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# Molecular basis of porin selectivity: membrane experiments with OmpC-PhoE and OmpF-PhoE hybrid proteins of *Escherichia coli* K-12

Roland Benz [a], Angela Schmid [1], Peter Van der Ley [2] and Jan Tommassen [2]

[1] *Lehrstuhl für Biotechnologie, Universität Würzburg, Würzburg (F.R.G.) and* [2] *Department of Molecular Cell Biology, State University of Utrecht, Utrecht (The Netherlands)*

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**Lipid bilayer experiments were performed with one OmpF-PhoE and several OmpC-PhoE hybrid porins of *Escherichia coli* K-12. All hybrid pores had approximately the same pore-forming activity, which indicated that the structure of the pores remained essentially unchanged by the genetic manipulation. This result was supported by single-channel experiments because all pores had similar single-channel conductances in potassium chloride. Measurements with other salts indicated a drastic change in the ionic selectivity when the fusion site in the *ompC-phoE* hybrid genes passed along the sequence of the porins from the N-terminal to the C-terminal end. Selectivity measurements using zero-current membrane potentials showed that the selectivity suddenly changed from anion to cation selectivity when a relatively short portion from the N-terminal end of PhoE was replaced by the corresponding part of OmpC. The replacement of increasing portions led to an increase in the cation selectivity until that of OmpC was reached. The change in the anion to cation selectivity is correlated with exchange of lysine-18 and serine-28 by aspartic acids. The anion selectivity of the phosphate starvation-inducible PhoE porin is closely related to the presence of several lysines spread along the primary sequence of the polypeptide chain.**

## Introduction

The outer membrane of Gram-negative bacteria acts as a molecular filter for hydrophilic solutes (see Refs. 1–3 for reviews). Active components of this filter are wide water-filled channels which are formed by a major class of proteins called porins. Porins are generally present in high copy numbers in the outer membrane of Gram-negative bacteria [1–3]. Porin channels of enteric bacteria are formed by trimers of identical polypeptide subunits. Porin function is either general (general diffusion pores), which means that the pores sort mainly according to molecular weight [2–5], or specific because of a binding site for a given class of solutes inside the channels [6–10]. The outer membrane of *Escherichia coli* contains under normal growth conditions two general diffusion porins, OmpF and OmpC, the synthesis of which is regulated via, for example, the osmolarity of the growth medium [11]. Both porins form cationically selective pores [4,5].

Another general diffusion porin, PhoE, is induced in the family of *Enterobacteriaceae* when the organisms are grown under phosphate limitation [12–14]. This porin forms anionically selective pores at neutral pH [4,5,15] but it does not contain a binding site specific only for phosphate or polyphosphate [16]. Despite the difference in the selectivity, the primary sequences of all general diffusion porins of *E. coli* outer membrane are highly homologous [17–20]. Chemical modification of PhoE or *E. coli* using different reagents has shown that the $\epsilon$-amino groups of lysines are presumably responsible for its anion-selectivity [21–22]. In fact, the primary sequence of PhoE contains some additional lysines as compared with the sequences of OmpF and OmpC [19]. On the other hand, all three porins contain a large number of positively charged amino-acid residues which are spread along the polypeptide chain. This means that it is not obvious which amino acid is responsible for the anion-selectivity of PhoE.

PhoE mutants [23,24] and hybrid proteins [25–27] between the closely related OmpF, OmpC and PhoE porins allowed the detection of surface-exposed groups in the primary sequence of PhoE via the interaction of these groups with phages and monoclonal antibodies.

Correspondence: R. Benz, Lehrstuhl für Biotechnologie, Universität Würzburg, Röntgenring 11, D-7750 Würzburg, F.R.G.

The negatively charged β-lactam antibiotic cefsulodin permeates much more quickly through PhoE pores than through OmpC pores [28]. The diffusion of the antibiotic cefsulodin has been used to characterize the pore properties of the hybrid pores [26]. The results have indicated that several regions of the PhoE sequence contribute to the selectivity of the pore-forming complex. On the other hand, it was not known whether the pores formed by the hybrid proteins had the same structure as PhoE or OmpC. Furthermore, the discussion of the results obtained with cefsulodin is dependent on the degree of the expression of the porins in the porin-deficient mutant. In this study, we used lipid bilayer experiments to show that the functional integrity of one OmpF-PhoE and nine OmpC-PhoE hybrid porins is retained. The ionic selectivity of the OmpC-PhoE hybrid porins is shifted from anionic to cationic selectivity when the fusion point is shifted from the N-terminal to the C-terminal end of the hybrid proteins, i.e., when larger N-terminal portions of PhoE are replaced by homologous fragments of OmpC. The results are consistent with the assumption that additional lysines are responsible for the selectivity of enterobacterial PhoE porins [29].

## Materials and Methods

### *Bacterial strain, plasmids, growth condition and porin isolation*

*E. coli* K-12 strain CE 1228 is an *ompF ompC* derivative of PhoE mutant strain AM 1157 [26]. Plasmid pJP47, containing an *ompF-PhoE* hybrid genes was constructed in vitro [25]. In the hybrid protein encoded by this gene, the 73 N-terminal amino acids on PhoE are replaced by the corresponding fragment of OmpF. Plasmid-containing *ompC-phoE* genes were obtained by employing in vivo recombination of these homologous genes [26]. The approximate positions of the fusion joint in the hybrid genes was determined by restriction enzyme analysis and allowed the classification of the hybrid genes into ten classes [26]. In some cases, the fusion joints were determined exactly by the nucleotide sequence analysis [27]. Table I shows the OmpC-PhoE hybrid proteins used in this study.

Porins were isolated from cells of strain CE 1228, containing the hybrid genes plasmids after growth overnight at 37 °C with aeration in L-broth supplemented with chloramphenicol (25 μg/ml). The porins were isolated and purified by a four-step procedure essentially as described in Ref. 31.

### *Lipid bilayer experiments*

Black lipid bilayer membranes were formed as described previously in Ref. 32. The apparatus consisted of a Teflon chamber with two aqueous compartments of 5 ml connected by small circular holes. The holes had a surface area of either 1 $mm^2$ (for the multi-channel measurements) or 0.1 $mm^2$ (in the case of the single channel experiments). Membranes were formed by painting onto the holes a 1% solution of diphytanoylphosphatidylcholine (Avanti Biochemicals, Birmingham, AL) in *n*-decane. The aqueous salt solutions (Merck, Darmstadt, F.R.G.) were used unbuffered and had a pH of approx. 6. The porins were added from the concentrated stock solution either to the aqueous phase bathing a membrane in the black state or immediately prior to membrane formation. The temperature was maintained at 25 °C throughout.

The membrane current was measured with a pair of calomel electrodes with salt bridges switched in series with a voltage source and an electrometer (Keithley 602). In the case of the single-channel recordings, the electrometer was replaced by a current-to-voltage converter based on a Burr Brown operational amplifier. The amplified signal was monitored with a storage oscilloscope and recorded with a tape or a strip-chart recorder. Zero-current membrane potential measurements were performed by establishing a salt gradient across membranes containing 100–1000 porin pores as described earlier in Ref. 33.

TABLE I

*Characteristics of OmpC-PhoE hybrid porins*

The fusion sites in the hybrid genes are localized in ten regions, numbered I to X, as has been shown previously in Ref. 26. The approximate position of the fusion joint in the primary sequence of PhoE is shown in the last column. In some cases, the fusion joint was determined exactly by nucleotide sequencing of the hybrid genes [27].

| *ompC-phoE* fusion numbers | Class | Fusion point from N-terminus of PhoE |
|---|---|---|
| 12 | I | 7 |
| 18 | II | 12–49 |
| 47 | II | 12–49 |
| 35 | III | 52 |
| 32 | V | 112 |
| 29 | VIII | 174–267 |
| 65 | X | 280–330 |
| 53 | X | 280–330 |
| 74 | X | 280–330 |

## Results

### *Multi-channel experiments*

Lipid bilayer experiments were performed to study the pore properties of the hybrid pores in more detail with respect to the well-characterized OmpC, OmpF and PhoE porins [5]. The different proteins were added in small quantities (10–100 ng/ml) to the aqueous solution bathing a lipid bilayer membrane while stirring to allow equilibration. After 1–2 min, most likely due to diffusion through unstirred layers, the specific membrane conductance started to increase by several orders

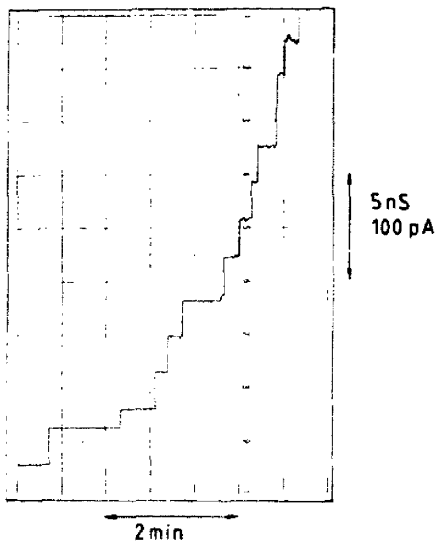


Fig. 1. Single-channel recording of a diphytanoylphosphatidylcholine/*n*-decane membrane in the presence of 10 ng/ml OmpC-PhoE hybrid porin 18 and 1 M KCl. The applied voltage was 20 mV; $T = 25\,^{\circ}C$.

TABLE II

*Average single-channel conductances of OmpF-PhoE and OmpC-PhoE hybrid proteins in different 1 M salt solutions*

Average single-channel conductance, $\Lambda$, measured with diphytanoylphosphatidylcholine/*n*-decane membranes in the presence of different hybrid porins. The pH of the unbuffered aqueous salt solutions was around 6; $V_m = 10$ mV. $\Lambda$ was determined by the recording and averaging of at least 100 conductance steps. Note that $\Lambda$ always corresponded with the mean of the maximum peak in the histograms (see Fig. 2).

| Porin | $\Lambda$ (nS) | | |
|---|---|---|---|
| | KCl | LiCl | KAc |
| OmpF-Phoe | 1.5 | 0.70 | 0.72 |
| OmpC-PhoE hybrids: | | | |
| 12 | 1.6 | 1.1 | 0.48 |
| 18 | 1.5 | 0.82 | 0.85 |
| 47 | 1.6 | 0.68 | 1.0 |
| 35 | 1.7 | 0.65 | 0.96 |
| 29 | 1.6 | 0.67 | 1.1 |
| 53 | 1.5 | 0.66 | 1.0 |
| 65 | 1.5 | 0.64 | 1.1 |
| 74 | 1.6 | 0.67 | 1.1 |

of magnitude (Fig. 1). The time-course of the conductance increase was very similar to that described earlier for other bacterial and mitochondrial porins [32,34]. After an initial rapid increase for 15–20 min, the membrane conductance increased at only a very slow rate. The specific conductance measured at this time for different porins varied little under otherwise identical experimental conditions. This result indicated that the structure of the hybrid pores was very similar to OmpC, OmpF or PhoE; otherwise, we would have expected a stronger variation of the reconstitution rate of the hybrid pores.

*Single-channel recordings*

The multi-channel experiments suggested that the structure of the OmpF-PhoE and OmpC-PhoE hybrid pores remained essentially unchanged as compared to the authentic pores. To test this assumption, we performed single-channel experiments. The addition of small concentrations of the hybrid porins (1–10 ng/ml) to membranes of small surface (0.1–0.2 $mm^2$) allowed the resolution of defined step increases in membrane conductance. These steps were specific for the presence of the different porins and were not observed if only the detergent SDS or Triton X-100 was present in the aqueous phase. The hybrid porins formed defined pores of long lifetime (see Fig. 1) in lipid bilayer membranes similar to those observed with other Gram-negative bacterial porins [34]. The sizes of the hybrid pores were very similar to those of OmpC, OmpF and PhoE because the single-channel conductances in 1 M KCl (see Table II) varied only little in the series PhoE, hybrid porins of class I to X and OmpC and for the OmpF-PhoE hybrid protein. Although the ionic selectivity of the pores should change in the same series from anion to cation selectivity, this would probably not affect single-channel conductances because potassium and chloride ions have the same mobility in the aqueous phase (limiting molar conductivities 73.5 mS/M and 76.4 mS/M, respectively [35]). In other words, a change in the ionic selectivity should not influence the single-channel conductance if the structure of the hybrid pores is identical to that of PhoE and OmpC.

Fig. 2 shows a histogram of the conductance steps observed with OmpC-PhoE hybrid 18 in 1 M KCl solution. The histogram has maxima between 1.25 and 1.75 nS and between 2.25 and 2.75 nS, indicating the formation of single and double pores in the membranes. Single-channel experiments were performed with three

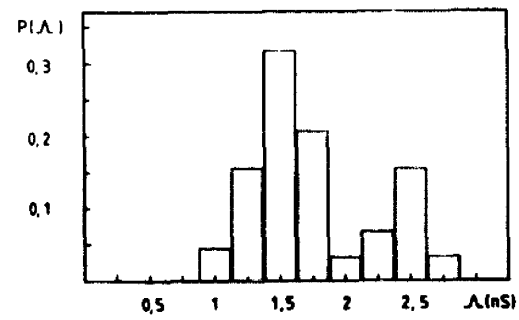


Fig. 2. Histogram of the conductance steps observed with diphytanoylphosphatidylcholine/*n*-decane membranes in the presence of OmpC-PhoE hybrid porin 18 and 1 M KCl. The mean of the conductance steps was 1.8 nS for 218 single events. The average single-channel conductance of the maximum peak was 1.5 nS for 175 single steps.

TABLE III

*Dependence of the single-channel conductance of the OmpC-PhoE hybrid porin 18 on the KCl concentration in the aqueous phase*

The average single-channel conductance, $\Lambda$, was determined using diphytanoylphosphatidylcholine/*n*-decane membranes by the recording and averaging of at least 100 conductance steps. The pH of the unbuffered aqueous salt solutions was around 6; $V_m = 20$ mV. Note that $\Lambda$ always corresponded with the mean of the maximum peak in the histograms (see Fig. 2).

| KCl (mM) | $\Lambda$ (pS) |
|---|---|
| 3 | 7 |
| 10 | 18 |
| 30 | 54 |
| 100 | 170 |
| 300 | 580 |
| 1000 | 1500 |

different 1 M salt solutions (see Table II). Furthermore, we measured the single-channel conductance for hybrid 18 at four different KCl concentrations to test whether the conductance versus concentration curve was linear. The results of these measurements are shown in Table III. The average single-channel data given in Tables II and III always correspond with the average conductance of the main peak of the histograms which probably represented the single (trimeric) pore. The data indicate that the hybrid porins formed general diffusion pores in lipid bilayer membranes. With 1 M LiCl, we found a decrease of the single-channel conductance from hybrid 12 (class I) to hybrid 47 (class II), whereas we observed the opposite for 1 M potassium acetate.

TABLE IV

*Zero-current membrane potentials of OmpF-PhoE and OmpC-PhoE hybrid porins*

Zero-current membrane potentials $V_m$ in the presence of a ten-fold salt concentration gradient across diphytanoylphosphatidylcholine/*n*-decane membranes in which the different hybrid porins were reconstituted. $V_m$ is the mean of the electrical potential of the dilute side (10 mM) minus the potential at the concentrated side (100 mM) obtained from at least four individual measurements. The temperature was 25°C. PhoE, OmpF (K-12) and OmpC had for KCl, under otherwise identical conditions, zero current membrane potentials of −24, 26 and 50 mV, respectively [5].

| | $V_m$ (mV) | | |
|---|---|---|---|
| | KCl | LiCl | KAc |
| OmpF-PhoE | 28 | 7.0 | 44 |
| OmpC-PhoE hybrids: | | | |
| 12 | −26 | −40 | −6.9 |
| 18 | 33 | 5.5 | 48 |
| 47 | 35 | 5.3 | 50 |
| 35 | 32 | 5.6 | 51 |
| 32 | 46 | 34 | 54 |
| 29 | 51 | 45 | 55 |
| 65 | 51 | 44 | 55 |
| 53 | 50 | 46 | 55 |
| 74 | 50 | 45 | 55 |

TABLE V

*Permeability ratios $P_c/P_a$ of different porins and hybrid porins as calculated from the data of Table IV using the Goldman-Hodgkin-Katz equation [33]*

The data for OmpF, OmpC and PhoE are taken from Ref. 5.

| Porin | $P_c/P_a$ | | |
|---|---|---|---|
| | KCl | LiCl | KAc |
| OmpF | 3.6 | 1.5 | 8.2 |
| OmpC | 26 | 15 | 54 |
| PhoE | 0.30 | 0.11 | 0.65 |
| OmpF-PhoE | 4.0 | 1.4 | 12 |
| OmpC-PhoE hybrids: | | | |
| 12 | 0.27 | 0.11 | 0.72 |
| [illegible] | 5.5 | 1.3 | 19 |
| 47 | 6.2 | 1.3 | 24 |
| [illegible] | 5.3 | 1.3 | 25 |
| [illegible] | 15 | 6.0 | 43 |
| [illegible] | 27 | 14 | 55 |
| [illegible] | 28 | 13 | 58 |
| [illegible] | 23 | 14 | 52 |
| [illegible] | 24 | 12 | 54 |

This result clearly indicated a selectivity change in the hybrid porins because anions and cations of these salts have different mobilities [35].

*Zero-current membrane potential measurements*

Zero-current membrane potential measurements were carried out to study the selectivity of the hybrid pores in more detail. These measurements were performed by creating a 10-fold salt gradient across the membranes containing at least 100 porin pores. The more dilute side became negative only for PhoE and the hybrid 12 of class I. For all the other hybrids (including the OmpF-PhoE hybrid), the more dilute side was positive with respect to the concentrated side, which indicated preferential movement of cations through the pores (see Table IV). The transmembrane potential caused by the 10-fold ion gradient can be used to calculate the permeability ratio of the cation permeability, $P_c$, divided by the anion permeability $P_a$ using the Goldman-Hodgkin-Katz equation [33]. The results of $P_c/P_a$ are given in Table V. The class I hybrid 12 had approximately the same selectivity as PhoE, while for all the other hybrids $P_c/P_a$ was larger than unity.

Several steps could be detected in the ionic selectivity of the OmpC-PhoE hybrids. The first step occurred already between hybrid 12 (class I) and hybrid 18 (II). Other steps were detected between hybrids 35 (III) and 32 (V) and between 32 (V) and 29 (VIII). Hybrid porins of classes VIII and X had the same selectivity as OmpC (see Table V). This result indicated that between PhoE and OmpC we passed on the way several amino acids which contribute to the overall selectivity of PhoE and OmpC. The OmpF-PhoE hybrid is in its characteristics

very similar to OmpF, which means that most groups responsible for its ionic selectivity may be located within the 74 amino acids exchanged from the N-terminal end.

## Discussion

PhoE, OmpF and OmpC of *E. coli* K-12 form large water-filled pores in the outer membrane. However, the pores are not merely water-filled cylinders because they exhibit a certain selectivity for ions [5,15] and for other charged solutes [4,36]. OmpF exhibits a moderate cation selectivity whereas OmpC is highly cationically selective (see Table V). PhoE is induced in *E. coli* K-12 under the conditions of phosphate starvation [12,13]. This porin forms moderate anionically selective pores for small ions [15] and hydrophilic solutes [4,28]. But it is not a specific porin similar to LamB [7,8] and Tsx [9] of *E. coli* K-12 or to protein P of *Pseudomonas aeruginosa* outer membrane [37]. The selectivity of PhoE is due to lysines, as has clearly been demonstrated by chemical modification of PhoE, OmpF and OmpC [21,22]. The primary sequence of PhoE is highly homologous to that of the other general diffusion pores of *E. coli* [17–20]. Despite the existence of different models for the arrangement of PhoE [24,38] and OmpC [39] in the outer membrane, we must assume that the pores formed by trimers of both proteins have more or less the same structure. Otherwise, we cannot explain why (i) functional pores are formed in the outer membrane by the hybrid proteins, (ii) the OmpC-PhoE hybrid pores had in KCl the same single-channel conductance as OmpC and PhoE and (iii) the reconstitution rate of the different porins into lipid bilayer membranes was similar. Assuming that PhoE and OmpC indeed have the same folding pattern, it should be noted that all available data on the topology of OmpC [39] easily fit the topology model of PhoE [24,38]. Therefore, there is no need for a separate OmpC model, especially since some amino acids of PhoE which have been shown to be cell-surface-exposed [24] are not exposed in the proposed OmpC model [39]. The results presented here indicate that the exchange of a short-portion of less than 49 amino acids of PhoE from the N-terminal end already results in a dramatic change of the selectivity because

```
          -----I----- ----------------------II-------
PhoE  AEIYNKDGNKL DVYGKVKAMHYMSDNASKDGDQSYIRFGF                  40
OmpC    V           L     DGL  F    KDV     T M L                40

          --------- -----------III---------- ------
          KGETQINDQ LTGYGRWEAEFAGNKAESDTAQQKT RLAFAG              80
               VT         Q  YQIQ  S  NENNS-W   V                 79

          ---------IV------------------- ----------
          LKYKDLGSFDYGRNLGALYDVEAWTDMFPE FGGDSSAQTD               120
            FQ V         Y VV   TS   VL        -TYGS              118

          -V--------- ----VI---- --------------------YII----------
          NFMTKRASGLA TYRNTDFFGV IDGLNLTLQYQGKNEN--------------RDV  160
             QQ GN F             L V     FAV       G PSGEGFTSGVTNNG  A  172

          ------------ ----------------------------
          KKQNGDGFGTSLT YDFGGSDFAISGAYTNSDRTNEQN-LQS              200
          LR      V G I     YE -- G G   ISS K   DA   TAAY          211

          -----------------VIII--------------------
          RGTGKRAEAWATGLKYDANNIYLATFYSETRKMTPIT-GGF               240
          I N D    TYTG              AQ TQ YNA RVGSLGW            252

          --------------------------- -------IX------
          ANKTQNFEAVAQYQFDFGLRPSLGYVL SKGKDIE-GIGDED              280
             A                      A LQ      NLGR YD             293

          ----------------------------X----------------
          LVNYIDVGATYYFNKNMSAFVDYKINQLDSDNKLN----INNDD            320
          ILK V        P      TY       L  -  QFTRDAG  T N         336

          ----------
          IVAVGMTYQF                                              330
             L LV                                                 346
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Fig. 3. Primary sequences of PhoE [18] and OmpC [19]. The mature proteins consist of 330 and 346 amino acids, respectively. The vertical bars indicate the ten regions or classes in which the OmpC-PhoE hybrid proteins can be divided according to the restriction sites in *phoE* and *ompC* (Ref. 25).

hybrid 12 (class I) is anionically selective whereas hybrid 18 (class II) has the opposite selectivity. Comparison of the primary sequences of PhoE and OmpC (see Fig. 3) with respect to this region shows that, in total, one positively charged group of PhoE (Lys-18) and one neutral amino acid (Ser-28) could be replaced by the negatively charged aspartic acids from the sequence of OmpC if the fusion site in hybrid proteins was beyond amino acid 30 (the exchange of lysine-29 into valine is counterbalanced by the exchange of alanine-27 by lysine). This could explain the rather strong change in the selectivity – by a factor of about 20 – which occurs for the hybrids 18 and 47 of class II. Furthermore, it is assumed in our model [38] that Lys-18 is located very close to the rim of the pore. This means that the replacement of this amino acid could have a large influence on the ionic composition of the pore interior. The other residue, Ser-20, is assumed to be exposed to the cell surface.

The assumption that some of the groups responsible for the ionic selectivity of PhoE are located within a relatively short region from the N-terminus is supported by the selectivity of the OmpF-PhoE hybrid. The exchange of 74 amino acids is already sufficient to obtain the selectivity of OmpF. This is consistent with the observation that all the other changes in the selectivity of the OmpC-PhoE hybrids are rather moderate as compared with the initial one. They occur between amino acids 52 (hybrid 35) and 112 (hybrid 32) and between amino acid 112 (hybrid 32) and the fusion joint in class VIII (hybrid 29). It should be noted that the results on the selectivity of the hybrid porins are in excellent agreement with the results of the in vivo experiments, in which the uptake of the negatively charged antibiotic cefsulodin was measured. Also in the in vivo experiments, the pore characteristics of the hybrid proteins changed in three distinct steps when the fusion joint moved to the C-terminus. Since more different hybrid proteins were analysed in vivo, the positions of the last two steps can be defined more closely by including the in vivo data. A strain-producing class IV hybrid protein 46, which has the fusion joint located at amino acid 91 of PhoE [27], showed a rate of cefsulodin uptake similar to a strain-producing class V hybrid protein 32. Thus, the second step in selectivity occurs between amino acids 52 (hybrid 35) and 91 (hybrid 46). The amino acids responsible in this region could be Lys-64, Lys-73 and Lys-84. In these positions, OmpC contains Ser, Trp and Gln residues, respectively. According to our topology model [38], Lys-64 and Lys-73 are surface-exposed, whereas Lys-84 is close to the periplasmic side of the membrane. A strain-producing class VI hybrid protein 68, which has the fusion joint between amino acids 132 and 141 of PhoE, showed a rate of cefsulodin uptake similar to a strain-producing class VIII hybrid 29. Thus, the third step in selectivity occurs between amino acids 112 (hybrid 32) and the fusion joint in hybrid 68. The responsible amino acid in this region is probably Lys-125, which is substituted by a Gln in OmpC. Lys-125 is located at the rim of the pore according to our topology model and may possibly play a major role in pore selectivity.

Our data provide strong evidence that the hybrid pores have the same overall structure as the pores formed by OmpC, PhoE and probably also by OmpF. Charged amino acids located on the surface of the pore-forming complex or at the rim of the pore, but not inside the pore, are responsible for the selectivity of the porins. On the other hand, small structural alterations within the pore-forming trimers as the consequence of the exchange of several amino acids cannot be completely rules out. The exact localization of the amino acids responsible for the selectivity of OmpC and PhoE, respectively, may be obtained by side-directed mutagenesis. This method allows the defined exchange of single amino acids and allows defined access to their function [23].

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