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Molecular Basis for Promoter Selectivity of the Transcriptional Activator OmpR of *Escherichia coli*: Isolation of Mutants That Can Activate the Non-Cognate *kdpABC* Promoter

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Escherichia coli **has two osmo-responsive two-component regulatory systems, the EnvZ-OmpR and KdpD-KdpE systems, each of which consists of a sensor histidine protein kinase and a response regulator. The OmpR and KdpE response regulators belong to the same family of DNA-binding proteins, and act as positive transcriptional factors in response to the medium osmolarity. However, OmpR specifically activates the *ompC* gene encoding the OmpC outer membrane protein, whereas KdpE exclusively activates the *kdpABC* operon encoding the high-affinity Kdp potassium-transporter. To gain insight into the molecular basis for such strict promoter selectivity, we isolated OmpR mutants that can activate the non-cognate *kdpABC* promoter *in vivo*. For these OmpR mutants, it was found that a few common and crucial amino acids are responsible for the altered property of OmpR (*e.g.*, Gly-164, Glu-193). *In vitro* properties of these OmpR mutants were further examined by means of DNA-binding assays and DNA-footprinting analyses with reference to the *kdpABC* promoter. These results were interpreted on the basis of the three-dimensional structure of the C-terminal half of OmpR, which consists of a DNA-binding helix-turn-helix motif and a RNA polymerase-interacting surface. The results of this study were best explained by assuming that the isolated OmpR mutants have an altered property with regard to the interaction with RNA polymerase on the *kdpABC* promoter. We propose that the promoter selectivity of OmpR is determined not only by its DNA-binding specificity, but also by the spatial configuration of the promoter on which OmpR must properly associate with RNA polymerase.**

Key words: *E. coli*, promoter selectivity, signal transduction, transcription factor, two-component system.

In both prokaryotes and eukaryotes, a common signal transduction mechanism, generally referred to as the "two-component system," is involved in a wide variety of cellular responses to environmental stimuli (*1*, *2*). The osmo-regulatory EnvZ-OmpR system is one of the well-known two-component systems in *Escherichia coli* (*3*), and OmpR is one of the best-characterized bacterial transcriptional regulators (or response regulators) (*4*, *5*), the function of which is modulated through phosphorylation (*6–8*). OmpR contains a common phospho-accepting receiver domain of approximately 120 amino acids at the N-terminal portion, followed by a C-terminal DNA-binding domain of approximately 120 amino acids (*9*, *10*). It is believed that OmpR directly associates with RNA polymerase and acts as a positive regulator (*11–14*). Notably, an inspection of the entire *E. coli* genome sequence revealed that this bacterium has 14 response regulators belonging to the OmpR family, the amino acids sequences of which show extensive similarities to each other in their C-terminal DNA-binding domains as well as their N-terminal receiver domains (*15*, *16*). In addition to OmpR, the family includes ArcA, BasR, BaeR, CopR, CreB, CpxR, CusR, KdpE, PhoP. PhoB, QseB, RstA, and TorR (*17*). Occurrence of OmpR family members has frequently been reported for many other bacterial species (*18*, *19*). These facts indicate that OmpR-like proteins are among the most widespread DNA-binding transcriptional regulators in bacteria.

Expression of the major outer membrane proteins OmpC and OmpF is regulated in response to the medium osmolarity (*3*). The EnvZ-OmpR two-component system is crucially involved in this osmo-regulation in *E. coli* (*2*, *20*, *21*). EnvZ is a membrane-located osmo-sensor that exhibits OmpR-specific histidine kinase activity (*6–8*, *22*), whereas phosphorylated-OmpR specifically binds to both the *ompC* and *ompF* promoters (*23*, *24*). As a result, OmpR specifically triggers the transcription from both the promoters in a manner dependent on the medium osmolarity (*25–32*).

A second osmo-responsive two-component system in *E. coli* consists of KdpD and KdpE (*33–36*). This two-component system is involved in the osmo-regulation of the *kdpABC* operon, which encodes a high-affinity potassium transport system (*35*, *36*). KdpD is also a membrane-located sensor kinase. It specifically phosphorylates KdpE, which acts as a positive transcription factor for the *kdpABC* operon (*37*, *38*).

In short, the EnvZ-OmpR and KdpD-KdpE two-component systems both respond to medium osmolarity. Furthermore, KdpE belongs to the OmpR family of transcrip-

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tion factors. However, OmpR specifically activates the *ompC* gene, whereas KdpE exclusively activates the *kdpABC* operon. The close structural similarity of OmpR and KdpE raises the question of how each of these analogous response regulators selectively activates its cognate target gene under the high osmolarity conditions. In general, this issue of promoter selectivity may be simply explained by assuming that OmpR binds to a specific target DNA sequence in the *ompC* promoter (*25–29*, *31*), while KdpE binds exclusively to a specific DNA sequence in the *kdpABC* promoter (*36*). However, is this only the mechanism underlying the promoter selectivity of OmpR and KdpE? Is it possible to generate OmpR mutants that are capable of activating the non-cognate *kdpABC* promoter? In this study, we attempted to address these issues by isolating certain OmpR mutants that can trigger the transcription from the non-cognate *kdpABC* promoter. The results of molecular characterization of such OmpR mutants suggest a new aspect of the mechanism underlying promoter selectivity among the OmpR family of response regulators.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The following *Esherichia coli* strains were mainly used. NKC002 (Δ*ompR*/*envZ*, *ompC-lacZ*$^+$) and HAK007 (Δ*kdpE*/*kdpD*, Δ*ompR*/*envZ*, *kdpA-lacZ*$^+$) are derivatives of CSH26 (F$^-$, Δ*lac*/*pro*, *ara, thi*) (*12*, *35*, *37*). Cells were grown in TY-medium, comprising 10 g of Bacto-tryptone (Difco) and 5 g of Bacto-yeast extract (Difco) per liter of distilled water. When required, 0.6 M NaCl (for high osmolarity medium), chloramphenicol (30 μg per ml) (for plasmid selection), and 1 mM IPTG (for β-galactosidase assay) were added to the medium.

Plasmids—Plasmids pACYA-*kdpE*/*kdpD*, pACYC-*ompR*/*envZ*, and pNK10-Z11 were used, all of which are derivatives of pACYC184. The original plasmids (pIN-DE and pNK012) were constructed previously (*12*, *35*, *37*) and slightly modified in this study.

β-Galactosidase Assay—β-Galactosidase activity was examined as described previously (*36*). The presented values are an average of triplicate experiments (at least).

Mutagenesis of Plasmid DNA with Polymerase Chain Reaction (PCR)—The *Sal*I–*Sac*I region encoding the C-terminal half of OmpR was isolated from pNK10-Z11 (see Fig. 3). The fragment was inserted into the *Sal*I–*Sac*I sites in the pUC19 multi-cloning site. The plasmid was digested with *Bam*HI to generate the linear DNA fragments, which were subjected to PCR in the presence of 0.3 mM $MnCl_2$ with the commercially available RV and M4 oligonucleotide primers (Takara Shuzo). Other conditions for PCR mutagenesis were essentially as described previously (*12*). The PCR products were treated with *Sal*I and *Sac*I, and then the corresponding fragment was recloned into the pNK10-Z11. *E. coli* HAK007 cells were transformed with the mutagenized plasmid DNAs, and transformants were spread on TY-agar medium containing IPTG (see Fig. 3).

Protein Purification—The OmpR and KdpE polypeptides were purified by the established procedures (*4*, *35*). *E. coli* cytoplasmic membranes enriched with the EnvZ histidine kinase were also purified as described previously for use in the *in vitro* OmpR-phosphorylation assay (*16*, *22*). Similarly, cytoplasmic membranes containing the KdpD histidine kinase were also isolated as described previously (*35*, *37*).

Phosphorylation Assay—The OmpR and KdpE polypeptides were phosphorylated *in vitro* by employing the cytoplasmic membranes containing either EnvZ or KdpD as described previously (*6*, *22*, *35*).

DNA-Binding Gel-Shift Assay—A short DNA fragment encompassing the *ompC* promoter region (tentatively named *ompC*-DNA) was isolated as described previously (*30*, *31*, *39*). Similarly, another DNA segment encompassing the *kdpABC* promoter (tentatively named *kdpA*-DNA) was also isolated as described previously (*36*). These fragments were end-labeled with ^{32}P at their 3′-ends. The purified OmpR polypeptides including mutant derivatives were phosphorylated *in vitro* as described above. Each DNA fragment and each protein (in varied concentrations) were mixed, then analyzed by DNA-binding gel-shift assay, as described previously (*37*, *38*). The samples were subjected to non-denaturing polyacrylamide gel electrophoresis as described previously (*4*). The shifted protein-DNA complexes were quantified by measuring with a phosphoimage analyzer (BAS2500, Fuji Film).

Footprinting Analysis—The *kdpA*-DNA fragment used for DNA-binding assay was incubated with either phosphorylated-KdpE or phosphorylted-OmpR. The conditions were the same as those described above for the DNA-binding gel-shift assay. In these experiments, the samples were subjected to footprining analysis with DNase I. The detailed conditions were as described previously (*31*, *36*, *40*). The results were analyzed with a phosphoimage analyzer (BAS2500, Fuji Film).

Analysis of the Three-Dimensional Structure—We previously determined the X-ray three-dimensional structure of the DNA-binding domain of OmpR (*41*). The structure was imaged on a personal computer with Rasmac V.26-C24 software.

RESULTS

The Structures of OmpR and KdpE—We previously determined the three-dimensional structure of the C-terminal DNA-binding domain of OmpR, which contains the DNA-binding helix (α2)-loop-helix (α3) motif (Fig. 1, also see Fig. 7) (*41*). In particular, helix-α3 is postulated to be the DNA recognition helix (*16*). KdpE is also a member of the OmpR family, and a number of amino acid residues are conserved in the C-terminal domains of OmpR and KdpE (Fig. 1). It is thus reasonable to assume that KdpE has a C-terminal structure similar to that of OmpR. However, these analogous transcription factors act differently from each other: OmpR specifically activates the *ompC* promoter, whereas KdpE activates the *kdpABC* promoter. Such events with regard to the promoter selectivity were first examined, as follows.

E. coli strain NKC002 carries an *ompC-lacZ* reporter gene on the chromosome, in which the OmpR-binding site is located just upstream of the –35 (TTGGAT) and –10 (GAGAAT) sequences of the *ompC* promoter (*29*). *E. coli* strain HAK007 carries a *kdpA-lacZ* reporter gene on the chromosome (*36*). Figure 2 compares the promoter sequence of the *kdpA-lacZ* gene with that of the *ompC-*

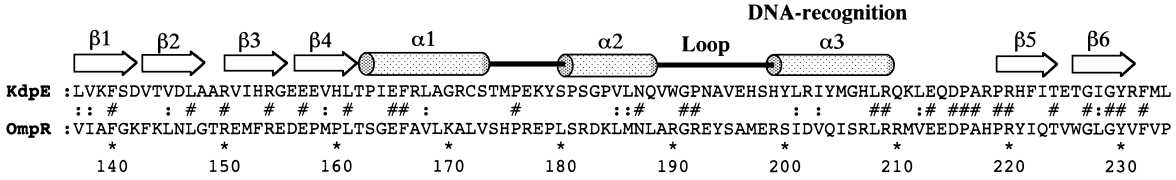

Fig. 1. **Alignment of the amino acid sequences of OmpR and KdpE.** The amino acid sequences of the C-terminal halves of OmpR and KdpE are aligned. Identical amino acids are indicated by (#), and similar amino acids by (:). The numbers indicate the amino acid positions of OmpR, where the first methionine was taken as 1. At the top, the secondary structure of OmpR is schematically shown, which consists of six β-strands and three α-helices. This structure is based on the X-ray three-dimensional structure of the C-terminal half of OmpR (*41*).

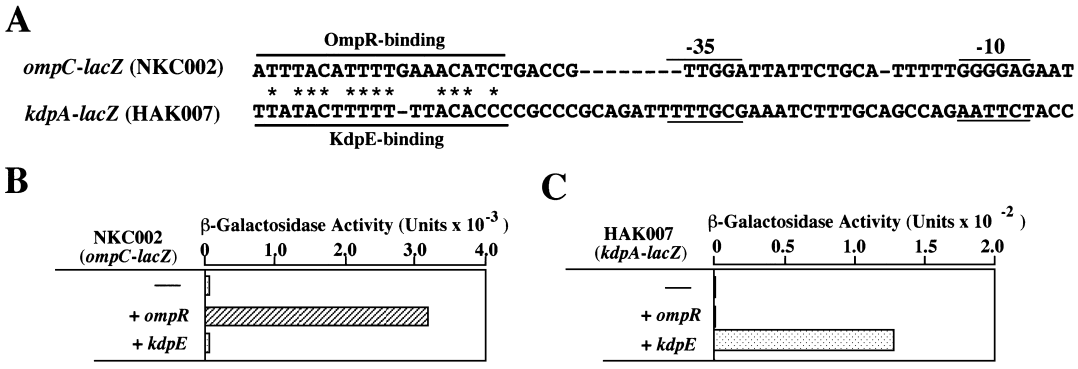

Fig. 2. **Promoter selectivity observed for OmpR and KdpE.** (A) *E. coli* strain NKC002 carries an *ompC-lacZ* fusion gene on the chromosome. The nucleotide sequence of the *ompC* promoter in the *ompC-lacZ* gene is shown, in which the –35 and–10 consensus sequences and the OmpR-binding sequence are marked. Another *E. coli* strain HAK007 carries a *kdpA-lacZ* fusion gene on the chromosome. The nucleotide sequence of the *kdpABC* promoter in the *kdpA-lacZ* gene is also marked. (B) A plasmid carrying the *ompR* and *envZ* genes (pACYC-*ompR*/*envZ*) was introduced into NKC002, which lacks both the genes. The transformed cells were grown in TY-medium containing 0.6 M NaCl, and then β-galactosidase activity was measured. (C) A plasmid carrying *kdpE* and *kdpD* genes (pACYC-*kdpE*/*kdpD*) was introduced into HAK007, which lacks both genes. The transformed cells were also subjected to β-galactosidase assay.

lacZ gene. In the *kdpABC* promoter, the KdpE-binding site is located upstream of the –35 (TTGCGA) and –10 (TCTACC) sequences. The structures of both promoters have previously been characterized (*25–32*, *35*, *36*). The OmpR-binding and KdpE-binding sequences share many common residues (asterisks in Fig. 2), although they differ in their locations relative to the respective –35 sequences: the KdpE-binding site is located slightly farther (8 bp) upstream than the OmpR-binding site. By employing these two *E. coli* strains, the selectivity of promoter recognition was examined with reference to OmpR and KdpE (Fig. 2, B and C).

When a low-copy-number plasmid (pACYC-*ompR*/*envZ*) carrying the *ompR*/*envZ* genes was introduced into NKC002 (Δ*ompR*/*envZ*, *ompC-lacZ*$^+$), a markedly high level of β-galactosidase activity was detected in the cells grown in high osmolarity medium (TY-medium containing 0.6 NaCl); but when a low-copy-number plasmid (pACYC-*kdpE*/*kdpD*) carrying the *kdpE*/*kdpD* genes was introduced into the same strain, no induction of β-galactosidase activity was observed (Fig. 2B). This indicates that the expression of the *ompC-lacZ* reporter gene in NKC002 is absolutely dependent on the function of OmpR. Conversely, when pACYC-*ompR*/*envZ* was introduced into HAK007 (Δ*kdpE*/*kdpD*, Δ*ompR*/*envZ*, *kdpA-lacZ*$^+$), the *kdpA-lacZ* gene was not activated, while it was fully activated when pACYC-*kdpE*/*kdpD* was introduced (Fig. 2C). This indicates that the expression of the *kdpA-lacZ* reporter gene in HAK007 is absolutely dependent on the function of KdpE. It was thus shown the OmpR transcription factor is highly specific to its cognate *ompC* promoter, whereas KdpE is specific to its cognate *kdpABC* promoter (compare between Fig. 2, B and C).

Isolation of OmpR Mutants That Can Activate the kdpABC Promoter—We have managed to generate OmpR mutants that can activate the *kdpABC* promoter by introducing amino acid substitutions into OmpR. The strategies are as follows (Fig. 3). We employed *E. coli* HAK007 (Δ*kdpE*/*kdpD*, Δ*ompR*/*envZ*, *kdpA-lacZ*$^+$) and plasmid pNK10-Z11 carrying the *ompR*/*envZ11* genes. This plasmid is a derivative of pACYC-*ompR*/*envZ*. It should be noted that the *envZ11* gene in pNK10-Z11 encodes a constitutive active form of EnvZ, which can function in a manner independent of medium osmolarity (thus we did not need to use a high osmolarity medium for the following experiments). Cells of HAK007 carrying pNK10-Z11 form white colonies on the selection medium (TY-containing X-Gal), because the wild-type OmpR transcription factor cannot activate the *kdpA-lacZ* reporter gene (see Fig. 2C). First, the *Sal*I–*Sac*I region of pNK10-Z11, which encompasses the coding sequence for the C-terminal half of OmpR, was mutagenized by means of localized PCR-mutagenesis (Fig. 3, upper right). The mutagenized plasmid DNA was introduced into HAK007, and the cells were spread on the selection medium. We then looked for blue colonies on the plates. Among

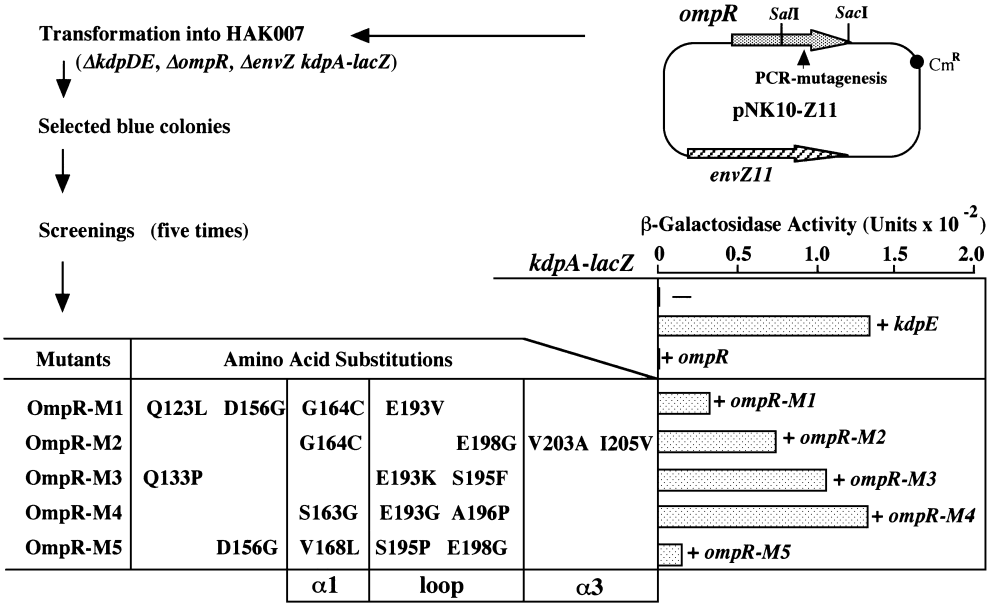

Fig. 3. **Isolation of OmpR mutants that can activate the *kdpABC* promoter.** The procedures for the screening of OmpR mutants that can activate the *kdpABC* promoter are schematically shown. The details are given in the text (see "MATERIAL AND METHODS"). Five independent OmpR mutants were isolated, each of which has a characteristic set of amino acid substitutions, as indicated. For instance, OmpR-M1 was found to have four amino acid substitutions, Q123L, D156G, G164C, and E193V, where Q123L means that the glutamine (Q) residue at the position of 123 has been replaced by leucine (L) (lower left). For these OmpR mutants, their abilities to activate the *kdpABC* promoter were assessed by means of β-galactosidase assays in the reporter HAK007 cells. Appropriate control experiments were also carried out, as shown at the top (lower right).

approximately 10^4 transformants, several dark-blue colonies appeared. We selected only one colony, from which plasmid DNA was recovered. This plasmid (tentatively designated as OmpR-M1) was re-introduced into HAK007 to quantitatively measure β-galactosidase activity. The result showed that HAK007 carrying this mutant plasmid indeed exhibited a high level of β-galactosidase activity, suggesting that the presumed mutant *ompR* gene on the plasmid is capable of activating the *kdpE-lacZ* reporter gene (Fig. 3, lower right). We repeated the PCR-mutagenesis and screening. In each screening, we selected only one candidate in order to obtain truly independent OmpR mutants. As a result, we isolated five independent candidates (OmpR-M1 to OmpR-M5), as summarized in Fig. 3.

For these five candidate plasmids, the nucleotide sequences of the mutagenized *Sal*I–*Sac*I region were determined. Several base substitutions were detected in each mutant gene, which resulted in multiple (three or four) amino acid substitutions at the protein level (Fig. 3, lower left). For instance, OmpR-M1 was inferred to have four amino acid substitutions: namely, Q123L [indicating that glutamine (Q) at position 123 has been replaced by leucine (L)], D156G, G164C, and E193V (the first methionine of the intact OmpR peptide was taken as 1). Such multiple amino acid substitutions were also found in the other candidates (OmpR-M2 to OmpR-M5), as summarized in Fig. 3. To exclude the possibility that there might be unexpected base substitutions in the backbone of plasmid DNA, the *Sal*I–*Sac*I segment was recovered from each candidate plasmid and re-inserted into the same position of non-mutagenized pNK10-Z11. We then confirmed that these OmpR mutants (M1 to M5), each carrying certain amino acid substitutions, indeed have the ability to activate the non-cognate *kdpABC* promoter.

Close Inspection of Crucial Amino Acid Substitutions—As shown above, each of these isolated OmpR mutants has multiple (three or four) amino acid substitutions in the DNA-binding domain. Interestingly, certain amino acid substitutions were commonly found in these inde-

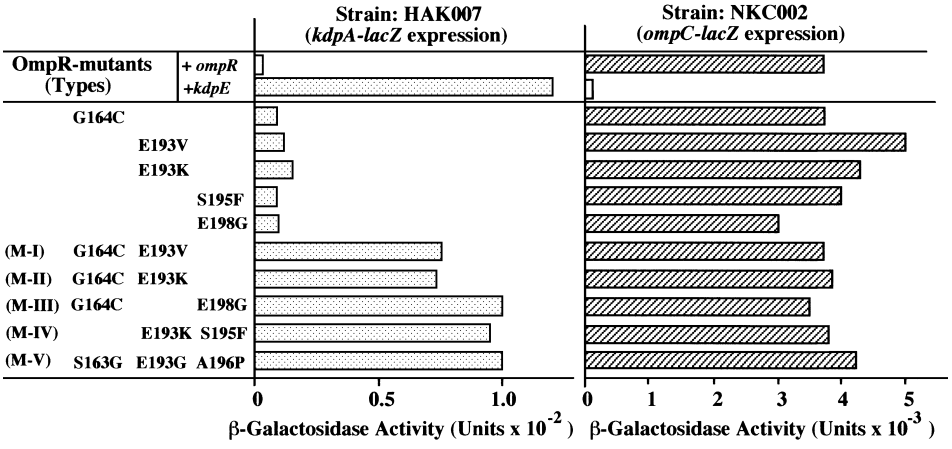

Fig. 4. **Clarification of the isolated OmpR mutants with regard to amino acid substituions.** Nine OmpR mutants were constructed on pNK10-Z11 (see Fig. 3) by means of site-directed mutagenesis, and their abilities to activate the *kdpABC* promoter were assessed by means of β-galactosidase assays in the reporter HAK007 cells (left panel). Appropriate control experiments were also carried out, as shown at the top. Similarly, the abilities of the mutants to activate the *ompC* promoter were assessed in the reporter NKC002 cells (right panel).

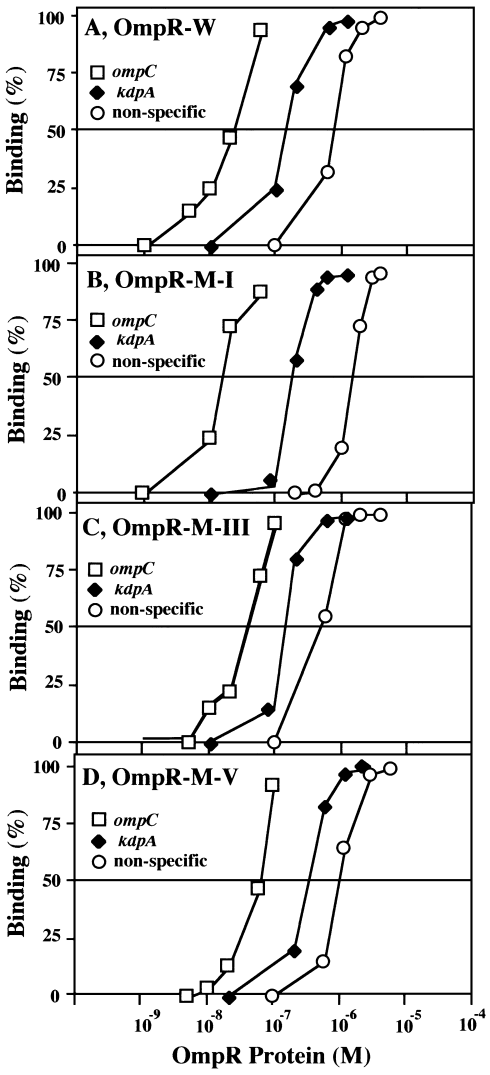

Fig. 5. **DNA-Binding assay for OmpR mutants with reference to the *kdpABC* promoter.** DNA-binding gel-shift assays were carried out, as follows. The purified proteins used were: (A), OmpR (wild-type); (B), OmpR-M-I; (C), OmpR-M-II; (D), OmpR-M-V, as indicated. The target DNAs used were: a short DNA segment encompassing the *ompC* promoter (*ompC*, open squares), a short DNA segment encompassing the *kdpABC* promoter (*kdpA*, closed diamonds), a DNA segment derived from the *lacZ* coding region (non-specific, open circles), as indicated. Employing these samples, semi-quantitative DNA-binding gel-shift assays were carried out (see "MATERIAL AND METHODS"). The concentration of phosphorylated-OmpR was varied, as indicated. The shifted bands were quantified on the basis of radioactivity of ^{32}P-labeled DNA. The values were expressed as the relative to those of input-DNA.

pendently isolated OmpR mutants (*e.g.*, G164, S195, E198) (see Fig. 3). Nevertheless, it was difficult to know which amino acid substitution is crucial for the functional alteration of OmpR. All of them might be crucial, or perhaps only a single amino acid change might be sufficient. To clarify this problem, we attempted to minimize the number of amino acid substitutions in these OmpR mutants. This was done by means of site-directed mutagenesis on pNK10-Z11, and the results of such intensive experiments are summarized in Fig. 4. The results indicated that a single amino acid substitution (G164C, E193V, E193K, S195F, or E198G) is not sufficient to render OmpR active toward the non-cognate *kdpABC* promoter. However, certain combinations of two or more amino acid substitutions were found to be sufficient (Fig. 4). These were M-I, a reconstructed OmpR mutant with the G164C and E193V substitutions, M-II (G164C and S193K), M-III (G164C and E198G), M-IV (E193K and S195F), and M-V (S163G, E193G, A196P). These OmpR mutants were fully potent to activate the non-cognate *kdpABC* promoter in HAK007 (Fig. 4, left panel). They were also introduced into NKC002 carrying the *ompC-lacZ* reporter. Interestingly, these mutants were still active toward the cognate *ompC* promoter (Fig. 4, right panel). This result suggested that the amino acid substitutions in OmpR do not result in a dramatic change of promoter specificity (or selectivity). Rather, these OmpR mutants have newly gained a broadened specificity that allows them to activate both the cognate and non-cognate promoters equally.

Characterization of OmpR Mutants In Vitro—How can we explain these interesting OmpR mutants in terms of the underlying molecular mechanism? The simplest explanation is that the altered OmpR molecules might have gained the ability to bind to the *kdpABC* promoter. To gain insight into the nature of these OmpR mutants at the molecular level, they were purified to near homogeneity. These purified proteins were characterized *in vitro* by means of DNA-binding gel-shift assays (Fig. 5). The target DNA segments used for these assays were: a short DNA segment encompassing the OmpR-binding site in the *ompC* promoter (designated as *ompC*-DNA), a short segment encompassing the KdpE-binding site in the *kdpABC* promoter (*kdpA*-DNA), and a non-specific DNA segment derived form the coding region of the *lacZ* gene (non-specific *lacZ*-DNA). The phosphorylated (or activated) form of OmpR used in these experiments was prepared *in vitro* as described previously (*18*). First, the binding of OmpR-W (*i.e.*, phosphorylated form of wild-type OmpR) to the cognate (*ompC*) and non-cognate (*kdpA* or *lacZ*) DNA segments was examined (Fig. 5A). OmpR-W showed the ability to bind preferentially to *ompC*-DNA. Surprisingly, OmpR-W was also capable of binding to *kdpA*-DNA with considerably higher affinities than to non-specific *lacZ*-DNA. The mutant OmpR proteins (OmpR-M-I, OmpR-M-III, and OmpR-M-VI) were also examined under essentially the same conditions (Fig. 5, B, C, and D). Essentially the same DNA-binding profiles were observed for these OmpR mutants as in the case of OmpR-W, although each mutant OmpR showed somewhat reduced affinity to each of three types of DNA segments. It may be also noted that the migration profiles (or positions) of the wild-type and mutant OmpR-DNA complexes on the gels were indistinguishable (data not shown). In any case, it is important to note that the affinity to *kdpA*-DNA was not markedly altered in the OmpR mutants, as compared with the wild-type OmpR molecule.

The results of *in vitro* DNA-binding assay did not support the idea that the mutant OmpR molecules have gained increased ability to bind to the KdpE-binding site. Rather, they suggested that the wild-type OmpR molecule itself has the intrinsic ability to bind to the KdpE-binding site. The estimated dissociation constant of

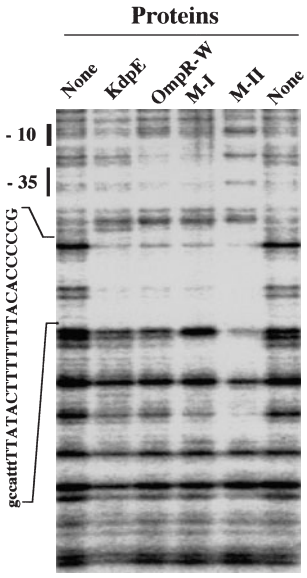

Fig. 6. **DNA-Footprinting analysis.** Footprinting analyses were carried out with a ^{32}P-labeled DNA segment encompassing the *kdpABC* promoter that have been the pre-incubated with purified proteins (KdpE, OmpR-W, OmpR-M-I, and OmpR-M-II) (see "MATERIAL AND METHODS"). Comparable amounts of purified proteins were used (2 μg of protein per reaction). The regions corresponding to the –35 and –10 sequences of the *kdpABC* promoter are indicated. The protected sequence is also indicated (the capitalized sequence represents the previously determined KdpE-binding site, see Fig. 1).

OmpR for the KdpE-binding site was approximately 10 times lower than that for non-specific DNA (Fig. 5A). We then attempted to determine the nucleotide sequence in the *kdpABC* promoter to which OmpR binds. This was done by DNase I footprinting analyses. As an appropriate reference, the KdpE protein was also purified. The *kdpA*-DNA segment was subjected to DNase I footprinting analyses with the purified and phosphorylated proteins (KdpE, OmpR-W, OmpR-M-I, OmpR-M-II). KdpE protected a stretch of nucleotides from DNase I digestion, and the protected region is located just upstream of the –35 and –10 sequences in the *kdpABC* promoter (Fig. 6). This protected region indeed encompasses the KdpE-binding site, as anticipated (also see Fig. 1). Interestingly, OmpR-W also protected essentially the same stretch of nucleotides in the *kdpABC* promoter. Essentially the same event was seen in the case of both OmpR-M-I and OmpR-M-II; although a close inspection revealed that the protection profiles with OmpR-W, OmpR-M-I, and OmpR-M-II are slightly different from each other. In any event, it was found that OmpR-W and its mutant derivatives are capable of binding to the specific nucleotide sequence (*i.e.,* KdpE-binding site) in the *kdpABC* promoter, at least, to certain extent.

In short, the *in vivo* and *in vitro* results of this study suggest a way in which the OmpR mutants can activate the non-cognate *kdpABC* promoter, implying an intriguing view of the molecular basis for the promoter selectivity of OmpR, as will be discussed (see Figs. 7 and 8).

DISCUSSION

E. coli has 14 members of the OmpR family of transcription factors, including OmpR and KdpE. How does each highly analogous DNA-binding protein precisely and specifically recognize its cognate promoter so as to selectively trigger the transcription in *E. coli* cells? In this study, we addressed this issue of promoter selectivity in transcriptional regulation with special reference to OmpR. To this end, we isolated a set of OmpR mutants that are capable of triggering the transcription from the non-cognate *kdpABC* promoter as efficiently as does KdpE, the cognate transcription factor for the *kdpABC* operon (Fig. 3). We identified five independent OmpR mutants, each of which shows the *in vivo* ability to activate the *kdpABC* promoter. Each OmpR mutant has certain (two or three) amino acid substitutions in the C-terminal DNA-binding domain (Fig. 4). The results of *in vitro* studies on these OmpR mutants (Figs. 5 and 6) suggested a new aspect of the molecular basis for the promoter selectivity of OmpR (Figs. 7 and 8).

In general, the simplest explanation of promoter selectivity would be that each OmpR family protein has a highly specific affinity to its own target DNA sequence in a given cognate promoter, but not to others. This idea appears to be true, *a priori*. One can thus assume that the isolated mutant OmpR molecules might have gained the ability to bind to the *kdpABC* promoter, while the wild-type OmpR molecule lacks such ability. The *in vitro* properties of these OmpR mutants, however, did not fit this simple idea (Figs. 5 and 6). Rather, it was found that OmpR (wild-type) has a latent and/or intrinsic ability to specifically recognize the KdpE-binding site in the *kdpABC* promoter (Figs. 5A and 6). Although its latent affinity to the KdpE-binding site, estimated in *vitro*, is weaker than that to the cognate OmpR-binding site, it is significantly stronger than that to non-specific DNAs (Fig. 5). This is not surprising because the OmpR-binding sequence is considerably similar to the KdpE-binding sequence (Fig. 1). In any case, the amino acid substitutions in a given mutant did not significantly affect such DNA-binding specificities of OmpR (Fig. 5). Thus, we needed an alternative explanation with regard to these OmpR mutants.

We next inspected the structure and function of OmpR more closely in the context of the three-dimensional structure of its C-terminal DNA-binding domain. As shown in Fig. 7, three tandem α helices (α1, α2, and α3) are folded into a characteristic structure that is involved in the DNA-binding (see also Fig. 1). In particular, helix-α2 and helix-α3 with an intervening loop together form a helix-turn-helix (HTH) structure (*41*), which is typical of many prokaryotic and eukaryotic DNA-binding proteins. It is generally believed that helix-α3 makes direct contact with certain bases in the major groove of the target DNA helix. In general, helix-α1 also plays an important role in stabilizing the HTH structure. In short, OmpR belongs to a large family of HTH DNA-binding proteins; but OmpR has a large loop flanked by the two canonical helices (α2 and α3), instead of a short turn found in typical HTH proteins (*e.g.*, λ repressor). This HTH structure

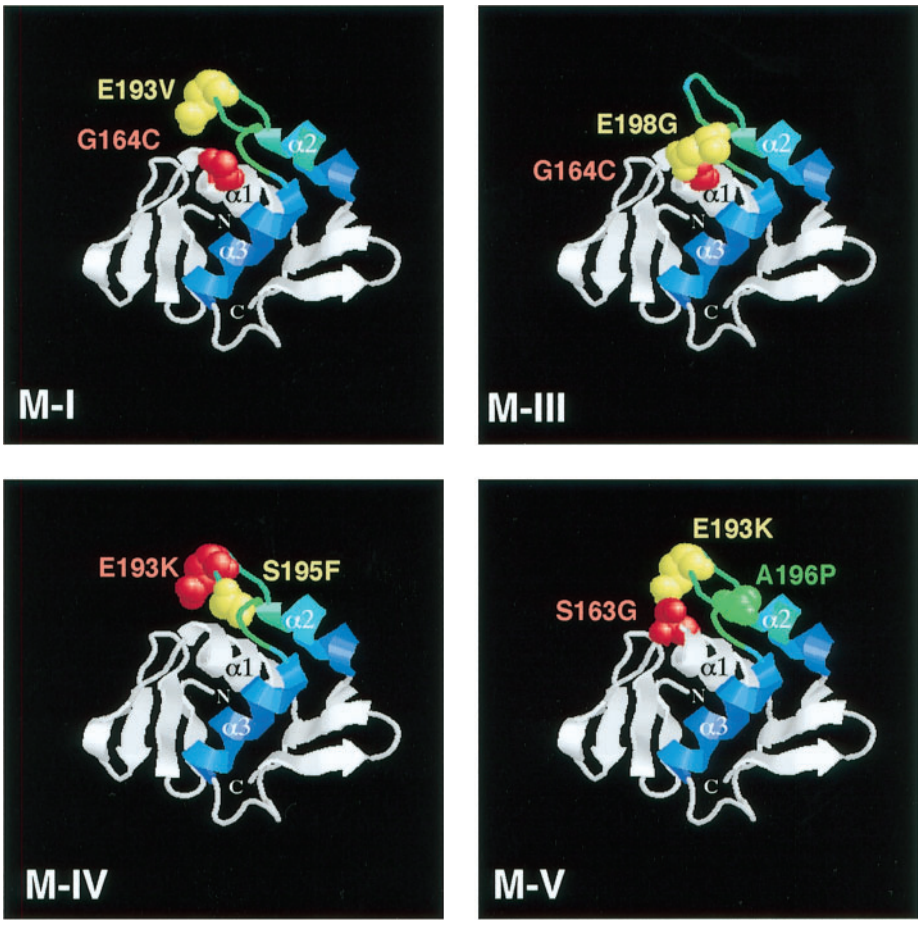

Fig. 7. **Inspection of the substituted amino acids in the OmpR mutants.** The X-ray crystal three-dimensional structure of the C-terminal half of OmpR has been determined previously. The structure is depicted with a ribbon model that highlights β-strands (as arrows) and α-helices (as coils) (see also Fig. 1A). "N" and "C" indicate the N-terminal and C-terminal ends, respectively. An HTH motif is further highlighted (α2- and α3-helices are blue, and the intervening large loop is green). The substituted amino acids are indicated with a space-filling model for each OmpR mutant (M-I, M-III, M-IV, and M-V).

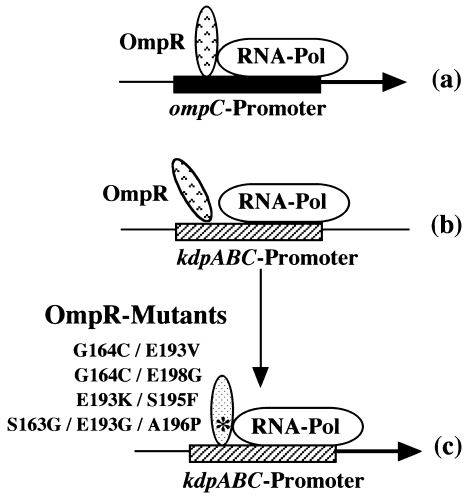

Fig. 8. **A hypothetical model that explains how the OmpR mutant can activate the non-cognate *kdpABC* promoter.** The OmpR protein, RNA polymerase (RNP), and *ompC* and *kdpABC* promoter regions are depicted. For details of each model (a, b, and c), see "DISCUSSION."

in OmpR is most likely responsible for its specific DNA-binding (*16*). However, OmpR must also interact with RNA polymerase to activate transcription (*9–12*). It was previously proposed that the large loop flanked by the helices (α2 and α3) provides a site that directly interacts with RNA polymerase (see the loop colored green in Fig. 7) (*41*). In fact, a set of positive control (PC) mutants has previously been reported (*13*, *27*, *40*). Such PC-OmpR mutants can normally bind to the OmpR-binding site in the *ompC* promoter, but cannot activate the transcription, presumably due to an impaired interaction with RNA polymerase. Indeed, certain PC mutations have been mapped within this loop (*e.g.*, E193, A196, and E198) (*27*). These results support the idea that this loop is a part of "activation domain (or RNA polymerase-interacting domain)". In summary, there are two distinctive domains in the C-terminal portion of OmpR: one is the DNA-interacting domain (the HTH motif with helices α2 and α3), and the other is the RNA polymerase-interacting domain (the extruded intervening loop, at least, in part).

Taking the three-dimensional structure of OmpR into consideration, we inspected the positions of the altered amino acid residues in each OmpR mutant (Fig. 7). OmpR-M-I has two amino acid substitutions at the positions of G164 and E193, which are distant from each other in the primary amino acid sequence (see Fig. 1). When plotted on the three-dimensional structure of OmpR, however, it is clear that these amino acids are closely located in space (Fig. 7, panel M-I). The same situation was seen for the other OmpR mutants (Fig. 7, panels M-III, M-IV, and M-V). No amino acid substitution was found in the DNA-recognition helix-α3. Interestingly, the amino acid substitutions in an OmpR mutants

occurred at a confined surface that is located closely to, or within, the presumed activation domain containing the loop. It is also noteworthy again that the E193 and E198 residues in question are indeed the ones that have previously been implicated in certain PC mutants, as mentioned above (*27*). These observations together suggested that the OmpR mutants might have an altered interaction with RNA polymerase, but not with DNA, as further discussed below.

Figure 8 illustrates our hypothetical view of how the OmpR mutants are capable of activating the non-cognate *kdpABC* promoter. (a) In the *ompC* promoter, OmpR binds to the specific OmpR-binding site just upstream of the –35 and –10 sequences, with which RNA polymerase associates. The bound OmpR molecule directly interacts with RNA polymerase (most likely with the α subunit) with a proper configuration in space (*9*). As the result, OmpR can help RNA polymerase to initiate the *ompC* transcription (Fig. 8a). (b) In the *kdpABC* promoter, wild-type OmpR molecules are able to recognize the KdpE-binding sequence (see Fig. 6), but OmpR molecules on the KdpE-binding site are unable to interact properly with RNA polymerase sitting on the –35 and –10 sequences on the *kdpABC* promoter (Fig. 8b). (c) However, the introduction of certain amino acid substitutions into the presumed RNA polymerase–interacting domain of OmpR alters its molecular conformation such that it can interact with RNA polymerase and help to initiate the *kdpABC* transcription (Fig. 8c).

As discussed above, the results of this study are well consistent with the proposed model (Fig. 8). Further indirect supporting evidence for this model is that when the wild-type OmpR proteins were overexpressed from a high-copy-number plasmid in the *E. coli* cells (*kdpDE*$^+$) carrying the *kdpA-lacZ* gene, the level of expression of β-galactosidase was significantly reduced (data not shown). The model explains this interesting *in vivo* event by postulating that the wild-type OmpR molecule would bind competitively to the *kdpABC* promoter, so as to interfere with the KdpE function. Nevertheless, it is difficult to prove the model without further experiments. For instance, the results of *in vitro* transcription analyses with OmpR mutants and RNA polymerase would rule out the possibility that as yet unidentified protein factors (*e.g.*, general nucleoid proteins, like H-NS) are somehow involved in the promoter selectivity of OmpR. It should also be noted that the *ompR* gene characterized in this study was on a low-copy-number plasmid. Thus, the *in vivo* concentration of OmpR must also be taken into consideration. For instance, we showed that the OmpR mutants are still capable of activating the *ompC* promoter *in vivo* as efficiently as in the case of OmpR-W (Fig. 4), although their *in vitro* binding affinities to the *ompC* promoter were considerably reduced (Fig. 5). This may be due to such a gene-dosage effect. Taking these considerations into account, the proposed model is intriguing from a general viewpoint with regard to the promoter selectivity of the OmpR family members. With regard to these homologous OmpR family members, it is reasonable to assume that their target DNA sequences might be considerably similar to each other, as indeed is observed for OmpR and KdpE (see Fig. 1). Such a situation would be very troublesome for *E. coli* cells in the sense that the promoter selectivity might not be ensured solely by the DNA-recognition specificity of a given transcription factor. It is thus tempting to speculate that the promoter selectivity is determined also by an overall promoter configuration in space, *e.g.,* the –35 and –10 sequences to which RNA polymerase binds, the upstream recognition sequence to which a given transcription factor binds, and also the distance between these sequences (compare the *ompC* and *kdpABC* promoters in Fig. 2). Indeed, we previously reported the *in vivo* evidence for that the distance between the –35 sequence and the OmpR-binding site is an important parameter for activation of the *ompC* promoter by OmpR (*25*, *29*). In conclusion, the results of this study with special reference to OmpR are compatible with the view proposed in the hypothetical and intriguing model.

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