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Interaction between Two Regulatory Proteins in Osmoregulatory Expression of *ompF* and *ompC* Genes in *Escherichia coli*: a Novel *ompR* Mutation Suppresses Pleiotropic Defects Caused by an *envZ* Mutation

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The *ompR* and *envZ* genes, which together constitute the *ompB* operon, are involved in osmoregulatory expression of the OmpF and OmpC proteins, major outer membrane proteins of *Escherichia coli*. The *envZ11* mutation results in the OmpF$^-$ OmpC-constitutive phenotype. A mutant which suppressed defects caused by the *envZ11* mutation was isolated. The suppressor mutation also suppressed the LamB$^-$ PhoA$^-$ phenotype caused by the *envZ11* mutation. The mutation occurred in the *ompR* gene and hence was termed *ompR77*. The *ompR77* mutation alone produced no obvious phenotype. Functioning of the *ompR77* allele remained *envZ* gene dependent. Although the *ompR77* mutation suppressed the *envZ11* mutation, it did not suppress a mutation that occurred in another position within the *envZ* gene (*envZ160*). These results indicate that OmpR and EnvZ, two regulatory proteins, functionally interact with each other.

The outer membrane of *Escherichia coli* K-12 contains two major proteins, OmpF and OmpC, which participate in the passive diffusion of small hydrophilic molecules (23). Although the functional and structural properties of these proteins are similar, the relative amounts of the two proteins are influenced by the osmolarity of the medium (13, 26). Increased osmolarity results in a decrease in the OmpF protein with a concomitant increase in the OmpC protein, whereas a decrease in osmolarity results in a decrease in OmpC with a concomitant increase in OmpF. This regulation is controlled by the *ompR* and *envZ* genes in the *ompB* operon at the transcriptional level (9, 10). Hall and Silhavy previously proposed a model for this osmoregulation (11). According to their model, the OmpR protein acts as a positive regulator for expression of the *ompF* and *ompC* genes, and the *envZ* product, a possible envelope protein, acts as an osmosensor. The *micF* gene, which codes for a small RNA molecule, was found to be a third regulatory gene (18). A recent study, however, indicated that one copy of the *micF* gene on the chromosome does not play an important role in osmoregulation (17).

Genetic analyses provided evidence that the OmpR protein acts as a positive regulator for osmoregulation through its binding to the upstream regions of the *ompF* and *ompC* promoters (5, 12, 19). Recently, the OmpR protein was purified, and its binding to the upstream regions was demonstrated biochemically (Y.-L. Jo, F. Nara, S. Ichihara, T. Mizuno, and S. Mizushima, J. Biol. Chem., in press). Several *ompR* mutants have been isolated, and their gene structures have been characterized (21, 22). These mutants exhibit different phenotypes as to expression of the OmpF and OmpC proteins, indicating that the OmpR protein is directly involved in osmoregulatory expression of the *ompF* and *ompC* genes.

Several *envZ* mutants which exhibit different phenotypes as to osmoregulation of the *ompF* and *ompC* genes have been isolated (7, 25, 28, 29). Some of them exhibited wider pleiotropic effects in that they made cells defective as to synthesis of the LamB, MalE, and PhoA proteins as well (28, 29). In contrast to the case of the OmpR protein, however, little is known about the functional role of the EnvZ protein. It is also unclear as to whether or not the two regulatory proteins, OmpR and EnvZ, cooperate, and if so, how.

This study was undertaken to detect possible interaction between the two regulatory proteins. A novel *ompR* mutation was found to suppress the pleiotropic phenotype caused by the *envZ11* mutation. Characterization of this suppressor mutation provided evidence that a specific interaction between the two regulatory proteins plays a role in osmoregulation of the *ompF* and *ompC* genes.

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. The *E. coli* K-12 strains, bacteriophages, and plasmids used in this study are listed in Table 1.

Media, chemicals, and enzymes. All experiments concerning expression of the *ompF* and *ompC* genes were carried out in medium A supplemented with different concentrations of sucrose, as described previously (13). M9 was used as the minimal medium. When required, ampicillin and kanamycin were added at concentrations of 50 and 30 μg/ml, respectively. Restriction endonucleases, bacteriophage T4 DNA ligase, *Bam*HI linker d (CGGATCCG), and a dideoxy sequencing kit were purchased from Takara Shuzo Co.

Transduction and transformation. Generalized transduction and transformation were performed as described previously (16).

DNA techniques. DNA sequencing was carried out by the dideoxy chain-terminating method (24). The isolated restriction fragments were cloned into pUC19. JM83 was used as the host strain. Other DNA techniques used were described previously (16).

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TABLE 1. Bacteria, bacteriophages, and plasmids

Strain	Relevant properties[a]	Reference or source
E. coli K-12		
MC4100	F⁻, Δ*lacU169 araD rpsL relA thi flbB*	3
MH1461	MC4100 *envZ11*	10
MH760 *recA*	MC4100 *ompR2 gyrA recA1*	12
MH19	MC4100 *malQ pyrD*	10
MH19 *aroB*	*molQ*⁺ *aroB* transductant of MH19; donor; AB2847 *mal*⁺	This study
SG480Δ76	MC4100 Δ(*malT-ompB*)	8
AT142	MC4100 Δ*envZ*	T. Mizuno[b]
AB2847 *mal*⁺	*aroB tsx supE*	21
YO160	F⁻, *thi rel rpsL ompC envZ160* Φ(*ompC-lacZ*)	16
JM83	*ara* Δ*lac pro rpsL thi* φ80d Δ*lacZ15*	27
Bacteriophage		
TuIa	Receptors, OmpF and lipopolysaccharide	6
TuIb	Receptors, OmpC and lipopolysaccharide	6
P1 *kc*	Used for generalized transduction	Our laboratory stock
Previously described plasmid		
pBR322	Ap^r Tc^r	1
pKEN403	Ap^r Km^r	17
pMF21	Km^r	14
pUC19	Ap^r	30
pAT224	Ap^r; vector, pBR322; cloned genes, *ompR* and *envZ*	20
pAT2004	Ap^r; vector, pIN-III; cloned gene, *envZ*	T. Mizuno[b]
pMAN043	Ap^r	22

[a] Ap, Ampicillin; Tc, tetracycline; Km, kanamycin.
[b] Construction and characterization will be published elsewhere.

Phage sensitivity test. Sensitivity to TuIa and TuIb of *E. coli* strains was tested by cross-streaking on medium A plates with or without 15% sucrose.

Preparation of cell envelopes and polyacrylamide gel electrophoresis of outer membrane proteins. Preparation of cell envelopes and outer membrane protein fractions and urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis were carried out as described previously (21) except for the use of sodium lauroyl sarcosinate in place of Triton X-100 in the preparation of outer membrane protein fractions.

Construction of plasmids. Cloning of the *ompB* region was carried out as follows. *Bam*HI-*Sal*I fragments (5.3 kilobases [kb]) in which the *ompB*-carrying fragment was supposed to exist (20) were isolated from SM6001 and YO160 and then cloned into pMAN043 to construct pMAN057 and pMAN115, respectively. The presence of the *ompB* operon in these plasmids was confirmed by restriction analyses. Further construction of plasmids carrying the *ompB* region with various mutations is summarized in Fig. 1. Changing of *Hpa*I sites to *Bam*HI sites was carried out by insertion of the *Bam*HI linker into the *Hpa*I sites. The *envZ11* mutation in pMAN102 and pMAN103 was confirmed by the presence of the *Hae*III site which was formed as a result of the *envZ11* mutation (see Fig. 5).

Alkaline phosphatase assay. Alkaline phosphatase activity was measured as previously described (2).

RESULTS

Isolation of *envZ11* suppressor mutants. MH1461 is an *envZ11* mutant with the OmpF⁻ OmpC-constitutive (OmpC^c) phenotype (10). From an old culture of this strain, OmpF⁺ OmpC⁺ revertants, in which the synthesis of the two proteins was normally regulated in response to the osmolarity of the medium, were isolated with high frequency (Fig. 2). One of them, SM6001, was used in the following experiments.

To determine whether SM6001 is a true revertant or a pseudorevertant with respect to *envZ*, P1 transduction was carried out. When SM6001 was used as the donor and MH19 *aroB* was used as the recipient, 5% of the *aroB*⁺ transductants were of the EnvZ11 phenotype (OmpF⁻ OmpC^c). When these transductants were transformed with pAT2004, which carried the entire *envZ* gene, they became the wild type (OmpF⁺ OmpC⁺). When MH1461 was used as the donor and MH19 *aroB* was used as the recipient, more than 50% of the *aroB*⁺ transductants were of the EnvZ11 phenotype. These results indicated that SM6001 still harbored the *envZ* mutation and that suppression was due to a second mutation linked to the *aroB-envZ* region. This suppressor mutation was tentatively designated as *sez* (suppressor of *envZ*).

Location of *sez* and *envZ11* loci on the chromosome. We first checked whether or not *sez* occurred in the *ompB* operon, which is composed of the *ompR* and *envZ* genes. The chromosomal DNA of SM6001 was digested with *Bam*HI and *Sal*I, and the resultant 5.3-kb fragments, which were assumed to contain an *ompB* operon-carrying fragment (20) (Fig. 1 and 3), were ligated into pMAN043. The ligated mixture was used to transform MH760 *recA*, an *ompR2* mutant (OmpF^c OmpC⁻). OmpC⁺ transformants were selected and confirmed by means of restriction analyses to harbor a plasmid carrying the *ompB* region. From pMAN057 thus obtained and pMAN059 carrying the wild-type *ompB* operon, the chimeric *ompB* operons shown in Fig. 3 were constructed through reciprocal exchange of fragment A. Then the *Bam*HI fragments carrying these chimeric *ompB* operons, the wild-type *ompB* operon, and that from pMAN057 were individually cloned into pMF21, a mini-F plasmid, to construct pMAN102, pMAN103, pMAN104, and pMAN101 shown in Fig. 3, respectively (see also Fig. 1). These plasmids were transferred into SG480Δ76, a Δ*ompB* strain, and expression of the *ompF* and *ompC* genes was examined.

Cells harboring pMAN104, which carries the wild-type *ompB* operon, synthesized the OmpF and OmpC proteins with a normal response to medium osmolarity (Fig. 4, lanes 7 and 8), whereas cells harboring pMAN103, which carries the wild-type fragment A and the mutant-type fragment B, exhibited the OmpF⁻ OmpC^c phenotype, as does the *envZ11* mutant (Fig. 4, lanes 5 and 6). These results indicated that the *envZ11* mutation occurred in fragment B. In spite of the existence of the *envZ11* mutation in pMAN101, cells harboring this plasmid normally synthesized OmpF and OmpC with a normal osmoresponse (Fig. 4, lanes 1 and 2). From these results, we concluded that the *sez* mutation is located in fragment A. The phenotype conferred by pMAN102, which is supposed to carry the *sez* mutation but not the *envZ11* mutation, was indistinguishable from that conferred by pMAN104 carrying the wild-type *ompB* region, indicating that the *sez* mutation alone has no obvious phenotype.

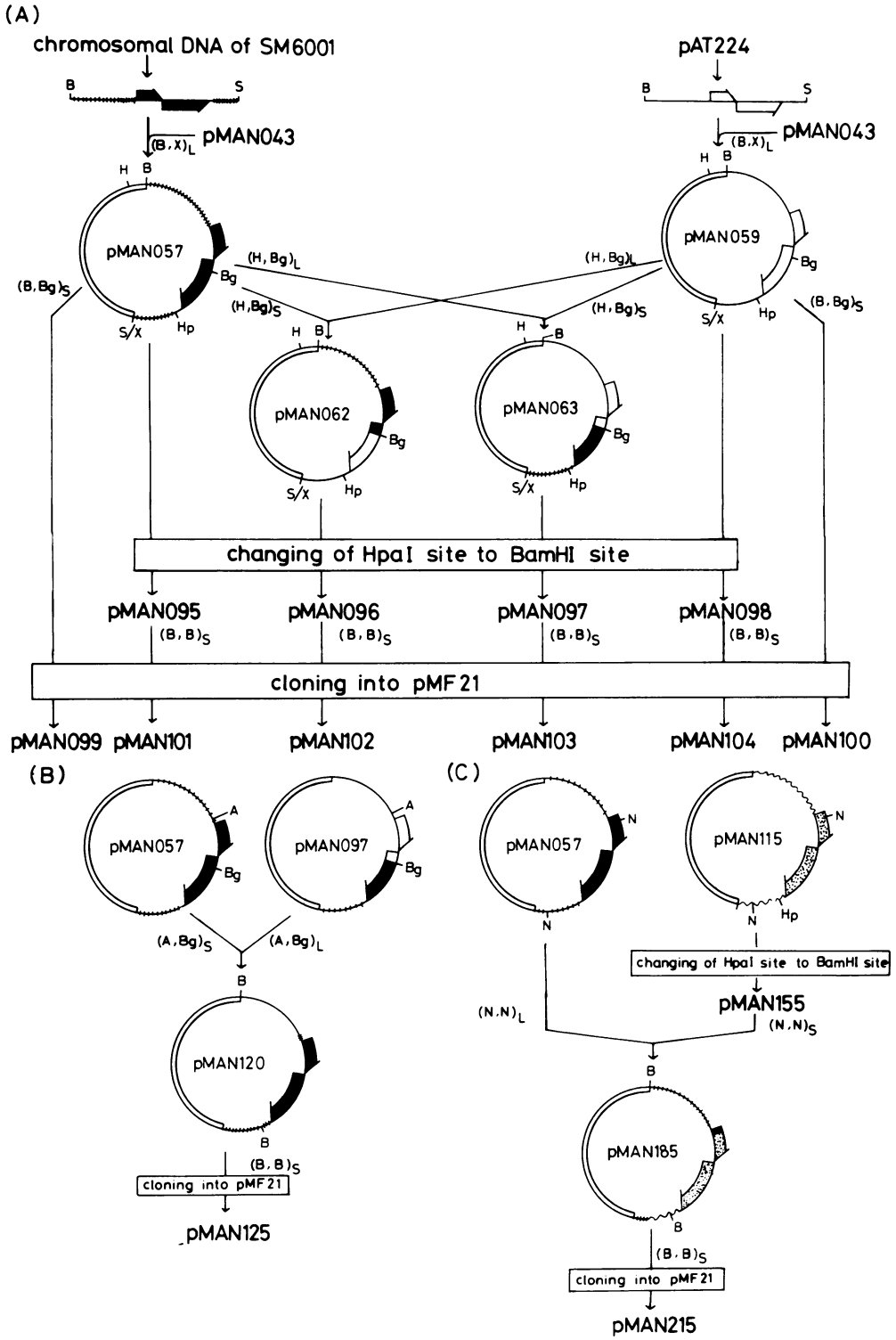

FIG. 1. Construction of plasmids carrying the *ompB* region with various mutations. Symbols: ◣ and ◤, *ompR* and *envZ* genes derived from SM6001, respectively; ◺ and ◸, *ompR* and *envZ* genes derived from wild-type cells; ◣ and ◤, *ompR* and *envZ* genes derived from YO160; ——, ——, and ∿∿∿, chromosomal DNA derived from SM6001, the *ompB*$^+$ wild-type strain, and YO160, respectively; ▭, a DNA fragment from pMAN043. H, *Hin*dIII; B, *Bam*HI; Bg, *Bgl*II; Hp, *Hpa*I; S, *Sal*I; X, *Xho*I; A, *Ava*I; N, *Nsp*V. Restriction sites are shown on plasmids. S and L outside the parentheses denote the smaller and larger fragments, respectively, formed on endonuclease digestion.

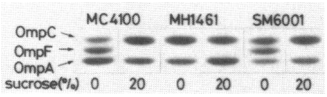

FIG. 2. Expression of *ompF* and *ompC* in the *envZ* suppressor (*sez*) mutant. Strains MC4100 (wild type), MH1461 (*envZ11*), and SM6001 (*envZ11 sez*) were grown with the indicated concentrations (wt/vol) of sucrose. Outer membrane protein fractions (25 μg of protein) were prepared from cell envelopes and analyzed on polyacrylamide gels. The positions of OmpC, OmpF, and OmpA are indicated.

In another experiment, fragment C shown in Fig. 3 was isolated from pMAN057 (*sez envZ11*), and the corresponding region of pMAN097, which carries the *envZ11* mutation alone, was replaced with it (Fig. 1B). The plasmid thus constructed (pMAN125) made SG480Δ76 (Δ*ompB*) of the *sez envZ11* type, suggesting that *sez* is located in the C region within fragment A (data not shown).

To determine the exact locations of the *sez* and *envZ11* mutations, the nucleotide sequences were determined for the 2.7-kb *Ava*I-*Hpa*I regions (Fig. 3) derived from SM6001 (*envZ11 sez*), MH1461 (*envZ11*), and the wild-type strain. With SM6001 and the wild-type strain, sequencing was carried out throughout the entire region of the *Ava*I-*Hpa*I fragment. With MH1461, only the *envZ* region was sequenced. Both SM6001 and MH1461 carried a missense mutation which results in a Thr-to-Arg conversion at amino acid 247 from the NH_2 terminus of the EnvZ protein (Fig. 5), indicating that this is the *envZ11* mutation. On the other hand, the *sez* mutation was localized in the *ompR* gene. The *sez*-carrying *ompR* allele was termed *ompR77*. The latter mutation resulted in a conversion of Leu to Gln at amino acid 16 of the OmpR protein. For the region covering the *envZ11* and *ompR77* mutations, both strands were sequenced to confirm the base alterations.

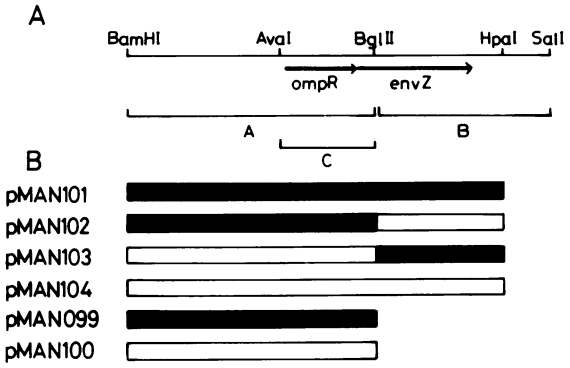

FIG. 3. Restriction map around the *ompB* region cloned into pMF21. (A) The coding regions for the *ompR* and *envZ* genes are indicated by arrows. Fragment A is a 3.1-kb *Bam*HI-*Bgl*II fragment which contains the entire *ompR* gene, the coding region for the NH_2-terminal portion of the EnvZ protein, and an unknown region located upstream from the *ompB* promoter. Fragment B is a 1.4-kb *Bgl*II-*Hpa*I fragment which contains the coding region for the EnvZ protein devoid of the NH_2-terminal portion. Fragment C is a 1.2-kb *Ava*I-*Bgl*II fragment carrying the entire *ompR* gene and the coding region for the NH_2-terminal portion of the EnvZ protein. (B) The *ompB* region cloned into pMAN101, pMAN102, pMAN103, pMAN104, pMAN099, and pMAN100 is indicated. The *Hpa*I site has been changed to a *Bam*HI site in all of these plasmids (cf. Fig. 1). ■ and □, Fragments derived from the *sez* mutant (SM6001) and the wild-type strain, respectively.

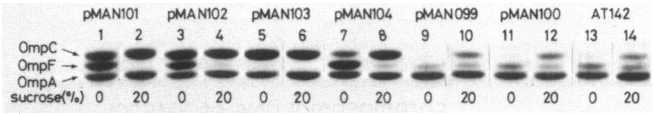

FIG. 4. Expression of the *ompF* and *ompC* genes in SG480Δ76, a Δ*ompB* strain harboring the indicated plasmids. The gene constructions for individual plasmids are shown in Fig. 3. These strains were grown with the indicated concentrations (wt/vol) of sucrose, and then the outer membrane proteins were analyzed on polyacrylamide gels. Lanes 13 and 14 contained the proteins from AT142, a Δ*envZ* strain. The positions of OmpC, OmpF, and OmpA are indicated.

***ompR77* suppresses the pleiotropic phenotype caused by the *envZ11* mutation.** The *envZ11* mutation causes the $OmpF^-$ $OmpC^c$ phenotype (28). It also renders the cells $LamB^-$ $PhoA^-$ (28). SM6001 (*envZ11 ompR77*) produced the LamB protein in the presence of maltose, as did the wild-type strain, whereas MH1461 (*envZ11*) did not (Fig. 6). The $PhoA^-$ phenotype conferred by the *envZ11* mutation was also suppressed by the *ompR77* mutation (data not shown). It was concluded, therefore, that the *ompR77* mutation suppresses pleiotropic defects caused by the *envZ11* mutation.

The *ompR77* function is *envZ* dependent. The *ompR77* mutation alone produced no obvious phenotype (Fig. 4, lanes 3 and 4); namely, the phenotypes of the *ompR77* mutant, *ompR77 envZ11* mutant, and wild-type strain were indistinguishable. Thus, the possibility arose that the *ompR77* allele exhibits the $OmpF^+$ $OmpC^+$ phenotype independently of the EnvZ protein. To examine this possibility, we constructed an *ompR77* Δ*envZ* strain (Fig. 1A). Fragment A (Fig. 3) was isolated from pMAN057 (*ompR77 envZ11*), cloned into pMF21 to construct pMAN099, and then transferred into SG480Δ76 (Δ*ompB*). SG480Δ76 (pMAN099), which is essentially *ompR77* Δ*envZ*, exhibited the same phenotype as did the Δ*envZ* strains [SG480Δ76 (pMAN100) and AT142] (Fig. 4, lanes 9 to 14), indicating that functioning of the *sez*-carrying OmpR protein remained *envZ* dependent. The phenotypes of these Δ*envZ* strains were the same as that of an *envZ* amber strain (7).

ompR77* suppresses only a particular mutation on the *envZ

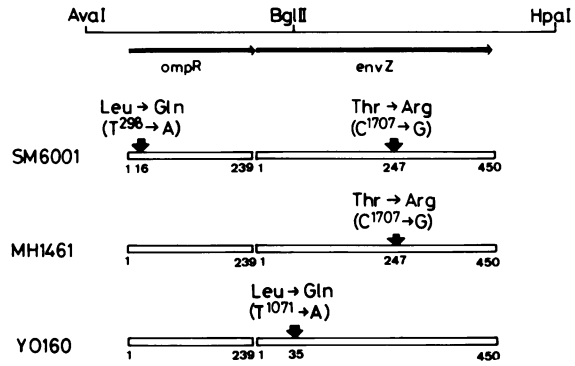

FIG. 5. Summary of the base alterations and amino acid changes in the *ompB* regions of SM6001, MH1461, and YO160. Alterations in the nucleotide sequences and those in the deduced amino acid sequences are shown. The positions of the bases are shown as suffixed numbers, which were taken from the published nucleotide sequence for the *ompB* operon (4). The positions of amino acid residues in the OmpR and EnvZ portions are numbered from the respective NH_2 termini.

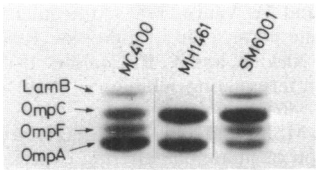

FIG. 6. Expression of the LamB protein in MC4100, MH1461, and SM6001. MC4100 (wild type), MH1461 (*envZ11*), and SM6001 (*envZ11 ompR77*) were grown in M9 in the presence of 0.2% maltose (and 0.2% glycerol), and then the outer membrane proteins (25 μg) were analyzed on polyacrylamide gels. The positions of LamB, OmpC, OmpF, and OmpA are indicated.

gene. YO160 is the *envZ160* mutant which exhibits the $OmpF^-$ $OmpC^c$ phenotype, as does the *envZ11* mutant. The *envZ* allele of YO160 cloned into pMAN115 was subjected to DNA sequencing. The *envZ160* mutation was found to cause a Leu-to-Gln conversion at position 35 of the EnvZ protein, which was different from the case of the *envZ11* gene (Fig. 5). The *ompB* operon carrying both the *ompR77* and *envZ160* mutations was then constructed from pMAN115 and pMAN057 and cloned into pMF21 (Fig. 1C). SG480Δ76 (Δ*ompB*) harboring this plasmid, pMAN215, was $OmpF^-$ $OmpC^c$, as is the *envZ160* mutant (data not shown). This result indicated that *ompR77* did not suppress the phenotype conferred by the *envZ160* mutation; namely, *ompR77* suppresses only a particular mutation on the *envZ* gene.

DISCUSSION

The OmpR and EnvZ proteins are involved in osmoregulatory expression of the *ompF* and *ompC* genes (11). The OmpR protein is a cytosolic positive regulator that binds to the upstream regions of the *ompF* and *ompC* promoters (5, 12, 19), whereas EnvZ is most likely a membrane protein, the function of which remains unknown. In the present work, we obtained evidence which strongly suggests that the two regulatory proteins interact with each other. The evidence is as follows. (i) The defects caused by the *envZ11* mutation were suppressed by *ompR77*, a mutation on the *ompR* gene, (ii) the *ompR77* mutation alone had no obvious phenotype, (iii) functioning of the *ompR77* allele remained *envZ* gene dependent, and (iv) the *ompR77* mutation did not suppress a mutation (*envZ160*) that occurred in another position within the *envZ* gene.

The *ompR77* mutation caused a Leu-to-Gln conversion at amino acid 16 from the NH_2 terminus of the OmpR protein. We have previously reported the *ompR3* mutation, which results in an Arg-to-Cys conversion at amino acid 15. It should be noted that the phenotype of the *ompR3* mutant is essentially the same as that of the *envZ11* mutant (22). Taking these results together, it is likely that the *ompR3-ompR77* region is responsible for interaction of the OmpR protein with EnvZ, and lack of proper interaction would result in the $OmpF^-$ $OmpC^c$ phenotype regardless of whether the mutation site is on the OmpR or EnvZ protein. This interaction must play an important role in transmission of the osmoregulatory signal from the external medium to the genes.

What is the possible role of the EnvZ protein then? The number of EnvZ molecules per cell is suggested to be far smaller than that of OmpR (15). This suggests that the function of the EnvZ protein may be catalytic for the OmpR protein. For example, the EnvZ protein may be involved in modification of the OmpR protein, which in turn positively regulates either the *ompF* or *ompC* gene, depending on the type of modification. Another possibility is that the EnvZ protein is a stimulator of OmpR-dependent transcription; it senses the osmolarity of the medium and then stimulates OmpR-dependent transcription of either the *ompF* or the *ompC* gene. For further studies on the functioning of the EnvZ protein in relation to that of the OmpR protein, an in vitro system for osmoregulatory expression of the *ompF* and *ompC* genes should be established.

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