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NOTES

Cloning of the Regulatory Genes (*ompR* and *envZ*) for the Matrix Proteins of the *Escherichia coli* Outer Membrane

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We have cloned the regulatory gene cluster of *Escherichia coli* which is composed of at least two distinct genes, *ompR* and *envZ*. These genes are known to regulate the production of the outer membrane matrix proteins. The newly formed plasmids were found to complement not only *ompR* mutations but also *envZ* mutations. The *ompR* gene product was identified as a protein of an apparent molecular weight of 28,500.

The outer membrane of *Escherichia coli* contains two major proteins called matrix proteins (4). These two proteins, OmpC and OmpF, form passive diffusion pores through the outer membrane for small hydrophilic molecules (9). The loci *ompC* and *ompF* are structural genes for these matrix proteins and map at 47 and 21 min, respectively, on the *E. coli* chromosome (12). The expression of these genes is known to be affected by the osmolarity of the culture medium; *ompF* is expressed at low osmolarity, and *ompC* is expressed at high osmolarity (1, 6). This complex gene expression is shown to be controlled by a third gene cluster (*ompB*) mapping at 74 min (10, 12). The mutations in this region show three different phenotypes; $OmpC^-$ $OmpF^-$, $OmpC^-$ $OmpF^+$, and $OmpC^+$ $OmpF^-$ (2, 3). Recently, Hall and Silhavy reported that mutations mapping at this region define at least two distinct genes, *ompR* and *envZ* (2). A mutation in the *envZ* gene is responsible for the $OmpC^+$ $OmpF^-$ phenotype. On the other hand, mutations in the *opmR* gene exhibit either the $OmpC^-$ $OmpF^-$ or the $OmpC^-$ $OmpF^+$ phenotype (2). In addition, the *tpo* mutation which affects transcription of *lamB* (a gene for an outer membrane protein) and *malE* (a gene for a periplasmic protein) have been found to map identically with the *envZ* mutation (2, 15). The *perA* mutation which affects production of alkaline phosphatase and several other periplasmic proteins is also identical to the *envZ* mutation (15, 16). Moreover, it was recently found that the *perA* mutation also affects production of iron-induced proteins found in the outer membrane (7). These facts indicate that the functions of the *ompB* cluster appear to be complex. In particular, the *envZ* gene seems to be involved in transcriptional, translational, or post-translational regulation of various outer membrane proteins as well as periplasmic proteins.

To elucidate the role(s) of these unique regulatory genes, we have attempted to clone the genes. We and Taylor et al. have already reported on the cloning of a part of the gene cluster (14, 17). However, as described below, the 3.2-kilobase (kb) *Eco*RI-*Bam*HI fragment cloned in our earlier study (originally described as 3.7-kb) (17) was able to complement the *ompR* function but not the *envZ* function. Therefore, to clone the entire gene cluster, especially the *envZ* gene, we first attempted to establish a more defined restriction map in this region. The restriction sites and the orientation of DNA containing the 3.2-kb *Eco*RI-*Bam*HI fragment were determined as shown in Fig. 1A. Since Mu insertions exhibiting the *envZ* phenotype mapped closely to Mu insertions in *ompR* (17), it seemed that the 7.8-kb *Bam*HI fragment, including the 3.2-kb *Eco*RI-*Bam*HI fragment, would contain not only the *ompR* gene but also the *envZ* gene. To clone the 7.8-kb *Bam*HI fragment, *Bam*HI fragments of 7.5 to 8.0 kb were isolated from *E. coli* W620 DNA and cloned into the *Bam*HI site of pBR322. Of 61 transformants tested, 2 contained the 7.8-kb fragment which hybridized with ^{32}P-labeled pEW007 DNA (data not shown), which contains the 3.2-kb *Eco*RI-*Bam*HI fragment (17). These two clones were different with respect to the orientation of the 7.8-kb fragment cloned into pBR322. Thus, these two clones have been denoted pAT003 and pAT039 (Fig. 1B). A detailed restriction map of the 7.8-kb fragment in pAT003 and pAT0039 was determined as shown in Fig. 1C. In addition to plasmid pEW007 isolated previously (17), plas-

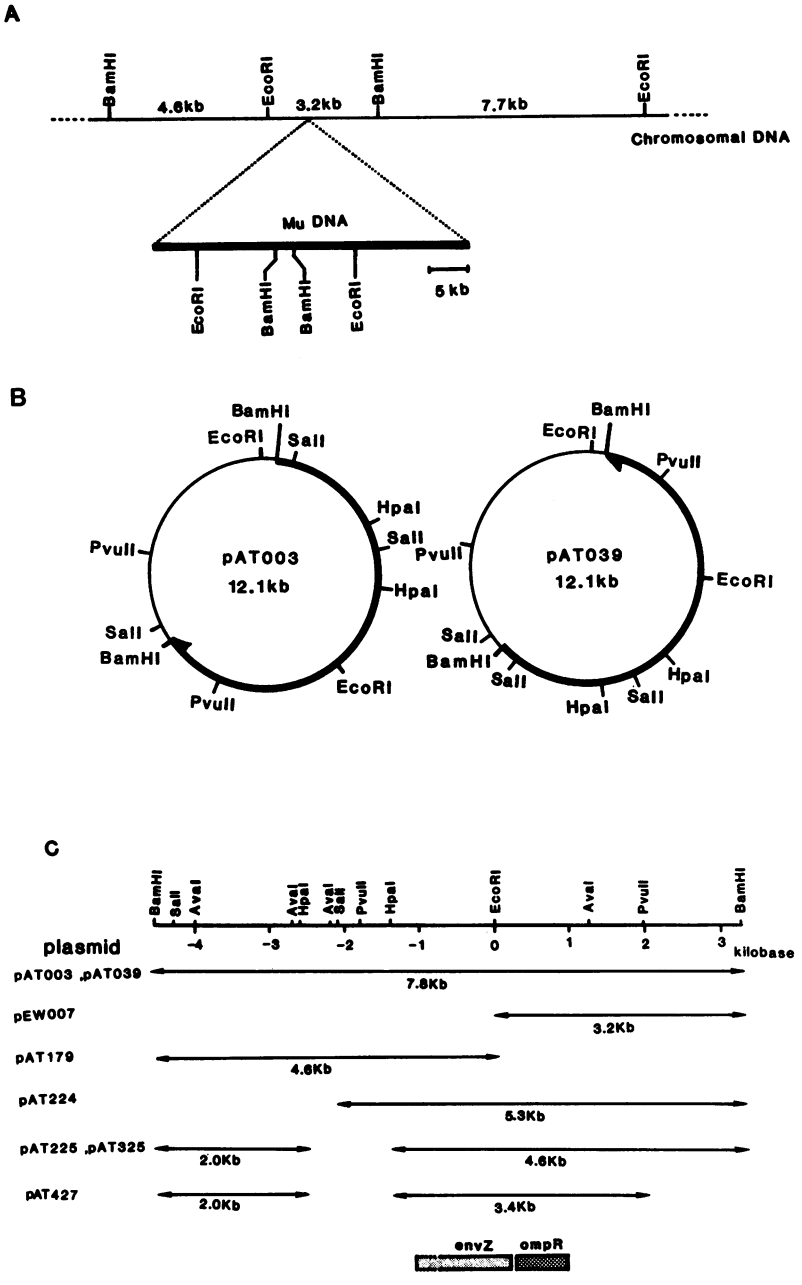

FIG. 1. (A) Identification of restriction sites of the DNA fragment carrying the *ompB* cluster. Total chromosomal DNA from an *ompB*$^+$ strain, Kb181/F'106, and an *ompB* strain containing a Mu insertion in *ompB*, Kb181 *ompB*::Mu no. 4/F'106 (17), were digested with *Eco*RI, *Bam*HI, or both *Eco*RI and *Bam*HI. To determine the restriction sites, the fragments were hybridized with a probe, pEW002 DNA (17), and analyzed (13). The pEW002 DNA is composed of approximately 11 to 15 kb of Mu DNA and approximately 10.9 kb of *E. coli* DNA, including a party of the *ompB* region as described previously (17). (B) Restriction map of plasmids pAT003 and pAT039. The 9.8-*Bam*HI DNA fragment (thick line) is inserted into plasmid pBR322 (thin line) at its *Bam*HI restriction site. (C) Schematic representation of isolated plasmids carrying the DNA surrounding the *ompB* cluster. Plasmids pAT179, pAT224, and pAT225 were isolated by removing the 3.5-kb *Eco*RI fragment, the 4.9-kb *Sal*I fragment, and the 1.2-kb *Hpa*I fragment, respectively, from pAT039 and religating. Plasmid pAT325, which was subcloned from pAT003, is essentially the same as pAT225 except for the reverse orientation of the fragment. Plasmid pAT427 was further subcloned from pAT325 by digesting it with *Pvu*II and religating. Arrows indicate the DNA fragments of each plasmid, which are cloned into pBR322.

mids pAT179, pAT224, and pAT225 were subcloned in pBR322 from pAT039. Plasmid pAT325, which was subcloned from pAT003, is essentially the same as pAT225 except for the reverse orientation of the fragment. Plasmid pAT427 was further subcloned from pAT325. These clones are schematically presented in Fig. 1C.

To analyze the phenotypic suppression of the *ompB* mutations by these clones, the following strains of isogenic background, except for the *ompB* mutations, were used as recipients: MC4100 *ompB*$^+$, MH760 *ompB*472 (*ompR*), MH1461 *tpo*11 (*envZ*), and MH1471 *ompB*473 (*envZ*) (2, 3). These mutant strains lack one of the matrix proteins (OmpC or OmpF). When these mutants were transformed with plasmids pAT003 or pAT427, the mutations (*ompR* and *envZ*) were completely suppressed as judged by their matrix proteins (Table 1). In addition, the response to variations in the osmolarity of the medium was identical to the wild-type response. Although we cloned the genes into a multicopy plasmid (pBR322), the production level of matrix proteins seems to be normal. As summarized in Table 1, pEW007 suppressed only the mutation of MH760 (*ompR*), but not that of MH1461 (*envZ*). Plasmid pAT179 was not able to suppress either of these mutations. To confirm these results, sensitivities to phage TuIb and colicin A, which utilize OmpC and OmpF proteins as receptors, respectively, were tested for each transformant. These results were completely consistent with the results described above (Table 1). Subclones pAT039, pAT224, pAT225, and pAT325 were also capable of suppressing both *ompR* and *envZ* mutations as judged by the membrane protein patterns and by sensitivity to phage TuIb and colicin A (data not shown).

These results indicate that the 7.8-kb *Bam*HI fragment cloned into pAT003 and pAT039 contains at least two genes, defined by the *ompR* and *envZ* genes, and these genes are located within the 3.4-kb *Pvu*II-*Hpa*I fragment cloned into pAT427. The 2.0-kb *Bam*HI-*Hpa*I fragment is not considered to be required for any of the *ompB* functions, since pAT179 could not complement the *envZ* mutation (Fig. 1C). The *ompR* gene was present within the 2.0-kb *Pvu*II-*Eco*RI fragment, and the *envZ* gene was located within the 1.5-kb DNA region between the *ompR* gene and the *Hpa*I site. These two genes are oriented as *ompR-envZ*, where the *ompR* gene is proximal to the *Pvu*II site. Hall and Silhavy suggested that the *ompR* and *envZ* genes are rather far apart as judged by their genetic recombination frequencies (2). However, our cloning results indicate that the *ompR* and *envZ* genes are closely positioned.

Figure 2 shows that, in addition to the OmpF protein, LamB protein and some iron-induced proteins were significantly reduced in the membrane fraction of MH1461 grown in iron-deficient glycerol medium (7, 15) (Fig. 2b). It was found that OmpF, LamB, and iron-induced proteins appeared concomitantly in the membrane fraction prepared from MH1461 carrying

TABLE 1. Phenotypic suppression of the *ompB* cluster by various clones

Strain[a]	Mutation	Plasmid	Protein composition[b]	TuIb[c]	ColA[c]	Response to osmolarity[d]
MC4100	Wild	None	OmpC$^+$ OmpF$^+$	S	S	Yes
MH760	*ompR*	None	OmpC$^-$ OmpF$^+$	R	S	No
		pAT003	OmpC$^+$ OmpF$^+$	S	S	Yes
		pEW007	OmpC$^+$ OmpF$^+$	S	S	Yes
		pAT179	OmpC$^-$ OmpF$^+$	R	S	No
		pAT427	OmpC$^+$ OmpF$^+$	S	S	Yes
MH1461	*envZ*	None	OmpC$^+$ OmpF$^-$	S	R	No
		pAT003	OmpC$^+$ OmpF$^+$	S	S	Yes
		pEW007	OmpC$^+$ OmpF$^-$	S	R	No
		pAT179	OmpC$^+$ OmpF$^-$	S	R	No
		pAT427	OmpC$^+$ OmpF$^+$	S	S	Yes

[a] These strains were gifts from T. J. Silhavy (3).

[b] Determined by an acrylamide gel electrophoresis (8) of the membranes prepared from the strains carrying each plasmid.

[c] Characterized by a cross-streak test. Twelve transformants of each were tested; S, sensitive; R, resistant.

[d] Examined for protein patterns of the membranes prepared from each strain grown in nutrient broth in the absence or presence of 2% NaCl. Preparation of membrane fractions and acrylamide gel electrophoresis were carried out as described previously (5, 8).

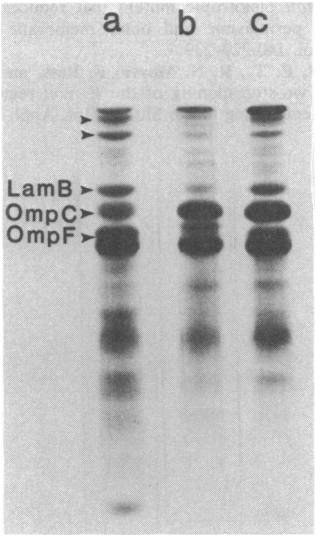

FIG. 2. Characterization of membrane protein composition of the *envZ* mutant carrying the plasmid pAT427 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Whole membrane fractions were prepared from (a) MC4100 (wild type), (b) MH1461 (*envZ*), and (c) MH1461 (*envZ*) carrying the plasmid pAT427. Cells were grown in M9 medium containing 2% glycerol, 0.1% Casamino Acids, and 0.2% maltose at 30°C. Membrane proteins were subjected to urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8). Arrows without letters indicate the position of iron-induced proteins.

pAT427 (Fig. 2c). This result indicates that the cloned genes are able to equally suppress *envZ* (OmpF protein), *tpo* (LamB protein), and *perA* (iron-induced proteins) mutations.

Analysis of products directed by the clones was carried out by using a minicell-forming mutant, *E. coli* χ984 (11). When the minicells carrying pAT427 were labeled with [^{35}S]methionine, a band was detected at a position just below the β-lactamase band. The band with an apparent molecular weight of 28,500 was also detected in transformants containing pAT003, pAT224, and pEW007, but not with pAT179 (data not shown). These results suggest that this protein is the *ompR* gene product. This conclusion is consistent with the results reported by Taylor et al. (14). This was confirmed by the results of DNA sequencing of this region. The entire DNA sequence of the 1.29-kb *Ava*I-*Eco*RI region, including the *ompR* gene, has been determined, and a gene coding for a protein with a molecular weight of 29,800 has been identified in the DNA sequence (Wurtzel et al., manuscript in preparation). On the other hand, no other significant bands for the putative *envZ* product were detected by pAT427, pAT003, and pAT224. Since the *envZ* gene is located within a 1.5-kb DNA fragment, the possible gene product should be less than 55,000 daltons. To resolve this problem, further subcloning and sequencing of the *envZ* gene are now in progress.

The *ompR* gene product seems to be a positive transcriptional regulator (2), but the role(s) of the *envZ* gene product is unknown. Mutations at *envZ* are pleiotropic, affecting the production of OmpC protein, OmpF protein, LamB protein (*tpo* [15]), alkaline phosphatase, and iron-induced proteins (*perA* [7, 16]). As described above, we have cloned the entire gene cluster (*ompR* and *envZ*), which is able to suppress these pleiotropic phenotypes. Therefore, these clones will facilitate further elucidation of the role(s) of these unique regulatory genes residing at the *ompB* region.

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