpR interaction as suggested in a recent report (18). OmpR-OmpR interaction would provide for cooperative binding of the protein to the multiple, in-phase-aligned OmpR-binding motifs in the *ompF* and *ompC* promoter regions (27). On the basis of all the previous studies, it is expected that a mutation in the N-terminal domain of OmpR could abolish DNA binding either by abolishing OmpR phosphorylation or by abolishing OmpR-OmpR interaction. Neither of these two seems to explain the loss of DNA binding by the G94D and E111K OmpRs.

Our results showed that both the G94D and the E111K OmpR were able to interact with EnvZ both in vivo (Fig. 6) and in vitro (Fig. 7) (in which the mutants underwent phosphorylation similar to that of wild-type OmpR). In addition, the dominant negative phenotype of these mutations (Fig. 3) suggests that phosphorylated mutant OmpRs sequester the wild-type OmpR molecule, probably by forming a heterodimer (or oligomer), thus preventing its functioning as a transcriptional activator. Currently, we cannot determine the fate of wild-type OmpR in such a complex with the mutant OmpRs, i.e., whether the wild-type OmpR is degraded. Alternatively, it is possible that the mutant OmpRs compete with wild-type OmpR for limiting factors such as EnvZ, so that wild-type OmpR cannot be phosphorylated. The latter is unlikely, however, since EnvZ was required for complete inhibition rather than releasing inhibition (Fig. 6). It is also possible that the production of wild-type OmpR from the chromosome is inhibited in cells producing high levels of the mutant OmpRs. In this regard, we did not observe any inhibitory effect of the mutant *ompB* plasmids on chromosomal *ompR-lacZ* expression (data not shown), indicating that the chromosomal *ompR* gene is normally expressed in the presence of the mutant OmpRs. Dominance of mutant OmpRs produced from multicopy plasmids over chromosomal wild-type OmpR has been observed previously. One N-terminal dominant negative OmpR mutant which is similar to the OmpR mutants in this study in terms of phenotype and the location of the mutation has been described (15). Whether the biochemical properties of this mutant OmpR are similar to those of the mutants in this study is not known. Otherwise, the only known OmpR mutations which are both dominant to wild-type OmpR and render the protein incapable of binding DNA have been located in the DNA-binding domain of OmpR (17, 19). Given that the *ompR* mutations in the present study are clearly outside of the DNA-binding domain and in light of the above evidence, the mutations must have either affected the activation function of the C-terminal domain by the N-terminal domain or the conformation of the DNA-binding domain. It is interesting that we have isolated a different mutation at amino acid residue 94 (G94S) in a previous study (5). The G94S substitution was found to suppress a defect in DNA binding of an OmpR phosphorylation site mutation (D55Q) in an EnvZ-independent manner. OmpR containing the G94S mutation alone showed a wild-type phenotype. The G94 position seems to play an essential role in the DNA-binding activity of OmpR, and mutations at this position exert both positive and negative effects on *ompF* and *ompC* expression. Both the G94 and the E111 residues lie within a highly conserved region among OmpR-like regulatory proteins (Fig. 1, black bar) (25). It is conceivable that this subdomain may have a common function among these DNA-binding regulatory proteins, possibly by playing a key role in communicating a conformational change to the C terminus of OmpR by the N-terminal domain.

During the preparation of this paper, another study reporting two similar OmpR mutations (E96A and R115S) to the G94D and E111K substitutions has been published (18). The isolation of the E96A and R111S OmpRs was not based on the selection of *ompF* or *ompC* expression. Nevertheless, the isolated mutant *ompR* genes gave an $OmpF^-$ $OmpC^-$ phenotype, and the mutant OmpRs were able to interact with EnvZ as judged by an in vitro phosphorylation and dephosphorylation test, although phosphorylation of the mutant OmpRs failed to increase DNA-binding ability, findings which were similar to the results of the present study for the G94D and E111K OmpRs. On the basis of an in vitro oligomeric cross-linking assay, the E96A and R115S substitutions were shown to affect OmpR-OmpR interaction, therefore leading to loss of ability to bind DNA in vivo. Both the E94A and R115S substitutions, coded in a single-copy plasmid, showed a codominant phenotype with the wild-type *ompR* on the chromosome that was somewhat similar to the present G94D and E111K mutations, and the authors acknowledge that it is difficult to explain the codominant phenotype on the basis of their model. In an attempt to confirm our in vivo observation on the mutant OmpR-mediated inhibition, we have purified all three mutant OmpRs and performed in vitro DNA-binding experiments. We found that the mutant OmpRs failed to bind to the *ompF* promoter, but we could not observe clear inhibition of wild-type OmpR binding to DNA by the mutant OmpRs at molar ratios as high as 10:1 (data not shown).

The mechanism by which the N-terminal domain of OmpR modulates the DNA-binding activity of the C-terminal domain remains an interesting challenge to further studies. Elucidation of the function of the highly conserved region where the G94D, E111K, E96A, and R115S mutations lie will be important for our understanding of OmpR function in osmoregulation of the *ompF* and *ompC* genes.

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