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# Mutations That Alter the Signal Sequence of Alkaline Phosphatase in *Escherichia coli*

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A *phoA-lacZ* gene fusion was used to isolate mutants altered in the alkaline phosphatase signal sequence. This was done by selecting $Lac^+$ mutants from a *phoA-lacZ* fusion strain that produces a membrane-bound hybrid protein and is unable to grow on lactose. Two such mutant derivatives were characterized. The mutations lie within the *phoA* portion of the fused gene and cause internalization of the hybrid protein. When the mutations were genetically recombined into an otherwise wild-type *phoA* gene, they interfered with export of alkaline phosphatase to the periplasm. The mutant alkaline phosphatase protein was found instead in the cytoplasm in precursor form. DNA sequence analysis demonstrated that both mutations lead to amino acid alterations in the signal sequence of alkaline phosphatase.

Many hydrolytic enzymes in *Escherichia coli* are in the periplasmic space, and thus, cytoplasmic components are not exposed to their degradative action. One of these periplasmic enzymes is alkaline phosphatase, a nonspecific phosphomonoesterase encoded by the *phoA* gene. Like other exported proteins, alkaline phosphatase is initially synthesized in precursor form with an amino-terminal signal sequence (11). To study the secretion of this protein and the consequences of preventing its export from the cytoplasm, we sought mutations which alter its signal sequence. It seemed possible that internalized alkaline phosphatase might be lethal to cells since many essential cytoplasmic metabolites are phosphomonoesters. Therefore, we devised a selection for *phoA* signal sequence mutations in which such a potentially lethal outcome could be avoided. To do this we took advantage of gene fusion technology.

In the accompanying paper (16), we described the construction of the gene fusion *phoA-lacZ* 456. This fusion encodes a hybrid protein containing the amino-terminal half of alkaline phosphatase joined to β-galactosidase. The hybrid protein is localized to the membrane, presumably because of its alkaline phosphatase signal sequence. It was anticipated that mutations which alter the signal sequence would lead to internalization of the hybrid protein. This would result in a cytoplasmic location for β-galactosidase activity, but not for phosphatase enzymatic activity, since the *phoA* gene is not intact in the fusion.

In this paper, we describe a selection for strains containing mutations in the *phoA* signal sequence region of the *phoA-lacZ* fusion. The amino acid alterations caused by two such mutations were determined by DNA sequencing. These mutations, when recombined into an otherwise wild-type *phoA* gene, prevent export of alkaline phosphatase to the periplasm. However, internalized alkaline phosphatase, which is present in precursor form, has no deleterious effect on *E. coli*.

## MATERIALS AND METHODS

**Media and chemicals.** TYE, MacConkey lactose agar, M63 minimal medium containing glucose, and LB broth have been described previously (17). Tris medium containing glucose and various amounts of phosphate is described in the accompanying paper (16). β-Glycerophosphate medium (when β-glycerophosphate is the sole carbon source present) contained Tris 121 buffer and salts (24), 0.25% β-glycerophosphate (Sigma Chemical Co.), 0.3 mM $KH_2PO_4$, and 1 μg of thiamine per ml. Amino acids were added at 40 μg/ml where required. 5-Bromo-4-chloro-3-indolyl phosphate (XP) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XG) were each added at 40 μg/ml. XP is a substrate for alkaline phosphatase and XG is a substrate for β-galactosidase. *p*-Nitrophenyl phosphate, the substrate for alkaline phosphatase assays, was obtained from Sigma.

**Bacterial strains and phages.** The bacterial strains and phages employed in this study are listed in Table 1.

**Bacterial strain and phage constructions.** The *prlA3* mutation was introduced into strains by P1 transduction, using P1 grown on strain SE4014. Since *prlA* and

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TABLE 1. Bacterial strains and bacteriophages

| Strain or bacteriophage | Genotype or bacterial genes carried[a] | Origin or reference |
|---|---|---|
| Strain | | |
| MC1000 | F⁻ *araD139* Δ(*ara-leu*)*7697* Δ*lac-74 galU galK rpsL* | (16) |
| MPh42 | MC1000 *phoR* | Beckwith collection |
| MPh44 | MC1000 Δ(*phoA-proC*) *phoR tsx*::Tn*5* | Beckwith collection |
| MPh51 | MPh44 *prlA3 rpsE* | This study |
| MPh1061 | MPh42 *phoA61* | This study |
| MPh1068 | MPh42 *phoA68* | This study |
| SM652 | MPh1061 *prlA3 rpsE* | This study |
| SM655 | MPh44/F′ *lac* | This study |
| SM656 | MPh51/F′ *lac* | This study |
| SE4014 | F⁻ *araD139* Δ*lac-169 relA rpsL lamB60 rpsE prlA3* | (7) |
| Bacteriophage | | |
| λ456 | Φ(*phoA-lacZ*)hyb456 *lacYA′* | (16) |
| λ456-61 | Φ(*phoA-lacZ*)hyb456-61 *lacYA′* | This study |
| λ456-68 | Φ(*phoA-lacZ*)hyb456-68 *lacYA′* | This study |
| λ *phoA* | *phoA*⁺ *proC*⁺ | (22) |
| λ *phoA* Δ*Pvu*II | *phoA* Δ*Pvu*II *proC*⁺ | (12) |
| f1 *phoA* | *phoA*⁺ | (10) |
| f1 *phoA* Δ*Pvu*II | *phoA* Δ*Pvu*II | H. Inouye (unpublished) |
| f1 *phoA61* | *phoA61* | This study |
| f1 *phoA68* | *phoA68* | This study |

[a] The abbreviation hyb indicates that the gene fusion encodes a hybrid protein.

*rpsE* (which confers resistance to spectinomycin) are greater than 99% cotransducible, nearly all spectinomycin-resistant transductants contain the *prlA3* mutation.

Mutation *phoA61* was recombined onto a λ specialized transducing phage in the following way. Strain MPh1061 was lysogenized with λ *phoA*, and a lysate was prepared by UV induction. λ *phoA* contains no *att* site and must therefore integrate and excise by homologous recombination in the region of *phoA*. Thus, the *phoA61* mutation from the chromosome will be present at high frequency among phages that have excised. When plated on strain MPh44 (*phoA phoR*), 10% of the plaques were white, indicating the presence of the mutant allele. One such phage, λ *phoA61*, was used for cloning.

Mutation *phoA68* was recombined onto a λ specialized transducing phage in a similar manner. Strain MPh1068 was lysogenized with λ *phoA* Δ*Pvu*II, which carries a deletion of the amino-terminal half of the *phoA* gene. After UV induction of the lysogen, blue plaques were sought since *phoA68* confers a partial PhoA⁺ phenotype. One such phage, λ *phoA68*, was used for cloning.

F′*lac* was mated (17) into strains to make them sensitive to the male-specific phage, f1.

**Obtaining cured derivatives of MPh42(λ456-61) and MPh42(λ456-68).** Lysogenic strains MPh42(λ456-61) and MPh42(λ456-68) were streaked on TYE media containing XG and subjected to UV irradiation for 60 s. After overnight incubation at 37°C, 0.1 to 1% of the colonies appeared white, indicating that they no longer contained the λ456 prophage. Absence of phage was confirmed by the inability of these strains to produce a zone of lysis on a λ-sensitive indicator lawn.

**Alkaline phosphatase assay.** Cultures to be assayed were grown to mid-log phase in M63 minimal medium containing glucose. Alkaline phosphatase assays were performed as described previously (4) except that cells were treated with chloroform and sodium dodecyl sulfate before assay. Units were calculated as described by Brickman and Beckwith (4).

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** Slab gel electrophoresis and autoradiography were performed as previously described (16) except that gels contained 10% acrylamide and 0.13% bisacrylamide. Gels were 28 cm in length to optimize separation between mature and precursor forms of alkaline phosphatase.

**[$^{35}$S]methionine labeling, cell fractionation, and immunoprecipitation.** These procedures were performed as described previously (16) except that in this case antiserum against periodate-treated alkaline phosphatase (provided by J. Garwin) was used for immunoprecipitation.

**Preparation of DNA.** λ *phoA61* and λ *phoA68* were grown lytically on MPh44. The phage lysates were precipitated with polyethylene glycol, and phage particles were purified by banding on a cesium chloride block gradient as described by Davis et al. (5). DNA was obtained from the phage by the formamide method (5).

Double-stranded replicative-form DNA from f1 *phoA* Δ*Pvu*II and the 190-base-pair *Hin*dIII-*Pvu*II restriction fragment used as primer for sequencing were provided by H. Inouye.

High-titer lysates of f1 *phoA61* and f1 *phoA68* were obtained by growth of the phage on SM655. Single-stranded phage DNA was purified for sequencing as described by Oliver and Crowther (19).

**Restriction endonuclease digestion, ligation, and transformation of DNA.** *Pvu*II was obtained from New England Biolabs. Digestions were carried out in 6 mM Tris (pH 7.4)–6 mM $MgCl_2$–60 mM NaCl–6 mM β-

mercaptoethanol. Ligations were performed overnight at 25°C in 60 mM Tris (pH 8)–10 mM $MgCl_2$–10 mM dithiothreitol–100 μM ATP. Transformation was performed as described previously (19).

**DNA sequencing procedures.** Sequencing reactions were carried out as described by Sanger and coworkers (21) as adapted to M13 (23). After completion of the chase reaction, primer was cleaved by addition of 0.5 μl of *Pvu*II for 5 min at room temperature. Sequencing gels were prepared and processed as described by Oliver and Crowther (19). The 6% sequencing gels were run at 30 mA of constant current for approximately 2 h.

## RESULTS

**Selection for mutants altered in localization of the hybrid protein encoded by *phoA-lacZ* 456.** The hybrid protein encoded by the gene fusion *phoA-lacZ* 456 is localized to the cell membrane in strain MPh42(λ456) (16). This hybrid protein exhibits abnormally low β-galactosidase activity so that strain MPh42(λ 456), which synthesizes it constitutively, is unable to grow on lactose as a sole carbon source. We considered the possibility that it was not the structure of the protein per se that was reponsible for this Lac⁻ phenotype, but rather its membrane location. Accordingly, selection for Lac⁺ derivatives of MPh42(λ456) might yield mutants in which the hybrid protein was cytoplasmic, and thus fully active.

Lac⁺ derivatives of MPh42(λ456) were obtained as red papillae which appeared on a white confluent lawn of cells on MacConkey lactose plates. After plates were incubated at 42°C for 2 days, 50 to 100 papillae were visible. Nearly all of these mutant derivatives could grow on minimal lactose medium. Two mutants, carrying *61* and *68*, were chosen for further analysis.

**Effect of Lac⁺ mutations on location of the hybrid protein.** To determine whether the cellular location of the hybrid protein was altered in the Lac⁺ mutants, cell fractions were examined fo the presence of the hybrid protein after [$^{35}$S]methionine labeling. Immunoprecipitation was performed on samples from whole cells and cell fractions by using antibody against alkaline phosphatase, which recognizes both the hybrid protein and the alkaline phosphatase produced in these strains. The immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). In the parental Lac⁻ fusion-bearing strain, a majority of the hybrid protein is in the membrane fraction. In contrast, the hybrid protein in the two Lac⁺ mutants is predominantly cytoplasmic. It appears that the mutations present in the Lac⁺ mutants prevent membrane localization of the hybrid protein. This supports the hypothesis that the hybrid protein in its membrane location

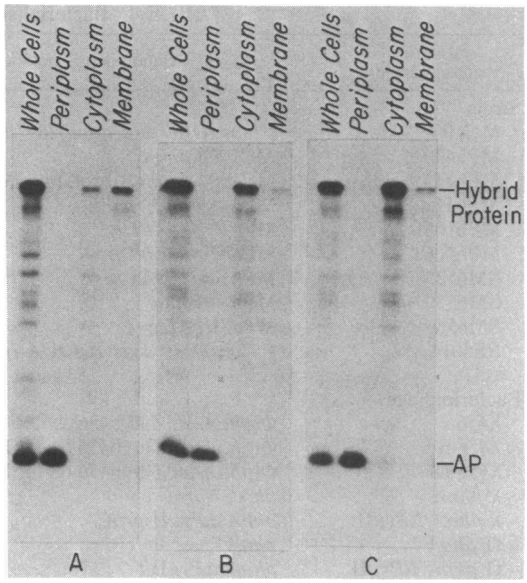


FIG. 1. Cellular location of hybrid protein in the parental Lac⁻ strain MPh42(λ456) and in two Lac⁺ mutant derivatives. Strains were labeled with [$^{35}$S]methionine for 5 min and cell fractions were obtained. Whole cells and cell fractions were subjected to immunoprecipitation with antiserum against alkaline phosphatase. Immunoprecipitates were electrophoresed in a 27-cm-long gel containing 10% acrylamide and 0.13% bis-acrylamide. Only the relevant portion of the gel is shown. (A) MPh42(λ456), (B) MPh42(λ456) Lac⁺ 61, (C) MPh42(λ456) Lac⁺ 68. The positions of the hybrid protein and alkaline phosphatase (AP) are indicated at right.

cannot efficiently cleave lactose, but can do so when localized to the cytoplasm.

**Linkage of the Lac⁺ mutation to the *phoA-lacZ* fusion.** If the mutations responsible for altering the export properties of the hybrid protein reside in the *phoA-lacZ* gene fusion, then they should be carried on the λ456 specialized transducing phage present in the Lac⁺ derivatives of MPh42(λ456). To determine whether this was the case, we prepared phage lysates by UV induction. Lysates obtained from the Lac⁺ mutant derivatives of MPh42(λ456) were capable of transducing the Δ*lac phoR* strain MPh42 to Lac⁺, whereas lysates from the Lac⁻ parent strain were not. These results indicated that the mutations reside within the *phoA-lacZ* gene fusion. The phages carrying mutant alleles were designated λ456-61 and λ456-68.

**Recombination of mutations *61* and *68* into the *phoA* gene.** Since it is the alkaline phosphatase portion of the hybrid protein which directs it to a membrane location, it seemed probable that the mutations causing internalization of the hybrid

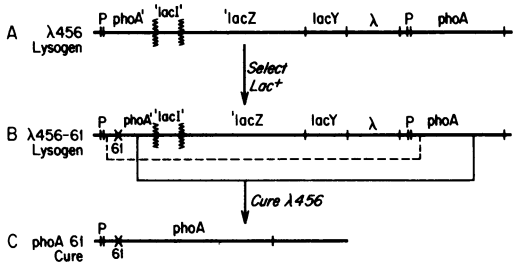


FIG. 2. Strategy for recombining mutations from the *phoA-lacZ* fusion gene into the chromosomal *phoA* gene. λ456, which carries a *phoA-lacZ* gene fusion, must integrate by a homologous recombination event into the *phoA* gene in strain MPh42 to form a stable lysogen. (This is because the phage does not have a λ *att* site.) (A) Chromosomal arrangement of genes in this lysogen. Bacterial genes and the prophage are not drawn to scale. In this lysogen, the *phoA-lacZ* fusion, *lacY*, and the λ prophage reside next to a wild-type *phoA* gene. Selection for Lac$^+$ derivatives of MPh42(λ456) yielded strains which contained a mutation in the *phoA* portion of the gene fusion, i.e, *phoA61*, indicated by an X in (B). For λ456 to excise, it must do so by recombination between homologous *phoA* DNA. Two possible crossover events are depicted in (B). If the phage excises by homologous recombination between the regions shown by the dashed line, a wild-type *phoA* gene is reconstituted. However, if the crossover occurs as shown by the solid line, the *phoA61* mutation is left behind in the chromosomal *phoA* gene (C).

protein would reside in the *phoA* region of the *phoA-lacZ* fusion. If this were the case, it should be possible to incorporate mutations *61* and *68* into an otherwise wild-type *phoA* gene to assess whether alkaline phosphatase export itself is altered.

The approach employed to recombine the mutations into *phoA* is outlined in Fig. 2. The phage λ456 had been integrated into MPh42 by homologous recombination in *phoA* to yield the lysogen depicted in Fig. 2A. The phage can also excise from the chromosome by a homologous recombination event. If the mutations reside in the *phoA* portion of the *phoA-lacZ* fusion carried on the phage (Fig. 2B), then, depending on the site of recombination, the excision event will yield either a wild-type or a mutant *phoA* gene as shown; the resulting strain is a "cured" nonlysogenic derivative.

We used a procedure which allowed us to detect cells in which such a recombination event had occurred between the *phoA* region on the fusion and the resident chromosomal *phoA* gene and which simultaneously resulted in loss of the prophage. The Lac$^+$ lysogens were streaked on agar medium and treated with a short pulse of UV irradiation. Bacteria cured of λ are enriched in the population after this procedure and can be detected as white colonies on agar plates containing XG.

Eight white, Lac$^-$, cured derivatives were obtained from each Lac$^+$ mutant. These were screened for their PhoA phenotype by streaking on (i) medium containing XP and (ii) minimal medium containing β-glycerophosphate as the sole carbon source. For mutation *61*, six of the eight cured derivatives appeared deep blue in the presence of XP and grew on β-glycerophosphate, which is the phenotype of *phoA*$^+$ *phoR* strains. In contrast, the remaining two cured derivatives appeared white in the presence of XP and could not grow on β-glycerophosphate. They were considered to have mutation *61* recombined into the *phoA* gene. In the case of mutation *68*, three of the eight cured derivatives also exhibited a mutant phenotype. These strains were blue on XP-containing media, but less blue than a *phoA*$^+$ strain, and they grew poorly on β-glycerophosphate.

It was necessary to ensure that the altered PhoA phenotype observed was due to the incorporation of mutations *phoA61* and *phoA68* into *phoA* and not the result of a new mutation generated by the UV irradiation. This was determined by relysogenizing the cured derivatives with λ456. To form a lysogen, λ456 must integrate by homologous recombination within the *phoA* region. In a significant proportion of the resulting lysogens, the mutation present in the *phoA* gene of the cured derivative will have recombined into the *phoA-lacZ* fusion. If the mutations responsible for the altered PhoA phenotype are the original *phoA61* and *phoA68* lesions, lysogens which are Lac$^+$ should arise at high frequency. When cured *phoA* derivatives were relysogenized with λ456, many of these lysogens were Lac$^+$, indicating that the cured derivatives did, in fact, contain the original mutations that had conferred the Lac$^+$ phenotype. The cured derivatives containing *phoA61* and *phoA68* were designated MPh1061 and MPh1068, respectively; both are *phoR*.

Assays were performed to measure the alkaline phosphatase activities of MPh1061 and MPh1068 (Table 2). The assays confirmed the qualitative results from the XP plate tests described above. The level of alkaline phosphatase activity produced by MPh1061 was 1.5% that of a wild-type *phoA*$^+$ strain, whereas MPh1068 produced 16% of the wild-type level.

To determine the location of residual enzymatic activity in the mutants, cell fractionation was performed as described above. Greater than 95% of the alkaline phosphatase activity in MPh1068 is found in the periplasmic fraction. Because of the low activity in strain MPh1061, it is difficult to accurately assay activity in cell

TABLE 2. Alkaline phosphatase activity in wild-type and mutant strains

| Strain[a] | Relevant genotype | Alkaline phosphatase activity (U)[b] |
|---|---|---|
| MPh42 | *phoA*$^+$ | 570 |
| MPh1068 | *phoA68* | 90 |
| MPh1061 | *phoA61* | 8 |
| SM652 | *phoA61 prlA3* | 34 |

[a] All strains carrying the *phoR* mutation.
[b] Units are expressed according to the formula in reference 4.

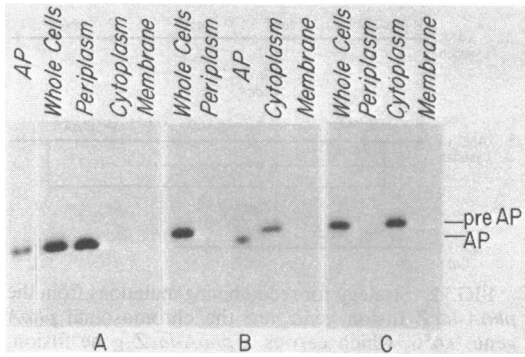


FIG. 3. Cellular location of alkaline phosphatase in wild-type or mutant strains containing the *phoA* gene. Strains were labeled with [$^{35}$S]methionine for 5 min and fractionated into periplasmic, cytoplasmic, and membrane fractions. Whole cells and cell fractions were subjected to immunoprecipitation with antiserum to alkaline phosphatase. Immunoprecipitates were electrophoresed in 27-cm-long gels containing 10% acrylamide and 0.13% bis-acrylamide. Only the relevant portion of the gel is shown. Cell fractions indicated are (A) MPh42 (*phoA*$^+$), (B) MPh1061 (*phoA61*), and (C) MPh1068 (*phoA68*). Lanes marked AP contain 2 μg of alkaline phosphatase standard. Positions of mature AP and precursor alkaline phosphatase (preAP) are marked at right.

fractions. In this case, at least 70% of the activity can be detected in the periplasm.

**Effect of mutations *phoA61* and *phoA68* on export of alkaline phosphatase.** Although some alkaline phosphatase enzymatic activity was detected in the periplasm of the mutants, it seemed likely that the major fraction of alkaline phosphatase made in these strains might be cytoplasmic, since these mutations cause internalization of the hybrid protein when present on the *phoA-lacZ* gene fusion. To examine this, wild-type and mutant cells were subjected to a 5-min [$^{35}$S]methionine labeling, cell fractionation, immunoprecipitation with antibody against alkaline phosphatase, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3). In the *phoA*$^+$ strain, MPh42, alkaline phosphatase was found in mature form in the periplasmic fraction. No precursor was detected. (Alkaline phosphatase precursor is normally short-lived in wild-type cells and can be visualized only after a very short pulse-labeling, 10 s or less [13].) A strikingly different pattern was observed for the mutants. In both cases, no mature alkaline phosphatase was detected. Instead, a higher-molecular-weight band was seen, which corresponded in size to alkaline phosphatase precursor. For both mutants, the precursor was found exclusively in the cytoplasmic fraction.

To determine the fate of internalized alkaline phosphatase precursor, cells were subjected to a 1-min pulse-label with [$^{35}$S]methionine followed by a chase period of up to 60 min in the presence of excess unlabeled methionine (Fig. 4). In a wild-type strain, the mature periplasmic alkaline phosphatase is completely stable (data not shown). In contrast, in MPh1061, the precursor is degraded and has a half-life of approximately 10 min. No mature phosphatase is observed. This absence of mature alkaline phosphatase correlates with the lack of enzymatic activity in this mutant. The pattern observed for MPh1068 is more complex. In this case, the precursor has two fates. A minor fraction of it is slowly converted to mature alkaline phosphatase and accounts for the residual periplasmic enzyme activity in MPh1068. The remainder of the precursor is degraded with the same kinetics as in MPh1061.

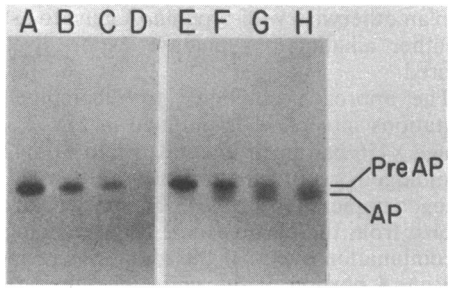


FIG. 4. Pulse-chase analysis of the fate of alkaline phosphatase precursor in mutants MPh1061 and MPh1068. Cells were labeled with [$^{35}$S]methionine for 1 min. The radioactivity was chased by addition of unlabeled methionine to a final concentration of 0.05% for the times specified. Whole cell extracts were immunoprecipitated with antiserum against alkaline phosphatase and electrophoresed. Lanes (A) through (D) show MPh1061 chased for (A) 0 min, (B) 10 min, (C) 30 min, and (D) 60 min. Lanes (E) through (H) show MPh1068 chased for (E) 0 min, (F) 10 min, (G) 30 min, and (H) 60 min. The positions of mature alkaline phosphatase (AP) and precursor alkaline phosphatase (PreAP) are indicated.

**Suppression of the alkaline phosphatase-negative phenotype of *phoA61* by *prlA*, a signal sequence-specific suppressor.** It seemed likely that mutations responsible for the inability of alkaline phosphatase to be exported would reside in the signal sequence-coding region of *phoA*. For maltose-binding protein, λ receptor protein, and β-lactamase, mutations which prevent export of these proteins alter the signal sequence and not the mature portion of the protein (1, 8, 14a). To obtain genetic evidence that this was the case for the *phoA61* mutation, we examined the effect of a signal sequence-specific suppressor (*prlA*) on alkaline phosphatase activity in the strain containing *phoA61*. *prlA* was introduced into MPh1061 by P1 transduction. When enzyme activity in the *prlA phoA61* double mutant (SM652) was measured, a fourfold increase in alkaline phosphatase activity was observed over that of the MPh1061 mutant alone (Table 2). After cells were fractionated, at least 70% of this activity was detected in the periplasmic fraction. This suppression of *phoA61* by *prlA* strongly suggests that the *phoA61* lesion resides in the signal sequence-coding region of *phoA*.

**Cloning mutations *phoA61* and *phoA68* into the single-stranded DNA bacteriophage f1.** To ascertain the precise location of the lesions in mutations *phoA61* and *phoA68*, it was necessary to analyze their DNA sequences. To use the dideoxy chain termination sequencing method of Sanger (21, 23), we cloned a restriction fragment containing the mutations onto the genome of the single-stranded DNA bacteriophage, f1.

To obtain DNA for cloning, we first attempted to grow λ456-61 and λ456-68, which contain the *phoA61* and *phoA68* mutations within the *phoA-lacZ* gene fusion. We were unable to obtain high-titer lysates of these phages. Instead, we recombined these mutations onto λ *phoA* transducing phages (see above), which did yield high-titer phage lysates. Lysates were made with λ *phoA61* and λ *phoA68*, and purified DNA was prepared from them.

The cloning strategy used to construct single-stranded DNA phages that carried mutation *phoA61* or *phoA68* is shown in Fig. 5. The single-stranded DNA phage f1 *phoA* Δ*Pvu*II (Fig. 5B), suitable as a vector for cloning DNA fragments containing *phoA* mutations, was constructed by H. Inouye (unpublished results). A *phoA* gene can be reconstituted by cloning the *Pvu*II-*Pvu*II fragment carrying the promoter and amino-terminal portion of *phoA* into this phage (Fig. 5C). If the cloned fragment contains a *phoA* mutation, then the mutation will be present in the reconstituted gene.

Double-stranded replicative-form DNA of phage f1 *phoA* Δ*Pvu*II was digested with *Pvu*II, which cuts it only once, and the resulting linear molecule was ligated to a *Pvu*II digest of λ *phoA61* or λ *phoA68* DNA. The ligated DNA was transfected into a recipient strain, and recombinant phage plaques were screened. f1 *phoA* Δ*Pvu*II forms white plaques on the Δ*phoA* indicator strain SM655. Because mutation *phoA68* results in a partial PhoA$^+$ phenotype, phages producing blue plaques were sought.

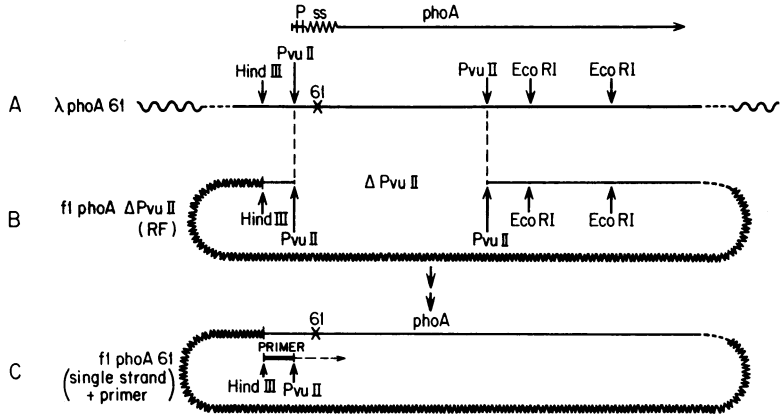


FIG. 5. Strategy used for cloning and sequencing *phoA* mutations. The position of the *phoA* promoter, signal sequence, and structural gene coding region relative to the indicated restriction sites (12, 16) is shown at top. λ *phoA61* is depicted in (A). Wavy lines represent λ DNA, and the solid line represents chromosomal DNA (including *phoA*) carried on the phage. The approximate site of *phoA61* is marked by an X. (B) The *Pvu*II-*Pvu*II fragment was cloned into f1 *phoA* Δ*Pvu*II as described in the text. The jagged line represents f1 phage DNA. The phage resulting from this cloning, f1 *phoA61* (C), contains a reconstituted *phoA61* gene. The *Hin*dIII-*Pvu*II primer and the direction of DNA sequencing are indicated in (C). An analogous approach was used for cloning and sequencing *phoA68*.

One such phage, f1 *phoA68*, was used for DNA sequence analysis. For the more stringent *phoA61* mutation, phages producing blue plaques were obtained by plating on strain SM656 (*prlA3*). One such phage, f1 *phoA61*, was used for DNA sequence analysis. Although it forms blue plaques on strain SM656 (*prlA3*), it produces white plaques on strain SM655, which lacks the *prlA* suppressor mutation. This *prlA*-dependent phenotype confirmed that mutation *phoA61* was present on the reconstituted *phoA* gene.

**DNA sequence analysis of the *phoA61* and *phoA68* mutations.** A 190-base-pair *Hin*dIII-*Pvu*II restriction fragment which includes a region near the beginning of the *phoA* gene was used as a primer for the DNA sequencing reactions (Fig. 5). With this primer, the nucleotide sequence of the 63-base-pair signal sequence-coding region for *phoA61* and *phoA68* was determined (data not shown). The DNA sequences obtained for these mutations were compared with the known DNA sequence for the *phoA*$^+$ gene (3, 10, 14).

Each of the mutations exhibited an alteration of a single base pair in the signal sequence-coding region (Fig. 6). For the *phoA61* mutation, the transversion T to G results in a change at amino acid residue 14 of the alkaline phosphatase precursor, CTG (leucine) to CGG (arginine). Thus, a hydrophobic residue is replaced by a positively charged residue. For the *phoA68* mutation, the transversion T to A results in a change in amino acid residue 8, CTG (leucine) to CAG (glutamine). In this case a hydrophobic residue is replaced by a hydrophilic but uncharged amino acid.

## DISCUSSION

In this paper, we have described two mutations which cause alterations in the alkaline phosphatase signal sequence. The mutations were selected in a strain containing a *phoA-lacZ* gene fusion and were then recombined into *phoA*. When present in an otherwise wild-type *phoA* gene, the signal sequence mutations *phoA61* and *phoA68* interfere with the export of alkaline phosphatase to the periplasm. In the mutants, most of the alkaline phosphatase polypeptide is found in the cytoplasm in precursor form.

It seemed possible that internalization of periplasmic enzymes which have degradative functions, such as phosphatase, would be lethal to the cell. Yet, with the mutants described here, the internalized alkaline phosphatase polypeptide is not lethal. This can be explained by the observation that in MPh1061, where export is completely blocked, enzymatic activity is almost completely absent. In this mutant, no mature alkaline phosphatase is detected on gels; instead, only the precursor is seen. The low enzymatic activity could be explained in two ways. First, the cytoplasmic precursor is unstable, with a half-life of approximately 10 min. However, this slow degradation may not be sufficient to account for the very low activity. A second factor involved may be the inability of cytoplasmic alkaline phosphatase precursor to form active enzyme. In previous studies, it has been demonstrated that the precursor synthesized in vitro is enzymatically active (11). Therefore, simply the presence of an uncleaved signal sequence could not account for absence of enzyme activity. It is possible that the relatively high reducing potential of the cytoplasm prevents formation of intrachain disulfide bridges and thus prevents proper folding or dimerization of alkaline phosphatase. It will be of interest to determine whether other periplasmic degradative enzymes such as RNase, DNase, or protease are active when internalized. It may be that these proteins, in general, have evolved in such a way that they would be inactive if they were cytoplasmic.

In contrast to MPh1061, mutant MPh1068 exhibits a considerable amount of alkaline phosphatase activity (16% of the wild-type level). This activity is in the periplasm and correlates with the presence of some mature-sized alkaline phosphatase on gels. It appears that a portion of the cytoplasmic precursor initially detected on gels is slowly processed and exported in a post-translational manner. Thus the altered signal

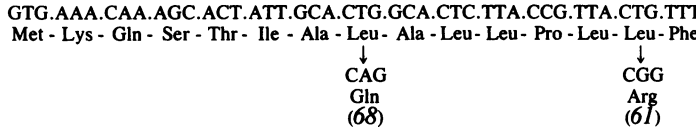


FIG. 6. DNA nucleotide sequence and amino acid sequence of the wild-type and mutant *phoA* signal sequences. DNA of the signal sequence-coding region of *phoA* (3, 10, 14) and the corresponding amino acids are shown. Codons altered by mutations *phoA61* and *phoA68* are indicated.

peptide in MPh1068 is weakly competent to facilitate the export of alkaline phosphatase, perhaps via some secondary cellular pathway.

Mutation *phoA61* was produced by a transversion event in codon 14 of the signal sequence-coding region. The CTG → CGG alteration leads to a substitution of the positively charged residue, arginine, for the hydrophobic residue, leucine. Eight of the nine point mutations obtained in the *lamB* and *malE* signal sequence-coding regions result in insertion of a charged residue within the hydrophobic core (1, 8). Like *phoA61*, these mutations, which greatly reduce the hydrophobicity of the signal peptide, result in a very strong inhibition of export of their respective gene products.

Mutation *phoA68* was produced by a transversion in codon 8, which is also in the hydrophobic core of the alkaline phosphatase signal sequence. The CTG → CAG change results in substitution of glutamine, a hydrophilic but uncharged residue, for leucine. This alteration also reduces the degree of hydrophobicity, but to a lesser extent than in the case of insertion of a charged residue. This less dramatic change in the extent of hydrophobicity of the signal sequence correlates with the weaker inhibition of export and higher level of enzyme activity seen for the *phoA68* mutant as compared with the *phoA61* mutant. Although the precise features of a signal sequence that contribute to its functional role in export are not understood, the properties of both *phoA61* and *phoA68* lend further support to the hypothesis (2, 15) that the hydrophobic nature of this peptide is critical for its export function.

The product of the *prlA* gene is thought to be a component of the cellular protein export machinery (7). Mutations at this locus have been shown to suppress *lamB* and *malE* signal sequence mutations (6). In the presence of *prlA*, the export-defective proteins are transported to their correct noncytoplasmic destinations and are also cleaved to the mature form. When the *prlA* mutation is introduced into MPh1061, the level of alkaline phosphatase increases fourfold over that of the *prlA*$^+$ parent strain. Since enzyme activity and successful export seem to be correlated in the case of alkaline phosphatase, it appears that *prlA* can also suppress a *phoA* signal sequence mutation. This result expands the spectrum of proteins which are affected by the *prlA* mutation and suggests that there is a common step in the export of alkaline phosphatase, maltose-binding protein, and λ receptor protein.

The procedure we used for isolating *phoA* signal sequence mutations employed a *phoA-lacZ* gene fusion. Since it was possible to select for the internalization of a hybrid protein instead of alkaline phosphatase itself, even mutations which could cause production of a lethal internal phosphatase could have been obtained. Thus, there was no bias for selection of only nonlethal classes of mutations. In this study, neither of the mutations analyzed caused lethality when present in *phoA* (as discussed above). However, if such a lethal class does exist, it should be possible to obtain mutations of this type by using Lac$^+$ selection.

It appears that the approach employed here may be generally applicable for obtaining signal sequence mutations in genes coding for other cell envelope proteins. In several cases, when the gene for an exported protein is fused to *lacZ*, the hybrid protein produced is membrane bound (15). It is now established for three different cases that such membrane-bound hybrid proteins exhibit abnormally low β-galactosidase activity. The Lac$^-$ phenotype of strains containing *phoA-lacZ* 456 has been discussed in this paper. Strains containing certain *lamB-lacZ* fusions are Lac$^-$ when the hybrid protein they produce is in the outer membrane (9). In the case of *malE-lacZ* fusion-bearing strains, which produce a hybrid protein localized to the inner membrane, an abnormally low basal level of β-galactosidase activity is observed (18). In all cases, selection for Lac$^+$ derivatives has yielded mutants which internalize the hybrid protein and contain signal sequence mutations in the *phoA*, *lamB* (T. Silhavy, personal communication), or *malE* (20) portions of the gene fusions. By using the *malE-lacZ* gene fusion, two additional classes of mutations have been selected which also internalize the hybrid protein. These mutations are unlinked to the gene fusion and have revealed two new genes, *secA* (18) and *secB* (20), whose products appear to be components of the cellular protein export machinery. Presumably mutations of this type can be obtained by Lac$^+$ selection with the *phoA-lacZ* fusion and may identify new *sec* genes.

One useful aspect of the Lac$^+$ selection for internalization of hybrid protein is its sensitivity. Even a slight restoration of Lac$^+$ activity permits papillation on MacConkey lactose medium. We have now obtained a collection of 60 Lac$^+$ derivatives which contain mutations linked to the *phoA-lacZ* 456 gene fusion (data not shown). When these mutations are recombined into the *phoA* gene, they cause a wide spectrum of PhoA phenotypes. Some mutants exhibit nearly wild-type levels of alkaline phosphatase activity, indicating that export may be only weakly blocked, whereas at the other extreme, some mutants have very low levels of enzyme activity, indicating that export is strongly blocked. DNA sequence analysis of these mutations should reveal considerably greater detail about the structural

aspects of the *phoA* signal sequence which are important for its export function.


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## LITERATURE CITED


1. **Bedouelle, H., P. J. Bassford, Jr., A. V. Fowler, I. Zabin, J. Beckwith, and M. Hofnung.** 1980. Mutations which alter the function of the signal sequence of the maltose binding protein of *Escherichia coli*. Nature (London) **285**:78–81.
2. **Bedouelle, H., and M. Hofnung.** 1981. On the role of the signal peptide in the initiation of protein export, p. 361–372. *In* B. Pullman (ed.), Intermolecular forces. D. Reidel Publishing Co., Dordrecht, England.
3. **Boidel, W., M. Simonis, M. Topert, and G. Siewert.** 1982. Recombinant plasmids with genes for the biosynthesis of alkaline phosphatase of *Escherichia coli*. Mol. Gen. Genet. **185**:510–512.
4. **Brickman, E., and J. Beckwith.** 1975. Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and $\phi$80 transducing phages. J. Mol. Biol. **96**:307–316.
5. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **Emr, S. D., and P. J. Bassford.** 1982. Localization and processing of outer membrane and periplasmic proteins in *Escherichia coli* strains harboring export-specific suppressor mutations. J. Biol. Chem. **257**:5852–5860.
7. **Emr, S. D., S. Hanley-Way, and T. J. Silhavy.** 1981. Suppressor mutations that restore export of a protein with a defective signal sequence. Cell **23**:79–88.
8. **Emr, S. D., J. Hedgpeth, J.-M., Clement, T. J. Silhavy, and M. Hofnung.** 1980. Sequence analysis of mutations that prevent export of $\lambda$ receptor, an *Escherichia coli* outer membrane protein. Nature (London) **285**:82–85.
9. **Hall, M. N., M. Schwartz, and T. J. Silhavy.** 1982. Sequence information within the *lamB* gene is required for proper routing of the bacteriophage $\lambda$ receptor protein to the outer membrane of *Escherichia coli* K-12. J. Mol. Biol. **155**:93–112.
10. **Inouye, H., W. Barnes, and J. Beckwith.** 1982. Signal sequence of alkaline phosphatase of *Escherichia coli*. J. Bacteriol. **149**:434–439.
11. **Inouye, H., and J. Beckwith.** 1977. Synthesis and processing of an *Escherichia coli* alkaline phosphatase precursor *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **74**:1440–1444.
12. **Inouye, H., S. Michaelis, A. Wright, and J. Beckwith.** 1981. Cloning and restriction mapping of the alkaline phosphatase structural gene (*phoA*) of *Escherichia coli* and generation of deletion mutants in vitro. J. Bacteriol. **146**:668–675.
13. **Josefsson, L. G., and L. L. Randall.** 1981. Different exported proteins in *E. coli* show differences in the temporal mode of processing *in vivo*. Cell **25**:151–157.
14. **Kikuchi, Y., K. Yoda, M. Yamasaki, and G. Tamura.** 1981. The nucleotide sequence of the promoter and the amino-terminal region of alkaline phosphatase structural gene (*phoA*) of *Escherichia coli*. Nucleic Acids Res. **9**:5671–5678.

14a. **Koshland, D., R. T. Sauer, and D. Botstein.** 1982. Diverse effects of mutations in the signal sequence on the secretion of $\beta$-lactamase in *Salmonella typhimurium*. Cell **30**:903–914.

15. **Michaelis, S., and J. Beckwith.** 1982. Mechanism of incorporation of cell envelope proteins in *Escherichia coli*. Annu. Rev. Microbiol. **36**:435–465.
16. **Michaelis, S., L. Guarente, and J. Beckwith.** 1983. In vitro construction and characterization of *phoA-lacZ* gene fusions in *Escherichia coli*. J. Bacteriol. **154**:356–365.
17. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Oliver, D. B., and J. Beckwith.** 1981. *E. coli* mutant pleiotropically defective in the export of secreted proteins. Cell **25**:765–772.
19. **Oliver, D. B., and R. A. Crowther.** 1981. DNA sequence of the tail fibre genes 36 and 37 of bacteriophage T4. J. Mol. Biol. **153**:545–568.
20. **Oliver, D., C. Kumamoto, M. Quinlan, and J. Beckwith.** 1982. Pleiotropic mutants affecting the secretory apparatus of *Escherichia coli*. Ann. Microbiol. (Paris) **133A**:105–110.
21. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**:5463–5467.
22. **Sarthy, A., S. Michaelis, and J. Beckwith.** 1981. Deletion map of the *Escherichia coli* structural gene for alkaline phosphatase, *phoA*. J. Bacteriol. **145**:288–292.
23. **Schreier, P. H., and R. Cortese.** 1979. A fast and simple method for sequencing. J. Mol. Biol. **129**:169–172.
24. **Torriani, A.** 1966. Alkaline phosphatase from *Escherichia coli*, p. 224–235. *In* G. L. Cantoni and R. Davies (ed.), Procedures in nucleic acid research. Harper & Row, Publishers, New York.