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Construction of a Series of *ompF-ompC* Chimeric Genes by In Vivo Homologous Recombination in *Escherichia coli* and Characterization of the Translational Products

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OmpF and OmpC are major outer membrane proteins. Although they are homologous proteins, they function differently in several respects. As an approach to elucidate the submolecular structures that determine the difference, a method was developed to construct a series of *ompF-ompC* chimeric genes by in vivo homologous recombination between these two genes, which are adjacent on a plasmid. The genomic structures of these chimeric genes were determined by restriction endonuclease analysis and nucleotide sequence determination. In almost all cases, recombination took place between the corresponding homologous regions of the *ompF* and *ompC* genes. Many of the chimeric genes produced proteins that migrated to various positions between the OmpF and OmpC proteins on polyacrylamide gel. On the basis of the results, a domain contributing to the mobility difference between the OmpF and OmpC proteins was identified. Some chimeric genes did not accumulate outer membrane proteins, despite the fact that the fusion of the *ompF* and *ompC* genes was in frame. Bacterial cells possessing the chimeric proteins were also tested as to their sensitivity to phages which require either OmpF or OmpC as a receptor component. The chimeric proteins were either of the OmpF or OmpC type with respect to receptor activity. Based on the observations, the roles of submolecular domains in the structure, function, and biogenesis of the OmpF and OmpC proteins are discussed.

OmpF and OmpC are major outer membrane proteins of *Escherichia coli* K-12. They share a number of biochemical and physicochemical characteristics. OmpF and OmpC are peptidoglycan-associated trimeric proteins (8, 15, 20, 26) and are thought to play roles in the maintenance of a stable cell surface structure (19, 25). In addition, both proteins form pores through which small hydrophilic nutrients cross the outer membrane, and hence they are called porins (18). Recently the entire nucleotide sequences of the genes coding for OmpF and OmpC were determined (9, 13). There is 69% homology between the sequences of the two genes (13).

Despite these similarities, OmpF and OmpC function rather differently in several respects. OmpF functions as a receptor component for phages TuIa and T2, whereas OmpC does so for phages TuIb and T4 (3, 5, 7). The pores which they form are different to some extent with respect to substrate specificity (17, 23). Their electrophoretic mobilities on sodium dodecyl sulfate (SDS)-polyacrylamide gel are also different (16, 22).

As an approach to elucidate the submolecular structures that make similar proteins functionally different, we attempted to construct a series of *ompF-ompC* chimeric genes. Since the *ompF* and *ompC* genes are highly homologous, recombination between the two genes might be able to occur in vivo at every homologous region throughout the genes. In the present work, we report the construction and characterization of a series of *ompF-ompC* chimeric genes.

MATERIALS AND METHODS

Materials. Restriction endonucleases, exonuclease *Bal* 31, T4 DNA polymerase, and T4 DNA ligase were purchased from Takara Shuzo Co.

Bacterial strains and plasmids. *E. coli* MH1160 (F⁻ *ΔlacU169 araD139 rpsL fibB relA ompB101*) (9) was used for the construction of plasmids carrying *ompF-ompC* chimeric genes. *E. coli* SM1005 (F⁻ Δ *lacU169 rpsL relA thiA fibB gyrA ompC ompF14*) (11) was used for the identification of products of the *ompF-ompC* chimeric genes. These bacterial strains were aerobically cultivated in L broth (12) and TY broth (10 g of tryptone [Difco Laboratories], 5 g of yeast extract per liter), respectively. Strains possessing plasmids were cultivated in L broth containing 100 μg of ampicillin per liter.

Plasmid pTUN8 used for the construction of *ompF-ompC* chimeric genes was constructed from plasmids pMAN008 (11) and pMY150 (14) as shown in Fig. 1. pMAN008 was digested with *Stu*I and religated in the presence of *Xba*I linker (dCTCTAGAG) to construct pTUN800X. Then pTUN8 was constructed by ligating the *Xba*I-*Hin*dIII large fragment of pTUN800X and the *Xba*I-*Hin*dIII small fragment of pMY150.

Phage sensitivity test. OmpF and OmpC are components of receptors for phages TuIa and TuIb, respectively (3). The receptor activity of the proteins coded for by *ompF-ompC* chimeric genes was examined on the basis of the sensitivity to these phages of the strains possessing the chimeric proteins. The sensitivity was tested by cross-streaking on medium A plates (10). Phages TuIa and TuIb were from our laboratory stock.

SDS-polyacrylamide gel electrophoresis. The outer membrane protein fraction was prepared by extraction of the cell envelope with sodium *N*-lauroylsarcosinate as described previously (4). Peptidoglycan fractions containing the OmpF and OmpC proteins were prepared as described previously (20), and the proteins were extracted by boiling in 2% SDS solution. SDS-polyacrylamide gel electrophoresis of outer membrane proteins was performed as described previously (14).

Other methods. Transformation was carried out by the

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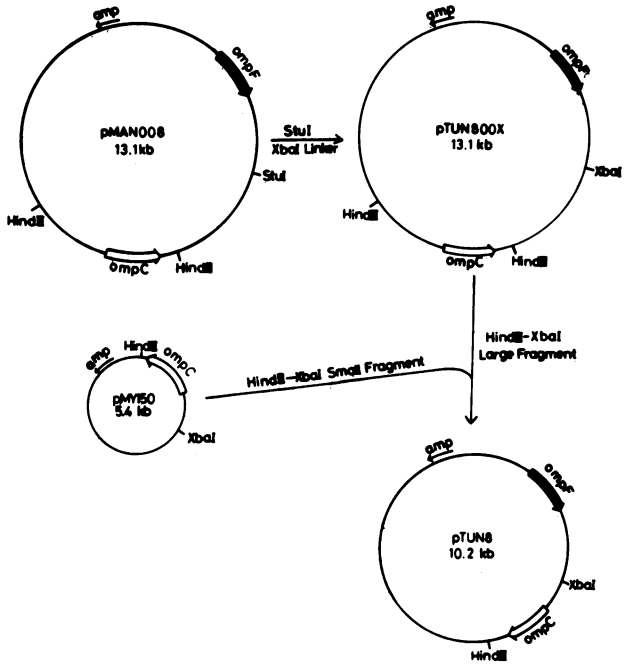

FIG. 1. Construction of pTUN8. The *ompF* and *ompC* genes and their directions are indicated by ▶ and ▷, respectively. *amp*, Ampicillin resistance gene. For more details, see Materials and Methods.

method of Dagert and Ehrlich (2). The plasmids were prepared by the method of Birnboim and Doly (1). Nucleotide sequences were determined by the dideoxy chain termination method of Sanger et al. (21). Other DNA techniques were described previously (11).

RESULTS

Construction of *ompF-ompC* chimeric genes in vivo. Plasmid pTUN8 carrying the *ompF* and *ompC* genes in the same direction was first constructed as shown in Fig. 1 (see Materials and Methods). It was then linearized with *Xba*I, further digested with *Bal* 31 so that the deletion proceeded to the promoter region of the *ompC* gene, and finally transferred to MH1160. Since the *ompF* and *ompC* genes are highly homologous as to their nucleotide sequences (9, 13) (see Fig. 3), it is possible that *recA*-dependent in vivo recombination between the two genes can take place at every homologous region to yield *ompF-ompC* chimeric genes. Transformants that appeared on ampicillin-containing plates were expected to contain plasmids that had been circularized via homologous recombination between the *ompF* and *ompC* genes on a linear plasmid. This recombination results in the deletion of a fixed length (3 kilobases) from the plasmid regardless of the site of recombination. The possibility of multiple crossovers between the two genes is expected to be very low, since a linear plasmid cannot be circularized by a double crossover event between these genes; at least a triple crossover is required.

Plasmids were prepared from 142 ampicillin-resistant transformants thus obtained, and their sizes were determined on agarose gels by electrophoresis after linearization with *Hin*dIII. Among them, 61 were found to show the 3-kilobase deletion, suggesting that they were most probably plasmids carrying *ompF-ompC* chimeric genes. All of the chimeric genes must consist of the *ompF* gene-derived upstream region and the *ompC* gene-derived downstream region. Restriction endonuclease analyses also revealed that circularization in most of the rest of the plasmids resulted from end-to-end ligation of linear plasmids.

Classification of the chimeric genes. The *ompF* and *ompC* genes have six unique restriction cleavage sites (*Mlu*I and *Pvu*II for the *ompF* gene; *Pvu*II, *Eco*RI, *Hin*cII, and *Pst*I for the *ompC* gene) (Fig. 2). By determining the presence or absence of these cleavage sites in each chimeric gene, it is possible to localize the site of recombination. Thus, the chimeric genes can be classified into seven classes (Fig. 2). First, the chimeric genes were divided into two groups, depending on the presence or absence of the *Eco*RI site that is unique to the *ompC* gene. Then the first group was further divided into three classes (I to III) according to the presence or absence of *Pvu*II and *Mlu*I sites, and the second group was divided into four classes (IV to VII) according to the presence or absence of *Hin*cII, *Pvu*II, and *Pst*I sites. The numbers of the chimeric genes thus assigned to classes I through VII were 17, 18, 2, 2, 8, 6, and 5, respectively. Representatives of them, which were used for further studies, are listed in Fig. 2. Hereafter, these chimeric genes and plasmids carrying these genes will be referred to by these numbers. The restriction endonuclease cleavage patterns of three other plasmids were not consistent with those expected from the presence of chimeric genes, and hence they were not analyzed further.

Determination of the sites of recombination in the individual chimeric genes. The DNA fragments which were found to cover the recombination sites were prepared from 11 chimeric gene-carrying plasmids (Fig. 2), and their nucleotide sequences were determined; thus the sites of recombination were determined (Fig. 3). In all the chimeric genes sequenced, except chimeric gene 523, recombination took

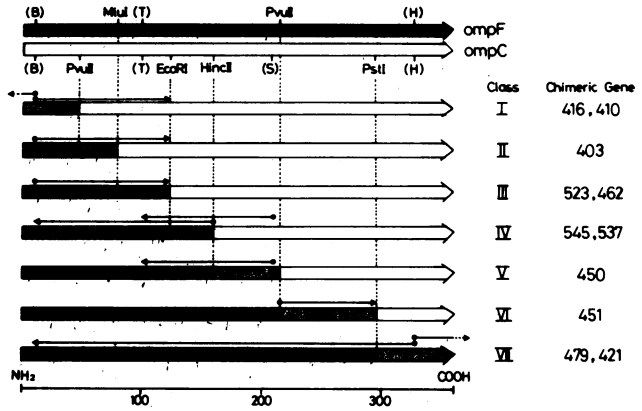

FIG. 2. Classification of *ompF-ompC* chimeric genes. The chimeric genes were classified into classes I through VII by means of restriction endonuclease analyses. Symbols: ▬, regions from the *ompF* gene; ▭, regions from the *ompC* genes; ▩, regions where homologous recombination was supposed to take place. Only the regions coding for the mature OmpF and OmpC proteins are presented. The scale at the bottom shows the numbers of amino acid residues from the N termini of the OmpF and OmpC proteins. The chimeric genes isolated were numbered; representatives are listed at the right. The fragments used for DNA sequencing to determine the recombination sites (Fig. 3) are indicated by ●⟶. Restriction endonuclease cleavage sites used for the preparation of fragments are indicated within parentheses as follows: B, *Bgl*II; S, *Sau*3A; T, *Taq*I; H, *Hin*cII.

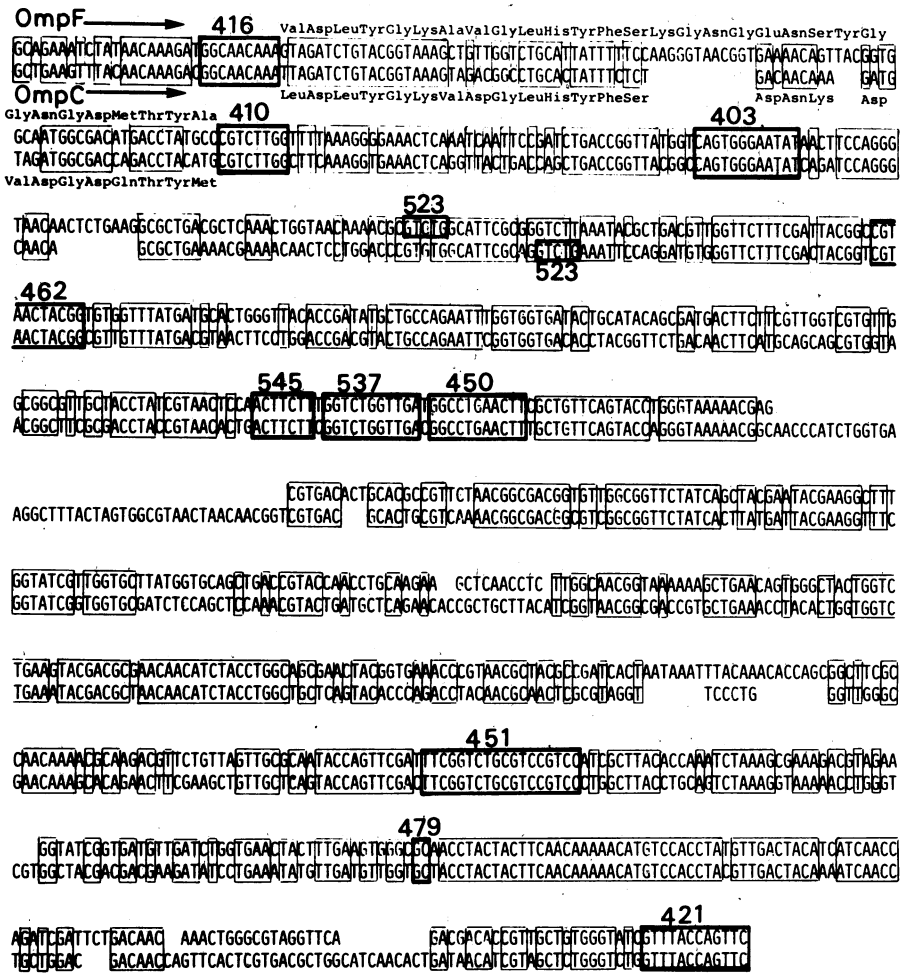

FIG. 3. Site of recombination in individual chimeric genes. The nucleotide sequences of the *ompF* (upper sequence) and *ompC* (lower sequence) genes are shown. Only the regions coding for the mature OmpF and OmpC proteins are presented. Homologous nucleotide sequences are boxed with thin lines. The homologous sequences determined to be the site of recombination for individual chimeric genes are boxed with thick lines with the chimeric gene numbers. The corresponding amino acid sequence is partly presented for comparison (see Discussion).

place between the corresponding homologous regions of the *ompF* and *ompC* genes. Neither addition nor deletion of bases was observed at the recombination sites; hence the reading frame was maintained, strongly suggesting that the chimeric genes thus constructed most likely code for OmpF-OmpC chimeric proteins. As to chimeric gene 421, the recombination occurred at the very ends of the coding regions. Therefore, the protein expressed by this chimeric gene should be OmpF. Chimeric gene 523 resulted from unequal recombination between the noncorresponding regions that have fortuitous base homology. Since the deletion that resulted from this unequal recombination event was 15 base pairs, this chimeric gene should code for an *ompF-ompC* chimeric protein that has a five-amino-acid deletion at the fusion site.

Identification and characterization of translational products of the chimeric genes. Figure 4A shows SDS-polyacrylamide gel electrophoretic profiles of the outer membrane proteins of the strains harboring one of the chimeric gene-carrying plasmids. The product of chimeric gene 416 showed the same mobility as that of OmpC. Upon a shift of the recombination site to that for chimeric gene 410, the migration position changed appreciably, whereas further shifts to the positions for genes 403 and 462 did not result in significant differences in mobility. It is very interesting that when the recombination took place at the central region between the genes (chimeric genes 545, 537, and 450), no translational product was detected in the outer membrane, despite the fact that the *ompF* and *ompC* genes were fused in frame in these chimeric genes. Significant amounts of translational products were also not detectable in either whole cell lysates or the culture media (data not shown). It should also be noted that accumulation of the protein product of chimeric gene 462 was also low. The products of chimeric genes 451, 479, and 421, which have the recombination site within their downstream regions, migrated to positions very close to that of the OmpF protein. Chimeric gene 523, which was formed as a result of a nonhomologous recombination, did not produce a protein.

The OmpF and OmpC proteins in the outer membrane are peculiar in that they are peptidoglycan associated (8, 20). All of the chimeric proteins expressed were peptidoglycan associated (Fig. 4B), indicating that these chimeric proteins were secreted through the cytoplasmic membrane and properly assembled into the outer membrane.

The strains harboring the chimeric gene-carrying plasmids

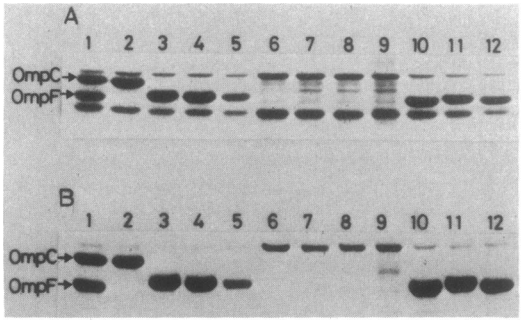

FIG. 4. SDS-polyacrylamide gel electrophoretic profiles of outer membrane proteins from cells carrying various chimeric genes. The *N*-lauroylsarcosinate-insoluble envelope fraction (A) and the peptidoglycan-associated protein fraction (B) were used as protein sources. Lanes show the chimeric genes carrying the following: 2, 416; 3, 410; 4, 403; 5, 462; 6, 523; 7, 545; 8, 537; 9, 450; 10, 451; 11, 479; 12, 421. Lane 1 contains the proteins from cells carrying pTUN8. The positions of the OmpF and OmpC proteins are indicated.

were also tested as to their sensitivity to phages TuIa and TuIb, which require OmpF and OmpC as receptor components, respectively. All the 35 strains harboring the plasmids of classes I and II and the strain harboring plasmid 462 of class III were sensitive to TuIb and resistant to TuIa, indicating that the products of these chimeric genes were of the OmpC type in terms of phage sensitivity. On the other hand, all of the 11 strains carrying the plasmids of classes VI and VII were sensitive to TuIa and resistant to TuIb, being of the OmpF type. None of the strains was sensitive or resistant to both phages. As can be expected from the gel profiles shown in Fig. 4, strains that did not exhibit detectable amounts of the chimeric proteins were found to be resistant to both TuIa and TuIb. The results of phage sensitivity tests are summarized in Table 1.

DISCUSSION

A simple and efficient method for constructing a series of *ompF-ompC* chimeric genes via in vivo homologous recombination is presented. The principle of this method was originally developed by Weber and Weissman and used for the studies on the α-interferon genes (24). Recently, we also succeeded in constructing a series of *ompF-ompC* chimeric genes that have the recombination site at the upstream noncoding region that includes the regulatory region and promoter region (T. Mizuno, M. Hasegawa, and S. Mizushima, manuscript in preparation).

The series of *ompF-ompC* chimeric genes enabled us to analyze the roles of the submolecular domains of the *ompF* and *ompC* genes and their translation products, the OmpF and OmpC proteins. First the translational products of these chimeric genes were analyzed (Fig. 4). Two things should be discussed here. OmpF and OmpC are very similar proteins with a slight difference (6-amino-acid difference in length) in molecular weight (9, 13). Nevertheless, their mobilities on polyacrylamide gel are appreciably different in the presence of SDS and urea. The mobility of chimeric protein 416 was the same as that of OmpC, whereas that of chimeric protein 410 was rather closer to that of OmpF than to that of OmpC, indicating that the 31 amino acid residues of OmpF and the 26 residues of OmpC that exist between the two recombination sites are largely responsible for the mobility difference between the OmpF and OmpC proteins. Although the OmpF proteins shares 16 homologous residues with the OmpC protein in this domain, the OmpF protein has 4 more glycine residues and 3 less negatively charged residues than OmpC (Fig. 3). The mobility difference on SDS-polyacrylamide gels may be due to these differences in amino acid sequence. Another point that should be discussed is the fact that the chimeric proteins from genes 547, 537, and 450 were not detected by the procedures used. Since the reading frames are conserved in these chimeric genes and they therefore code for OmpF-OmpC chimeric proteins, these genes should be expressed in *E. coli* cells. A preliminary pulse-labeling experiment with [^{35}S]methionine revealed that most of these chimeric genes expressed proteins that can be immunoprecipitated with both the anti-OmpF and anti-OmpC antisera (C. Hikita, H. Yamada, T. Mizuno, and S. Mizushima, unpublished observations). It is possible, therefore, that the translation products of these chimeric genes have defects which prevent secretion through the cytoplasmic membrane or assembly in the outer membrane. The low level of accumulation of chimeric gene 462 may also be due to similar defects.

The phage receptor function of the chimeric proteins was also examined. All chimeric proteins synthesized were active as a receptor to either one of TuIa or TuIb and inactive toward the other; none was active toward or inactive toward both phages. This suggests that the domain specifying the phage receptor function many be localized at corresponding regions in the OmpF and OmpC proteins. The phage receptor domain may be localized at the central part of the proteins, since the replacement of either the N-terminal or C-terminal region of one protein by the corresponding region of the other did not alter the receptor specificity. Unfortunately, in the present work we were unable to identify chimeric genes that express stable chimeric proteins having the junction point in the central region. The domain specifying the receptor function to TuIa and TuIb as well as other phages should be further studied after the establishment of more complete collection of the chimeric genes.

Success in the construction of a series of *ompF-ompC* chimeric genes opened a new way for studying the structure, function, and biogenesis of the OmpF and OmpC proteins. In the present work, we demonstrated a few examples of their usefulness. We also found that these chimeric proteins can be effectively used for the determination of target domains for monoclonal antibodies raised against the OmpC protein (unpublished experiments). The usefulness of chimeric genes will be significantly enhanced if another series of chimeric genes having the *ompC* and *ompF* sequences in the opposite order to those described in this paper becomes

TABLE 1. Summary of phage sensitivity of chimeric gene-carrying cells

Chimera gene class	Sensitivity to[a]:	
	TuIb	TuIa
I	S	R
II	S	R
III 462[b]	S	R
III 523[b]	R	R
VI	R	R
V	R	R
VI	R	S
VII	R	S

[a] S, Sensitive; R, resistant.
[b] 462 and 523 represent two different types of chimera genes in class III.

available. We are currently constructing such *ompC-ompF* chimeric genes.

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