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Role of the Arg[158] residue of the outer membrane PhoE pore protein of *Escherichia coli* K 12 in bacteriophage TC 45 recognition and in channel characteristics

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In order to study the structure-function relationship of the PhoE protein pore we have isolated five independent, TC 45-resistant, *phoE* mutants all of which appeared to produce normal amounts of an electrophoretically altered PhoE protein, designated as PhoE* protein. Nucleotide sequence analysis of the DNA fragments carrying the mutations showed that the mutations all correspond to a G · C to A · T transition at the same place within the *phoE* gene resulting in a deduced change of amino acid residue arginine 158 into histidine. This result shows that the arginine 158 residue plays an important role in the interaction of the PhoE protein pore with phage TC 45. Moreover, studies on the channel properties of the PhoE* protein showed that the PhoE channel has lost part of its preference for negatively charged solutes, as a result of the arginine to histidine change. The results are discussed in terms of the structure-function relationship of PhoE protein as well as in terms of the topological organization of the protein channel in the outer membrane.

In the outer membrane of *Escherichia coli* K 12 two constitutively synthesized proteins, OmpF protein and OmpC protein, form non-specific pores which facilitate the permeation of small hydrophilic nutrients [1 – 6]. In addition, these proteins are recognized by phages as part of the phage receptor [7, 8].

PhoE protein of *E. coli* K 12 is an outer membrane protein, the synthesis of which is derepressed upon phosphate starvation [9]. Mutations in one of the genes *phoR*, *phoS*, *phoT* or *pst* lead to the constitutive synthesis of PhoE protein [10]. Like OmpF protein and OmpC protein, PhoE protein is involved in the formation of aqueous channels which allow the entry of small hydrophilic molecules into the periplasmic space [5, 11 – 14]. Besides general pore properties, PhoE protein has a preference for phosphate-containing nutrients [15], as well as for most other negatively charged solutes [16 – 18]. A recognition site for these solutes on the PhoE pore is likely to be responsible for this property [16]. PhoE protein constitutes part of the cell surface receptor for bacteriophage TC 45 [19]. The nucleotide sequence of the *phoE* gene appeared to be very similar to the sequences of the *ompF* [21] and the *ompC* gene [22]. As one would expect from this homology, a strong similarity of the predicted amino acid sequences was found for the three pore proteins [22].

The relationship between the amino acid sequence of the PhoE protein, its structure and its functioning can be studied by isolating mutants in the *phoE* gene, which affect the functioning of the PhoE channel but which do not significantly affect its conformation or its embedding in the outer membrane. By determining the alterations in the nucleotide sequence of the *phoE* gene, amino acid residues involved in a particular function of the PhoE protein are likely to be identified. In this paper we describe the isolation and characterization of TC 45-resistant, PhoE-protein-producing, *phoE* missense mutants as an approach to study the structure-function relationship of PhoE protein.

MATERIALS AND METHODS

Strains, phages and growth conditions

All bacterial strains used in this study are derivatives of *E. coli* K 12. Their sources and relevant characteristics are listed in Table 1. The *recA* 56 strain CE 1248 was obtained by crossing strain CE 1238 with Hfr strain PC 1505 and selecting for His$^+$, ultraviolet-light-sensitive, transconjugants. The PhoE-specific phage TC 45 has been described by Chai and Foulds [19]. Phage N_3 was isolated in our laboratory as a TC 45 host range mutant phage which recognizes the electrophoretically altered PhoE protein produced by strain CE 1202, as well as wild-type PhoE protein.

Cells were grown overnight at 37 °C under vigorous aeration in L-broth, which contains 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.002% thymine, final pH 7.0. Cells containing plasmid pBR 322 and/or derivatives of plasmid pACYC 184 were grown in medium supplemented with the antibiotics chloramphenicol (50 µg/ml) and/or ampicillin (25 µg/ml).

Genetic techniques

Conjugation was carried out as described previously [26]. Sensitivity to phages TC 45 and N_3 was determined by the double-layer technique [26]. Strains were scored as phage-sensitive if plaques were formed after incubation at 37 °C for about 16 h. The cross streak method was used for rapid screening of mutants for sensitivity to phages TC 45 and N_3.

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Abbreviations. Cm^R, chloramphenicol resistance; kb, 10^3 basis; SDS, sodium dodecyl sulphate.

Table 1. *Bacterial strains*
Genotype descriptions follow the recommendations of Bachmann and Low [25]. The Phabagen Collection is at the State University of Utrecht (Department of Molecular Cell Biology, section Microbiology, Utrecht, The Netherlands). $MeSO_3Et$ is ethyl methanesulfonate

Strain	Relevant characteristics	Source or reference
PC0479	F⁻, *thr leu thi pyrF thy ilvA his lacY argG tonA rpsL cod dra vtr glpR*	Phabagen collection
PC1505	Hfr KL16, *thr ilv phx recA* 56	Phabagen collection
CE1107	*ompB*471 derivative of PC0479	[23]
CE1108	*phoS*200 derivative of CE1107	[13]
CE1110	*ompR*472 derivative of PC0479	[23]
CE1202[a]	$MeSO_3Et$ – induced TC45-resistant *phoE*202 derivative of CE1108	this study
CE1220	*phoR*18 *recA* 56 derivative of CE1107	[24]
CE1237	*phoR*69 derivative of CE1107	[15]
CE1238	*phoE proA, B* derivative of CE1237	[15]
CE1241	SDS-resistant Tula-resistant derivative of CE1238	[15]
CE1248	*recA* 56 derivative of CE1238	this study
CE1265	TC45-resistant *phoE proA, B* derivative of CE1220	this study

[a] Strain CE1202 produces a PhoE protein with an altered electrophoretic mobility in SDS/polyacrylamide gels.

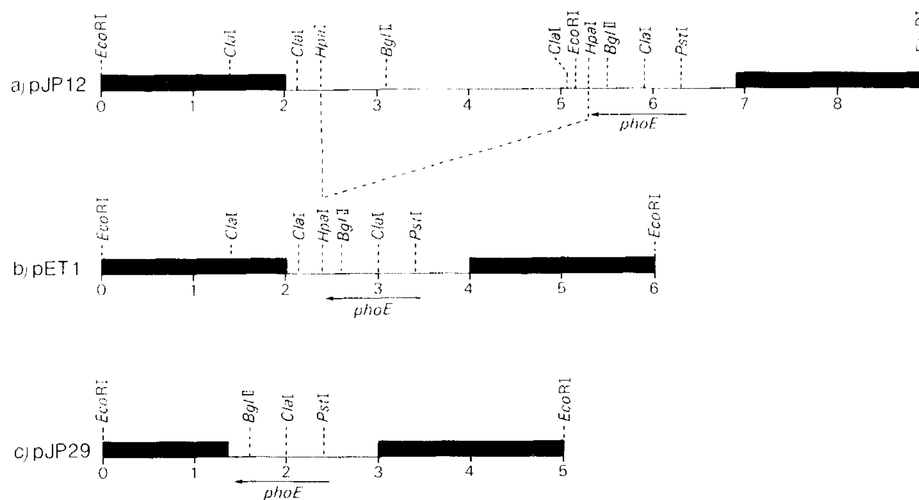

Fig. 1. *Schematic representation of the restriction map of plasmid pJP12 and its derivatives, plasmids pET1 and pJP29.* The plasmids are linearized in the *Eco*RI site at 0 kb. Plasmid pJP12 contains a 4.9-kb fragment on which the *phoE* gene is located, cloned in the *Sal*I site of pACYC184 [20]. The vector part of the plasmid is indicated by the black bar. The arrow represents the localization and the direction of transcription of the *phoE* gene. Plasmid pET1 consists of the 6.0-kb *Hpa*I fragment of plasmid pJP12 and has unique restriction sites for *Eco*RI and *Bgl*II. Plasmid pJP29 contains unique restriction sites for *Eco*RI, *Bgl*II and *Cla*I and was constructed by Tommassen et al. (unpublished)

Bacteriophage adsorption

Exponentially growing cells of strain CE1265 containing wild-type or mutagenized pJP12 were resuspended to a cell density of 10^8 cells/ml in yeast broth supplemented with KCN (1.25 mM) and rifampicin (5 µg/ml). At zero time bacteriophage TC45 was added to the cell suspension at a final concentration of about 10^7 plaque-forming units/ml. Samples were taken at appropriate time intervals and filtered through a membrane filter (0.45 µm pore size; Millipore Corp., Bedford, MA, USA). Various dilutions of non-adsorbed phages in the filtrate were mixed with bacteria of strain CE1265 containing wild-type pJP12 and applied as top layer of soft yeast agar on yeast agar plates. After incubation at 37°C for about 16 h the plates were scored for plaque formation.

DNA techniques and plasmids

Plasmid DNA was isolated by the cleared lysate procedure of Clewell and Helinski [27], followed by CsCl/ethidium bromide isopycnic centrifugation. For rapid screening of plasmids the alkaline extraction procedure of Birnboim and Doly [28] was used. Restriction endonucleases *Eco*RI, *Cla*I, *Bgl*II, *Pst*I and *Hpa*I were obtained from Boehringer Mannheim, FRG. Endonuclease reactions were performed according to the instructions of the manufacturer. Plasmid DNA digests were analyzed by electrophoresis in a horizontal 0.6% agarose slab gel. A *Hin*dIII digest of bacteriophage λ DNA was used as the molecular mass standard. Ligation with T4 ligase was performed as described by Tanaku and Weisblum [29].

Plasmids used in this study and their relevant genes and restriction sites are shown in Fig. 1. Plasmids pJP12 [20] and pJP29 (unpublished) were constructed by Tommassen et al. Plasmid pET1 consists of the 6.0-kb *Hpa*I fragment of plasmid pJP12 and was constructed as follows. Plasmid pJP12 was digested with *Hpa*I and after subsequent ligation with T4 ligase the mixture of recombinant plasmid DNA was used to transform strain CE1265, selecting for chloramphenicol-resistant (Cm®) colonies. Plasmid DNA was extracted from

the transformants and analyzed on agarose gels after digestion with *Hpa*I, *Eco*RI and *Bgl*II. Plasmid pET1 is the recombinant plasmid with unique sites for the latter endonucleases.

Transformation of strains with plasmid DNA was carried out as described by Brown et al. [30]. When strains were transformed with mixtures of recombinant plasmid DNA, the transformation procedure of Kushner [31] was used, in order to obtain high yields of transformants.

Hydroxylamine mutagenesis

Mutagenesis of plasmid pJP12 DNA was carried out as described by Humphreys et al. [32]. The incubation mixture consisted of 5–10 µg plasmid DNA, 60 µl 100 mM sodium phosphate buffer, pH 6.0/1 mM EDTA, and 40 µl 1.0 M hydroxylamine, pH 6.0. The mixture was kept on ice for 45 min and then incubated without shaking for 30 min at 75°C. After incubation the mixture was dialysed extensively against 0.1 mM Tris, pH 7.5, 5 mM EDTA, 50 mM NaCl. Finally the DNA was precipitated with ethanol and dissolved in 10 µl buffer containing 50 mM Tris, pH 8.0, 0.5 mM EDTA.

DNA sequence analysis

DNA sequence analysis was performed according to the method of Maxam and Gilbert [33]. The strategy used for sequencing the *phoE* gene has been described by Overbeeke et al. [21].

Uptake of nutrients and β-lactam antibiotics

The rate of permeation of glucose and glucose 6-phosphate through the outer membrane of intact cells was measured as described previously [15] and expressed as pmol min^{-1} (× µg pore $protein)^{-1}$.

The rate of permeation of β-lactam antibiotics through the outer membrane of intact cells and its inhibition by polyphosphate was measured as described by Overbeeke et al. [16].

Isolation and characterization of cell fractions

Cell envelopes were isolated by differential centrifugation after disintegration of cells by ultrasonic treatment [34]. Protein-peptidoglycan complexes were isolated by ultracentrifugation after incubation of cell envelopes at 60°C in buffer containing 2% SDS [35]. The protein patterns of all fractions were analyzed by SDS/polyacrylamide gel electrophoresis as described previously [34]. The amounts of pore protein per cell were calculated from gel scans [36].

RESULTS

Isolation and characterization of TC45-resistant mutants

The first step in our approach to identify amino acid residues involved in determining the receptor site of phage TC45 was the isolation of *phoE* mutants, resistant to the PhoE specific phage TC45. Most spontaneous TC45-resistant mutants were found to be affected in the expression of PhoE protein, presumably because these mutations are mostly due to deletions. Therefore, mutations in the *phoE* gene were introduced by mutagenesis. After *in vitro* mutagenesis of *phoE*-containing plasmid pJP12 DNA with hydroxylamine, the plasmid DNA was transformed into strain CE1265 which contains a *phoR* mutation and therefore expresses the *phoE* gene constitutively. Transformants were selected as Cm®, TC45-resistant colonies. In an initial experiment using 5 µg plasmid pJP12 DNA, 22 TC45-resistant mutants were isolated and further characterized. According to their cell envelope protein pattern the mutants correspond to two phenotypic classes. The three class I mutants produce a PhoE protein which migrates slower in SDS/polyacrylamide gels than wild-type PhoE protein (compare lanes a and b of Fig. 2). This protein was designated as PhoE* protein. All 19 class II mutants lack PhoE protein (Fig. 2A, lane d). Apparently, mutations belonging to this class affect the expression of PhoE protein. Except for the difference in electrophoretic mobility between PhoE protein and PhoE* protein the gel does not show significant differences (Fig. 2B lanes a, b, c and d).

As the three mutants producing PhoE* protein may not be independent, in the next experiment a number of independent

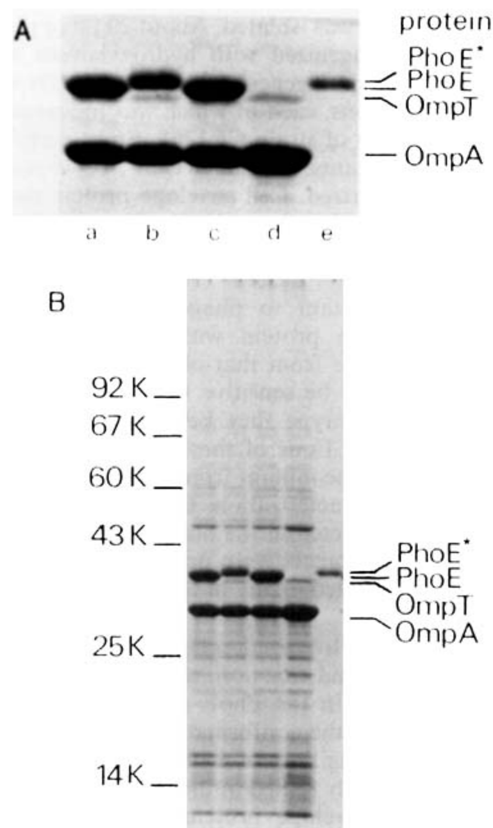

Fig. 2. *SDS/polyacrylamide gel electrophoresis patterns of cell envelope proteins of various strains.* (A) Strain CE1265 containing plasmid pJP12 (lanes a and c); strain CE1265 containing a plasmid pJP12 derivative which carries a class I *phoE* mutation resulting in the synthesis of PhoE* protein (lane b); strain CE1265 containing a plasmid pJP12 derivative which carries a class II *phoE* mutation resulting in the lack of PhoE protein (lane d); and peptidoglycan-associated proteins of strain CE1265 containing the pJP12 derivative which carries a class I *phoE* mutation (lane e). Only the relevant part of the gel, showing the proteins with apparent molecular masses between 39 kDa (PhoE* protein) and 35 kDa (OmpA protein) is shown. (B) Full electrophoretic protein patterns of cell envelope preparations as defined in the legend of Fig. 2A. Positions of the molecular mass markers are indicated at the left

TC45-resistant mutants was isolated. About 20 μg of plasmid pJP12 DNA was mutagenized with hydroxylamine as described before. After mutagenesis the plasmid DNA was divided into four portions, each of which was independently used for transformation of strain CE1265. From each transformation, 50 transformants, selected as Cm®, TC45-resistant colonies, were characterized. Cell envelope protein patterns of the mutants were analyzed on SDS/polyacrylamide gels and all mutants were tested for sensitivity to phage N_3. 179 TC45-resistant mutants lacked PhoE protein, and consequently, were resistant to phage N_3. The other 21 mutants all produced a protein with an electrophoretic mobility indistinguishable from that of PhoE* protein (not shown) and appeared to be sensitive to phage N_3. On the basis of the latter phenotype they belong to the formerly isolated class I mutants. Four of these independent class I mutants, together with one mutant from the first experiment, were used for further characterization. To check whether these mutants were indeed affected in the binding of phage TC45, phage adsorption experiments were performed. No binding of phage TC45 was detected in any of the mutants producing the PhoE* protein.

In order to contribute to considerations about the relationship between structure and function, mutations should only produce subtle changes in the PhoE protein molecule and should not grossly alter the conformation or localization of the protein. The following lines of evidence indicate that the PhoE* protein is normally folded in the outer membrane. (a) Analysis of the cell envelope protein pattern shows that normal amounts of PhoE* protein are found in the cell envelope fraction (e.g. compare lanes a and b in Fig. 2). (b) Cells producing PhoE* protein appear to be sensitive to phage N_3, indicating that the receptor site for this phage is well conserved in the PhoE* protein. (c) Similar to wild-type PhoE protein, PhoE* protein can be isolated complexed with peptidoglycan (Fig. 2A, lane e). (d) As we will show later, PhoE* protein functions as a pore for several nutrients and antibiotics. In conclusion, using TC45 resistance as a selection criterion, a class of mutants is obtained that is likely to be useful for a study on the structure-function relationship of PhoE protein.

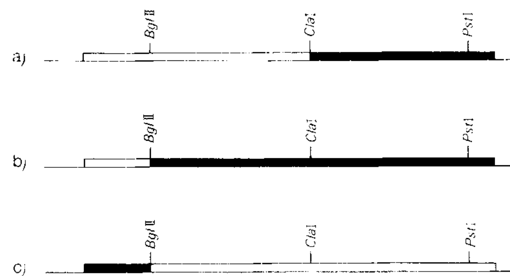

Fig. 3. *Recombinant* phoE *genes in which part of the wild-type gene is replaced by the corresponding part of the mutagenized gene.* The bar represents the complete structural gene. The black part of the bar represents the fragment of the mutagenized *phoE* gene, the open part the fragment of the wild-type *phoE* gene. The construction of the genes indicated in Fig. 3 was a follows. Plasmid pJP29, carrying unique restriction sites (Fig. 1c) and the mutagenized pJP12 plasmid were separately digested with *Cla*I and *Eco*RI. Plasmid DNA fragments were analyzed on agarose gels and the 3.0-kb *Cla*I-*Eco*RI fragment of the mutagenized pJP12 (Fig. 1a) and the 2.0-kb *Cla*I-*Eco*RI fragment of pJP29 (Fig. 1c) were extracted from the gel and subsequently ligated with T4 ligase. The resulting plasmid carries a *phoE* gene, in which the DNA fragment right from the *Cla*I cleavage site has been replaced by the corresponding part of the mutagenized *phoE* gene (a). For the construction of the other two genes, the mutagenized plasmid pJP12 was first deleted for the 2.9-kb *Hpa*I fragment (Fig. 1a). The resulting plasmid has unique sites for *Bgl*II (Fig. 1b). Subsequently, this purified plasmid and pJP29 DNA were separately digested with *Bgl*II and *Eco*RI. Plasmid DNA fragments were analyzed on agarose gels and the relevant fragments were extracted from the gels. The 3.4-kb *Bgl*II-*Eco*RI fragment of the plasmid carrying the mutation was ligated with the 1.6-kb *Bgl*II-*Eco*RI fragment of pJP29. In the recombinant plasmid, the part of the gene right from the *Bgl*II cleavage site is replaced by the corresponding part of the mutagenized *phoE* gene (b). The third recombinant plasmid is composed of the 2.6-kb *Bgl*II-*Eco*RI fragment of the plasmid carrying the mutation ligated with the 3.4-kb *Bgl*II-*Eco*RI fragment, and contains a gene of which the part left from the *Bgl*II site is replaced by the corresponding part of the mutagenized *phoE* gene (c)

Localization of the mutations within the phoE *gene*

In order to map the mutations more precisely, fragments of the *phoE* gene were replaced by the corresponding fragments of the mutagenized *phoE* gene. Expression of the resulting recombinant genes was obtained by transforming the plasmids carrying the recombinant genes into the pore-deficient strain CE1248. Transformants, selected as Cm® colonies, were tested for sensitivity to phages TC45 and N_3. In addition, the cell envelope protein pattern of the transformants was analyzed on SDS/polyacrylamide gels to test which transformants were of the PhoE* phenotype.

Using the *Cla*I and *Bgl*II cleavage sites in the *phoE* gene and the *Eco*RI cleavage of the vector, three recombinant plasmids were constructed carrying *phoE* genes of which DNA fragments are replaced by the corresponding DNA fragments of the mutagenized *phoE* gene (Fig. 3). Transformants containing plasmids with recombinant genes as shown in Fig. 3a and 3c all appeared to be sensitive phages TC45 and N_3. Analysis of the cell envelope protein patterns showed that the recombinant gene products coded for by these plasmids had the same electrophoretic mobility as wild-type PhoE protein. Apparently, the mutation resulting in the PhoE* phenotype is not localized on the DNA fragment right from the *Cla*I site (Fig. 3a) or on the DNA fragment left from the *Bgl*II site (Fig. 3c). Only transformants containing the recombinant gene of the second type (Fig. 3b) produce the electrophoretically altered PhoE protein which renders the cell resistant to phage TC45. Therefore we conclude that the mutation must be localized on the *Bgl*II-*Cla*I fragment of the *phoE* gene. For each of the mutants the procedure as described above was followed. All mutations were found to be localized in the *Bgl*II-*Cla*I fragment of the structural gene.

The 400-base-pair *Bgl*II-*Cla*I DNA fragments carrying the mutations were sequenced and the only change was found to be a G · C to A · T transition at the same location for each of the five studied mutants (Fig. 4). The nature of the base change is in agreement with observations that hydroxylamine predominantly induces G · C to A · T base-pair transitions [37]. The deduced amino acid change is from an arginine residue at position 158 into a histidine.

Pore properties of PhoE protein*

Besides being a receptor for phage TC45, PhoE protein functions as a pore for various nutrients and antibiotics. Phosphate-containing nutrients [15] and most other negatively charged solutes [18] permeate preferentially through PhoE channels. To determine whether the change of arginine 158 into histidine affects the characteristics of the pore, the rates of permeation of glucose 6-phosphate and glucose through

PhoE* protein pores were measured and compared with those through PhoE and OmpC protein pores. Consistent with our previous results [15], PhoE channels are about six times more efficient for the permeation of glucose 6-phosphate than OmpC channels (Table 2). When glucose 6-phosphate is replaced by glucose, the rate of permeation through OmpC channels strongly increases, whereas the rate of permeation through PhoE channels is not significantly influenced (Table 2). The pore characteristics of the PhoE* channel were found to be intermediate. Compared to PhoE channels, PhoE* channels exhibit a 30% reduced efficiency for glucose 6-phosphate which makes them only 4.5 times more efficient for this solute than OmpC channels. In addition, the results show that the permeation of glucose through PhoE* protein pores does not significantly differ from that through PhoE protein pores. The latter result indicates that the reduced efficiency of PhoE* channels for glucose 6-phosphate is not the result of a decreased effective diameter of the pore, as in that case the PhoE* protein pore would also have a reduced efficiency for glucose. Apparently, by substituting histidine for arginine 158 the PhoE protein pore loses part of its preference for the phosphate-containing nutrient glucose 6-phosphate.

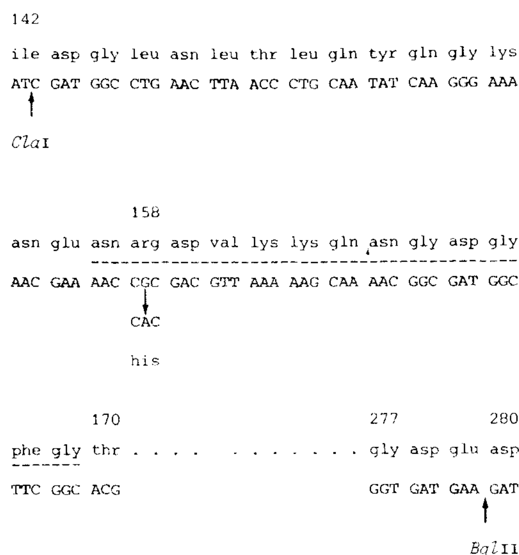

Fig. 4. *Nucleotide sequence of the ClaI-BglII fragment of the wild-type phoE gene, together with the corresponding predicted amino acid sequence.* Only the DNA strand with the same polarity as the messenger RNA is shown. The numbering of the residues was deduced from the sequence of the complete *phoE* gene [21]. The codon and amino acid change resulting from the hydroxylamine-induced mutation is indicated under the nucleotide sequence. The base substitution in codon 158 corresponds to an arginine into histidine change and was found for all five mutants. The hydrophilic peptide corresponding to residues 157–169 is indicated by the dotted line and is one of five pronounced hydrophilic regions of PhoE protein [21]

For a further characterization of the pore properties of PhoE* protein, the rates of permeation of cephaloridine and cephsulodin through PhoE* channels were measured and compared with those through PhoE and OmpF channels. The chemical structures of cephaloridine and cephsulodin are closely related but the latter antibiotic contains an additional sulphate residue and its molecular mass is higher. Table 3 shows that, consistent with previous results [16, 38], cephaloridine permeates about 30 times faster through OmpF channels than through PhoE channels, whereas cephsulodin permeates about twice as fast through PhoE protein channels than through OmpF protein channels. The major part of this difference has been attributed to the additional negative charge on cephsulodin [16, 38]. As can be seen from Table 3, substitution of histidine for arginine 158 reduces the rate of penetration of cephsulodin through PhoE channels to a level which is still slightly faster than that through OmpF protein pores. Moreover, replacement of PhoE channels by PhoE* channels slightly accelerates the permeation of cephaloridine across the outer membrane. The ratio of the rate of cephaloridine permeation over that of cephsulodin permeation, which is independent on the influence of the amount of pore protein produced per cell, confirms the intermediate behaviour of PhoE* protein pores with respect to OmpF and PhoE protein pores. These results suggest that, in addition to the reduced efficiency for phosphate residues (Table 2), PhoE* protein pores also have a significantly reduced efficiency for other negatively charged solutes like cephsulodin (Table 3).

The preference of the PhoE protein pore for anions has been explained by assuming that the PhoE protein pore contains a site which is recognized by phosphate-containing compounds and by other anionic solutes [16]. Experiments showing competitive inhibition of the permeation of β-lactam antibiotics by anionic solutes through PhoE channels, but not through OmpF channels, confirmed the presence of such a site [16]. To check whether the amino acid substitution under

Table 2. *Rates of permeation of glucose 6-phosphate and glucose through PhoE protein pores, OmpC protein pores and PhoE* protein pores*
Experiments were carried out with strains producing PhoE protein, OmpC protein or PhoE* protein as the only type of pore protein. Rates of uptake were measured at pH 7.0. Nutrient concentrations used are below the apparent K_m values for uptake of glucose 6-phosphate and glucose. Uptake data listed for PhoE and OmpC protein represent the averages of five experiments performed with independent batches of cells. Uptake data listed for PhoE* protein represent the average values of uptake experiments with five independent mutants, which later were found to carry the same mutation. The last line gives the ratio of glucose 6-phosphate permeation over glucose permeation, which is independent of the influence of the amount of pore protein produced per cell

Nutrient	Concn	Rate of uptake by intact cells		
		CE1248(pJP12) PhoE protein	CE1241 OmpC protein	CE1248(pJP12) PhoE* protein
	μM	pmol min^{-1} (μg pore protein)$^{-1}$		
Glucose 6-phosphate (A)	1.0	120 ± 8	19 ± 4	87 ± 5
Glucose (B)	1.0	110 ± 10	105 ± 7	116 ± 10
Ratio A/B		1.09 ± 0.12	0.19 ± 0.04	0.69 ± 0.07

Table 3. *Rate of permeation of cephaloridine and cephsulodin through PhoE protein pores, OmpF protein pores and PhoE* protein pores in the presence and absence of polyphosphate*
Experiments were carried out with strains producing PhoE protein, PhoE*, protein and OmpF protein as the only type of pore protein. Rates of uptake were measured at pH 7.0. In case of PhoE protein and OmpF protein the data represent the average of five experiments with independent batches of cells. Uptake data listed for PhoE* protein are the averages of experiments performed with five independent mutants. Data given in parentheses represent percentages of inhibition calculated as 100 × (uptake without inhibitor-uptake in the presence of inhibitor)/uptake without inhibitor. The last line gives the ratio of cephaloridine permeation over cephsulodin permeation, which is independent of the influence of the amount of pore protein produced per cell

β-Lactam antibiotic (0.8 mM)	Poly(-*P*) type P15 (0.2 mM)	Rate of uptake by intact cells		
		CE1248/ (pJP12, pBR322) PhoE protein	CE1110/ (pBR322) OmpF protein	CE1248/ (pJP12, pBR322) PhoE* protein
		nmol min^{-1} (μg pore protein)$^{-1}$		
Cephaloridine (A)	−	5.6 ± 0.2	160 ± 6	10.1 ± 0.4
Cephsulodin (B)	−	9.4 ± 0.5	5.0 ± 0.3	6.9 ± 0.5
Cephsulodin	+	2.6 ± 0.2 (72 ± 5)	4.8 ± 0.4 (3 ± 1)	3.5 ± 0.3 (49 ± 5)
Ratio A/B		0.60 ± 0.04	32.0 ± 2.3	1.5 ± 0.1

study, which results in a reduced anion-selectivity, indeed affects the recognition site for negatively charged solutes, the influence of polyphosphate on the rate of permeation of cephsulodin was measured. The rate of permeation of this antibiotic through PhoE protein channels was found to be significantly inhibited by polyphosphate but the inhibitory effect was considerably less in the case of PhoE* channels (Table 3). In conclusion, substitution of histidine for arginine 158 results in a less efficient recognition of negatively charged solutes. This is the most likely explanation for the reduced rates of glucose 6-phosphate (Table 2) and cephsulodin (Table 3).

Whereas it is clear that the rate of permeation of the neutral solute glucose is not significantly changed by the mutation (Table 2) and those for the strongly negatively charged solutes glucose 6-phosphate (Table 2) and cephsulodin (Table 3) are considerably decreased, the results obtained for the zwitterion cephaloridine, which permeates faster through the mutant channel, are harder to explain. It is likely that the positive of the molecule meets less repulsive forces due to the amino acid change but we think that the full explanation will be much more complex.

DISCUSSION

The approach we adopted here to study the structure-function relationship of PhoE protein consists in the isolation of mutants affected in the functioning of the PhoE protein pore and the subsequent determination of the corresponding nucleotide sequence alterations. Using TC45 resistance as a selection, five independently isolated mutants were obtained, all of which produced an electrophoretically altered PhoE protein, designated as PhoE* protein. For all the mutants the sequence alterations appeared to correspond to a G · C to A · T transition at exactly the same position within the *phoE* gene, corresponding to a deduced amino acid change of arginine 158 into histidine. From this result we conclude that arginine 158 plays an important role in the interaction of the PhoE protein with phage TC45. Arginine 158 could be directly involved in binding the phage. Alternatively, it is possible that phage TC45 binds to other residues in the spacial vicinity of arginine 158 and that the amino acid alteration results in a change of the secondary structure of the phage binding site. Indeed, in an attempt to identify part of a phage binding site on the LamB protein pore, such a mutant was isolated [39]. However, as discussed in Results, gross alterations in the conformation of PhoE protein, as a result of the studied mutations, are not to be expected.

It is striking that all five independently isolated mutants carry exactly the same nucleotide change. In the only other pore protein studied in detail, the LamB protein, resistance to phage lambda can be caused by any of several different changes affecting various regions of the primary structure of the protein molecule [40]. Whether the difference is due to the procedure used or to drastically different receptor requirements, e.g. as the result of the differences in morphology between the two phages, remains to be established.

As far as the pore properties of PhoE* protein are concerned, it was shown that PhoE* channels are less efficient for the permeation of the negatively charged solutes glucose 6-phosphate (Table 2) and cephsulodin (Table 3) than PhoE channels whereas no significant effect was found on the permeation of the neutral glucose molecule (Table 2). This result indicates that arginine 158 is involved in the recognition site for phosphate residues and other negatively charged solutes. The observation that polyphosphate has a stronger effect on the permeation of cephsulodin through PhoE channels than through PhoE* channels confirms the notion that the first interaction between solute and channel molecule in the permeation process is affected by the mutation. Although an indirect effect of the amino acid charge on the recognition of negatively charged solutes presently cannot be excluded, the above-mentioned results strongly suggest a direct influence of the positive charge of arginine 158 in the recognition of the PhoE protein pore by negatively charged solutes. It therefore seems likely that arginine 158 is exposed to the exterior of the cell. This is consistent with the earlier observation that arginine 158 is located in a hydrophilic region of the PhoE protein (Fig. 4, residues 157−169), as it can reasonably be assumed that hydrophilic parts of pore proteins are either surface-exposed, exposed to the periplasmic space of located in the hydrophilic channel [41].

It seems unlikely that only one amino acid constitutes the complete receptor for phage TC45 and at the same time

constitutes part of the recognition site for negatively charged solutes. Recently, a hybrid pore protein was described in which the 73 amino-terminal amino acids of PhoE protein were replaced by the homologous part of the closely related OmpF protein [42]. The hybrid protein does not function as the receptor for TC45 and has lost part of its preference for anions with respect to PhoE protein. These results indicate that at least part of the receptor site for TC45 is located in the 73 amino-terminal amino acids and that the anion preference of PhoE protein is partly determined by this part of PhoE protein. As our results show that arginine 158 is also involved in both functions, it must be concluded that at least two regions of the PhoE protein pore are required for TC45 phage adsorption and for recognition of negatively charged solutes. As these regions are separated from each other by at least 85 amino acids, the binding site of the phage and the recognition site for negatively charged solutes must be created by the secondary structure of the protein in the membrane.

In future, additional information on the TC45 binding site and the recognition site for negatively charged solutes may be obtained by an extension of the approach described in this paper. Moreover, the use of hybrid pore proteins, monoclonal antibodies against PhoE protein pores [43] and site-specific mutagenesis on cloned DNA may be of great help for the study on the structure-function relationship of PhoE protein. The determination of sequence alterations in the *lamB* gene has shown that at least ten amino acids located in four different hydrophilic regions of the protein are involved in the binding of the LamB-specific phage [43]. These amino acids are supposed to face the outside of the cell. Together with other structural assumptions and conventions, a working model was proposed for the molecular organization of the LamB protein in the outer membrane. Such a secondary structure prediction will be possible for the PhoE protein pore when more data are available about amino acids involved in particular functions of PhoE protein.

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