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Cloning of *phoE*, the Structural Gene for the *Escherichia coli* Phosphate Limitation-Inducible Outer Membrane Pore Protein

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The hybrid plasmid pLC44-11 from the Clarke and Carbon collection, which was known to carry the *proA* gene, was shown also to contain the *phoE* gene. In vitro recombination techniques were used to subclone a 4.9-kilobase-pair DNA fragment of pLC44-11 into the plasmid vectors pACYC184 and pBR322. Expression of this fragment in a minicell system showed that it codes for the PhoE protein and for polypeptides with apparent molecular weights of 47,000 and 17,000. These results supply definite proof for the earlier supposition that the *phoE* gene is the structural gene for the outer membrane PhoE protein. Overproduction of the PhoE protein in a *phoS* strain resulted in reduced amounts of OmpF and LamB proteins.

The PhoE protein of *Escherichia coli* K-12 is an outer membrane pore protein which is inducible by phosphate limitation (14). It is constitutively produced in *phoR*, *phoS*, *phoT*, and *pst* mutants (18). By mapping mutations which cause an electrophoretically altered PhoE protein, we have recently localized the structural gene for this protein at min 6 of the chromosomal map, very close to *proA* (19). In this paper we describe the cloning of this *phoE* gene.

MATERIALS AND METHODS

Strains and growth conditions. All bacterial strains were derivatives of *E. coli* K-12. Table 1 lists the characteristics of the relevant strains. Except where noted, cells were grown in yeast extract broth (12) at 37°C under aeration. For growth of cells containing plasmid pBR322 or pACYC184 and their derivatives, the medium was supplemented with penicillin G (80 μg/ml) or chloramphenicol (25 μg/ml), respectively.

Genetic techniques. Transfer of the ColE1 hybrid plasmid pLC44-11 was performed by F-factor-mediated transfer (7). Conjugation (9), P1 transduction (22), and transformation (5) were carried out as described previously. Sensitivity to bacteriophages was determined by cross-streaking.

DNA techniques. Plasmid DNA was isolated by the cleared-lysate technique of Clewell and Helinski, followed by CsCl-ethidium bromide isopycnic centrifugation (8). The alkaline extraction procedure of Birnboim and Doly (2) was used for rapid screening of plasmids. Restriction endonucleases *Eco*RI, *Bam*HI, *Hin*dIII, *Sal*I, *Bgl*II, *Pst*I, *Cla*I, and *Sma*I were obtained from Boehringer Mannheim Corp. *Kpn*I was obtained from Bethesda Research Laboratories. The conditions for restriction endonuclease reactions were those proposed by the manufacturers. Analyses of plasmid DNA fragments were performed by electrophoresis in a horizontal 0.6% agarose slab gel (21). *Hin*dIII- and *Eco*RI-generated fragments of bacteriophage λDNA were used as molecular weight standards in gels. T4 DNA ligase was a generous gift from P. Weisbeek. Ligation was performed as described by Tanaku and Weisblum (17).

Isolation and characterization of cell fractions. Cell envelopes were isolated by differential centrifugation after ultrasonic disintegration of the cells (11). Periplasmic proteins were isolated by the EDTA-lysozyme method described previously (18). The protein patterns of the cell fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (11).

Analysis of protein synthesis in minicells and fractionation of the labeled minicells. Minicells were isolated by three cycles of sucrose gradient centrifugation from stationary-phase cultures and labeled with [^{35}S]methionine as described by Andreoli et al. (1). After centrifugation, the minicells were suspended in sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (11). Radioactive proteins were detected in the gels by exposure of the dried gel to a Fuji X-ray film for 15 to 65 h. In this paper, several labeled proteins are indicated by their molecular weights multiplied by 10^{-3} and followed by the letter K.

For isolation of cell envelopes of the labeled minicells, the same procedure was used as described for the isolation of cell envelopes of normal cells (11) except that sonication was prolonged to eight periods of 10 s and the cell envelopes were collected by centrifugation for 75 min at 10,000 × *g* in an Eppendorf centrifuge. The peptidoglycan-associated cell envelope fraction was subsequently isolated from the cell envelopes as described previously (10), except that somewhat harsher extraction conditions (45 min at 60°C in 2% SDS) were used.

For immunoprecipitation reactions, the proteins were dissolved by heating the cell envelope fractions for 10 min at 96°C in 2% SDS. The SDS-concentration was subsequently reduced to 0.1% by dilution, and immunoprecipitation was carried out as described

TABLE 1. Characteristics of bacterial strains

Strain	Characteristics	Source
CE1197	F⁻, *thr leu thi pyrF thy ilvA lacY argG tonA rpsL cod dra vtr glpR ompB471 phoS200 phoE205 Δ(phoE-proAB) recA56*	This study
AM1602	F⁻, *thr leu lacI lacZ recA171*	This study
CE1211	F⁻, *gal xyl mtl mal lam tonA phx rpsL azi minA minB phoR18 phoE206 Δ(phoE-proAB), ompB483*	This study
AB1157	F⁻, *thr leu proA2 Δ(proA-phoE-gpt) his thi argE lacY galK xyl rpsL*	Adelberg
CE1194	F⁻, *bgl phoS21* derivative of AB1157	This study

(15), using rabbit anti-PhoE protein serum and goat anti-rabbit immunoglobulin G.

RESULTS

Identification of a hybrid plasmid carrying the *phoE* gene. The hybrid plasmid pLC44-11 of the Clarke and Carbon colony bank is known to carry the *proA* gene (7). To investigate whether this plasmid also contains the *phoE* gene, which is located very close to *proA* (19), we transferred this plasmid to the *phoS phoE* mutant strain CE1197, selecting for *pro*⁺ *rpsL* transconjugants. In contrast to the plasmidless strain CE1197, all 12 transconjugants tested were sensitive to the PhoE protein-specific phage TC45, and their cell envelopes contained a protein band with the same electrophoretic mobility as the PhoE protein (not shown). Therefore, we concluded that plasmid pLC44-11 contains the *phoE* gene.

Subcloning of the *phoE* gene. Analysis of the DNA fragments of pLC44-11, generated by several restriction enzymes, revealed that the size of the entire plasmid is 29.7 kilobase pairs (kb), 23.4 of which comprise chromosomal DNA. The restriction enzyme *SalI* cleaves the plasmid at three sites, generating fragments of 23.3, 4.9 and 1.5 kb. For subcloning the *phoE* gene, *SalI* fragments of pLC44-11 were cloned into the miniplasmids pACYC184 and pBR322. The first cloning vector renders the cells resistant to chloramphenicol and tetracycline (6), whereas the latter vector renders the cells resistant to ampicillin and tetracycline (3). Both plasmids have a unique site for the endonuclease *SalI* in the tetracycline gene (3, 6).

For cloning the *SalI* fragments of pLC44-11 into pACYC184, both plasmids were mixed and digested with endonuclease *SalI*, and the fragments were subsequently ligated with T4 DNA ligase. The ligated DNA was then used to transform the *pho*⁺ strain AM1602 as well as the *phoS phoE* strain CE1197 to chloramphenicol resistance. From each recipient strain, 15 chloramphenicol-resistant, tetracycline-sensitive transformants were further analyzed. All 15 transformants of strain CE1197 were resistant to phage TC45 and contained a hybrid plasmid in which the 1.5-kb *SalI* fragment of pLC44-11 was cloned in pACYC184. As expected, all 15 transformants of the *pho*⁺ strain AM1602 were resistant to phage TC45. Three types of hybrid plasmids were found in these transformants. Seven transformants contained a hybrid plasmid, e.g., pJP9, in which the 1.5-kb *SalI* fragment of pLC44-11 was cloned in pACYC184. Seven transformants contained a plasmid, e.g., pJP12 and pJP14, in which the 4.9-kb *SalI* fragment of pLC44-11 was cloned, and one transformant contained a plasmid, i.e., pJP10, in which both the 1.5-kb and the 4.9-kb *SalI* fragments were cloned. When plasmid pBR322 was used as the cloning vector, similar results were obtained. Plasmid pJP20 is a hybrid plasmid in which the 4.9-kb *SalI* fragment of pLC44-11 is cloned into pBR322.

These results suggest that there is a selective disadvantage for transformants of *phoS* strain CE1197 carrying a hybrid plasmid containing the 4.9-kb *SalI* fragment of pLC44-11. This can be explained by assuming that the *phoE* gene is located on this DNA fragment and that overproduction of the PhoE protein in this *phoS* strain is harmful. To investigate this possibility, the hybrid plasmids pJP12 and pJP14 and vector pACYC184 were transformed into strain AB1157 and into its *phoS* derivative CE1194, and the cell envelope protein patterns were analyzed (Fig. 1). Neither strain AB1157 (lane a) nor its pACYC184-, pJP14-, or pJP12-containing derivatives (lanes d, e, and f) produced the PhoE protein. Also, *phoS* strain CE1194 (lane b) and its pACYC184-containing derivative (lane g) did not produce this protein because of the presence of the *proA2* mutation, a deletion of part of the chromosome including the genes *proA*, *phoE*, and *gpt* (20). *pro*⁺ transductants of strain CE1194, e.g., CE1195 (lane c), produced the PhoE protein constitutively. Transformants of strain CE1194, containing pJP14 (lane h) or pJP12 (lane i), overproduced the PhoE protein. This result shows that indeed the *phoE* gene is cloned on pJP12 and pJP14 and that the gene is still subject to the regulation mechanism of the *pho* regulon. In addition, it should be noted that the presence of pJP12 or pJP14 in strain CE1194 led to strongly decreased amounts of at least one other outer membrane protein, i.e., the OmpF

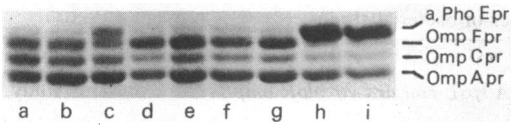

FIG. 1. SDS-polyacrylamide gel electrophoresis patterns of the cell envelope proteins of strains AB1157 (a); CE1194 (b); CE1195 (c); AB1157 containing pACYC184 (d), pJP14 (e), or pJP12 (f); and CE1194 containing pACYC184 (g), pJP14 (h), or pJP12 (i). Only the relevant part of the gel, showing the proteins with apparent molecular weights between 40,000 (PhoE protein) and 35,000 (OmpA protein), is shown.

protein, whereas the amounts of OmpA protein and OmpC protein were less affected (Fig. 1). Growth in maltose-containing media revealed that the amount of the LamB protein also was strongly decreased by the presence of pJP12 and pJP14 (not shown). To determine whether the presence of pJP12 or pJP14 also influenced the amounts of certain periplasmic proteins, which could be expected when a common step in the translocation of these proteins is blocked by overproduction of the PhoE protein, the periplasmic proteins of derivatives of CE1194 containing either pACYC184, pJP12, or pJP14 were isolated and compared on SDS-polyacrylamide gels. However, no differences in the protein patterns were observed (not shown).

Restriction map of pJP14. To determine a restriction enzyme cleavage map of the *phoE* region of the chromosome, restriction fragments of pJP14, generated by digestions and suitable double digestions with several enzymes, were analyzed on agarose gels. Figure 2 presents restriction map of pJP14. The position of the pACYC184 vector was deduced from its known restriction map (6). Plasmid pJP12 is identical to pJP14, except that the 4.9-kb *Sal*I fragment is present in the opposite orientation relative to the pACYC184 sequences.

For a preliminary localization of *phoE* on pJP14, the 2.4-kb DNA fragment between the *Bgl*II cleavage sites at 1.4 and 3.8 kb on the pJP14 map was subcloned in pACYC184. Since the resulting plasmid, pJP16, did not complement *phoE* mutations, the 2.4-kb *Bgl*II fragment does not carry the (complete) *phoE* gene. A mutant plasmid, pJP21, which carries a 400-base-pairs insert in the *Pst*I site of pJP14, did not complement *phoE* mutations either. Therefore this *Pst*I site is most likely located in the *phoE* gene.

Synthesis of PhoE protein in minicells. The synthesis of plasmid-coded proteins was studied in minicells of the *phoR phoE* minicell-producing strain CE1211. Compared with minicells isolated from the strains carrying the cloning vectors pACYC184 (Fig. 3, lane a) and pBR322 (lane f), the minicells from the strains carrying the hybrid plasmids pJP12 (lane b), pJP14 (lane c), and pJP20 (lane g) produced at least three additional labeled polypeptides with apparent molecular weights of 47,000, 40,000, and 17,000. Also, additional bands of 34,000 and 16,000 molecular weights were visible, but it is not clear whether these products are coded by the cloned fragment, since faint bands were sometimes also observed in these positions in minicell preparations from strains carrying the vector plasmids. The 40K protein had exactly the same electrophoretic mobility as the PhoE protein and was not visible in preparations of minicells which contained the plasmids pJP16 and pJP21 (lanes d and e), which did not complement *phoE* mutations. The 40K protein was found in the cell envelope fraction of labeled minicells containing pJP20 (lane h), and the protein was peptidoglycan associated (lane i) as is known for the PhoE protein (13). Finally, the 40K protein could specifically be precipitated from the solubilized cell envelope preparation with rabbit anti-PhoE protein serum (lane j) but not with preimmune serum (lane k). From these results, it is obvious that the structural gene for the PhoE protein had been cloned.

DISCUSSION

We cloned the *phoE* gene of *E. coli* K-12 starting from a hybrid plasmid pLC44-11 which was known to carry the *proA* gene (7). The results show that the *phoE* gene is indeed located at min 6 close to *proA*, as shown earlier (19), and confirm the supposition that this gene is the structural gene for the PhoE protein.

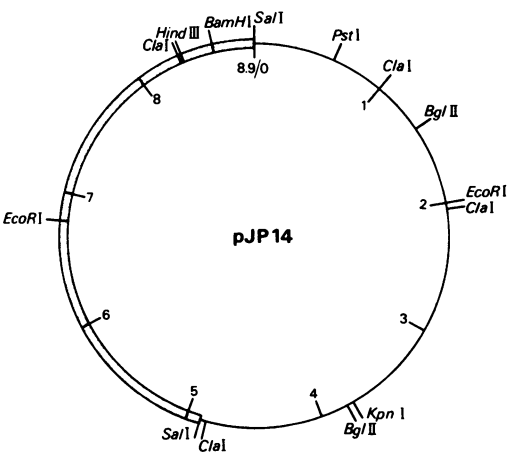

FIG. 2. Restriction enzyme cleavage map of pJP14. The pACYC184 vector is indicated by the dotted area; the chromosomal *E. coli* DNA is represented by a thin line. Map units are in kilobases. Restriction enzyme *Sma*I does not cleave pJP14.

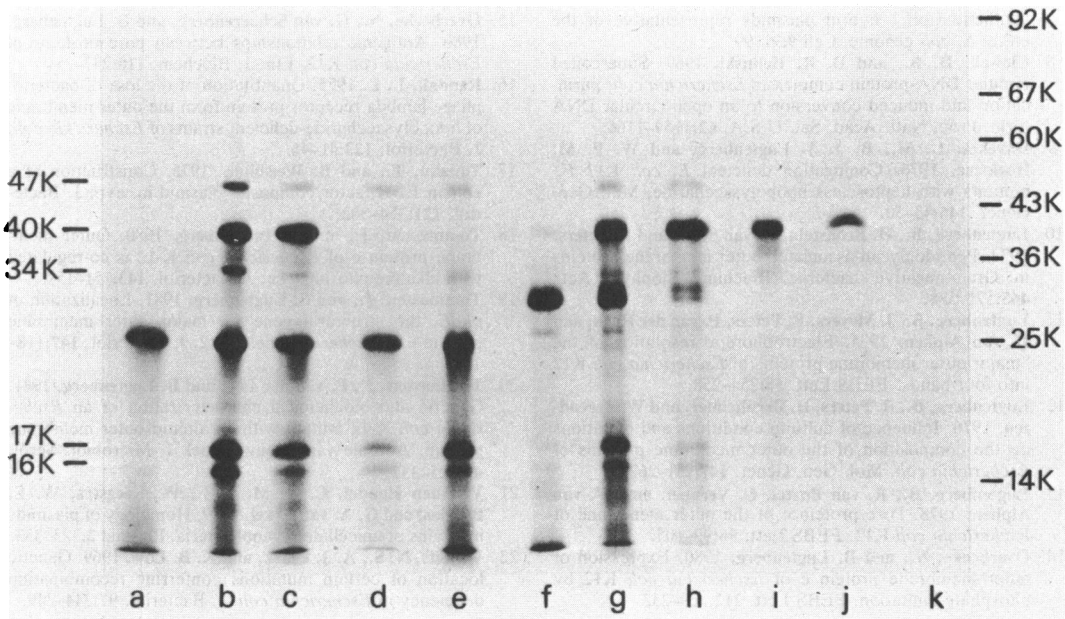

FIG. 3. Autoradiogram of ^{35}S-labeled proteins from minicells, separated by SDS-polyacrylamide gel electrophoresis. The slots represent total ^{35}S-labeled proteins from minicells containing plasmids pACYC184 (a), pJP12 (b), pJP14 (c), pJP16 (d), pJP21 (e), pBR322 (f), and pJP20 (g); a cell envelope preparation of ^{35}S-labeled minicells containing pJP20 (h); the peptidoglycan-associated proteins isolated from this cell envelope preparation (i); and an immunoprecipitate of the cell envelope preparation of ^{35}S-labeled minicells containing pJP20 obtained with anti-PhoE protein serum (j) or preimmune serum (k). The positions of the molecular weight standard proteins are indicated at the right.

Bremer et al. (4) reported that they did not succeed in cloning the *ompA* gene on a multicopy plasmid, probably due to lethal overproduction of this outer membrane protein. Our experiments show that it is possible to clone the *phoE* gene on multicopy plasmids in a *pho*$^+$ background. When these *phoE*-containing plasmids are introduced into *phoS* strains, the resulting transformants grow poorly and overproduce the PhoE protein (Fig. 1). Cell envelopes of these strains contain strongly decreased amounts of OmpF protein and, when the cells are grown in maltose-containing media, also of LamB protein. In contrast, the periplasmic proteins are unaffected and the OmpA and OmpC proteins are affected only slightly; therefore, the results can be interpreted as a competition between the former three proteins for common sites in the translocation process or in the outer membrane itself. In this respect, it is interesting to note that, in contrast to the amounts of OmpA and OmpC proteins, the amounts of OmpF (9), LamB (16), and PhoE (13, 19) proteins in the cell envelopes are dependent on the structure of the lipopolysaccharide in that mutants with a heptose-deficient lipopolysaccharide have strongly decreased amounts of these proteins.

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LITERATURE CITED

1. **Andreoli, P. M., N. Overbeeke, E. Veltkamp, J. D. A. van Embden, and H. J. J. Nijkamp.** 1978. Genetic map of the bacteriogenic plasmid CloDF13 derived by insertion of the transposon Tn901. Mol. Gen. Genet. **160**:1–11.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleid Acids Res. **7**:1513–1524.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlak, H. L. Heynecker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
4. **Bremer, E., E. Beck, I. Hindennach, I. Sonntag, and U. Henning.** 1980. Cloned structural gene (*ompA*) for an integral outer membrane protein of *Escherichia coli* K-12. Localization on hybrid plasmid pTU100 and expression of a fragment of the gene. Mol. Gen. Genet. **179**:13–20.
5. **Brown, M. G. M., A. Weston, J. R. Saunders, and G. O. Humphreys.** 1979. Transformation of *Escherichia coli* C600 by plasmid DNA at different phases of growth. FEMS Microbiol. Lett. **5**:219–222.
6. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A crytic miniplasmid. J. Bacteriol. **134**:1141–1156.
7. **Clarke, L., and J. Carbon.** 1976. A colony bank containing

synthetic ColE1 hybrid plasmids representative of the entire *E. coli* genome. Cell **9**:91–99.

8. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. U.S.A. **62**:1159–1166.
9. **Havekes, L. M., B. J. J. Lugtenberg, and W. P. M. Hoekstra.** 1976. Conjugation deficient *E. coli* K12 F^- mutants with heptoseless lipopolysaccharide. Mol. Gen. Genet. **146**:43–50.
10. **Lugtenberg, B., H. Bronstein, N. van Selm, and R. Peters.** 1977. Peptidoglycan-associated outer membrane proteins in Gram-negative bacteria. Biochim. Biophys. Acta **465**:571–578.
11. **Lugtenberg, B., J. Meyers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the "major outer membrane protein" of *Escherichia coli* K12 into four bands. FEBS Lett. **58**:254–258.
12. **Lugtenberg, B., R. Peters, H. Bernheimer, and W. Berendsen.** 1976. Influence of cultural conditions and mutations on the composition of the outer membrane proteins of *Escherichia coli*. Mol. Gen. Genet. **147**:251–262.
13. **Lugtenberg, B., R. van Boxtel, C. Verhoef, and W. van Alphen.** 1978. Pore protein e of the outer membrane of *Escherichia coli* K12. FEBS Lett. **96**:99–105.
14. **Overbeeke, N., and B. Lugtenberg.** 1980. Expression of outer membrane protein e of *Escherichia coli* K12 by phosphate limitation. FEBS Lett. **112**:229–232.
15. **Overbeeke, N., G. van Scharrenberg, and B. Lugtenberg.** 1980. Antigenic relationships between pore proteins of *Escherichia coli* K12. Eur. J. Biochem. **110**:247–254.
16. **Randall, L. L.** 1975. Quantitation of the loss of bacteriophage lambda receptor protein from the outer membrane of lipopolysaccharide-deficient strains of *Escherichia coli*. J. Bacteriol. **123**:41–46.
17. **Tanaku, T., and B. Weisblum.** 1975. Construction of a colicin E1-R factor composite plasmid in vivo. J. Bacteriol. **121**:354–362.
18. **Tommassen, J., and B. Lugtenberg.** 1980. Outer membrane protein e of *Escherichia coli* K-12 is co-regulated with alkaline phosphatase. J. Bacteriol. **143**:151–157.
19. **Tommassen, J., and B. Lugtenberg.** 1981. Localization of *phoE*, the structural gene for major outer membrane protein e of *Escherichia coli* K-12. J. Bacteriol. **147**:118–123.
20. **Tommassen, J., P. van der Ley, and B. Lugtenberg.** 1981. Genetic and biochemical characterization of an *Escherichia coli* K-12 mutant with an altered outer membrane protein. Antonie van Leeuwenhoek J. Microbiol. Serol. **47**:325–337.
21. **Van den Hondel, C. A. M. J. J., W. Keegstra, W. E. Borrias, and G. A. van Arkel.** 1979. Homology of plasmids in strains of unicellular cyanobacteria. Plasmid **2**:323–333.
22. **Willetts, N. S., A. J. Clark, and K. B. Low.** 1969. Genetic location of certain mutations conferring recombination deficiency in *Escherichia coli*. J. Bacteriol. **97**:244–249.