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Mechanism of gene activation by the *Escherichia coli* positive regulator, OmpR: a mutant defective in transcriptional activation

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Abstract The OmpR protein is an activator specific for the *E. coli ompC* and *ompF* genes. This protein functions in a phosphorylation-dependent manner through a presumed interaction with RNA polymerase. In this study we isolated OmpR mutants which were suggested to be defective for transcriptional activation, but not for DNA binding. Two such mutants, that we isolated, have a single amino acid alteration at positions 131 [P131S], and 179 [P179L], respectively, of OmpR, comprising 239 amino acids. These altered amino acids in OmpR may be implicated, directly or indirectly, in the presumed RNA polymerase/OmpR interaction that is important for transcriptional activation.

Key words: Positive regulator of *E. coli*; Transcriptional activation; Mutation analysis; Osmotic regulation

1. Introduction

In *Escherichia coli*, expression of the genes for major outer membrane proteins, OmpC and OmpF, is regulated at the transcriptional level in response to the medium osmolarity (for a review, see [1]). Whereas OmpR is the activator protein which binds to both the cognate *ompC* and *ompF* promoters, EnvZ is presumably a membrane-located osmotic sensor which exhibits OmpR-specific kinase and apparent phosphatase activities. The EnvZ-mediated phosphorylation and dephosphorylation of OmpR are crucial events in the osmoregulation.

The molecular mechanism by which transcription is triggered through the coordinate functions of RNA polymerase and positive regulators has been the general subject of longstanding debate in both prokaryotes and eukaryotes. In this context, OmpR is one of the best characterized bacterial positive regulators that somehow enhance the transcriptional ability of RNA polymerase. The N-terminal half of OmpR, comprising about 120 amino acids, contains the site involved in phosphorylation, whereas the C-terminal half, containing about 120 amino acids, exhibits latent DNA-binding ability specific for cognate DNAs [2]. The DNA-binding ability of the C-terminal domain is modulated through phosphorylation of the N-terminal domain, i.e. the phosphorylation of OmpR results in remarkable enhancement of its DNA-binding ability [3]. Several lines of evidence suggested that OmpR functions through a direct interaction with RNA polymerase [4–7]. A crucial question then arose, namely, whether or not there is any contact site (or activation site) with RNA polymerase in the primary sequence of the OmpR molecule. To address this mechanistic question, in this study we attempted to isolate an OmpR mutant which maintains its phosphorylation-dependent DNA-binding ability, but lacks the ability to help RNA polymerase to activate the cognate promoters. Here we isolated such OmpR mutants and characterized them with emphasis on the mechanism underlying gene activation by OmpR.

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Abbreviations: PCR, polymerase chain reaction; bp, base pair(s); PC-mutant, positive control mutant.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli K-12 strain SG480Δ76, which lacks both the *ompR* and *envZ* genes, was mainly used in this study, unless otherwise noted [8]. Plasmid pCP-CS1 was constructed previously [9]. Plasmid pBRC006 was constructed from pSAC112B [10], in which the ampicillin resistance gene was replaced by the chloramphenicol resistance gene.

2.2. Mutagenesis of the ompR gene with polymerase chain reaction (PCR)

The *SalI–Eco*RI fragment from plasmid pBRC006 was subcloned into the corresponding sites in pUC19. The resultant plasmid was cut with *Sca*I and subjected to PCR in the presence of 0.3 mM $MnCl_2$ with commercially available oligonucleotide primers (RV and M4, Takara Shuzo Co.). Other PCR-conditions were essentially the same as those described previously [11]. PCR products were treated with *SalI–Bgl*II, and then cloned into the corresponding region of pBRC006 to yield a set of *ompR* mutants.

2.3. DNA-binding assay

Total cell lysate, used for DNA binding assay, was prepared as follows. *E. coli* DB225 [12], carrying each plasmid examined, were grown to the mid-logarithmic phase. The cells were suspended in buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 2 mM DTT, 5% glycerol, 50 mM KCl and 0.05% Nonidet P40, and then disrupted by sonication. Using these lysates directly, DNA-binding assay was performed as follows. To phosphorylate OmpR in the cell lysate, 2 μl of EnvZ11* (1 μg) [13], 1.5 μl of 4 mM ATP and 1.5 μl of 50 mM $MgCl_2$ were added to 10 μl of cell lysate, and then incubated for 20 min at 37°C (lanes denoted by + in Fig. 2). As a control experiment, EnvZ11* and ATP were not added (lanes denoted by − in Fig. 2). To this sample, 3.7 μl of ^{32}P-labelled DNA probe was added. This DNA solution contained 0.56 ng of DNA, 240 ng of poly(dI–dC)·poly(dI–dC) and 115 mM EDTA. After incubation for 20 min at 24°C, the mixtures were immediately subjected to non-denaturing polyacrylamide gel electrophoresis (6% acrylamide), followed by autoradiography, as described previously [2].

3. Results

We used a composite *ompC* promoter, as an appropriate reporter, which was fused to the *lacZ* gene on a plasmid (pCP-CS1), as shown in Fig. 1. In this promoter, a 20-bp sequence that functions as a high affinity OmpR-binding site was placed in front of the −35 and −10 sequences of *ompC* with an appropriate spacer between them. This plasmid was introduced into strain SG480Δ76 (*ΔompR, ΔenvZ, ΔlacZ*). In the cells, this promoter-*lacZ* fusion gene was fully active, provided that the cells

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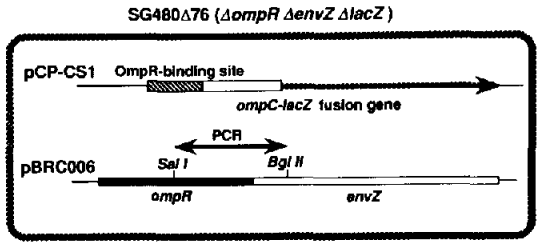

Fig. 1. Schematic representation of experimental design. The structure of the plasmids, pCP-CS1 and pBRC006, were illustrated. The one (pCP-CS1) carries a simplified *ompC* promoter fused to the *lacZ* gene, whereas the other (pBRC006) carries both the *ompR* and *envZ* genes. The region subjected to PCR-mutagenesis was indicated for pBRC006. For other details, see the text.

were concomitantly transformed with a plasmid (pBRC006) carrying both the *ompR* and *envZ* genes (Fig. 1). In other words, its transcription was efficiently triggered in a phospho-OmpR-dependent manner, as judged from β-galactosidase activity expressed (for example, see Fig. 4). The reason we used this particular promoter is as follows. Both the natural *ompC* and *ompF* promoters contain multiple OmpR-binding sites [14]. A complex interplay of these OmpR-binding sites appears to be implied not only in the activation, but also in the osmoregulation of *ompC* and *ompF*. However, the simplified promoter should allow us to focus particularly on the process of the OmpR-dependent transcriptional activation, circumventing any other complexity (e.g. a presumed cooperative OmpR–OmpR interaction) with regard to the osmoregulation. It is worth mentioning that the level of β-galactosidase activity, expressed in SG480Δ76 carrying both pCP-CS1 and pBRC006, was slightly higher (less than 2-fold) in Luria-broth containing 0.4 M NaCl than in Luria-broth without NaCl.

Based on the experimental rationale described above, we attempted to isolate an OmpR mutant that fails to activate the promoter-*lacZ* fusion gene, but binds to the cognate OmpR-binding site. It was previously suggested that the C-terminal half of OmpR appears to possess the activation domain (Tsuzuki, Aiba and Mizuno, unpublished results). Thus, here plasmid pBRC006 was subjected to in vitro PCR-mutagenesis with appropriate primers, which together correspond to the region encoding the C-terminal half of OmpR. The *SalI–BglII* region encompassing the mutagenized region was isolated and ligated into the original plasmid (see Fig. 1). SG480Δ76 carrying the reporter plasmid was transformed with the resultant mixture of plasmids and then spread on lactose-MacConkey plates. The osmolarity of lactose-MacConkey plate was estimated to be intermediate, because both the *ompC* and *ompF* genes are efficiently expressed on this plate. Among about 10^4 transformants, a number of white colonies appeared. They were suspected to contain a mutant plasmid producing an altered OmpR. Among them, those producing no or a reduced amount of OmpR in cells were discarded, through selecting by means of immuno-blotting analysis with an anti-OmpR antiserum. The remainder were subjected to the next critical selection procedure.

To examine the ability of a given OmpR mutant to bind to the cognate DNA in an EnvZ (or phosphorylation)-dependent manner, we conducted an in vitro gel-shift assay. A crude cell lysate containing a given OmpR mutant was isolated on a small scale and then directly incubated with a 71-bp 3′-end radiolabelled DNA fragment encompassing the OmpR-binding sequence. In order to assess the phospho-OmpR dependency, the binding assay was carried out in either the absence or presence of purified form of EnvZ11* and ATP. The samples were subjected to nondenaturing polyacrylamide gel electrophoresis, followed by autoradiography (Fig. 2). The results for a set of appropriate reference experiments showed that one can easily assess whether or not a given OmpR mutant retains the ability to bind to the cognate DNA in phosphorylation-dependent manner (Fig. 2A). Under these conditions, for example, the wild-type OmpR exhibited a substantial DNA-binding ability in an phosphorylation-dependent manner (lanes 3 and 4), whereas the OmpR mutant, which lacks the crucial phosphorylated site [Asp-55 to Gln], failed to do so (lanes 5 and 6). A number of putative OmpR mutants was then subjected to this analysis. Most of them were found to have lost their DNA-binding ability, suggesting that these OmpR mutants are defective in either their intrinsic DNA-binding ability or in the process of their phosphorylation-dependent DNA-binding. They were also discarded, because in this study we did not intend to isolate these types. Out of 30 OmpR mutants examined, only two appeared to retain their phosphorylation-dependent DNA-binding ability. The results of gel-shift assaying of these two (tentatively named OmpR-4A and -10A) and a negative instance (named OmpR-10C) are shown in Fig. 2B. These results were confirmed further by mean of a quantitative DNA-binding assay using various amounts of OmpR in cell lysates (Fig. 3). Both OmpR-4A and -10A exhibited a significant ability to bind to the cognate DNA, as compared in the case of OmpR-10C. The ability of OmpR mutants to activate the promoter-*lacZ* fusion gene was finally confirmed in SG480Δ76 cells carrying pCP-CS1 by monitoring β-galactosidase activity (Fig. 4). The cells producing each of OmpR-4A and -10A did indeed fail to express the promoter-*lacZ* fusion gene. From these results, we assumed that OmpR-4A and -10A may represent ones whose function is defective in a process of transcriptional activation, but not in the process of DNA-binding.

To examine the amino acid substitutions in these OmpR mutants, the nucleotide sequence was determined for the entire mutagenized region of each mutant plasmid (Fig. 5). In par-

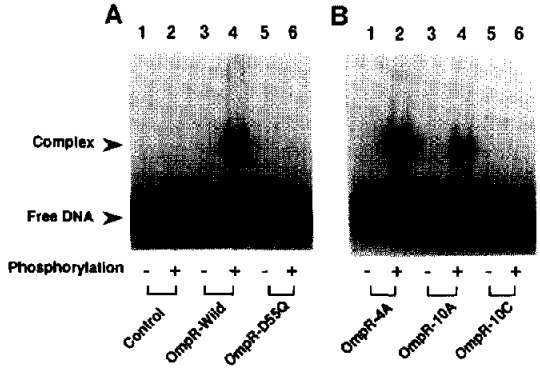

Fig. 2. Phosphorylation dependent DNA binding assay. DNA-binding assay was carried out with a ^{32}P-labeled DNA fragment encompassing the synthetic OmpR binding site, which was derived from pCP-CS1. Total cell lysates, containing approximately same amount of OmpR (15 ng) was subjected to DNA-binding assay, as described in section 3. Note that OmpR-D55Q was characterized previously, which lacks the presumed phospho-accepting aspartate site [Asp-55].

ticular, OmpR-4A and -10A were inferred to have a single amino acid substitution at positions 131, [Pro-131 to Ser] and179, [Pro-179 to Leu], respectively. These amino acid substitutions are most likely responsible for the altered in vivo and in vitro properties observed for OmpR-4A and -10A, respectively.

Here, the following questions remained to be answered. First, what would happen when these OmpR mutants were assessed as to the natural *ompC* and *ompF* promoters. Second, what would happen when these were assessed in the presence of the wild-type OmpR. To address these issues, a plasmid specifying either [P131S] or [P179L] was introduced into some strains, namely, DB225 (*ΔompR, ΔenvZ*) carrying an *ompC-lacZ* fusion gene; DB513 (*ΔompR, ΔenvZ*) carrying an *ompF-lacZ* fusion gene; and MC4100 carrying pCP-CS1 (wild-type with respect to both the *ompR* and *envZ* genes). It should be noted that the *ompC-lacZ* and *ompF-lacZ* fusion were previously constructed by means of Muc(Ts)-*lac* insertion on the chromosome, and were characterized as a transcriptional fusion. β-Galactosidase activity expressed by these cells, transformed by the set of plasmids each specifying [P131S] or [P179L], were examined (Fig. 6). It was found that OmpR[P179L] failed to activate both the *ompC* and *ompF* promoters, whereas OmpR[P131S] failed to activate the *ompC* promoter as expected, but could fully activate the *ompF* promoter. Essentially the same results were obtained irrespective of the medium osmolarity tested (data not shown, and see discussion). It was also found that these OmpR mutants, expressed from the high copy-number plasmid, exhibited a negative dominant character with respect to the wild-type OmpR, thereby MC4100 carrying pCP-CS1 exhibited a very low level of β-galactosidase activity after the OmpR mutants had been introduced.

4. Discussion

It has been reported that certain amino acid substitutions in the α subunit of RNA polymerase severely affect OmpR-dependent activation of in vivo *ompC* and/or *ompF* transcription [5–7]. A reconstituted RNA polymerase containing C-terminally truncated α subunits does not respond to in vitro transcription activation by certain activator proteins including OmpR [4]. These two independent lines of evidence strongly suggested that OmpR interacts with RNA polymerase (e.g.

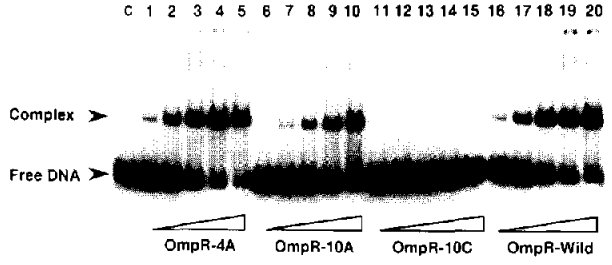

Fig. 3. Quantitative DNA-binding assay. Total cell lysates, containing various amounts of OmpR were incubated with EnvZ11* and ATP, and then subjected to DNA-binding assay, as described in section 3. Amounts of OmpR in the lysates used were estimated to be 1.5 ng (lanes 1, 6, 11, 16), 4.5 ng (lanes 2, 7, 12, 17), 15 ng (lanes 3, 8, 13, 18), 45 ng (lanes 4, 9, 14, 19) and 150 ng (lanes 5, 10, 15, 20). These estimations were based on the results of the quantitative immunoblotting analysis with an OmpR anti-serum.

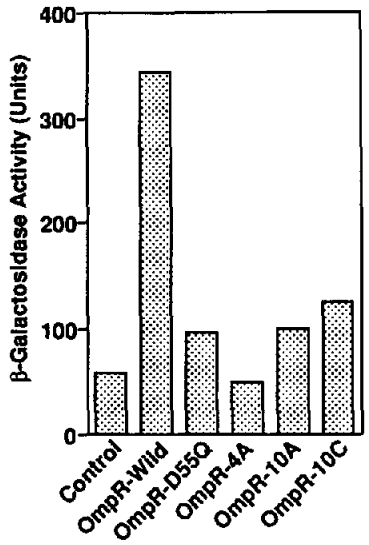

Fig. 4. β-Galactosidase activity expressed by the composite *ompC-lacZ* fusion gene. Each mutant plasmid, including the original one (pBRC006), was transformed into SG480Δ76, which had carried plasmid pCP-CS1. These cells were grown in Luria-broth to the mid-logarithmic phase. β-Galactosidase activity expressed in these cells was examined as described by Miller [25] with modifications [9]. The values are an average of triplicated examinations.

α-subunit) during the processes of transcription initiation. OmpR may have certain contact sites (or activation domain) with RNA polymerase. An approach to verify such an activation site of OmpR would be the isolation of mutants that bind to the cognate promoter normally, but fails to enhance one or more steps of transcriptional initiation (i.e., a positive control (PC) mutant). Several instances of such PC-mutants have been reported for the *E. coli* cyclic AMP receptor protein (CRP) [15–17]. In fact, such an approach allowed the identification of certain amino acids that may be involved in the interaction between CRP and RNA polymerase. Our OmpR mutants, isolated in this study, may be categorized as such PC-mutants.

OmpR[P179L] seems more likely to be a PC-mutant than OmpR[P131S], because the former cannot activate transcription from not only the composite *ompC* promoter but also both the natural *ompC* and *ompF* promoters. However, the results of in vitro assaying showed that the DNA-binding ability of OmpR[P179L] is slightly less efficient, as compared with in the case of the wild-type. In this context, it is known that a crucial DNA-contact site is located in the C-terminal half of OmpR, within which the mutation of [P179L] is occurred (see Fig. 5). Therefore, it is tempting to assume that both the presumed transcriptional activation site and the DNA-contact domain may be in close proximity, from both structural and functional points of view. OmpR[P131S] is also of interest in the sense that it retains normal ability to bind to the cognate DNA tested, but lacks the ability to activate transcription from both the composite and natural *ompC* promoters, nevertheless it can activate the natural *ompF* transcription normally. This can be interpreted formally as meaning that OmpR[P131S] is a PC-mutant specific for the *ompC* promoter. However, it is actually possible to envision several other complicated explanations, as discussed further below. If the former is the case, it is then interesting to assume that the activation site(s) in OmpR (or activation mech-

anisms for OmpR) are redundant, depending upon the type of promoter. An activation site(s) in OmpR for *ompC* may be somewhat different from the one for *ompF*. We also learned from the previous papers from Silhavy's laboratory, in which a number of OmpR mutants have been systematically isolated and genetically characterized, that one such mutant is identical to OmpR[P131S] [18,19]. Since their mutant, OmpR[P131S], can trigger the *ompF* expression constitutively regardless of the medium osmolarity, they categorized this mutant as a '*ompF* repression mutant' (it should be noted that *ompF* transcription was proposed to be repressed under certain conditions through the function of OmpR that binds to 'repression sites') [18,19]. They also reported that OmpR[P131S] can trigger the *ompC* transcription particularly in the high osmolarity medium (or in a certain *envZ* mutant background), albeit with a lower efficiency as compared with in the case of the wild-type OmpR [18]. In fact, we also found that when DB225 producing our OmpR[P131S] was grown in Luria-broth containing 0.4 M NaCl, the cells exhibited significantly higher β-galactosidase activity (data not shown, and see Fig. 6). However, the level of β-galactosidase activity observed for OmpR[P131S] in the high osmolarity medium was still markedly lower than that observed for the wild-type OmpR (data not shown). It should be also emphasized that we further demonstrated quantitatively in vitro that OmpR[P131S] retains its DNA-binding ability as well as the wild-type, but is unable to trigger the *ompC* transcription from pCP-CS1. These results are compatible with the view that OmpR[P131S] is defective, if not completely, in the ability to trigger the *ompC* transcription. In this context, the repression mutant model (proposed for *ompF*) and the positive control mutant model (proposed for *ompC*) are not mutually exclusive. In any case, it is of interest to isolate extragenic suppressor mutations for our OmpR mutants in order to see if they are found in any one of the components of RNA polymerase.

It should be briefly mentioned that both OmpR[P131S] and OmpR[P179L] exhibit a negative dominant character with respect to the wild-type OmpR. This observation is compatible with the view that they are PC-mutants. However, this issue must carefully be examined, because we used a high copy-number plasmid carrying these *ompR* mutant alleles. Therefore, a number of other explanations might be possible.

In conclusion, we isolated OmpR mutants which are tentatively categorized in a type of PC-mutants. A number of OmpR mutants, exhibiting a wide variety of phenotypes as to *ompC* and *ompF* expression, has been reported by several groups including ours [18–23]. Among them, however, no obvious PC-mutant has been characterized in detail so far, although Silhavy and his colleagues have noted some candidates for PC-mutants [18]. It should be noted that OmpR[P179L] is a novel one in this

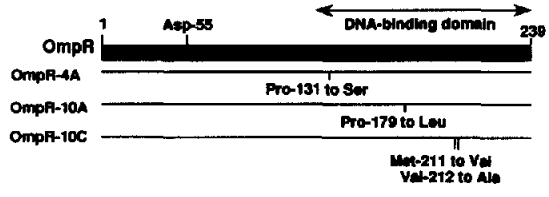

Fig. 5. Amino acid substitutions of the OmpR mutants. Amino acid substitutions, identified for the set of OmpR mutants, are shown in the schematic structure of OmpR. Numbers indicate positions of altered amino acids, the N-terminal methionine being taken as 1.

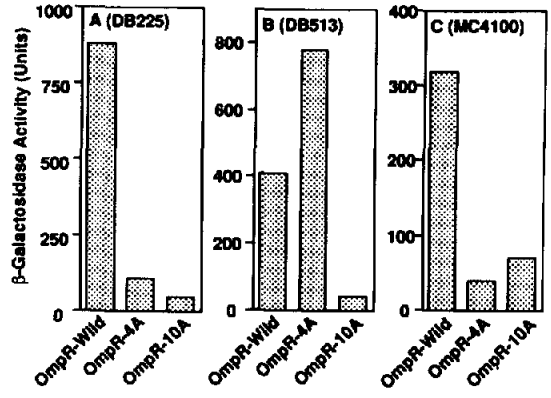

Fig. 6. β-Galactosidase activity expressed by the *ompC-lacZ* and *ompF-lacZ* fusion genes. Each mutant plasmid was transferred into either DB225 carrying an *ompC-lacZ* fusion gene (panel A), DB513 carrying an *ompF-lacZ* fusion gene (panel B), or MC4100 carrying pCP-CS1 (panel C). Cells were grown either in Luria-broth (panels A and C) or NaCl-deprived Luria-broth (panel B) (a reference for these strain is reference [12]). β-Galactosidase activity expressed in these cells was examined as described by Miller [25] with modifications [9]. The values are an average of triplicated examinations.

sense. Further characterization of these intriguing OmpR mutants should shed light on the general mechanism underlying transcriptional activation in prokaryotes, since a number of *E. coli* activator proteins belongs to the so-called OmpR-subfamily, including 8 known members so far, whose amino acid sequences are extensively similar to each other not only in the N-terminal phosphorylation domain but also in the C-terminal DNA-binding domain [24].

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