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Prolipoprotein signal peptidase of *Escherichia coli* requires a cysteine residue at the cleavage site

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A signal peptidase specifically required for the secretion of the lipoprotein of the *Escherichia coli* outer membrane cleaves off the signal peptide at the bond between a glycine and a cysteine residue. This cysteine residue was altered to a glycine residue by guided site-specific mutagenesis using a synthetic oligonucleotide and a plasmid carrying an inducible lipoprotein gene. The induction of mutant lipoprotein production was lethal to the cells. A large amount of the prolipoprotein was accumulated in the outer membrane fraction. No protein of the size of the mature lipoprotein was detected. These results indicate that the prolipoprotein signal peptidase requires a glyceride modified cysteine residue at the cleavage site.

Key words: protein secretion/signal peptide/site-specific mutagenesis/outer membrane

Introduction

Signal peptidases are unique proteases which are involved in the secretion of proteins across the membrane. They cleave a signal peptide from a secretory precursor protein. The signal peptides, particularly in bacterial systems, have several common features, which are considered to play an important role during protein secretion (Inouye and Halegoua, 1980). In *Escherichia coli* there are at least two distinct signal peptidases, one for M13 procoat protein along with several other secretory precursor proteins (Wolfe *et al.*, 1982), and the other for the major outer membrane protein precursor, prolipoprotein (Yamagata *et al.*, 1982; Tokunaga *et al.*, 1982). In contrast to the M13 procoat protein signal peptidase, the prolipoprotein signal peptidase requires a much higher specificity at the cleavage site. It cleaves the peptide bond between the glycine residue at the 20th position and the cysteine residue at the 21st position of the prolipoprotein (Inouye *et al.*, 1977). The cysteine residue is modified by a glyceride group, forming a glyceride cysteine residue (Hantke and Braun, 1973). A cyclic peptidic antibiotic, globomycin, specifically inhibits the prolipoprotein signal peptidase resulting in the accumulation of the glyceride-modified prolipoprotein (Inukai *et al.*, 1978; Hussain *et al.*, 1980). The glyceride-modified prolipoprotein (prolipoprotein III) can serve as a substrate for the prolipoprotein signal peptidase upon removal of globomycin.

We have examined whether the prolipoprotein signal peptidase requires the cysteine residue at the cleavage site for its function. For this purpose, we have substituted the cysteine residue with a glycine residue by guided site-specific mutagenesis with use of a synthetic oligonucleotide and a plasmid carrying the lipoprotein (*lpp*) gene. We found that the mutant prolipoprotein cannot be processed by the prolipoprotein signal peptidase nor by the M13 procoat protein signal peptidase, resulting in the accumulation of the unmodified prolipoprotein (prolipoprotein I). These results indicate that the cysteine residue, most likely the glyceride cysteine residue, is required for the prolipoprotein signal peptidase.

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Results

Isolation of the mutant

The DNA sequence of the *E. coli lpp* gene at the region corresponding to the cysteine residue at the 21st position of the prolipoprotein has been determined to be:

$^{5'}$GCAGGTTGCTCCAG$^{3'}$
AlaGlyCysSerSer

(Nakamura and Inouye, 1979). In order to change the cysteine residue to a glycine residue, the DNA sequence should be changed to $^{5'}$GCAGGTGGCTCCAG$^{3'}$ (the base that is altered is underlined). For guided site-specific mutagenesis, a 14-mer oligonucleotide, $^{5'}$CTGGAGCCACCTGC$^{3'}$, which is complementary to the DNA sequence described above was synthesized and used for mutagenesis.

Since the alteration of the cysteine residue to a glycine residue may have a severe inhibitory effect on bacterial growth even at a basal level of gene expression when the plasmid pKEN125 is used in the absence of IPTG, pMI001 DNA was used for mutagenesis instead of pKEN125 DNA. pMI001 is identical to pKEN125 except that in pMI001 the tandemly repeated promoters upstream of the *lpp* gene were deleted (see Figure 1 and Materials and methods).

After mutagenesis, ^{32}P-labeled oligonucleotide was used as a probe to detect mutant colonies. Two positive colonies were obtained from 1200 transformants. One of them was further characterized, and final identification was carried out by DNA sequence analysis (Figure 2). The sequence of the mutant DNA was identical to that predicted from mutagenesis.

Expression of the mutant lpp gene

To express the mutated *lpp* gene, a 0.56-kb *Xba*I-*Eco*RI fragment carrying the mutant *lpp* gene was isolated from the mutant plasmid and inserted into an expression cloning vector, pIN-II (Nakamura and Inouye, 1982), which was then digested with both *Xba*I and *Eco*RI. The resultant plasmid, pC_2 [pIN-II-*lpp*(^{21}Cys→Gly)] has the mutant *lpp* gene under the control of the *lpp* promoter and the *lac*UV5 promoter-operator (see Figure 1). Thus, the expression of the mutant *lpp* gene could be induced by *lac* inducers such as isopropyl thiogalactoside (IPTG, Nakamura *et al.*, 1982; Inouye *et al.*, 1982). It was found that JA221 *lpp*$^-$ F′*lac*I^q carrying pC_2 [pIN-II-*lpp*(^{21}Cys→Gly)] could not form colonies on a plate containing IPTG. When IPTG was added to the culture, the Klett units slowly doubled during the first 1.5 h and then decreased gradually (data not shown). These data indicate that the product from the mutant *lpp* gene is toxic for cell growth.

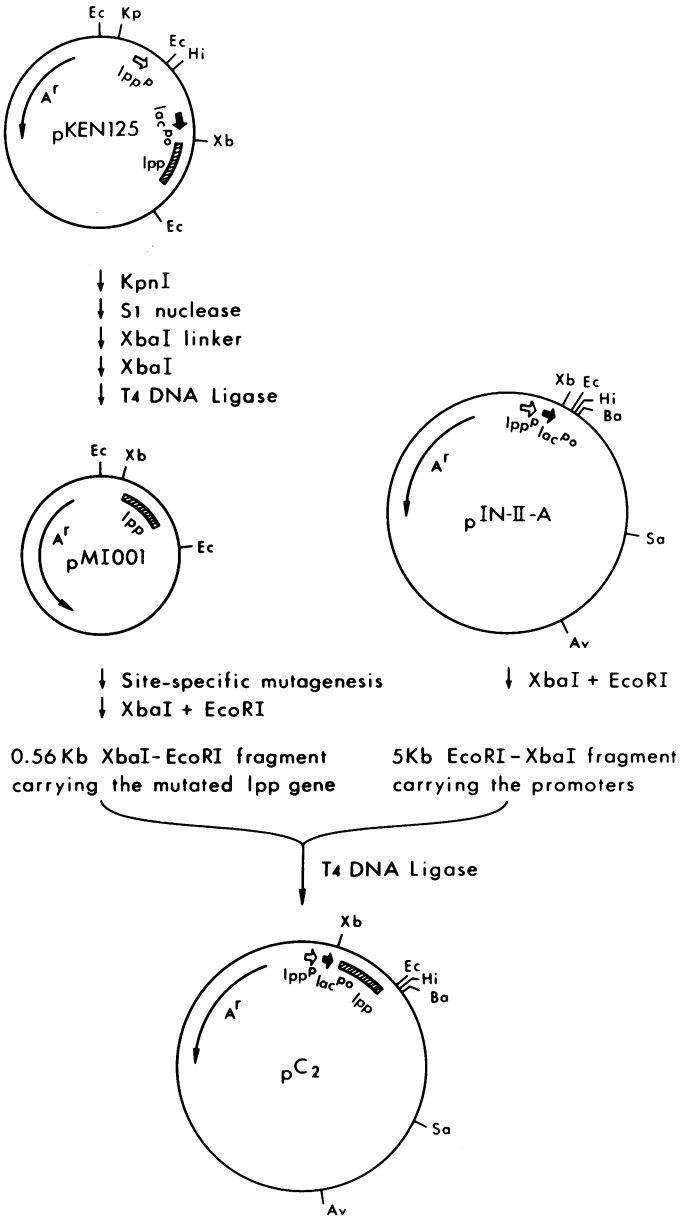

Fig. 1. Construction of pMI001 and pC_2 [pIN-II-*lpp*(Cys^{21}→Gly)]. Details of constructions are described in the text. The map location of restriction enzyme cleavage sites and the size of DNA fragments are not drawn to exact scales. The coding region of the *lpp* gene is indicated by a shaded bar. The approximate location and the direction of transcription of the lpp^P (⇨) and the lac^{PO} (➡) are indicated. A^r, the gene for β-lactamase; EC, *Eco*RI; Kp, *Kpn*I; Hi, *Hind*III; Xb, *Xba*I; Ba, *Bam*HI; Sa, *Sal*I; and Av, *Ava*I site.

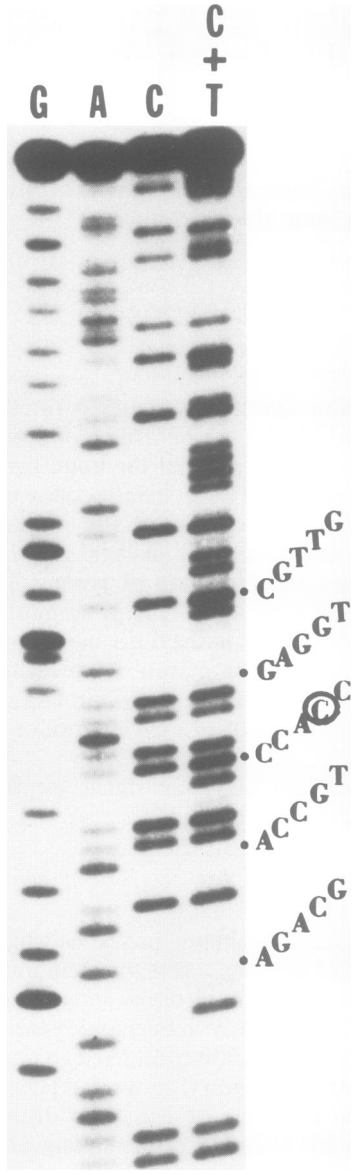

Fig. 2. DNA sequence gel corresponding to the region of the cleavage site of the lipoprotein signal peptide of pC_2 [pIN-II-*lpp*(Cys^{21}→Gly)]. The plasmid DNA was digested with *Xba*I at the unique *Xba*I site in the ribosome binding site of the *lpp* gene (Nakamura and Inouye, 1979) and labeled with [^{32}P]dNTP as described previously (Inouye *et al.*, 1982). The labeled DNA was cleaved with *Hinf*I and the ~120-bp *Xba*I/*Hinf*I fragment was purified and used for sequencing. The cleavage products were applied to a 10% polyacrylamide gel and subjected to electrophoresis. Only the region around the signal peptide cleavage site is shown. The sequence at the side of the gel is complementary to the sense sequence. The circled base indicates the base that is different from the wild-type *lpp* gene.

Characterization of the product from the mutant lpp gene

When cells were pulse labeled for 5 min with [^{35}S]methionine at 20 min after induction with IPTG, a product was observed in the membrane fraction which migrated at the same position as the mature lipoprotein in SDS-polyacrylamide gel electrophoresis (Figure 3, lane 1). When chased for another 30 min, the amount of the product in the membrane fraction decreased significantly as shown in Figure 3, lane 2. When cells were pulse labeled at 2 h after induction, the production of all the membrane proteins appeared to be suppressed except for the band indicated by arrow b and the mutant *lpp* gene product indicated by arrow a (Figure 3). The protein at band b was identified as OmpA protein by its unusual mobilities in SDS-gel electrophoresis when treated at different temperatures (data not shown; OmpA protein, see review by DiRienzo *et al.*, 1978).

Since the gel system used in Figure 3 (Tris-HCl system) cannot separate the mature lipoprotein from the unmodified prolipoprotein (prolipoprotein I), immunoprecipitates were prepared from the experiments described in Figure 3 and applied to another gel system which utilizes a sodium phosphate buffer. In this system, one can clearly separate the two proteins (Inouye *et al.*, 1982). In Figure 4, the mutant *lpp* gene product was examined in the membrane fraction as well as in the cytoplasmic membrane. The mutant *lpp* gene product in

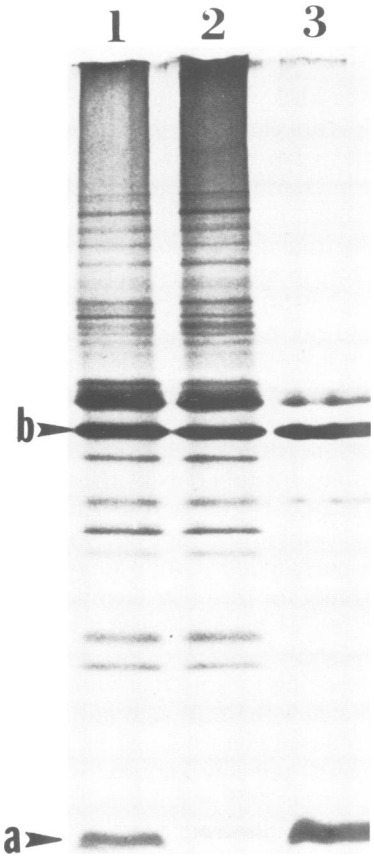

Fig. 3. SDS-polyacrylamide gel electrophoresis of the membrane fractions of JA221 *lpp*$^-$/F′ *lac*I^q/pIN-II-*lpp*(Cys^{21}→Gly) with Tris-HCl system (Anderson *et al.*, 1973). **Lane 1**, cells were pulse labeled for 5 min after 20 min induction with IPTG; **lane 2**, same as **lane 1** except that cells were chased for 30 min, and; **lane 3**, cells were pulse labeled for 5 min after 2 h induction with IPTG. An arrow with letter a indicates the position of the mature lipoprotein and the unmodified prolipoprotein, and an arrow with letter b, the position of OmpA protein.

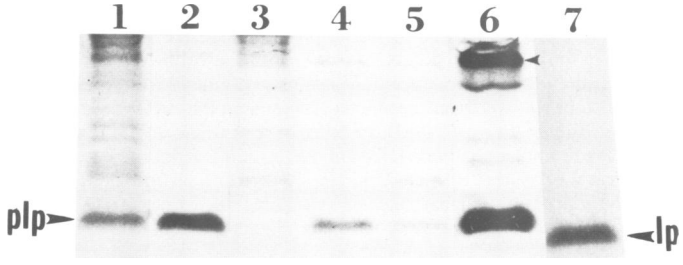

Fig. 4. SDS-polyacrylamide gel electrophoresis of immunoprecipitates with anti-lipoprotein serum with sodium phosphate system (Inouye *et al.*, 1982). With use of the same cells labeled with [^{35}S]methionine as described in Figure 3, the cytoplasmic and the membrane fractions were treated with anti-lipoprotein serum, and the immunoprecipitates were applied to the gel. **Lanes 1** and **2**, the immunoprecipitates from the cytoplasmic and membrane fractions, respectively, of cells pulse labeled for 5 min after 20 min induction with IPTG; **lanes 3** and **4**, the immunoprecipitates from the cytoplasmic and the membrane fractions, respectively, of cells chased for 30 min after pulse labeling as described above; **lanes 5** and **6**, immunoprecipitates from the cytoplasmic and the membrane fractions, respectively, of the cells pulse labeled for 5 min after 2 h induction with IPTG; and **lane 7**, Coomassie brilliant blue R stained, purified mature lipoprotein. An arrow with plp indicates the position of the unmodified prolipoprotein (prolipoprotein I). A small arrow in **lane 6** indicates a protein co-immunoprecipitated with the mutant protein.

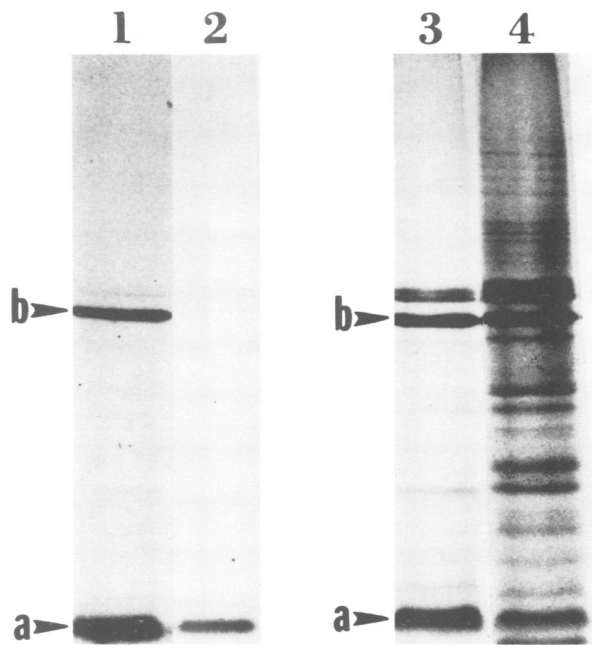

Fig. 5. SDS-polyacrylamide gel electrophoresis of the same immunoprecipitates used in Figure 4 with the Tris-HCl system. **Lane 1**, the same immunoprecipitate as applied to **lane 6** in Figure 4; **lane 2**, the same immunoprecipitate as applied to **lane 2** in Figure 4; **lane 3**, the membrane fraction for **lane 1**; and **lane 4**, the membrane fraction for **lane 2** before immunoprecipitation. Arrows a and b indicate the positions of the mature lipoprotein and prolipoprotein I and OmpA protein, respectively.

all cases migrated at the same position as prolipoprotein I, which migrated slower than the mature lipoprotein (lane 7) in the phosphate gel system. The mutant prolipoprotein was localized mainly in the membrane fraction (lanes 1 and 2) and chasing the mutant prolipoprotein did not generate any other immunoprecipitable product in the membrane fraction nor in the cytoplasmic fraction (lanes 3 and 4). These results indicate that the mutant prolipoprotein is very unstable in contrast to the wild-type prolipoprotein (Hussain *et al.*, 1980) and other mutant prolipoproteins (Inouye *et al.*, 1982). When pulse labeled at 2 h after induction, the major amount of the product was still in the membrane fraction (lanes 5 and 6). It is interesting to note that a large amount of another membrane protein was co-immunoprecipitated with the mutant prolipoprotein only when cells were pulse labeled at 2 h after induction as indicated by a small arrow in Figure 4, lane 6. This band was identified as a protein migrating at the same position as band b as indicated in Figure 3 by applying the membrane fractions (Figure 5, lanes 3 and 4) and the immunoprecipitates (Figure 5, lanes 1 and 2) to the Tris-HCl gel system. Furthermore, this protein showed heat-modifiability identical to and characteristic for OmpA protein (data not shown), indicating that it is OmpA protein.

Localization of the mutant prolipoprotein

To examine the localization of the mutant prolipoprotein in the membrane fraction, the total membrane fraction was treated with sodium sarcosynate, which is known to solubilize specifically the cytoplasmic membrane (Filip *et al.*, 1973). As shown in Figure 6, the mutant lipoprotein was found almost exclusively in the outer membrane fraction. Similar results were obtained when the outer and the cytoplasmic membranes were separated by sucrose-density gradient centrifugation (data not shown).

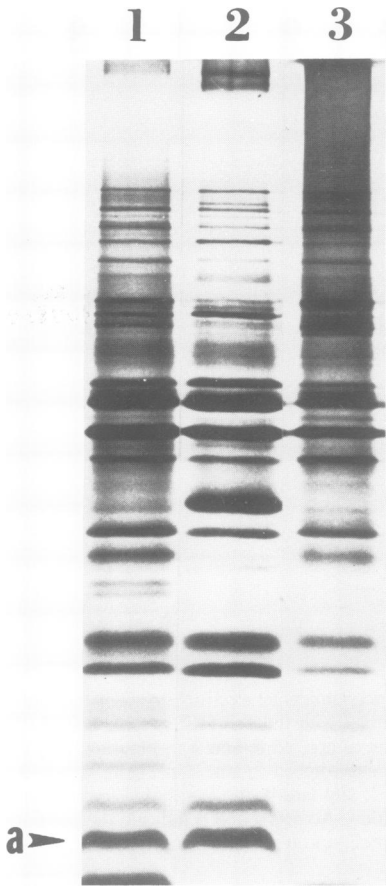

Fig. 6. Separation of the cytoplasmic and the outer membrane fractions with use of sodium sarcosynate. The membrane fraction was prepared from the cells pulse-labeled for 5 min after 20 min induction with IPTG. SDS-polyacrylamide gel electrophoresis was carried out with the Tris-HCl system. **Lane 1**, total membrane; **lane 2**, the sarcosynate-insoluble outer membrane; and **lane 3**, the sarcosynate-soluble cytoplasmic membranes. An arrow with letter a indicates the position of the mature lipoprotein and prolipoprotein I.

Discussion

As a result of the alteration of the cysteine residue at the 21st position to a glycine residue, the mutant prolipoprotein cannot be modified with a glyceride group. Since the glyceride-modified prolipoprotein (prolipoprotien III) appears to be the substrate for the prolipoprotein signal peptidase (Yamagata *et al.*, 1982), the inability of the signal peptide of the mutant prolipoprotein to be cleaved is most likely due to the lack of a glyceride group at the cleavage site. Thus, the prolipoprotein signal peptidase appears to require a glyceride cysteine residue at the carboxy-terminal side of the cleavage site.

The fact that prolipoprotein III is accumulated in the presence of globomycin (Inukai *et al.*, 1978; Hussain *et al.*, 1980) or in a temperature-sensitive mutant of the prolipoprotein signal peptidase (Yamagata *et al.*, 1982) indicated that prolipoprotein III cannot be a substrate for the M13 procoat protein signal peptidase (Wolfe *et al.*, 1982). It should be noted that even if the glyceride group is removed from the cleavage site, the mutant prolipoprotein cannot be cleaved by the M13 procoat protein signal peptidsae. This is rather surprising since the signal peptide of the M13 procoat protein has similar features to the signal peptide of the mutant prolipoprotein (see review by Inouye and Halegoua, 1980). Since IPTG-resistant revertants are easily obtained from JA221 lpp^-/F′ $lacI^q$/pIN-II-*lpp*(Cys^{21}→Gly), analysis of plasmid DNAs carrying revertant mutants may provide a clue to understanding the specificities of the prolipoprotein signal peptidase and the M13 procoat protein signal peptidase.

At present, it is not clear how the accumulation of the mutant prolipoprotein causes a lethal effect on the cell growth. However, it is interesting to note that the mutant prolipoprotein was localized mainly in the outer membrane fraction similar to another mutant prolipoprotein in which the glycine residue at the 14th position was altered to an aspartic acid residue (Lin *et al.*, 1978). In the case of the latter mutant prolipoprotein it did not have a lethal effect on cell growth. It should also be noted that the present mutant prolipoprotein was found in the outer membrane fraction by two different methods, sarcosynate solubilization and sucrose-density gradient centrifugation, whereas prolipoprotein III was found in the outer membrane fraction only by the sarcosynate solubilization method but not by the sucrose-density gradient centrifugation method (Inukai and Inouye, 1982). These results indicate that the polypeptide portion of the lipoprotein itself has a strong affinity for the outer membrane. This raises the question of the role of the lipid portion of the lipoprotein in the outer membrane.

It is very interesting to note that OmpA protein was coimmunoprecipitated with the mutant prolipoprotein at a later stage of induction (see Figures 4 and 5). The reason for this result is unknown at present. Site-specific mutagenesis at the glycine residue at the 20th position of the prolipoprotein is now in progress. Results from these experiments will shed light on the exact specificity of the prolipoprotein signal peptidase.

Materials and methods

Bacterial strains and plasmids

E. coli JA221 lpp^- F^- *had*R^- *had*M^+ *trp*E5 *leu*B6 *lac*Y *rec*A1/F′ $lacI^q$ lac^+ pro^+ (Nakamura *et al.*, 1982) was grown as described previously (Inouye *et al.*, 1982). Plasmids pKEN125 (Nakamura *et al.*, 1982) and pIN-II (Nakamura and Inouye, 1982) were also used.

Construction of pMI001

For guided site-specific mutagenesis, pMI001 was used, which was constructed from pKEN125 by removing the promoter region required for the expression of the *lpp* gene in pKEN125 (see Figure 1) as follows: pKEN125 has two tandemly repeated promoters, one upstream promoter from the *lpp* gene and the other downstream promoter containing the *lac*UV5 promoter-operator (Nakamura *et al.*, 1982). In order to remove the 0.9-kb region containing the promoter region described above, a unique *Kpn*I site upstream of the region was converted to an *Xba*I site by digesting pKEN125 DNA with *Kpn*I (New England Biolabs) followed by treating the digested DNA with S1 nuclease and then inserting an *Xba*I linker oligonucleotide (dCTCTAGAG; Collaborative Research) according to the method described previously (Nakamura and Inouye, 1982). The complete digestion of the resulting DNA with *Xba*I (New England Biolabs) removed the 0.9-kb promoter region since a unique *Xba*I site exists in the ribosome-binding site of the *lpp* gene in pKEN125 (Nakamura *et al.*, 1982). Religation of the *Xba*I-digested DNA produced pMI001.

Guided site-specific mutagenesis

Oligonucleotide site-directed mutagenesis was carried out with use of pMI001 DNA as described previously (Inouye *et al.*, 1982). The DNA sequence of the oligonucleotide used was dCTGGAGCCACCTGC (14-mer), which was synthesized by a solid-phase triester method (Miyoshi *et al.*, 1980). Colony hybridization was performed at 43°C.

Labeling experiments

Cells were pulse labeled by a method similar to that described previously (Inouye *et al.*, 1982). Cultures were grown in M9 medium (Miller, 1972) supplemented with glucose (4 mg/ml), leucine (20 μg/ml), trytophan (20 μg/ml), thiamine (2μg/ml), $MgSO_4.7H_2O$ (200 μg/ml) and ampicillin (50 μg/ml) at 37°C. At 2 x 10^8 cells/ml, IPTG (Sigma) was added to a final concentration of 2 mM. 20 min or 2 h after induction, 10 ml of the culture was pulse labeled

with 50 μCi of [^{35}S]methionine (Amersham; 1390 Ci/mmol) for 5 min at 37°C. Incorporation was stopped by adding 10 ml of 40 mM sodium phosphate buffer, pH 7.1 containing 0.8% formaldehyde and non-radioactive methionine (2 mg/ml) (stopping solution). Chase experiments were carried out by adding 400 μl of methionine (5 mg/ml) at 5 min and stopped at 35 min (30 min chase) by adding 10 ml of stopping solutions. The membrane fraction was prepared as described (Inouye and Guthrie, 1969). Immunoprecipitation was carried out with rabbit anti-lipoprotein serum as described (Inouye *et al.*, 1976). The separation of the outer and the cytoplasmic membrane was performed by specific solubilization of the cytoplasmic membrane with sodium sarcosynate (Filip *et al.*, 1973) and by sucrose-density gradient centrifugation (Osborn and Munson, 1974).

SDS-polyacrylamide gel electrophoresis

Two different systems for SDS-polyacrylamide gel electrophoresis were used; one with use of Tris-HCl buffer (Anderson *et al.*, 1973) and the other with use of sodium phosphate buffer (Inouye *et al.*, 1982). With the former system, the mature lipoprotein can be separated from glyceride-modified prolipoprotein (prolipoprotein III) but not from unmodified prolipoprotein (prolipoprotein I). With the latter system, prolipoprotein I can be separated from the mature lipoprotein but not from prolipoprotein III (Inouye *et al.*, 1982).

Other methods

DNA sequencing was carried out according to the method of Maxam and Gilbert (1977).

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