Transcription Regulation of *ompF* and *ompC* by a Single Transcription Factor, OmpR*

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The *ompF* and *ompC* genes of *Escherichia coli* are reciprocally regulated by a single transcription factor, phosphorylated OmpR (OmpR-P), depending upon medium osmolarity. This regulation involves activation of *ompF* and its repression with concomitant activation of *ompC*. This occurs through OmpR-P binding to four (F1, F2, F3, and F4) and three (C1, C2, and C3) sites located upstream of the *ompF* and *ompC* promoters, respectively, through a novel mechanism. Here we show that there is a distinct OmpR-P binding hierarchy within F1, F2, and F3 sites as well as within C1, C2, and C3 sites. Each of these sites contains two tandem 10-bp OmpR-P-binding subsites, a-site and b-site (from 5′ to 3′ direction). OmpR-P has higher affinity to the downstream b-site than to the upstream a-site in each case. Six OmpR-P molecules bind to F and C sites two-by-two in a discontinuous “galloping” manner. We propose that this tight hierarchical binding of a transcription factor, OmpR, allows distinct stepwise regulation of *ompF* and *ompC* transcription, which minimizes their overlapping expression upon changes in the medium osmolarity to achieve the reciprocal expression of *ompF* and *ompC*.

*Escherichia coli* contains two outer membrane porin proteins, OmpF and OmpC, which serve as passive diffusion pores across the outer membrane. Expression of these two proteins is reciprocally regulated by medium osmolarity. OmpF is preferentially produced at low osmolarity, whereas OmpC is almost exclusively produced at high osmolarity. Because OmpF forms a larger pore than OmpC (1), the regulation of the size of the passive diffusion pores across the outer membrane appears to be vital for *E. coli*, which is able to live in two distinctly different environmental conditions: one under nutrient-rich high osmolarity conditions in animal gut at 37 °C and the other under poor nutritional and low osmolarity conditions outside animals and thus at low osmolarity at ambient temperatures. It has been proposed that the larger OmpF pore size is important for efficient nutrient uptake from nutritionally poor media, whereas the smaller OmpC pore size is important to exclude the passage of toxic bile salt across the outer membrane (2). *E. coli* has a highly sophisticated regulatory system to regulate the reciprocal expression of *ompF* and *ompC*. Both are controlled at the transcriptional level by the histidine kinase EnvZ, a transmembrane osmosensor, and the response regulator OmpR, a transcriptional factor (3), and also at the translational level by antisense RNAs, *micF* RNA for *ompF* mRNA (4) and *micC* RNA for *ompC* mRNA (5).

The EnvZ-OmpR system is a typical two-component His-Asp phosphorelay signal transduction system in *E. coli* (see review in Ref. 6). Because EnvZ not only has kinase activity to phosphorylate OmpR but also phosphatase activity toward phosphorylated OmpR (OmpR-P), it is able to regulate the level of OmpR-P in the cell depending upon the medium osmolarity. At low medium osmolarity, the phosphatase activity is relatively higher than the kinase activity so that the cellular concentration of OmpR-P is maintained at a lower concentration, which is sufficient to activate expression of *ompF* but not of *ompC*. At high medium osmolarity, the EnvZ phosphatase activity becomes relatively lower than its kinase activity, resulting in higher cellular concentration of OmpR-P. Under this condition, *ompF* expression is repressed, whereas *ompC* expression is activated.

In our recent study on the interaction between the cytoplasmic domain of EnvZ (EnvZC) and OmpR or OmpR-P (7), we proposed that the reciprocal expression of *ompF* and *ompC* occurs through a finely tuned mechanism. In this model, osmotic signals regulate the levels of OmpR-P by modulating the ratio of kinase to phosphatase activity of EnvZ. Most importantly, of the large pool of OmpR (3,500 molecules/cell), only a very small portion is proposed to be phosphorylated (8). On the other hand, the *Kₐ* value for OmpR-P binding to the regulatory regions of the *ompF* and *ompC* promoters (9, 10) is less than 1% of the total cellular concentration of OmpR and OmpR-P. This was proposed to be a critical factor in the reciprocal expression of *ompF* and *ompC*. For example, even with a minor change in the ratio of the kinase to phosphatase activity of EnvZ, the resulting small shift in the cellular concentration of OmpR-P would be sufficient to substantially change the number of OmpR-P molecules bound to the regulatory regions of the *ompF* and *ompC* promoters. There is an approximately a few hundred-fold difference between the *Kₐ* value for OmpR-P binding to the regulatory regions of the *ompF* and *ompC* promoters versus the total cellular concentration of OmpR and OmpR-P (7).

There are four F sites (F1, F2, F3, and F4) and three C sites (C1, C2, and C3) in the upstream region of the *ompF* and *ompC* promoters, respectively. Each site consists of 20 base pairs, providing a tandem *Kₐ* value for OmpR-P binding to these sites. Because OmpR-P binding to these F and C sites was determined to be F1, C1, C2, and C3 sites bound to OmpR-P (9, 10, 12). The hierarchy of OmpR-P binding to the *F* and *C* sites was determined to be F1, C1 > F2, F3 > C2 > C3 (9). At low osmolarity, OmpR-P cooperatively binds to F1-F2/F1-F2-F3 to activate *ompF* transcription. Under this condition, only the C1 site is occupied by OmpR-P, which is not sufficient to activate *ompC* transcription. Only when the OmpR-P concentration elevates in response to increased medium osmolarity do the C2 and C3 sites become occupied by OmpR-P, resulting in *ompC* expression. Under this condition, OmpR-P also binds to the F4 site (−380 to −361), a weak OmpR-P-binding site located 260-bp upstream of the F1 site (−100 to −81). OmpR-P binding to the F4 site is proposed to form a loop that interacts with OmpR-P molecules binding to F1, F2, and F3, thereby blocking *ompF* transcription (9, 13, 14).

Here, we investigated how two OmpR-P molecules cooperatively bind to F1, F2, and F3 as well as C1, C2, and C3. The 20-bp F1 site is
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EXPERIMENTAL PROCEDURES

Oligonucleotides—The OmpR-binding sites of F (F1, F1-F2, and F1-F2-F3), C (C1, C1-C2, and C1-C2-C3) and various mutated sequences were synthesized by Integrated DNA Technologies, Inc. Complementary strands were first annealed, and double-stranded DNA was purified for DNA gel mobility shift assay. DNA concentration was determined by UV absorbance at 260 nm.

Purification of Proteins—EnvZc[T247R] previously termed EnvZc11, OmpR, and PrS2-OmpR were purified as described previously (11, 15). The C-terminal domain of OmpR (OmpRc) was purified as described previously (16).

DNA Gel Mobility Shift Assay—Each purified 20 pmol of double-stranded DNA was labeled by using T4 kinase and [γ-32P]ATP (3000 Ci/mmol and 10 mCi/ml) for 1 h at 37 °C. Free [γ-32P]ATP was removed by phenol/chloroform extraction followed by ethanol precipitation. After air drying, labeled DNA was resuspended in 100 μl of water containing 70 pmol of unlabeled double-stranded DNA.

The binding reactions were carried out in 50 mM Tris-HCl (pH 7.2) buffer containing 50 mM KCl, 5 mM CaCl2, 5% glycerol, 0.05% Nonidet P-40, and 100 ng of poly(dI-dC) at room temperature. First, EnvZc11 was phosphorylated using 1 mM ATP for 5 min. EnvZc11 mixture was transferred to the tube containing different amounts of OmpR or PrS2-OmpR, and the reaction mixture was further incubated for 20 min to generate phosphorylated OmpR or PrS2-OmpR. Under this condition, 100% of OmpR or PrS2-OmpR was phosphorylated as judged from C4 reverse phase high pressure liquid chromatography. Fifteen μl of the reaction mixture was mixed with 1.5 μl of labeled DNA (~45 fmol/μl) and incubated for 30 min (final volume, 16.5 μl). After the prerun for 1 h, the samples were subjected to 5% acrylamide/bisacrylamide (40:1.2) gel. The gel was run at 4 °C in TE buffer (10 mM Tris-HCl, pH 7.2, 1 mM EDTA) containing 70 pmol of unlabeled double-stranded DNA.

DNase I Footprinting—Each purified 30-pmol sense oligonucleotide was labeled at 5'-end by using T4 kinase and [γ-32P]ATP (3000 Ci/mmol and 10 mCi/ml) for 1 h at 37 °C. Free [γ-32P]ATP was removed by phenol/chloroform extraction followed by ethanol precipitation. Labeled oligonucleotide was annealed by mixing its complementary sequence (40 pmol) in 50 μl of the annealing buffer (25 mM Tris-HCl, pH 8.0, and 400 mM NaCl).

The DNA binding reaction was carried out as described in DNA gel mobility shift assay by mixing OmpR or OmpRc with 1 μl of labeled DNA (~600 fmol/μl); however, 50 mM Tris-HCl (pH 8.0) buffer containing 50 mM KCl, 10 mM MgCl2, and 1 mM ATP, was used. Partial DNase I digestion was carried out at room temperature by adding 1 μl of 0.2 unit/μl DNase I (Promega) into the reaction mixture (final reaction volume, 1 μl). After 1-min incubation, the reaction was stopped by adding 1 μl of 200 mM EDTA, followed by incubating in the boiling water bath for 4 min. 6 μl of the loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol FF) was added to the sample. The samples were incubated in the boiling water bath for 4 min, and 2.5 μl of the sample was subjected to 12% acrylamide/bisacrylamide (19:1), 8 μl urea gel after prerun for 30 min. The gel was run in TBE buffer (50 mM Tris-HCl, 50 mM boric acid, and 1 mM EDTA) at 45 W. The residues were positioned with the A + G ladders.

RESULTS

Hierarchical Binding of OmpR-P to F1 Subsites—Each 20-bp F- and C-binding site for OmpR-P is composed of two 10-bp repetitive subunits, the a-site and the b-site, as shown in Fig. 1A. Earlier PCR selection of nine randomized sequences of the 3’-end of the F1 site revealed a consensus sequence of 12TTAcAXXX20 (the numbers indicate the base positions in the 20-bp F1 site from the 5’-end), in which the 14AC15 sequence is tightly conserved (11). In addition to this consensus sequence, it is also known that the highly conserved G at the 11th position is important for OmpR-P binding (11, 17). Compared with these consensus sequences, the 10-bp F1b site, the 3’-end of the F1 site (GTTCATATT), contains all six consensus bases, whereas the 10-bp F1a site, the 5’-end of the F1 site (TTTACTTTTG), contains four of six consensus bases (Fig. 1A), suggesting that OmpR-P may have higher affinity for the F1b site than for the F1a site. To examine this possibility, two 20-mer DNA fragments, F1aF1a and F1bF1b, were synthesized. Note that most of the short constructs in the present study have four C-G base pair extensions (CCCC) at both the 5’- and 3’-ends to stabilize duplex formation as shown in Fig. 1B. As shown in Fig. 1B, binding to F1aF1a was barely detectable even at the highest concentration of OmpR-P used (80 nM; lane 12), whereas almost 90% of F1bF1b was bound at a concentration as low as 5 nM OmpR-P (lane 14). With the wild-type F1aF1b fragment, 50% binding to OmpR-P was observed between 5 and 10 nM OmpR-P (lanes 2 and 3, respectively). These observations are consistent with the previously reported Kd value of OmpR-P binding to the F1 site (10). These results indicate that OmpR-P binding to the F1a site in the F1 site depends on the initial binding of OmpR-P to the F1b site because OmpR-P is unable to bind to F1aF1a under the same condition. However, binding of one OmpR-P molecule to the F1b site is not enough to form a stable complex with the F1 site, because the mutation of the F1a site abolishes the binding of OmpR-P to the F1 site (11). Therefore, for OmpR-P to form a stable complex with the F1 site, binding of two OmpR-P molecules to both F1a and F1b sites is necessary. In this sense the binding of two OmpR-P molecules to the F1 site is cooperative.

We also carried out DNase I footprinting using the same 28-bp oligonucleotides used in the gel shift assay to monitor the binding of OmpR-P to the F1a and the F1b site. Because the amount of OmpR-P was increased, both the F1a and F1b sites in the wild-type F1 site (F1aF1b) were protected from DNase I digestion, in a cooperative manner (Fig. 1C, lanes 1–6). The protection of F1aF1a by OmpR-P (lanes 7–12) was significantly less compared with that of F1bF1b (lanes 13–18). This supports the hierarchy in the binding affinity of OmpR-P for these sequences (F1bF1b > F1aF1b > F1aF1a) consistent with the results of the gel mobility shift assay (Fig. 1B).
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**A**

![Diagram A](image)

**B**

![Diagram B](image)

**C**

![Diagram C](image)

**FIGURE 1.** Hierarchy between 10-bp F1a and F1b sites within the 20-bp F1 site. A, the sequences from the upper strands from the 5' to 3' direction of the *ompF* and *ompC* regulatory regions are shown: F1, F2, F3, and F4 sites for *ompF* and C1, C2, and C3 sites for *ompC*. Each 20-bp OmpR-P-binding site is further dissected into two 10-bp subsites: the a-site and the b-site. Highly conserved bases are shown in **bold** type. The arrangements for these sites are from Bergstrom et al. (9), except that the F3 site is shifted by two bases to the left, so that the highly conserved G, C, and A residues in F3b can be identified.

**B**

![Graph B](image)

**C**

![Graph C](image)

**Legend:**
- **A**: The sequences from the upper strands from the 5' to 3' direction of the *ompF* and *ompC* regulatory regions are shown: F1, F2, F3, and F4 sites for *ompF* and C1, C2, and C3 sites for *ompC*. Each 20-bp OmpR-P-binding site is further dissected into two 10-bp subsites: the a-site and the b-site. Highly conserved bases are shown in **bold** type. The arrangements for these sites are from Bergstrom et al. (9), except that the F3 site is shifted by two bases to the left, so that the highly conserved G, C, and A residues in F3b can be identified.

**B**: The gel mobility shift assay was carried out with 5'-end-labeled 28-bp DNA fragments; wild-type F1 (F1aF1b), F1aF1a or F1bF1b. OmpR-P was incubated with F1aF1b at different OmpR-P concentrations (0, 5, 10, 20, 40, and 80 nM for lanes 1–6, respectively). F1aF1a (lanes 7–13), or F1bF1b (lanes 13–18). The gel was dried and exposed for autoradiography. The result shown is representative of three independent experiments. C, DNase I footprinting experiments were carried out using oligonucleotides of 28 base pair as in the case for the gel mobility shift assays; F1aF1b, F1aF1a, or F1bF1b. Different concentrations (0, 0.325, 0.75, 1.5, and 3 μM) of OmpR were incubated with EnvzC11 in 50 mM Tris-HCl (pH 8.0) containing 50 mM KCl, 10 mM MgCl2, and 1 mM ATP. OmpR-P was incubated with F1aF1b (lanes 1–6), F1aF1a (lanes 7–12), or F1bF1b (lanes 13–18). Partial DNA digestion was carried out by adding DNase I to the reaction mixture. The samples were applied to 12% PAGE, 8 M urea gel. The gel is representative of three independent trials. ND indicates naked DNA without DNase I digestion. The residues were positioned with respect to the A+G ladders of the F1aF1b, F1aF1a, or F1bF1b fragments (data not shown). The positions of bases are indicated on the right. The numbering does not include the extra four bases (CCCC).

**C**: OmpR-P binding was substantially improved with a T11G mutation (lanes 7–12), albeit weaker than wild type (lanes 1–5). This suggests that similar to the F1 site, the G residue at position 10 in the C1 site can also interact with OmpR-P.

**G** and **ACA** Are Essential for Optimal Binding of OmpR-P—We noticed that the G residue is highly conserved and is located at every 11th position of each F and C site except in the case of the F4 site (Fig. 1A). Because G11 has been shown to be important for OmpR-P binding to the F1 site (11), we next examined its role in OmpR-P binding using the F1 and C1 sites. Because the F1 site has two G residues in the center at positions 10 and 11 (Fig. 1A), we tested which G residue contributes more to OmpR-P/F1 site interaction. Substitution of G10 (G10A; Fig. 2A, lanes 13–18) hardly affected the binding of OmpR-P to the DNA fragment as compared with the wild-type F1 sequence (lanes 1–6). On the other hand, the G11A mutation weakened the binding with a *C*~50~ value of 80 nM (lanes 7–12), ~10 times greater than the C~50~ value observed with the wild-type F1 fragment. Mutation of both G residues (G10A/G11A; lanes 19–24) further destabilized the binding of OmpR-P, resulting in a C~50~ value of higher than 80 nM. These results indicate that contribution of the G11 residue is more significant in OmpR-P/F1 site interaction than that of the G10 residue.

We performed a similar experiment with the C1 site, which has a single G residue at position 11 (Fig. 1A). When G11 was substituted with either C (Fig. 2B, lanes 6–10) or T (lanes 16–19), OmpR-P binding was significantly affected. These results further support the importance of the G11 residue both in F1 and C1 sites in OmpR-P binding. Interestingly, in the absence of G at position 11, a second mutation at position 10, T10G, elevated the affinity for OmpR-P (Fig. 2B, lanes 11–15), or T11G/T16A mutations (lanes 16–19), OmpR-P binding was further improved with a T11G/T16A mutation. Indeed, when the T16A mutation was introduced in to create the ACA consensus sequence in the second F1a subsite, OmpR-P binding was substantially improved with a C~50~ value of ~10 nM (Fig. 2C, lanes 7–12). Similarly, the T11G mutation also improved the C~50~ value to ~10 nM (lanes 13–18). However, interestingly, if these mutations were combined together, they exerted a synergistic effect as the C~50~ value was lowered to less than 5 nM as can be seen with the T11G/T16A mutations (lanes 19–24), reaching a C~50~ value similar to that with the F1bF1b fragment (Fig. 1B,
lanes 13–18). These results suggest that the G residue at position 1 of a subsite and the ACA consensus sequence are key for OmpR-P binding and are sufficient to elevate the affinity of F1αF1α to that of F1αF1β (Fig. 2A, lanes 1–6).

Because the G11 residue is more important than G10, we speculated that having the G residue as the first position in each tandem repeated subsite is critical. Therefore, we further examined the role of the G residue for OmpR-P binding using an F1βF1β fragment. This fragment has the G residue at the first position of each subsite at G1 and G11, respectively. As shown in Fig. 2D, the G11A mutation slightly increased the $K_{50}$ value. The G1A/G11A double mutant further increased the $K_{50}$ value to ~20 nM (lanes 13–18). This result indicates that the G residue located at the first position of each subsite is important for OmpR-P binding.

**Importance of the C5 and G11 Residues on the Upper Strand for OmpR-P Binding**—In all F and C b-sites, the C:G pair at the fifth position is highly conserved, and it also exists in some of the a-sites (Fig. 1A). It has been shown that mutation of this C:G pair abolishes the binding of OmpR-P (11). When the C:G pair at the 5th position in F1 (F1αF1β) was substituted with an A:T pair, binding to OmpR-P became undetectable under the conditions used (700 nM OmpR-P) (Fig. 3A, compare lanes 2 and 4). By exchanging one strand between the two duplexes used above, two other heteroduplexes having a C:T or A:G mismatched base pair at position 5 in the F1 site were formed. OmpR-P was still able to bind to the DNA fragment with the C:T mismatched pair (lane 6) but not with the A:G pair (lane 8), indicating that in the conserved C:G pair at the 5th position in the a-site, it is the C residue in the upper strand that is essential for OmpR-P binding; this is most likely the case for the b-site as well.

A similar experiment was carried out to elucidate which residue in the G:C pair at the 11th position plays an important role for OmpR-P binding using the C1 site. When the G:C pair was substituted with the C:G or T:A pair, binding was very weak, even at concentrations of OmpR-P as high as 190 nM (Fig. 2B), suggesting that the G residue in this G:C pair is important for binding. Using the wild-type and mutated oligonucleotides, two heteroduplex DNA fragments were constructed. OmpR-P was still able to bind to the C1 fragment with the G:T mismatched pair at the 11th position (Fig. 3B, lanes 6–10) but not with the A:C pair (lanes 16–20). This confirmed that similar to the C:G pair at the 5th position, the G residue on the upper strand of the G:C pair at the 11th position is involved in OmpR-P binding. The replacement of the G residue with an Ile residue had little effect on OmpR-P binding (lanes 11–15), suggesting that the carbonyl group at position 6 of the purine ring may be directly involved in the interaction with OmpR-P.

**Nonphosphorylated OmpR Is Capable of Binding to F1βF1β—Nonphosphorylated OmpR is capable of binding to the F1 site with low affinity (more than a 10-fold difference in the $K_{50}$ value as compared with that of OmpR-P (10)). However, interestingly, nonphosphorylated OmpR was able to bind to the F1βF1β fragment with a $K_{50}$ value of ~10 nM (Fig. 4A, lanes 19–24), which is very similar to the binding of OmpR-P to F1 (lanes 1–6). On the other hand, the affinity of OmpR to the F1 site was very weak, and its $K_{50}$ value appears to be higher than 80 nM (lanes 13–18). Importantly, the high affinity of OmpR for the F1βF1β fragment can be
further enhanced by its phosphorylation because more than 90% of the DNA is bound at 5 nM concentration of OmpR-P (lane 8).

The N-terminal Domain Is Required for Cooperative Binding of OmpR to the F1 Site—We next compared the DNA binding activity of the C-terminal DNA-binding domain of OmpR (OmpRc) with that of OmpR and OmpR-P by DNase I footprinting. OmpR was able to protect both the F1a and F1b sites from DNase I digestion in a cooperative manner (Fig. 4B, lanes 2–6). When the F1bF1b fragment was used, the protection of both F1b sites was dramatically increased (lanes 13–17) (compare the F1b site in the F1aF1b fragment and the upper F1b site in the F1bF1b site). Phosphorylation of OmpR further enhanced protection of the F1aF1b (lanes 7–11) and F1bF1b (lanes 18–22) fragments. These results using DNase I footprinting and a 28-bp DNA fragment are in accordance with those obtained using the gel shift assay for OmpR-P binding to DNA (Fig. 4A).

Therefore, DNase I footprinting experiments were then carried out using the F1, F1aF1a, or F1bF1b fragment with OmpRc. Interestingly, the F1b site in the F1 site was only protected at high concentrations of OmpRc (Fig. 4C, lanes 1–6), and the F1a site was not protected at all even at the highest OmpRc concentration (lane 6). Consistent with this result, both F1a sites in the F1aF1a fragment were poorly protected from DNase I digestion (lanes 7–12), whereas both F1b sites in the F1bF1b fragment were well protected (lanes 13–18), confirming that OmpRc has higher affinity to F1b than to F1a. These results indicate that the N-terminal domain is required for OmpR to bind to the F1a site in the F1 fragment and are consistent with the fact that OmpR-P has higher affinity for the F1b site than for the F1a site.

OmpR-P Binding to DNA: a Two-by-Two Mode—We previously showed that only an even number of OmpR-P molecules can bind to the F1-F2-F3 fragment, because the OmpR-P/DNA complexes detected contained only two, four, or six OmpR-P molecules (11). To further examine this two-by-two mode of OmpR-P binding to DNA, we synthesized an oligonucleotide consisting of three tandem F1b sequences, F1bF1bF1b. As a control, another oligonucleotide, F1-F2a (F1aF1bF2a), was synthesized that was previously shown to bind only two OmpR-P molecules (11).

To accurately estimate the number of OmpR-P molecules binding to the DNA fragments, mixtures of OmpR-P and PrS2-OmpR-P were used (11). PrS2-OmpR is a hybrid protein, in which two tandemly repeated N-terminal domains (a total of 184 amino acid residues) from Myxococcus xanthus protein S is linked to the N-terminal end of the OmpR molecule. The resulting PrS2-OmpR is 20 kDa larger than OmpR. Importantly, this hybrid protein exhibited binding activity similar to that of OmpR-P upon phosphorylation with EnvZc (11). As shown in lanes 1–8 of Fig. 5, only three complexes were detectable with increasing concentrations of PrS2-OmpR-P and concomitantly decreasing concentrations of OmpR-P. The protein/DNA complex with the fastest mobility contained only OmpR-P molecules, whereas the slowest mobility complex contained only PrS2-OmpR-P molecules. Therefore, the center complex should contain one molecule each of

![Figure 3](image-url)
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OmpR-P and PrS2-OmpR-P. We conclude that only two OmpR-P molecules are able to bind to the F1F2a fragment.

When a similar experiment was carried out with the F1bF1bF1b fragment, again only three complexes were detectable (Fig. 5, lanes 9–16). This result demonstrates that even if a DNA fragment contains three high affinity OmpR-P-binding sites, not more than two OmpR-P molecules can bind to the fragment, likely in two different modes as illustrated in Fig. 5A.

All b-sites Have Higher Affinity for OmpR-P than Their Cognate Upstream a-sites—Thus far we demonstrated that there is a distinct OmpR-P binding hierarchy between F1a and F1b subsites consistent with the F1b sequence having the consensus G1 and ACA residues relative to F1a. A similar tendency is observed in all F and C sites without exception (Fig. 1A), indicating that all of the downstream b-sites have a higher affinity than their cognate upstream a-sites. Thus, we next examined OmpR-P binding to all of the other F and C sites using the same strategy used for the F1 site.

We synthesized 40-bp F1-F2aF2a and F1-F2bF2b for studying the F2 site and 60-bp F1-F2-F3aF3a and F1-F2-F3bF3b for studying the F3 site. Note that to detect the binding of OmpR-P to the F2 site and the F1-F2 site, respectively, was required. Binding of OmpR-P to the F1-F2aF2a fragment was significantly reduced (Fig. 6A, lanes 8–14), because even at the highest concentration of OmpR-P used (lane 14), the binding of four OmpR-P molecules to this fragment was marginal in comparison with that to the wild-type F1-F2 fragment (lane 7). Surprisingly, the binding of two OmpR-P molecules to the F1 site in this F1-F2aF2a was also affected. However, this reduction of OmpR-P binding to the F1 site in F1-F2aF2a was abolished by a point mutation in either highly conserved C5 (in first F2a site) or C15 (in second F2a site) in F1-F2aF2a (data not shown), suggesting that...
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FIGURE 5. Two OmpR-P molecules bind to the three tandemly repeated F1b sequence, F1bF1bF1b. OmpR-P and PrS2-OmpR-P were mixed at different concentrations as indicated in the figure and incubated with F1F2a (lanes 1–8) or F1bF1bF1b (lanes 9–16). The symbols (○) and (●) represent OmpR-P and PrS2-OmpR-P, respectively. The bars indicate the 30-bp DNA fragments having three OmpR-P-binding subsites. The gel mobility shift assay was carried out with 5’-end-labeled DNA fragments. The gel was dried and exposed for autoradiography. The gel is representative of three independent experiments.

somewhat the weak binding of OmpR-P to F2aF2a negatively affects the overall stability of complex formation between four OmpR-P molecules and F1-F2aF2a. On the other hand, binding of OmpR-P to the F1-F2F2b fragment was similar to (or slightly better than) its binding to the F1-F2 fragment, resulting in highly cooperative binding of four OmpR-P molecules to this fragment (lanes 16–21). These results demonstrate that F2b has a higher affinity to OmpR-P than to F2a. Similarly, six molecules of OmpR-P exhibited greater cooperative binding to the F1-F2-F3bF3b fragment (Fig. 6B, lanes 17–24), relative to the F1-F2-F3aF3a fragment (lanes 9–16), again supporting that OmpR-P has a higher affinity to F3b than to F3a.

Similar experiments were carried out with C2 (Fig. 6C) and C3 (Fig. 6D) sites, demonstrating that OmpR-P has a higher affinity to the C2b2b sequence or the C3bC3b sequence than to the C2a2a sequence or the C3aC3a sequence, respectively. Interestingly, OmpR-P binding to either C2a2a or C3aC3a sequences was completely abolished, because complexes with the lowest mobility were not detected at the positions indicated by “4 OmpR-P” for C1-C2aC2a (Fig. 6C, lanes 9–14) and by “6 OmpR-P” for C1-C2-C3aC3a (Fig. 6D, lanes 9–16), respectively. In contrast, C2b2b in C1-C2 (Fig. 6C, lanes 16–21) significantly improved cooperative binding of OmpR-P compared with the C1-C2 (lanes 1–7). A similar effect was also observed with C3bC3b in C1-C2-C3 (Fig. 6D, lanes 17–24).

Interestingly, if the order of F1a and F1b sites in F1-F2 was reversed to that of F1bF1a-F2, four OmpR-P molecules were still able to bind to the DNA fragment; however their cooperative binding (Fig. 6D, lanes 8–14) was poorer relative to the wild-type F1-F2 fragment (lanes 1–7), indicating that having the site for which OmpR-P has weaker affinity (F1a) upstream of the site for which it has stronger affinity (F1b) is important for the most effective two-by-two OmpR-P binding mechanism.

These results unambiguously established the hierarchies for OmpR-P binding affinity in all F and C sites as follows: F1b > F1a, F2b > F2a, F3b > F3a, C1b > C1a, C2b > C2a, and C3b > C3a. Furthermore, the present results indicate that OmpR-P cooperatively binds to the a-sites in F2 and F3 than for the a-sites in C2 and C3. This may have important implications in the reciprocal regulation of ompF and ompC genes as discussed below.

DISCUSSION

Hierarchical Arrangement of Two OmpR-P-binding Subsites—It has been shown that two OmpR-P molecules cooperatively bind to the 20-bp F1 site (11). This F1 sequence consists of two 10-bp direct repeats, F1a at the 5’-end and F1b at the 3’-end, which share a number of common bases (Fig. 1A). In the present study, we demonstrated that OmpR-P binds more tightly to downstream F1b than to upstream F1a, because OmpR-P is able to bind to F1bF1b but not to F1aF1a (Fig. 1, B and C). This OmpR-P binding hierarchy is due to the fact that the F1b site contains more conserved bases (eight bases; Fig. 1A) than the F1a site (six bases) in the 10-base consensus sequence (G1)-T2-T3-A4-C5-A6-T7-X-T9-X, derived by PCR-based enrichment technology (Fig. 1A and Ref. 11). We showed that G1, A4, C5, and A6 residues in this consensus sequence play a key role for optimal OmpR-P binding (Figs. 2C and 3, A and B). Similarly, in all of the other OmpR-P-binding sites (F2, F3, C1, C2, and C3) except for F4, the b-sites have more consensus bases than their cognate a-sites (Fig. 1A). Notably, all of the b-sites contain both G1 and C5 residues. Consistently, the gel shift assay demonstrated that OmpR-P has a higher affinity to the b-sites than to the a-sites (Fig. 6). Thus, the following hierarchy was established for OmpR-P binding affinity within each OmpR-P-binding site: F1b > F1a, F2b > F2a, F3b > F3a, C1b > C1a, C2b > C2a, and C3b > C3a. As for the F4 site, the G1 residue in F4b is absent, whereas F4a has the G1 residue. It is currently not known whether F4b has higher affinity than that of F4a for binding to OmpR-P.

Stimulation of OmpR Cooperative Binding by Phosphorylation—Although both OmpR and OmpR-P exist as monomers in solution, two OmpR-P molecules cooperatively bind to the F1 site (11, 12). There are two modes of cooperative binding of OmpR-P F and C sites: one between two OmpR-P molecules in each 20-bp OmpR-P binding unit and the other between OmpR-P dimer binding to F1 and F2, to F2 and F3, to C1 and C2, or to C2 and C3. It is not known at present how cooperative binding occurs between two OmpR-P dimers on the F and C sites. In the present study, we found that nonphosphorylated OmpR is also capable of cooperatively binding to F1aF1b (F1) and F1bF1b (Fig. 4, A and B). The C-terminal DNA-binding domain (OmpRc) by itself was capable of binding to the F1b site in F1, but it could not cooperatively bind to F1aF1b (Fig. 4C). This indicates that the N-terminal domain of OmpR is responsible for its cooperative binding to DNA, and its phosphorylation is likely to play an important role in enhancing the interaction between two N-terminal domains of the OmpR-P dimer on DNA.

Recent structural studies of proteins in the OmpR/PhoB family suggest that the N-terminal domain is important to cooperative OmpR-P DNA binding activity. The x-ray structures of DrrD and DrrB, OmpR
homologues from *Thermotoga maritima*, revealed that the N-terminal domain of the molecule interacts with its C-terminal domain (18, 19). Because the α4-β3-α5 structure of the N-terminal domain that interacts with the C-terminal domain is highly conserved among the OmpR/PhoB family, it was proposed that this region may also serve as an interface to stabilize dimer formation on DNA (18). This proposal was

FIGURE 6. Hierarchical binding of OmpR-P at two binding sites within the F2, F3, C2, and C3 sites. A, hierarchy between the F2a and F2b sites. OmpR-P was incubated at different concentrations as indicated on top, with wild-type F1-F2 (lanes 1–7), F1-F2aF2a (lanes 8–14), or F1-F2bF2b (lanes 15–21) fragment. B, hierarchy between the F3a and F3b sites. OmpR-P was incubated at different concentrations with wild-type F1-F2-F3 (lanes 1–8), F1-F2-F3aF3a (lanes 9–16), or F1-F2-F3bF3b (lanes 17–24) fragment. C, hierarchy between the C2a and C2b sites. OmpR-P was incubated with wild-type C1-C2 (lanes 1–7), C1-C2aC2a (lanes 8–14), or C1-C2bC2b (lanes 15–21) fragment. D, hierarchy between the C3a and C3b sites. OmpR-P was incubated at different concentrations with wild-type C1-C2-C3 (lanes 1–8), C1-C2-C3aC3a (lanes 9–16), or C1-C2-C3bC3b (lanes 17–24) fragment. E, OmpR-P was mixed at different concentrations as indicated and incubated with F1bF1aF2 (lanes 1–7) or F1bF1aF2 (lanes 8–14). The gel mobility shift assay was carried out with 5′-end-labeled DNA fragments. The gels were dried and exposed for autoradiography. The gel is representative of three independent experiments. The arrow indicates the number of OmpR-P molecules bound in an OmpR-P/DNA complex.
supported by recent structural studies of the dimerized N-terminal domain of ArcA (20) and PhoB (21), suggesting an intriguing insight into the role of phosphorylation and subsequent interaction between OmpR-P molecules through the N-terminal \( \beta \)-strands.

**DNA-mediated Dimerization of OmpR-P**—We previously showed that certain substitution mutations in F1a completely abolished OmpR-P binding to the mutated F1 fragments even if F1b was kept intact, indicating that one OmpR-P molecule is unable to make a stable complex with DNA (11). In the present study, we showed that only two OmpR-P molecules bind to a 30-bp DNA fragment consisting of three optimized binding subsites (F1bF1bF1b), indicating that OmpR-P dimer formation is mediated by DNA.

This pair-wise binding of OmpR-P is not due to preformed OmpR-P dimers that subsequently bind to DNA, because switching the order of the a- and b-sites in F1 significantly reduced cooperative OmpR-P binding to the F1bF1a-F2 fragment (Fig. 6E). It appears that OmpR-P dimer formation occurs only on DNA such that the binding of an OmpR-P molecule to the downstream (stronger) b-site assists binding of a second OmpR-P molecule to the upstream (weaker) a-site. This DNA-mediated OmpR-P dimer formation is highly cooperative and synergistic, being mediated by interaction between the N-terminal domains (possibly the \( \alpha4-\beta5-\alpha5 \) regions) of two OmpR-P molecules and the interaction of the C-terminal domain of the second OmpR-P molecule with DNA at the a-site. In this model, the C-terminal DNA-binding domains are tandemly (or in a head-to-tail manner) aligned on DNA, whereas the N-terminal domains interact in a head-to-head manner as proposed by Stock and co-workers (12, 18, 20, 21). Thus, the order of OmpR-P interaction with the subsites appears to play a critical role in the formation of the most stable OmpR-P/DNA complex.

**Significance of Hierarchical OmpR-P Binding in Reciprocal Gene Regulation of ompF and ompC**—On the basis of the data presented, we termed the two-by-two binding mechanism of OmpR-P to DNA as a galloping model because this OmpR-P binding mode is similar to the way the front and rear legs move when a horse gallops. The key feature of this model is that according to hierarchical arrangement of OmpR-P-binding sites at the regulatory elements, F1-F2-F3 and C1-C2-C3, six OmpR-P molecules bind discontinuously (two-by-two) because a downstream b-subsite in individual F and C sites serves as the primary OmpR-P-binding site, and simultaneously a second OmpR-P molecule binds to a weaker upstream a-subsite (Fig. 7A).

The tandem arrangement of two 10-bp sites is essential for two OmpR-P molecules to form a stable complex with DNA. If both sites have a weak affinity for OmpR-P, it severely reduces the binding of OmpR-P to these sites, requiring a significantly high concentration of
Hierarchical Binding of OmpR-P Regulates \textit{ompF} and \textit{ompC}

OmpR-P to detect OmpR-P binding to DNA (Figs. 1 and 6). On the other hand, if both sites have a high affinity for OmpR-P, even nonphosphorylated OmpR is able to bind to DNA (Fig. 4). Therefore, the arrangement of a weaker (a-subsite) followed by a higher (b-subsite) affinity found in all OmpR-P-binding sites in F1-F2-F3 and C1-C2-C3 is important for \textit{ompF} and \textit{ompC} regulation. As proposed in a galloping model, discontinuous arrangement of the a-subsite with weak affinity and the b-subsite with high affinity probably ensures that one OmpR-P molecule first binds to the b-subsite. This also regulates the final binding of two OmpR-P molecules by modulating the affinity of a second a-subsite. It is also important to note that the different affinity between F2a and C2a (which lacks C at 5th position) for OmpR-P, plays a key role in the reciprocal regulation of \textit{ompF} and \textit{ompC}. This is because F2a has a higher affinity for OmpR-P than C2a, because two OmpR-P molecules are able to bind to F2aC2a site at a high concentration of OmpR-P, but not to the C2aC2a site. This results in the binding of four OmpR-P molecules to F1-F2-F3 (Fig. 6A) but binding of only two OmpR-P molecules to C1-C2-C2a (Fig. 6C). The same effect was also observed with F3a and C3a (Fig. 6, B and D), so that only after OmpR-P occupies all F1-F2-F3 sites, will the C1-C2-C3 sites be occupied with OmpR-P, thus achieving the reciprocal regulation of \textit{ompF} and \textit{ompC}.

Earlier footprinting studies established that OmpR-P binding to each of the regulatory elements for the \textit{ompF} and \textit{ompC} promoters occurs differentially in four steps (9, 14): (i) F1, C1 (\textit{ompF} and \textit{ompC} are not expressed); (ii) F1-F2, C1 (\textit{ompF}, but not \textit{ompC} is expressed); (iii) F1-F2-F3, C1 (\textit{ompF}, but not \textit{ompC} is expressed); and (iv) F1-F2-F3 plus F4, C1-C2-C3 (\textit{ompF} repressed and \textit{ompC} is expressed). This suggests that there are four critical cellular concentrations of OmpR-P that regulate the induction and repression of \textit{ompF} and the induction of \textit{ompC}. Fig. 7B illustrates the sequential regulation of \textit{ompF} and \textit{ompC} expression, wherein the cellular concentration of OmpR-P increases from KI (low) to KIV (high) upon medium osmolarity changes. At KI, only F1 and C1 are occupied with two OmpR-P molecules. Note that two OmpR-P molecules bind to both F1 and C1 sites in the order depicted by the numbered molecules and according to the galloping model. Both \textit{ompF} and \textit{ompC} are not transcribed under this condition. When the OmpR-P concentration increases from KI to KII, two more OmpR-P molecules bind to the F2 site again in the order from molecule 3 to molecule 4. At KII, two more OmpR-P molecules (molecules 5 and 6) bind to the F3 site. On the other hand, the OmpR-P concentration of KII or KIII is not high enough to occupy C2 and C3 sites, allowing exclusive transcription of \textit{ompF}. As medium osmolarity further increases, the OmpR-P concentration finally reaches KIV allowing the binding of two more OmpR-P molecules (molecules 7 and 8) to F4 to repress \textit{ompF} transcription. This repression is assumed to be due to DNA loop formation between F4 and F1-F2-F3 mediated by OmpR-P bound to these regions (9). Six OmpR-P molecules (molecules 1–6) are now able to bind to C1-C2-C3 in a discontinuous and cooperative manner to activate \textit{ompC} transcription.

This well orchestrated gene regulation of \textit{ompF} and \textit{ompC} by OmpR-P is achieved by the combination of the two-domain structure of OmpR, the cellular concentration of OmpR-P, and the highly sophisticated hierarchical arrangement of OmpR-P-binding sites. Each N- and C-terminal domain of OmpR has a distinct role in the DNA binding of OmpR-P. We have demonstrated that \textit{E. coli} cells contain a large pool of OmpR, whereas only a minor fraction is presumed to be phosphorylated by EnvZ, a transmembrane osmosensing histidine kinase (7, 8). The cellular level of OmpR-P is thus finely tuned by dual EnvZ functions, OmpR kinase and OmpR-P phosphatase, whose ratios are regulated by the medium osmolarity. Finally, because of the hierarchical arrangement of OmpR-P-binding sites, OmpR-P occupy differently the regulatory element of \textit{ompF} and \textit{ompC} promoters depending on the concentration of OmpR-P, allowing a reciprocal expression of these genes to avoid overlapping expression from both genes at the same time.

\textbf{Conclusion}—In this study, we demonstrated a regulatory mechanism for transcription using a single transcription factor. The reciprocal transcription of \textit{ompF} and \textit{ompC} is regulated by OmpR-P and is finely tuned by virtue of hierarchical OmpR-P binding to DNA. This simple but highly sophisticated transcriptional regulation performed by a single transcription factor may be widely used in prokaryotes and is an interesting contrast to the eukaryotic systems, where a large number of transcription factors are involved in regulating the expression of a single gene.

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