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Activation of the Osmoregulated *ompC* Gene by the OmpR Protein in *Escherichia coli*: A Study Involving Synthetic OmpR-Binding Sequences[1]

Sumio Maeda,* Katsuhiko Takayanagi,* Yoshifumi Nishimura, Takemi Maruyama,** Kou Sato,** and Takeshi Mizuno*,[2]**

**Laboratory of Microbiology, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya, Aichi 464; and* ***Graduate School of Integrated Science, Yokohama City University, Kanazawa-ku, Yokohama, Kanagawa 236*

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Expression of the *Escherichia coli* outer membrane proteins, OmpC and OmpF, is regulated in response to the medium osmolarity. The OmpR and EnvZ proteins are transcriptional factors involved in this osmotic regulation of the *ompC* and *ompF* genes. In particular, expression of the *ompC* gene is activated by the positive regulator, OmpR, in response to high osmolarity of the medium. In this study, we succeeded in defining a functional OmpR-binding sequence by analyzing a set of synthetic oligonucleotides, and propose a consensus motif for OmpR-binding. It was also demonstrated that the asymmetric OmpR-binding sequence, thus identified, can activate the canonical *ompC* promoter in an orientation independent-manner, providing that this sequence is placed closely and stereospecifically with respect to the −35 region.

The OmpR protein is the transcriptional activator which is involved in the expression and regulation of the *ompC* and *ompF* genes in response to the medium osmolarity in *Escherichia coli* (see Refs. *1-3* for reviews and references therein). The function of OmpR is modulated by EnvZ, which is an OmpR-specific protein kinase located in the cytoplasmic membrane, so the phosphorylated form of OmpR is capable of binding efficiently to *ompC* and *ompF* promoter DNAs (*4-7*). Recent extensive studies on this particular osmoregulatory system provided us with a paradigm of not only signal transduction, but also gene activation in procaryotes.

In general, the definition of transcriptional regulatory DNA domains, including promoters, and their functions is an essential step for an understanding of the molecular mechanism underlying any gene expression. In this respect, the canonical −35 and −10 regions of the *ompC* and *ompF* promoters appear to show poor degrees of sequence homology with the respective consensus sequences (TTGACA and TATAAT) (*8, 9* and see Fig. 1), although these promoters are two of the most efficiently expressed ones in *E. coli* cells, In fact, in addition to the canonical −35 and −10 regions, a long *cis*-acting sequence located just upstream of the −35 region is essentially required for both the genes to be fully activated (*8, 9*). *In vitro* DNase I and *in vivo* chemical footprinting studies established previously that OmpR binds to these upstream sequences comprising more than 60 nucleotides (nucleotide positions around −40 and −100 in Fig. 1, where +1 corresponds to the first nucleotides of the *ompC* and *ompF* mRNAs) (*10, 11*). It was then suggested that the upstream sequence of the *ompC* promoter comprises three distinct OmpR recognition sites, mainly based on the results of successive deletion analyses (*12*). This is most likely true even for the *ompF* promoter. To date, however, it has been rather difficult to define each OmpR recognition motif (or consensus motif) located within these upstream sequences of both the *ompC* and *ompF* promoters, because the results of DNase I footprinting revealed that OmpR covers the entire stretch of more than 60 nucleotides consecutively and completely (*10, 11*). In this respect, we previously suggested that three repetitive sequences (5′-TGAAACATCT motif) found in the *ompC* promoter region may be important for the OmpR-binding (*8, 13*). Previously, Tsung *et al.* also proposed relatively short sequences, 5′-TG(A/T)ANCAT-NT and 5′-TTTAC(A/T)TTTT, as consensus motifs specific for the *ompC* and *ompF* promoters, respectively, mainly based on their results as to *in vivo* chemical footprinting and simple inspection of sequence homology (*11, 14*). However, the functional significance of these relatively short sequences has never been assessed further. In this study, we succeeded in defining a functional OmpR-binding sequence by analyzing a set of synthetic oligonucleotides. We also demonstrated that the asymmetric OmpR-binding sequence, thus identified, can activate the canonical *ompC* promoter in an orientation-independent manner, providing that this sequence is placed closely and stereospecifically with respect to the −35 region.

EXPERIMENTAL PROCEDURES

Bacteria and Growth Media—The following *E. coli* K-12 strains were used (*15*): MC4100 [F⁻ *Δ(argF-lac)U169 araD139 rpsL150 flbB5301 relA1 ptsF25*] and MH1160 (*ompR101*). The latter is a derivative of MC4100 (*16*). Unless otherwise specified, bacteria were grown in Luria broth. For the β-galactosidase assay, medium A supplemented with 15% (w/v) sucrose was used (*17*).

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[2] To whom correspondence should be addressed.

DNA Techniques—The reagents used for recombinant DNA techniques, such as restriction endonucleases, the Klenow large fragment of *E. coli* DNA polymerase I and bacteriophage T4 ligase were all purchased from Takara Shuzo. The conditions used for these reagents were those suggested by the supplier. Recombinant DNA manipulations were mainly carried out according to conventional laboratory methods described by Maniatis *et al.* (*18*).

Plasmid Construction—Strategies of construction of plasmids used in this study were as follows (see Figs. 1 and 3). First of all, oligonucleotides-1 and -4 were inserted into a *Hin*cII site in the polylinker region of Bluescript M13$^+$ in both orientations with respect to the polylinker sequence. From the plasmids thus constructed, each *Xba*I-*Xho*I polylinker sequence containing the respective oligonucleotide was isolated, and then inserted into the *Xba*I-*Xho*I sites of plasmid pCX-0 carrying the −35 and −10 regions of the *ompC* promoter fused to the *lacZ* gene, which was constructed previously (unpublished). The resultant plasmids were designated as pCP-C, pCP-CR, pCP-F, and pCP-FR, respectively. These plasmids were digested with *Xho*I, and treated with S1 nuclease, and then ligated to construct plasmids pCP-CS1, pCP-CRS1, pCP-FS1, and pCP-FRS1, respectively. On the other hand, the same restriction fragments from plasmids pCP-C and pCP-F were ligated in the presence of a *Bam*HI linker (dCCGGATCCGG) to construct plasmids pCP-CB and pCP-FB, respectively. The nucleotide sequences of the manipulated regions of each plasmid were confirmed as shown in Fig. 3.

OmpR-Phosphorylation—The experimental conditions for OmpR-phosphorylation were essentially the same as those given previously (*19*).

DNA-Binding Assay—*In vitro* binding of OmpR to the synthetic oligonucleotides was assayed by means of non-denaturing gel electrophoresis, as described previously (*4*).

Assay of β-Galactosidase Activity—β-Galactosidase was assayed as described by Miller with slight modifications (*20*). Cells grown to mid-logarithmic phase were collected and then suspended in a volume of 250 mM sodium phosphate (pH 7.2) to accurately determine the cell density. The cells were permeabilized with toluene before the assay.

RESULTS

Synthetic Oligonucleotides for OmpR-Binding Sequences—A set of synthetic oligonucleotides (20-mers) corresponding to various portions of the upstream sequence of the *ompC* promoter were prepared, as shown in Fig. 1. Oligonucleotide-1 covers the region which was suggested to be essential for the functioning of the *ompC* promoter (positions −99 to −80), *i.e.*, when this region was removed by successive deletion mutagenesis, the OmpR-dependent transcription of the *ompC* promoter was almost completely abolished (*8, 12*). Oligonucleotide-2 has a sequence which overlaps that of oligonucleotide-1, but slightly differs from the latter at its end (positions −94 to −75). A similar sequence to that of oligonucleotide-1 was found in the corresponding region of the upstream sequence of the *ompF* promoter (positions −101 to −82). Since this sequence was also demonstrated previously to be required for the full

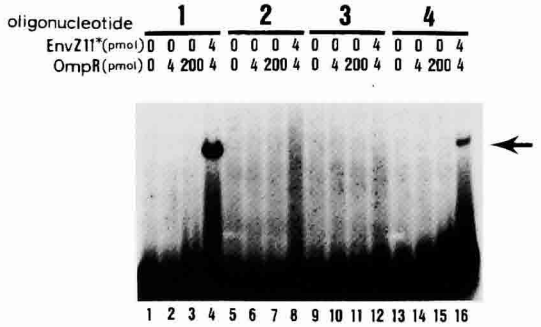


Fig. 2. **Binding of the phosphorylated form of OmpR to the set of synthetic oligonucleotides.** Various amounts of OmpR (0, 4, and 200 pmol) were incubated with 0.1 mM ATP in the presence or absence of EnvZ11* (4 pmol) in 20 μl of a buffer, as described previously (*4*). Note that upon incubation with ATP and EnvZ11*, which is a truncated form of the OmpR-specific kinase (*19*), OmpR is known to be efficiently phosphorylated (*4*). Each synthetic oligonucleotide (1 through 4), which was radioactively pre-labeled with ^{32}P, was added to the reaction mixtures and then subjected to DNA-binding assay by means of non-denaturing gel electrophoresis, followed by autoradiography. The arrow indicates the position of OmpR-DNA complex.

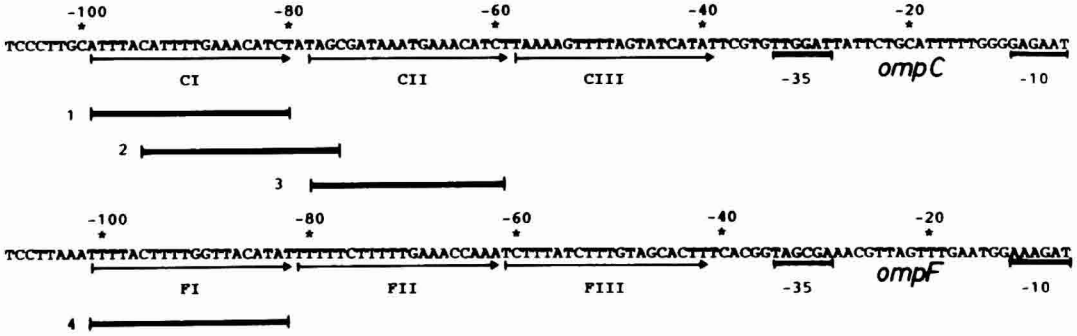
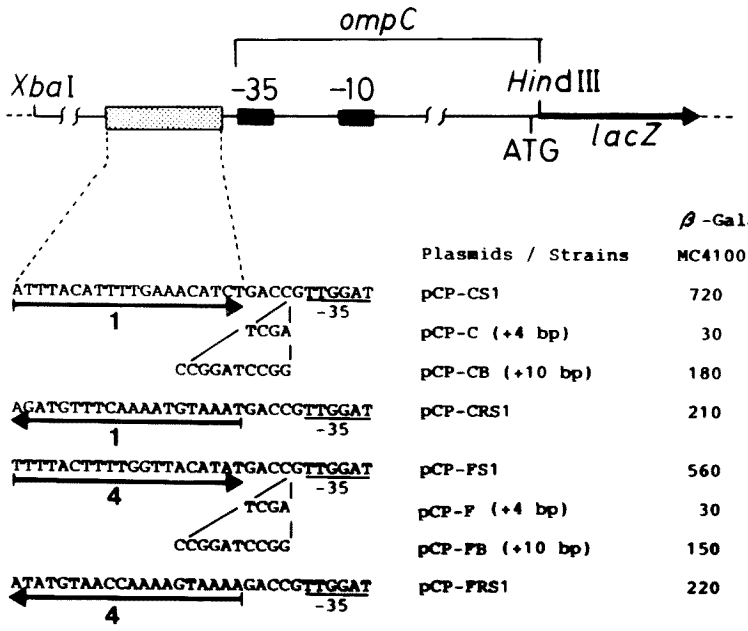


Fig. 1. **DNA sequences of the *ompC* and *ompF* promoter regions.** The upper and lower sequences represent the *ompC* and *ompF* promoter regions, respectively, where the sequences are numbered with their mRNA start sites taken as +1. The canonical promoter sequences (−35 and −10 regions) are indicated. Synthetic oligonucleotides (20-mers) used in this study are denoted by the horizontal bars with numbers 1 through 4. The horizontal arrows (CI through CIII and FI through FIII) represent 20-bp consensus motifs, which were proposed as OmpR-recognition sequences in this study (see Fig. 4).



Plasmids / Strains	β-Galactosidase (Units) MC4100	MH1160
pCP-CS1	720	50
pCP-C (+4 bp)	30	20
pCP-CB (+10 bp)	180	60
pCP-CRS1	210	70
pCP-FS1	560	50
pCP-F (+4 bp)	30	40
pCP-FB (+10 bp)	150	60
pCP-FRS1	220	80

Fig. 3. **β-Galactosidase activities expressed by the series of composite *ompC* promoters fused to the *lacZ* gene.** The structure of the composite *ompC* promoters is schematically shown, in which the shaded rectangle denotes the oligonucleotides inserted (1 or 4, see Fig. 1). The relevant sequences for the series of composite *ompC* promoters are also shown. These composite *ompC* promoters fused to the *lacZ* gene were cloned on a low-copy-number plasmid, as listed. The series of plasmids were introduced into the wild-type (MC4100) and *ompR101* mutant (MH1160) strains, and then β-galactosidase activity expressed by these cells was measured. The cells for the assay were grown in medium A supplemented with 15% sucrose.

activation of the *ompF* promoter (*21*), we decided to synthesize oligonucleotide-4 comprising this particular sequence. Several other oligonucleotides were also prepared (*e.g.*, oligonucleotide-3 and others not shown).

Binding Ability of OmpR to the Set of Synthetic Oligonucleotides—First of all, the synthetic oligonucleotides were examined in terms of their OmpR-binding abilities by means of non-denaturing gel retardation analysis with purified OmpR, OmpR being either non-phosphorylated or phosphorylated (Fig. 2). Even when a large amount of the non-phosphorylated form of OmpR was used, *e.g.*, 200 pmol per reaction mixture, none of the oligonucleotides exhibited detectable OmpR-binding ability. Next, a small amount of OmpR (4 pmol per reaction mixture) was preincubated with a truncated form of EnvZ in the presence of ATP, under which conditions OmpR is known to be efficiently phosphorylated (*4*), and then subjected to the binding assay. It was revealed that oligonucleotide-1 and -4 exhibited significant OmpR-binding ability, whereas the others, oligonucleotides-2 and -3, did not. It should be noted that several other synthetic oligonucleotides were also subjected to the same OmpR-binding assay, *e.g.*, a 20-mer oligonucleotide covering the region from −61 to −42 in the *ompF* promoter. None of those we tested exhibited detectable OmpR-binding ability, even when the phosphorylated form of OmpR was used (data not shown). We thus concluded that both oligonucleotides-1 and -4, at least, contain a high-affinity OmpR-binding sequence.

Functional Assessment of the Synthetic OmpR-Binding Sequences—The crucial question then arose as to whether these high-affinity OmpR-binding sequences, thus defined *in vitro*, are able to function *in vivo* to activate the transcription of the canonical *ompC* promoter or not. To answer this crucial question directly, a series of composite *ompC* promoters fused to the *lacZ* coding sequence were constructed on a low-copy-number plasmid (Fig. 3). Oligonucleotides-1 and -4 were, respectively, placed immediately upstream of the −35 region of the *ompC* promoter in either the correct or inverted orientation with respect to the −35 region. The distance between them was also varied by inserting short spacer sequences (Fig. 3). The plasmids thus constructed were introduced into the wild-type and *ompR*$^-$ mutant strains, MC4100 and MH1160 (*ompR101*), and then β-galactosidase activity expressed in these cells was assayed (Fig. 3). Such analyses provided us with several intriguing results as follows. 1) The short sequences exhibited OmpR-binding ability *in vitro* can indeed efficiently trigger the *ompC* transcription *in vivo* in an OmpR-dependent manner. 2) These sequences can function in both orientations with respect to the −35 region, although they are apparently asymmetric. 3) The distance between these sequences and the −35 region is a crucial parameter for the composite promoters to be fully functional, suggesting that their stereospecific positioning with respect to the −35 region is required. It should be noted that when the distance between them was increased to +20 base pairs, no activation of the *ompC* promoter was observed (data not shown). It is also worth mentioning that oligonucleotide-2 was also used to construct a set of composite promoters, but none of them was found to be functional (data not shown). In conclusion, taking all these results together, the 20-mer asymmetric OmpR-binding sequences defined in this study can function to trigger *ompC* transcription in an orientation-independent manner, providing that the sequences are placed closely and stereospecifically with respect to the −35 region.

DISCUSSION

Based on the results presented in this paper as well as those reported previously (*8, 13*), we can now consider the possible OmpR-binding sequence located within the upstream sequences of both the *ompC* and *ompF* promoters. First of all, oligonucleotides-1 and -4 show a high degree of sequence homology with each other. It was then noticed that similar sequences to them, each comprising 20 nucleotides, are tandemly repeated three times within each upstream sequence of the *ompC* and *ompF* promoters (see

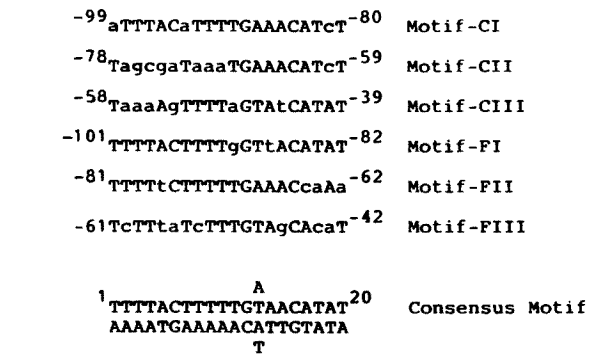


Fig. 4. **Consensus motif found in the *ompC* and *ompF* promoter regions.** Stretches of nucleotides with similar 20-bp sequences with each other, each of which was proposed to be involved in OmpR-binding, were aligned. Motifs CI through CIII were located in the *ompC* promoter region, whereas motifs FI through FIII were in the *ompF* promoter region (see Fig. 1). Bases different from the consensus motif are shown in the lower case letters.

Fig. 1, namely, CI to CIII and FI to FIII, respectively). When these sequences were aligned, a consensus sequence could be deduced for the putative OmpR recognition motif, as shown in Fig. 4. In this respect, it is worth mentioning that the two invariant G-C base pairs found at positions 12 and 16 in the motifs were previously suggested to be important for the OmpR-binding, based on the results of studies involving base substitution mutagenesis (*8*) and *in vivo* chemical footprinting (*11*). It is also worth mentioning that the motifs proposed in this study contain the short repetitive sequences, which were previously suggested as the putative OmpR-binding sequences (*8, 11, 14*).

Motifs CI and FI represent a high-affinity OmpR-binding site, as demonstrated in this study, whereas motifs CII, CIII, and FIII were considered to represent a low-affinity OmpR-binding site, as judged from the results of DNase I footprinting (*13, 21*) as well as those presented in this study. Motif FII seems to be a high-affinity OmpR binding-site, since its sequence well matches the consensus sequence, although this remains to be proven. The binding of OmpR to these low-affinity binding sites may cooperatively depend on its binding to the most upstream high-affinity binding site (CI or FI). In any case, it is of interest to note that such alignment of three OmpR recognition motifs within the upstream sequences can be envisioned as being not only repetitive but also periodical as to the helical topology of the DNA structure, since each motif comprising 20 nucleotides is roughly equivalent to two helical turns of ordinary B-form DNA. In other words, OmpR molecules, when bound to each motif, should line up on one side of the DNA helix. Such topological alignment of OmpR on the DNA helix is most likely required for the proper interaction with RNA polymerase. This is probably the reason why the stereospecific positioning of the OmpR-binding sequence with respect to the −35 and −10 region is required, as demonstrated in the previous study (*22*) and confirmed in this study. It should also be emphasized that the distance between the most downstream motif, CIII, and the −35 region is 5 base pairs, and this distance appears to be crucial even for the artificial composite promoter (see pCP-CS1 in Fig. 3). In any event, the results presented in this paper suggest that the stereospecifically aligned multiple OmpR-binding sequences, each of which exhibits different affinity to OmpR, play important roles in the complex mechanism underlying activation of the *ompC* and *ompF* genes in response to the medium osmolarity.

In this paper, we were mainly concerned with the molecular mechanism underlying activation of the *ompC* promoter by OmpR. It is also known that expression of *ompC* and *ompF* responds in opposite directions in response to the medium osmolarity (*1, 16*). In this respect, it is of interest to examine whether the composite *ompC* promoters constructed in this study respond to the medium osmolarity or not. In order to do so, however, these composite promoters fused to the *lacZ* gene on a low-copy-number plasmid should be introduced into the *E. coli* chromosome, one by one, because a gene-dosage-effect should be taken into consideration to carefully examine fine regulation of any gene *in vivo*. Experiments along these lines are currently under way in our laboratory.

Finally, the interesting but bothersome fact that the apparently asymmetric OmpR-binding sequence can function in an orientation-independent manner should be mentioned. Given the importance of the OmpR-RNA polymerase interaction (*13*), this suggests, at least, that an equivalent OmpR-RNA polymerase contact can be established regardless of the orientation of the OmpR-binding sequence. In order to clarify this paradoxical problem, however, the molecular structure of the nucleoprotein complex composed of OmpR and its cognate DNA motif should be resolved at the atomic level by means of nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography. Experiments along these lines are also under way in our laboratory.

REFERENCES

1. Csonka, L.N. (1989) *Microbiol. Rev.* **53**, 121-147
2. Mizuno, T. & Mizushima, S. (1990) *Mol. Microbiol.* **4**, 1077-1082
3. Stock, J.B., Ninfa, A.J., & Stock, A.N. (1989) *Microbiol. Rev.* **53**, 450-490
4. Aiba, H., Nakasai, F., Mizushima, S., & Mizuno, T. (1989) *J. Biochem.* **106**, 5-7
5. Aiba, H. & Mizuno, T. (1990) *FEBS Lett.* **261**, 19-22
6. Forst, S.A., Delgado, J., & Inouye, M. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6052-6056
7. Igo, M.M., Ninfa, A.J., & Silhavy, J. (1989) *Gene Dev.* **3**, 598-605
8. Mizuno, T. & Mizushima, S. (1986) *J. Bacteriol.* **168**, 86-95
9. Dairi, T., Inokuchi, K., Mizuno, T., & Mizushima, S. (1985) *J. Mol. Biol.* **184**, 1-6
10. Mizuno, T., Kato, M., Jo, Y.-L., & Mizushima, S. (1988) *J. Biol. Chem.* **263**, 1008-1012
11. Tsung, K., Brissette, R.E., & Inouye, M. (1989) *J. Biol. Chem.* **264**, 10104-10109
12. Maeda, S. & Mizuno, T. (1990) *J. Bacteriol.* **172**, 501-503
13. Maeda, S., Ozawa, Y., Mizuno, T., & Mizushima, S. (1988) *J. Mol. Biol.* **202**, 433-441
14. Tsung, K., Brissette, R.E., & Inouye, M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5940-5944
15. Casadaban, M.J. (1976) *J. Mol. Biol.* **104**, 541-555
16. Hall, M.N. & Silhavy, T.J. (1981) *J. Mol. Biol.* **151**, 1-5
17. Kawaji, H., Mizuno, T., & Mizushima, S. (1979) *J. Bacteriol.* **140**, 843-847
18. Maniatis, T., Fritsch, E.F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Aiba, H., Mizuno, T., & Mizushima, S. (1989) *J. Biol. Chem.* **264**, 8563-8567
20. Miller, J.H. (1972) *Experiments in Molecular Genetics*, pp. 201-205, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Kato, M., Aiba, H., & Mizuno, T. (1989) *J. Biochem.* **105**, 341-347
22. Maeda, S. & Mizuno, T. (1988) *J. Biol. Chem.* **263**, 14629-14633