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Localization of functional domains in *E. coli* K-12 outer membrane porins

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The genes *ompC* and *phoE* of *Escherichia coli* K-12 encode outer membrane pore proteins that are very homologous. To study the structure-function relationship of these proteins, we have constructed a series of *ompC-phoE* hybrid genes in which the DNA encoding part of one protein is replaced by the corresponding part of the other gene. These hybrid genes were easily obtained by using *in vivo* recombination. The fusion sites in the hybrid genes were localized by restriction enzyme mapping. The hybrid gene products were normally expressed and they were characterized with respect to functions and properties in which the native OmpC and PhoE proteins differ, such as pore characteristics, the receptor activity for phages and the binding of specific antibodies. Three regions within the N-terminal 130 amino acids were localized which determine pore characteristics and a segment between residues 75 and 110 contains amino acids which determine specificity for PhoE phages. A major cell surface-exposed region is located between residues 142 and 267. This region contains residues which are required for the binding of monoclonal antibodies directed against the cell surface-exposed part of PhoE and residues which determine specificity for OmpC phages.

Key words: functional domains/hybrid genes/*in vivo* recombination/outer membrane/pore proteins

Introduction

The outer membrane of Enterobacteriaceae protects these bacteria by creating a barrier for harmful compounds. To allow the influx of nutrients, this membrane contains a number of proteins which form pores through which small hydrophilic solutes can pass (for a review, see Nikaido, 1979). Under standard laboratory conditions, *Escherichia coli* K-12 produces two distinct pore proteins, namely OmpC protein and OmpF protein. The synthesis of these proteins is under the control of the *ompR* gene product. The synthesis of another pore protein, PhoE protein, is induced when cells are grown under phosphate limitation (Overbeeke and Lugtenberg, 1980; Tommassen and Lugtenberg, 1980). The structural genes for these proteins, *ompC, ompF* and *phoE,* have been sequenced (Inokuchi *et al.*, 1982; Mizuno *et al.*, 1983; Overbeeke *et al.*, 1983) and comparison of these sequences revealed an extensive homology. In the predicted primary structures of the proteins ~60% of their amino acid residues are identical. Therefore, it seems likely that these genes are derived from a common ancestral gene and that those sequences which are involved in the export of the proteins and their incorporation into the outer membrane have been conserved.

The latter notion was the basis of our approach to study the structure-function relationship of pore proteins by using hybrid genes. If part of one pore protein gene is replaced by the homologous part of another pore protein gene, the translocation of the hybrid gene product to the outer membrane will probably not be influenced. Thus, subsequent characterization of the hybrid gene product will allow the localization of functional domains in the native pore proteins.

In a previous study (Tommassen *et al.*, 1984), a hybrid *ompF-phoE* gene was constructed with the aid of restriction enzymes that cleave the constituent genes at homologous sites. To avoid the dependency on the very limited number of identical restriction sites, we have now chosen another approach to construct hybrid genes, i.e., by making use of *in vivo* recombination between these homologous genes. Such an approach has been employed by Weber and Weissman (1983) to obtain hybrid α-interferon genes. In this paper we describe the construction of hybrid *ompC-phoE* genes by *in vivo* recombination. Through functional characterization of the hybrid gene products, functional domains of the original PhoE and OmpC porins could be localized.

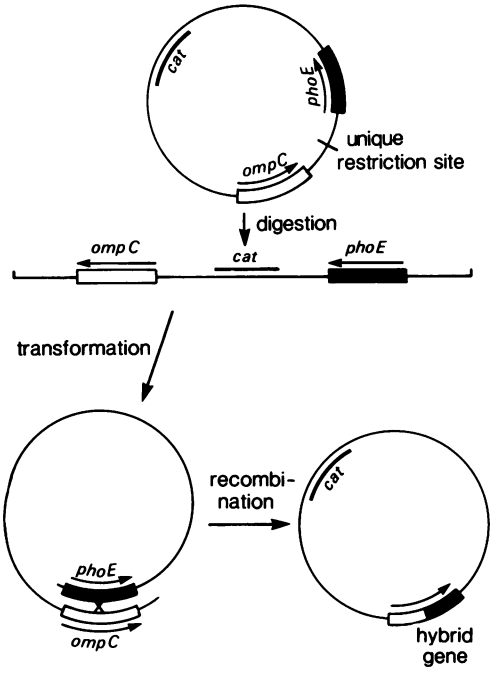

Fig. 1. General scheme for the construction of hybrid genes. Two homologous genes (e.g., *ompC* and *phoE*) are located in tandem on a plasmid. The arrows indicate the direction of transcription of these genes. An antibiotic resistance marker (e.g., chloramphenicol resistance, indicated as *cat*) is also located on the plasmid. Further steps are described in the text. In this example, hybrid genes are obtained with a promoter proximal *ompC* moiety.

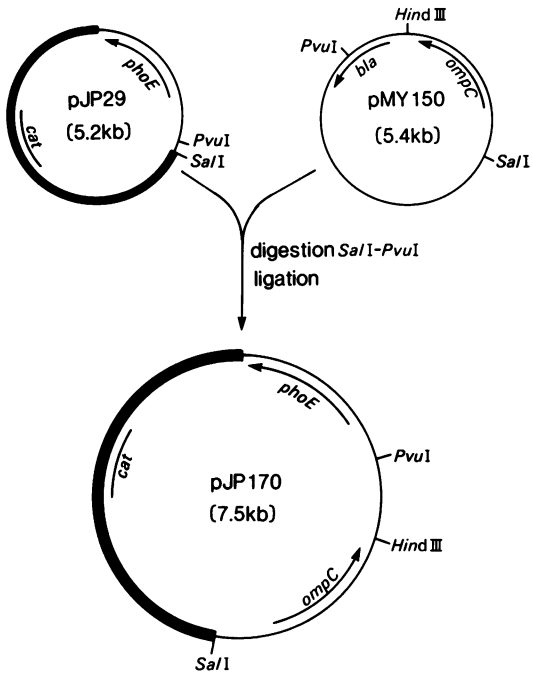

Fig. 2. Construction of pJP170, containing *ompC* and *phoE* in tandem. *cat* and *bla* indicate the genes conferring resistance to the antibiotics chloramphenicol and ampicillin, respectively. The arrows indicate the direction of transcription of the genes.

Results

In vivo construction of ompC-phoE hybrid genes resulting in hybrid porins

The general approach to construct hybrid porin genes is outlined in Figure 1. First, a plasmid is constructed which contains two of these genes in tandem. A unique recognition site for a restriction enzyme should be located between these genes. Then, the plasmid is cleaved with this restriction enzyme and the linear DNA is transformed into an *E. coli* K-12 strain, selecting for a vector-encoded antibiotic resistance marker. The linear DNA cannot be maintained in the host, but stable circular plasmids can arise by a recombination event between homologous regions in the porin genes. Thus, transformants are expected to contain plasmids with hybrid porin genes.

Plasmid pJP170 (Figure 2), which contains the *ompC* and *phoE* genes in tandem, was constructed as described in Materials and methods. The plasmid is 7.5 kb in size and contains unique *Hind*III and *Pvu*I sites between the porin genes. The plasmid was digested with both of these enzymes and the linear DNA was transformed into *ompR* strain CE1224, selecting the chloramphenicol-resistant colonies. Plasmid DNA was isolated from the transformants and analyzed on agarose gels. In two independent experiments, 50% and 90%, respectively, of the transformants contained a plasmid of the expected length of 5.1 kb, i.e., pJP170 deleted for the length of a complete porin gene and the DNA located in between these genes.

To determine whether true hybrid genes were obtained and to study the expression of these hybrid genes, 25 independent plasmid preparations were introduced by transformation in *ompR*$^+$ strain CE1228, which lacks all pore proteins due to mutational alterations in the corresponding structural genes. All transformants were found to contain a major cell envelope protein with an apparent mol. wt. between 38 000 and 40 000, the apparent mol. wts. of OmpC and PhoE, respectively (Figure 3). Since transcription from *phoE* promoters does not occur in strain

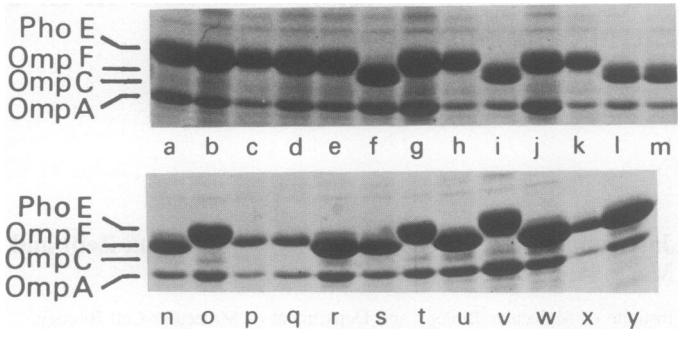

Fig. 3. SDS-polyacrylamide gel electrophoresis patterns of the cell envelope proteins of derivatives of strain CE1228 containing the plasmids pJP171 to 195 (**lanes a-x**, respectively) each of which contains a hybrid *ompC-phoE* gene. Only the relevant part of the gel containing the proteins with apparent mol. wts. between 42 000 and 35 000 is shown. At the left, the positions of the normal porins, PhoE, OmpF and OmpC, as well as of OmpA are indicated.

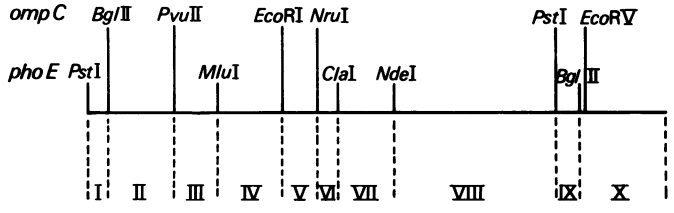

Fig. 4. Relative positions of restriction sites in *ompC* (upper line) and *phoE* (lower line). On the basis of these restriction sites, the porin genes can be divided in 10 regions, indicated as I-X. The mature PhoE and OmpC proteins consist of 330 and 346 amino acid residues, respectively. The regions I–X and the corresponding amino acid segments in PhoE (OmpC) are: I, 1–11 (1–11); II, 12–49 (12–49); III, 50–74 (50–73); IV, 75–110 (74–109); V, 111–131 (110-129); VI, 132-141 (130-139); VII, 142-173 (140-185); VIII, 174–267 (186–279); IX, 269–279 (280–292); X, 280–330 (293–346).

CE1228 grown in L-broth, the synthesis of these proteins must be under the control of the *ompC* promoter. All the new proteins detected in the cell envelope preparations had a slightly lower electrophoretic mobility than OmpC (Figure 3), suggesting that all were hybrid proteins. This was confirmed by restriction enzyme analysis of the plasmids.

Localization of the fusion sites in the hybrid genes

The fusion sites in the hybrid genes were mapped by using restriction enzymes which cleave either in *ompC* (*Bgl*II, *Pvu*II, *Eco*RI, *Nru*I, *Pst*I and *Eco*RV) or in *phoE* (*Pst*I, *Mlu*I, *Cla*I, *Nde*I and *Bgl*II). In Figure 4, the porin genes are aligned and the relative positions of these restriction sites are indicated. Thus, digestions of the plasmids containing the hybrid genes with these enzymes and analysis of the DNA fragments on agarose gels, allowed us to allocate the fusion joints in the distinct plasmids to either one of the 10 regions, indicated as I–X in Figure 4, defined by the positions of differing restriction sites (Table I).

Physical characterization of the hybrid gene products

OmpC protein has a mol. wt. of 38 306 (Mizuno *et al.*, 1983) and has a comparable apparent mol. wt. as determined by polyacrylamide gel electrophoresis (Lugtenberg *et al.*, 1975). However, PhoE protein has a mol. wt. of 36 782 (Overbeeke *et al.*, 1983), but an apparent mol. wt. of 40 000 (Van Alphen *et al.*, 1978). The apparent mol. wts. of the hybrid proteins are summarized in Table I. Even the class X hybrid proteins have a larger apparent mol. wt. than OmpC, showing that the aberrant electrophoretic mobility of PhoE protein is in part deter-

Table I. Characterization of OmpC-PhoE hybrid porins

ompC-phoE fusion numbers	Class[a]	Apparent mol.wts[b] (K)	Cefsulodin uptake[c] -	MeI, TuIb PA-2, TuIa*hrc*[d]	TC45 TC45*hr*N3[d]
12	I	40	11	R	S
28	I	40	5	R	S
4, 18, 47	II	40	5	R	S
8	III	40	5	R	S
35	IV	40	5	R	S
46, 73	IV	40	2	R	R
32, 49	V	40	2	R	R
68	VI	40	<1	R	R
61	VII	40	<1	R	R
60, 67, 75	VIII	39	<1	S	R
29, 66, 80	VIII	39	<1	S	R
41	IX	39	<1	S	R
64, 65	X	39.5	<1	S	R
53, 54, 70	X	39	<1	S	R
Controls					
CE1321 (PhoE$^+$)		40	12	R	S
CE1324 (OmpC$^+$)		38	<1	S	R

[a]The fusion sites in the hybrid genes were localized in 10 regions as shown in Figure 4 and the fusions are classified accordingly.
[b]Apparent mol. wts. were determined on SDS-polyacrylamide gels. K represents 10^3.
[c]Rate of uptake of cefsulodin was measured in cells of CE1228 containing either one of the plasmids pJP171 to pJP195, which encode the hybrid proteins, and pBR322 to provide a high β-lactamase level. The rate of uptake is expressed as nmol/min/10^8 cells.
[d]Resistance (R) and sensitivity (S) to the phages are indicated.

Table II. Binding of PhoE protein-specific antibodies to cells producing hybrid porins[a]

ompC-phoE fusion number	Class	Monoclonals PP1-1 to PP1-5[b]	Monoclonal PP2-1[b]	Polyclonal Anti-PhoE[b]
12	I	++	++	++
28	I	++	+	++
4, 18, 47	II	++	+	++
8	III	++	+	++
35	IV	++	+	++
46, 73	IV	++	+	++
32, 49	V	++	+	++
68	VI	++	+	++
61	VII	++	+	++
60, 67, 75	VIII	−	−	+
29, 66, 80	VIII	−	−	+
41	IX	−	−	+
64, 65	X	−	−	−
53, 54, 70	X	−	−	−
Controls				
CE1321 (PhoE$^+$)		++	++	++
CE1324 (OmpC$^+$)		−	−	−

[a]CIRA experiments were performed to study the binding of PhoE protein-specific antibodies to cells of CE1228 derivatives producing the hybrid proteins indicated in the first column.
[b]++, + and − indicate, respectively, 90−160%, 5−25% and <1% radioactive protein A bound to the cells as compared with the PhoE producing control strain.

mined by amino acid residue(s) very close to the C terminus. Similarly, part of the protein between the class VII and class VIII fusion joints influences the electrophoretic mobility of the protein.

Functional characterization of the hybrid proteins

PhoE protein pores, in contrast to OmpC protein pores, are more permeable for anionic solutes than for cationic solutes (Benz *et al.*, 1984; Korteland *et al.*, 1984). Probably as a result of this anion selectivity, the β-lactam antibiotic cefsulodin permeates much faster through PhoE pores than through OmpC pores (Nikaido *et al.*, 1983). Therefore, measurement of the rate of uptake of this antibiotic by cells producing the hybrid proteins as the only porins could be used to characterize the pore properties of the hybrid proteins (Table I). A rate of uptake similar to the PhoE-producing control strain was found only for strain CE1228 containing pJP173, which produces class I hybrid protein 12. In all other cases, the rate of uptake of cefsulodin was reduced. In three steps the rate of uptake is reduced to a level comparable with OmpC protein pores; this level is reached in the class VI fusions. These results suggest that at least three regions in the PhoE protein contribute to its specificity.

PhoE protein functions as the receptor for phage TC45 (Chai and Foulds, 1978) and its host range derivative TC45 *hr* N3 (Tommassen *et al.*, 1984), whereas OmpC protein functions as the receptor for the phages Mel (Verhoef *et al.*, 1977), TuIb (Datta *et al.*, 1979), PA-2 (Bassford *et al.*, 1977) and a host range derivative of the OmpF-specific phage TuIa. To localize segments of the proteins which contribute to the receptor sites of these phages, strains producing the hybrid proteins were tested for sensitivity to the phages. All class I−III hybrid proteins, and some class IV hybrid proteins, functioned as receptor for phages TC45 and TC45 *hr* N3 (Table I), suggesting that at least part of the receptor site for these phages is located on region IV of PhoE protein. All strains producing the class I−VII hybrid proteins were resistant to all the OmpC-specific phages tested, in contrast to the strains producing the class VIII−X hybrid proteins (Table I). Thus, at least part of the receptor sites for these phages is located in the region of OmpC between the fusion joints of the class VII and class VIII proteins. Plaques of PA-2 on strains producing the class VIII−X proteins were smaller and less clear than on strains producing native OmpC protein, suggesting that a part of OmpC very close to the C-terminus is also involved in the phage receptor function.

Mapping antigenic determinants with the aid of the hybrid proteins

We have isolated six monoclonal antibodies which recognize the cell surface-exposed part of PhoE protein (Van der Ley *et al.*, 1985). These antibodies do not recognize OmpC protein. The cell immunoradioassay (CIRA) technique was applied to determine whether the antibodies recognized the OmpC-PhoE hybrid proteins. Monoclonal antibodies PP1-1, PP1-2, PP1-3, PP1-4 and PP1-5 bound to cells producing the class I−VII hybrid porins even slightly more efficiently than to the PhoE-producing control strain (Table II). This more efficient binding can be explained as a result of a slight overproduction of the hybrid proteins which are encoded on multicopy plasmids. Cells producing the class VIII−X hybrid proteins did not bind these five monoclonal antibodies (Table II). Thus, the antigenic determinants for these antibodies are at least partly located in the region of PhoE protein between the fusion joints of the class VII and VIII hybrid proteins. Different results were obtained for monoclonal antibody PP2-1 (Table II). This antibody recognizes only the product of *ompC-phoE* fusion 12 as efficiently as phoE protein (Table II). The binding to the other hybrids varied from 5% to 25% for class I−VII fusions and <1% for class VIII−X fusions. This result suggests that part of the antigenic determinant also for this monoclonal antibody is located between the fusion joints of the

class VII and VIII hybrids.

We have found that a polyclonal antiserum directed against PhoE protein was specific for this protein with respect to cell surface-exposed epitopes (Van der Ley *et al.*, 1985). Therefore, the hybrid proteins could also be used to localize these epitopes in CIRA experiments. Again a breakpoint was observed in the binding of the antibodies between cells producing the class VII hybrid proteins and cells producing the class VIII hybrid proteins (Table II), suggesting that there is a major immunogenic epitope exposed to the cell surface in the part of PhoE protein between the fusion sites in regions VII and VIII. When the fusion joint shifts further to the C terminus, the binding of the antiserum gradually decreased to zero, suggesting that there are also some cell surface-exposed immunogenic epitopes near the C terminus.

Discussion

To study the structure-function relationship of outer membrane pore proteins of *E. coli* K-12, we have constructed *ompC-phoE* hybrid genes by making use of *in vivo* recombination between these homologous genes. Weber and Weissmann (1983) used a comparable approach to obtain hybrid α-interferon genes. The homology between the two constituting α-interferon genes was ~80%. The homology between *phoE* and *ompC* genes is ~60% (Mizuno *et al.*, 1983) which appeared to be sufficient to obtain fusion sites throughout these genes.

The products of the hybrid genes were characterized for those features in which the two original porins differ. This approach allowed the localization of domains in the native proteins involved in particular properties and functions. Two segments are responsible for the aberrant mol. wt. of PhoE protein, i.e., one in the region between the fusion joints of class VII and VIII hybrids and one close to the C terminus. Interestingly, in both regions *phoE* contains a deletion of 14 and four codons, respectively, as compared with *ompC*. Thus, although the hybrid proteins really become larger when the fusion joint shifts up to the C terminus, their apparent mol. wt. decreases.

The efficiency of the uptake of cefsulodin by cells producing the hybrid proteins was decreased in three steps when the fusion joint shifts up to the C terminus (Table I). Thus, region I, region IV and the region between the fusion joints of class V and VI hybrids seem to determine the pore characteristics of the proteins. Interestingly, only the amino acid residue in position 3 is different in PhoE and OmpC in region I, namely an isoleucine in PhoE and a valine in OmpC. Thus, the exchange of Ile-3→Val in PhoE apparently influences the pore characteristics. However, this isoleucine is unlikely to play a direct role in the anion selectivity of the PhoE pores in which arginine or lysine residues are presumed to be involved (Overbeeke *et al.*, 1983; Benz *et al.*, 1984). An Ile →Val replacement is also not normally expected to result in gross conformational distortion of the protein. However, monoclonal antibody PP2-1 binds to class I hybrid protein 28 less efficiently than to PhoE protein (Table II). Since the extreme N terminus of PhoE protein is thought to be located at the periplasmic side of the membrane (Tommassen and Lugtenberg, 1984), the amino acid residue in position 3 cannot directly be involved in the antigenic determinant which supposedly is located at the outside surface. Thus, the effect of replacement of Ile-3→Val on the binding of the monoclonal antibody is most likely by influencing the conformation of the binding site. Similarly, the Ile-3→Val replacement might only indirectly influence the anion selectivity of the PhoE pores. DNA sequence analysis will have to reveal whether hybrid protein 28 differs from PhoE indeed only by the amino acid in position 3. Residues in region IV and in the region between the fusion joints of class V and VI hybrids seem also to be involved in determining the pore characteristics of PhoE. Likely candidates for the relevant residues in these regions are lysine-84 and lysine-125, respectively, which are both replaced by glutamine residues in OmpC.

The region between the fusion joints of class VII and class VIII hybrid proteins seems to contain a major cell surface-exposed part of the porins, since antigenic determinants of all six monoclonal antibodies tested are located in this region. These antibodies recognize at least three distinct epitopes (Van der Ley *et al.*, 1985). Also, part of the antibodies directed against the cell surface-exposed region of PhoE protein in a polyclonal antiserum recognize an antigenic determinant in this region of the protein. Furthermore, this region contributes to the receptor sites for all OmpC-specific phages tested. In region VII, *ompC* contains an insert of 14 codons as compared with *phoE*. If the fusion joint in class VII OmpC-PhoE hybrid protein 61 is located in front of this insert, it would be reasonable to assume that the segment of the porins in the neighbourhood of this insert determines the major cell surface-exposed properties of the porins. Indeed, Korteland *et al.* (in preparation) have isolated five independent TC45 resistant mutants that produced an altered PhoE protein. All the mutations resulted in the exchange of the arginine residue in position 158, which is very close to the insert in *ompC*.

In summary, the approach of constructing hybrids between the OmpC and PhoE porins allowed a fairly precise assignment of several segments of the amino acid sequences to certain functions. The preference of the PhoE porin for anionic substrates is determined by the three segments of the polypeptide chain corresponding to amino acid residues 1–11, 75–110 and 111–141, while the segment 75–110 contributes to the receptor site for PhoE-specific phages. On the other hand, the receptor site for OmpC-specific phages is at least partially defined by the segment comprising amino acids 140–279. The homologous segment of PhoE protein (amino acids 142–267) appears to be responsible for the interaction with antibodies raised towards its part exposed at the cell surface. To determine the exact extent of the antigenic epitopes as well as of the phage receptor structures, the inverse hybrid porins, PhoE-OmpC fusions, however, will have to be analyzed.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used are *E. coli* K-12 derivatives. Strain CE1228 is an *ompF ompC* derivative of *phoE* strain AB1157 and was isolated as a mutant resistant to phages Me1 and TuIa. Strain CE1224 (Tommassen *et al.*, 1983) is also a derivative of AB1157 and does not produce OmpF and OmpC due to a mutation in the regulatory gene *ompR*. Strains CE1321 and CE1324 (Van der Ley *et al.*, 1985) produce PhoE protein and OmpC protein, respectively, as the only porin.

Cells were grown overnight at 37°C with aeration in L-broth (Tommassen *et al.*, 1983). Where necessary, the medium was supplemented with chloramphenicol (25 μg/ml) or ampicillin (50 μg/ml).

Plasmids

Plasmid pJP170, which contains the $ompC^+$ and $phoE^+$ genes in tandem, was constructed as outlined in Figure 2. The $phoE^+$ plasmid pJP29 (Tommassen *et al.*, in preparation), which is a derivative of pJP12 (Tommassen *et al.*, 1982), and the $ompC^+$ plasmid pMY150 (Mizuno *et al.*, 1984) were digested with *Sal*I and *Pvu*I; after inactivation of the restriction enzymes, the DNA preparations were mixed, ligated with T4 DNA ligase and transformed into the $ompC^-$ $ompF^-$ $phoE^-$ strain CE1228 selecting for chloramphenicol-resistant colonies. Plasmid DNA of a transformant that was sensitive to the OmpC-specific phage Me1 was isolated and restriction fragments were checked on gels to confirm that the correct construction had been made.

In this paper a number of derivative plasmids of pJP170 are described containing *ompC-phoE* hybrid genes. The fusion numbers and the corresponding

plasmid numbers are: φ4, pJP171; φ8, pJP172; φ12, pJP173; φ18, pJP174; φ28, pJP175; φ29, pJP176; φ32, pJP177; φ35, pJP178; φ41, pJP179;φ46, pJP180; φ47, pJP181; φ49, pJP182; φ53, pJP183; φ54, pJP184; φ60, pJP185; φ61, pJP186; φ64, pJP187; φ65, pJP188; φ66, pJP189; φ67, pJP190; φ68, pJP191; φ70, pJP192; φ73, pJP193; φ75, pJP194; φ80, pJP195.

Genetic techniques

Transformation was carried out as described by Kushner (1978). Sensitivity of strains to phages was determined by cross-streaking and by testing for plaque formation.

Isolation and characterization of cell envelopes

Cell envelopes were isolated as described by Lugtenberg *et al.* (1975). The protein patterns of the cell envelopes were analyzed by SDS-polyacrylamide gel electrophoresis (Lugtenberg *et al.*, 1975).

Rate of permeation of cefsulodin

The rate of permeation of the β-lactam antibiotic cefsulodin was measured using a method originally described by Zimmerman and Rosselet (1977) and modified by Overbeeke and Lugtenberg (1982).

Binding of antibodies to whole cells

The binding of antibodies to whole cells was measured using the CIRA technique (Van der Ley *et al.*, 1985). In short, whole cells were incubated with antibodies and, after washing, with ^{125}I-labeled protein A. The amount of radioactivity bound to the cells after washing is a measure for the amount of antibodies bound to the cells.

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