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Localization of *phoE*, the Structural Gene for Outer Membrane Protein e in *Escherichia coli* K-12

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To localize the structural gene for outer membrane protein e, mutants resistant to the protein e-specific phage TC45 were isolated and characterized. Three classes of TC45-resistant strains were found: (i) mutants in *phoB*, a regulatory gene for protein e, (ii) mutants with an altered lipopolysaccharide, and (iii) mutants unaltered in the regulation of the *pho* regulon and producing an apparently normal lipopolysaccharide. Mutations in the latter class of mutants are probably altered in the structural gene for protein e and are cotransducible with *proA,B* at min 6 on the chromosomal map. As class (iii) includes mutants with an electrophoretically altered protein e, in both an *nmpA* and an *nmpB* background, we conclude that the structural gene for protein e, designated as *phoE*, is localized at min 6 on the chromosomal map, the gene order being *phoE proA argF*.

Several proteins of the outer membrane of *Escherichia coli* K-12 are involved in the formation of aqueous pores through which small hydrophilic molecules can pass this membrane (2, 15, 17, 25, 26). Two of them, the products of the genes *ompC* and *ompF*, are synthesized constitutively in wild-type strains of *E. coli* K-12. Another outer membrane protein, protein e (26) (also called Ic [8] or E [5]), was detected in pseudorevertants of mutants lacking both the OmpC and the OmpF protein (26). This protein also has porin properties (14, 21, 26). In wild-type cells of *E. coli* K-12, the synthesis of protein e can be induced by phosphate limitation (18). Mutations leading to constitutive synthesis of protein e have been localized in either of two loci, *nmpA* and *nmpB* at min 83 (6, 21) and min 9 (10, 21), respectively. Recently, we showed that the synthesis of protein e is controlled by the same regulation mechanism as that which controls the synthesis of alkaline phosphatase and that *nmpA* is identical to one of the genes, *phoS*, *phoT*, or *pst*, which are involved in the uptake of inorganic phosphate, whereas the *nmpB* gene is identical to the *phoR* gene, which is a regulatory gene of the *pho* regulon (23).

Recently, Pugsley et al. (20) reported that probably both the *nmpA* and *nmpB* loci contain structural genes for protein e. Starting with *nmpA* and *nmpB* mutant strains, they isolated TC45-resistant mutants which produce an electrophoretically altered protein e, and these secondary mutations were reported to map in or close to *nmpA* and *nmpB*, respectively. These data were interpreted as strong evidence for the presence of two structural genes at min 83 and 9, respectively (20). When this information was published, we were analyzing mutants resistant to the protein e-specific phage TC45 to localize the structural gene for this protein. Our results, which are described in this paper, indicate that there is only one structural gene for protein e and that this gene is located at min 6.

MATERIALS AND METHODS

Strains, phages, and growth conditions. All bacterial strains are derivatives of *E. coli* K-12. Their sources and relevant characteristics are listed in Table 1. Cells were grown overnight in yeast broth (13) under vigorous aeration, usually at 37°C. A growth temperature of 30°C was used to check for the presence of protein e in cell envelopes, because protein a, which is another outer membrane protein with the same electrophoretic mobility as protein e in the gel system used (14), is hardly produced at the latter growth temperature (13, 16).

Laboratory stocks of coliphages T3, T4, T7, P1, C21, and TC45 were used.

Genetic techniques. P1 transduction (28) and conjugation (7) were carried out as described previously. Sensitivity to bacteriophages was determined by cross-streaking.

Assays for alkaline phosphatase. Assays for alkaline phosphatase were performed as described previously (23).

Isolation and characterization of cell fractions. After ultrasonic disintegration of the cells, cell envelopes were isolated by differential centrifugation (12). For the isolation of periplasmic proteins, a slightly modified procedure (23) of the EDTA-lysozyme

TABLE 1. *Characteristics of bacterial strains*[a]

Strain	Characteristics	Source[b] or reference
PC0479	F⁻ *thr leu thi pyrF thy ilvA his lacY argG tonA rpsL cod dra vtr glpR*, protein e⁻	PC
CE1108	*ompB471 phoS200*, protein e⁺ derivative of PC0479	14, 23
CE1174	*phoB201*, protein e⁻ derivative of CE1108	23
CE1182	TC45-resistant protein e⁻ derivative of CE1108, resistant to T3, T7, and P1	This study
CE1183	TC45-resistant protein e⁻ derivative of CE1108, sensitive to C21	This study
CE1184	TC45-resistant protein e⁺ derivative of CE1108, sensitive to C21	This study
CE1185	TC45-resistant protein e⁻ *phoE203 pro* derivative of CE1108	This study
CE1186	TC45-resistant protein e⁻ *phoE204 pro* derivative of CE1108	This study
CE1187	TC45-resistant protein e⁻ *phoE205 pro* derivative of CE1108	This study
CE1202	TC45-resistant protein e* *phoE202*, derivative of CE1108	N. Overbeeke
CS809	F⁻ *metB65 his-53 proC24 cyc-1 xyl-14 lacY29 rpsL77 tsx-63 ompF ompC171 nmpA104 nmpA114*, protein e*	20
CS812*[c]	F⁻ *metB65 his-53 proC ilvY227 cyc-1 xyl-14 lacY29 rpsL77 tsx-63 ompF ompC171 nmpB105 nmpB116*, protein e*	20
JF694	*ilv his purE proC metB rpsL cyc xyl lacY tsx ompF ompC nmpA*, protein e⁺	5
CE1188	*proA*, protein e⁺ derivative of CE1108	This study
PC0031	Hfr R4 *argF gal tonA phx rel*	PC
C9[d]	Hfr Cav *relA1 tonA22 pit-10 spoT1 phoR18* T2ʳ	A. Garen via CGSC
CE1196	Hfr R4 *thr leu proA argF argI phx rel bgl phoS21*	This study

[a] Genotype descriptions follow the recommendations of Bachmann and Low (1).

[b] PC, Phabagen Collection, Department of Molecular Cell Biology, Section Microbiology, State University of Utrecht, Utrecht, The Netherlands; CGSC, *E. coli* Genetic Stock Center, Department of Human Genetics, Yale University School of Medicine, New Haven, Conn. (B. J. Bachmann, curator).

[c] This strain is a derivative of CS812, obtained from A. Pugsley. In contrast to strain CS812, this strain carries an additional *proC* mutation.

[d] As the mutation which causes resistance to phage T2 and disappearance of the *ompF* protein is 40% cotransducible with *pyrD*, it probably is localized in the *ompF* gene (R. van Boxtel, J. Tommassen, and B. Lugtenberg, unpublished data).

method of Willsky and Malamy (29) was used. The protein patterns of the cell fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (12). For the isolation of ^{32}P-labeled lipopolysaccharide (LPS) and its characterization by means of paper chromatography, we used the method described by Boman and Monner (3). Isobutyric acid–1 N NH_4OH (5:3, by volume) was used as the solvent in paper chromatography.

RESULTS

Isolation and characterization of TC45-resistant mutants.

Starting with strain CE1108, a *phoS* strain which produces protein e constitutively, spontaneous mutants resistant to the protein e-specific phage TC45 were isolated. Three classes of TC45-resistant mutants were found. The characteristics of these mutants are summarized in Table 2. Class 1 mutants lack, in addition to protein e, alkaline phosphatase (Table 2) and the other periplasmic proteins of the *pho* regulon (data not shown). The mutation in one of these mutants, strain CE1174, has previ-

ously been located in the *phoB* gene, which codes for a positive regulatory protein of the *pho* regulon (23). Class 2 mutants have an altered LPS, as indicated by their changed sensitivity towards the LPS-specific phages T3, T7, and P1 or C21 (11). One subclass of these mutants, of which strain CE1182 is an example, has largely decreased quantities of protein e (<10%) and has become simultaneously resistant to the phages T3, T7, and P1. Like the parental strain, it is still resistant to phage C21. This phage pattern indicates that these strains have a heptose-deficient LPS (7, 24). The isolation of such mutants could be expected since it has been reported that mutants with a heptose-deficient LPS contain strongly decreased amounts of protein e (14). A second subclass of TC45-resistant LPS mutants has become sensitive to phage C21 and is still sensitive to phages T3, T7, and P1, suggesting that their LPS is altered such that it is galactose deficient but contains heptose (27). This subclass is heterogeneous with respect to the residual amounts of protein e, as some strains, e.g., strain CE1183, have very low levels of this protein (<10%), whereas in others, e.g., strain CE1184, the amount of protein e is slightly or not at all affected (>80%). It should be noted that also in the latter case, phage TC45 resistance is due to a decreased rate of phage adsorption.

All 15 isolated class 3 mutants, e.g. CE1185, CE1186, and CE1187, lack protein e. They have an apparently wild-type LPS, as judged by their sensitivity pattern towards the described phages and by the chromatographic mobilities of the isolated ^{32}P-labeled LPS. Since all mutants still produce alkaline phosphatase constitutively, the lack of protein e cannot be due to reversion to *phoS*$^+$ or to mutation of the *phoB* gene. Thus, the most likely explanation is that these strains have an altered structural gene for protein e.

Genetic localization of the structural gene for protein e. In contrast to their parental strain CE1108, 14 out of 15 class 3 TC45-resistant mutants appeared to be dependent on exogenous proline for growth. In transduction experiments, this proline auxotrophy was always strongly coupled with TC45 resistance, indicating that the mutations are caused by deletions of part of the genome including the structural gene for protein e and at least one of the *pro* genes. On the *E. coli* K-12 chromosome, three genes are known to be involved in proline synthesis, namely, *proA* and *proB* at min 6 and *proC* at min 8.8 (1). *proA* and *proB* are proximal markers in crosses with Hfr strain R4, whereas *proC* is a late marker (Fig. 1). To localize the proline auxotrophy and TC45 resistance markers in class 3 mutants, we crossed three mutant strains, namely, CE1185, CE1186 and CE1187, with Hfr R4. Proline prototrophy appeared to be an early marker in these conjugation experiments, and for each cross, all 50 tested *pro*$^+$ transconjugants were TC45 sensitive. So we conclude that the TC45 resistance marker in the class 3 mutants is localized close to *proA* and

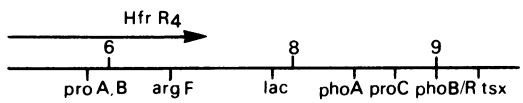

FIG. 1. *Marker positions in the region of min 6 to 9 of the E. coli chromosome (1). The origin and direction of transfer of Hfr strain R4 are indicated.*

TABLE 2. *Characteristics of three classes of TC45-resistant mutants of strain CE1108*[a]

Class	Strain	Sensitivity to:			AP	Protein e	Defective in:
		TC45	T3, T7, P1	C21			
Parent	CE1108	S	S	R	c	+	*phoS*
1	CE1174	R	S	R	unind	−	*phoB*
2	CE1182	R	R	R	c	−	LPS
	CE1183	R	S	S	c	−	LPS
	CE1184	R	S	S	c	+	LPS
3	CE1185	R	S	R	c	−	*phoE*[b]
	CE1186	R	S	R	c	−	*phoE*[b]
	CE1187	R	S	R	c	−	*phoE*[b]
	CE1202	R	S	R	c	*	*phoE*[b]

[a] R and S, Resistant and sensitive, respectively; AP, synthesis of alkaline phosphatase; c and unind., constitutive and uninducible, respectively; + and −, presence or absence, respectively, of protein e in cell envelope preparations; *, a protein e with altered mobility in sodium dodecyl sulfate-polyacrylamide gels.

[b] *phoE* is the proposed abbreviation for the structural gene for protein e.

proB at min 6.

Recently, Pugsley et al. (20) isolated TC45-resistant mutants from *nmpA* and *nmpB* strains, and they localized these mutations in or close to *nmpA* at min 83 and *nmpB* at min 9, respectively. Some of their mutants produced an electrophoretically altered protein e. Since mutations that cause an altered protein are very likely to be in the structural gene for this protein, they suggested that there are two structural genes for protein e, one at min 83 and one at min 9. Assuming that the existence of three structural genes for protein e is unlikely, we therefore reinvestigated the localization of the two mutations which cause an electrophoretically altered protein e (designated as protein e* in this paper) in two mutant strains obtained from Pugsley. A third strain with an e* protein, strain CE1202, which was isolated in our lab, was also included in these experiments. The major outer membrane proteins of the e^+ strain CE1108 (Fig. 2b, d, and h) and of three TC45-resistant mutant strains which produce an altered protein e (Fig. 2a, c, and e) are shown in Fig. 2. Strain CS809 (Fig. 2a) is a TC45-resistant mutant isolated from an *nmpA* strain. This strain was isolated by Pugsley et al., and the TC45 resistance mutation (*nmpA**) was mapped at min 83 in or close to *nmpA* (20). Strain CS812* (Fig. 2c) is a TC45-resistant mutant isolated from an *nmpB* (*phoR*) strain. The TC45 resistance mutation (*nmpB**) in this strain was localized at min 9 in or close to the *nmpB* gene (20). The third protein e* strain, strain CE1202 (Fig. 2e), was isolated in our laboratory as a TC45-resistant strain.

To determine whether the *nmpA** mutation in strain CS809 is indeed localized at min 83 in or close to the *nmpA* gene, we transduced strain PC0479 to ilv^+, using P1 grown on CS809. A total of 30 ilv^+ transductants were tested, and 16 of them produced alkaline phosphatase constitutively, indicating that they received the *nmpA* (*pho*) gene from donor strain CS809. These 16 transductants were all TC45 sensitive. The protein pattern of the cell envelopes of these transductants was analyzed on gels, and, as is shown for one of the transductants in Fig. 2 (slot f), they appeared to have a normal (wild-type) protein e. In addition, by selecting for ilv^+ transductants, it was not possible to transduce the e^+ strains CE1108 and JF694 to TC45 resistance with P1 grown on *nmpA** strain CS809. For these reasons, we conclude that the *nmpA** mutation in strain CS809 is not localized in or close to the *nmpA* gene at min 83.

To determine whether the *nmpB** mutation in strain CS812* is indeed localized at min 9 in or close to the *nmpB* (*phoR*) gene, as reported in reference 20, we transduced strain CS812* to $proC^+$ with P1 grown on strain C9. Two hundred pro^+ transductants were tested. No contransduction of TC45 sensitivity with $proC^+$ was found, whereas normal cotransduction percentages for $lacY^+$ (14%) and tsx^+ (43%) were obtained. Therefore, we conclude that the *nmpB** mutation of strain CS812* is not localized between *lacY* and *tsx* in or close to *nmpB* (*phoR*) at min 9.

To determine whether the mutations which cause an e* protein are localized in the same region of the chromosome where the TC45-resistant *pro* mutations are mapped (class 3 mutants), we transduced e^+ strain CE1188 to $proA^+$ with P1 grown on strain CE1202. A total of 200 pro^+ transductants were tested, and 194 (97%) appeared to be TC45 resistant. Also, the TC45 resistance markers in the strains CS809 and CS812* were cotransducible with *proA* (97%). The cell envelope protein patterns of two $proA^+$ TC45-resistant transductants of strain CE1188 with P1 grown on strains CS809 and CS812* are shown in Fig. 2g and i, respectively. The results show that an altered protein e was transduced into strain CE1188 together with $proA^+$. Therefore, the mutations resulting in an altered electrophoretic mobility of protein e in the three

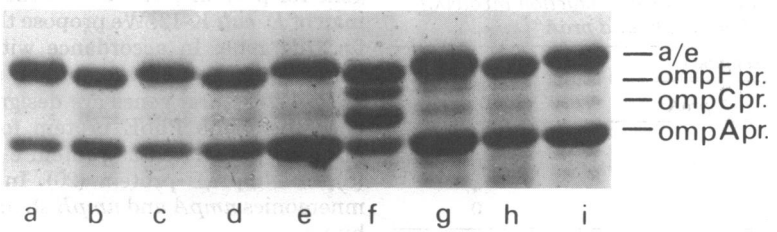

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of the cell envelope proteins of strain CE1108 (b, d, and h), nmpA* strain CS809 (a), nmpB* strain CS812* (c), e* strain CE1202 (e), an ilv^+ pho transductant of PC0479 with P1 grown on CS809 (f), a $proA^+$ TC45-resistant transductant of CE1188 with P1 grown on CS809 (g), and a $proA^+$ TC45-resistant transductant of CE1188 with P1 grown on CS812* (i). Only the relevant part of the gel showing the proteins with apparent molecular weights between 40,000 (40K) (protein e) and 35K (OmpA protein) is presented.*

strains CS809, CS812*, and CE1202 are all localized at min 6, very close to *proA*. The mutations are therefore probably all in the same gene, for which we propose the name *phoE*.

To see whether the *phoE* gene is localized clockwise or counterclockwise from *proA*, three-factor transductions were carried out with strain CE1196 as the recipient and strain CS809 as the donor strain. A total of 100 pro^+ transductants were tested, and 98 appeared to be TC45 resistant, whereas 29 were $argF^+$. This shows that *argF* is cotransducible with *proA*, although it is located more distant than *phoE* from *proA*. Therefore, the gene order is *phoE proA argF* or *proA phoE argF*. To discriminate between these two possibilities, we also selected for arg^+ transductants in the same transduction experiment. By selecting for arg^+ transductants, $argI^+$ as well as $argF^+$ transductants can be expected. Since *argI* is located in a clearly distinct region of the chromosome, at min 96 (1), this influences only the absolute but not the relative cotransduction percentage of *proA* and *phoE* with arg^+. The results of this transduction experiment are summarized in Table 3. Since $proA^+$ is linked more closely than *phoE* to arg^+, the gene order is *phoE proA argF*. Similar results were obtained when strains CS812* or CE1202 were used as donor strains.

DISCUSSION

To localize the structural gene for outer membrane protein e, mutants resistant to the protein e-specific phage TC45 were isolated. Three classes of TC45-resistant mutants were found: (i) *phoB* mutants, (ii) LPS mutants, and (iii) mutants probably altered in the structural gene for protein e. The results with TC45-resistant LPS mutants (Table 2) show that a considerable part of the LPS molecule is somehow required for the presence of normal amounts of protein e in the outer membrane. Heptose-proficient LPS obviously does not suffice for this purpose. An even larger part of the LPS, probably including the galactose residue, is required for phage TC45 receptor activity of protein e in vivo, consistent with and extending the observation that LPS stimulates the phage TC45 receptor activity of protein e in vitro (4).

TABLE 3. *Three-factor transduction involving phoE, argF, and proA*[a]

Unselected marker		No. of transductants
phoE	*proA*	
+	−	150
−	+	46
+	+	4
−	−	0

[a] P1 transduction was carried out with strain CS809 (*phoE* $proA^+$ $argF^+$) as donor, with strain CE1196 (*phoS* $phoE^+$ *proA argF argI*) as the recipient, and with selection of Arg^+ transductants. The presence of the wild-type or mutant *phoE* allele was determined by testing for sensitivity to phage TC45.

Out of 15 strains expected to have an altered structural gene for protein e, 14 had been simultaneously mutated in *proA* or *proB*, probably because the mutations arose by deletions extending over the structural gene for protein e and one or both of these *pro* genes. The observation that no pro^+ revertants of the mutant strains could be obtained supports this notion. A possible explanation for the many deletion mutations may be the presence of insertion elements in this region of the chromosome (9), since insertion elements can generate deletions (22).

Recently, Pugsley et al. (20) isolated TC45-resistant mutants from *nmpA* and *nmpB* strains, and they localized the mutations in or close to the *nmpA* and *nmpB* genes, respectively. Some of their mutant strains produced a protein e with an altered mobility on sodium dodecyl sulfate-polyacrylamide gels (e* proteins). Since mutations that cause an altered protein are most likely localized in the structural gene for this protein, the authors suggested that there are two structural genes for protein e, one at min 83 and one at min 9. Since our data on TC45-resistant mutants suggested a position at min 6 for the structural gene for protein e, we reinvestigated the localization of two mutations which cause an altered protein e. In addition, we localized the mutation of strain CE1202 which results in a similar phenotype. Our results showed that all of these mutations, no matter whether they had been isolated from an *nmpA* or an *nmpB* strain, were localized very close to and counterclockwise from *proA*. We therefore conclude that there is only one locus for the structural gene for protein e at min 6 of the chromosomal map of *E. coli* K-12. We propose the name *phoE* for this gene. In accordance with the way in which other outer membrane proteins with known structural genes are designated, we propose the name PhoE protein for the protein formerly designated as protein e (26), Ic (8), E (5), or NmpA,B protein (20). In addition, the mnemonics *nmpA* and *nmpB* should be replaced by *pho*.

Protein e has in common with the constitutively formed porins OmpC protein and OmpF protein that it has general pore properties (14, 21, 26). Recent experiments have shown that it is even immunologically related to the other two

porins (19). It therefore is clear that experiments with a cloned porin gene will provide information on porins in general. A potential problem with cloning of porin genes is that overproduction of porins could be lethal to the cell. However, cloning of the *phoE* gene may be easier than cloning of the structural genes for the other two porins as protein e is an inducible protein which usually is not expressed in a *pho*$^+$ background.

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