THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 260, No. 30, Issue of December 25, pp. 16192-16199, 1985
Printed in U.S.A.

Signal Sequence Mutants of β-Lactamase*

(Received for publication, April 1, 1985)

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The function of the NH_2-terminal signal peptide in the translocation of β-lactamase across the inner membrane of *Escherichia coli* has been studied by characterization of 15 signal sequence mutants. Three amino acid substitutions (Pro 20 to Ser, Pro 20 to Phe, and Cys 18 to Tyr) in the 23-amino acid signal sequence each cause, to varying degrees, a defect in the proteolytic processing of pre-β-lactamase, abnormal growth of the host strain, and a severe reduction in the expression of β-lactamase *in vivo* but not *in vitro*. The results are consistent with a model for protein secretion in *E. coli* that parallels the pathway proposed for translocation across the endoplasmic reticulum in eucaryotic cells.

Proteins that are secreted across the inner membrane of the Gram-negative bacterium *Escherichia coli* are synthesized with a NH_2-terminal extension known as the signal (or leader) sequence (for recent reviews, see Refs. 1–3). Sometime during the secretory process, this signal sequence is removed, and the mature protein is exported to its proper cellular compartment. While the signal peptides of proteins secreted by *E. coli* show little if any sequence homology, these peptides do possess several common features. Signal peptides of *E. coli* proteins are 18–26 amino acid residues long and contain 1 or more positively charged amino acid residues at the NH_2 terminus, a hydrophobic core in the middle, and amino acid residues with small side chains at positions −3 and −1 from the signal sequence cleavage site (4–6).

In eucaryotic cells, the mechanism of protein translocation across the endoplasmic reticulum, which also requires an NH_2-terminal signal sequence, has been recently clarified (see Ref. 3). A nucleoprotein complex, the signal recognition particle (SRP[1]), appears to participate in the secretion of proteins as follows. The synthesis of an exported protein is initiated, and the signal sequence emerges from the ribosome; the SRP recognizes the signal sequence, binds to the ribosome, and causes an arrest of translation; the conglomerate (ribosome, mRNA, SRP, and nascent polypeptide) attaches to the endoplasmic reticulum and interacts with a membrane-bound receptor; the SRP detaches from the ribosome and releases the translation arrest; the nascent polypeptide is cotranslationally extruded across the membrane, and the signal sequence is proteolytically removed. The SRP selectively regulates the synthesis of exported proteins and thus prevents the accumulation of precursors of secreted proteins in the cytoplasm.

The mechanism by which signal sequences function in the secretion of proteins in *E. coli* is not yet understood. To study this function, the behavior of signal sequence mutants has been analyzed. Most of these mutations were obtained either by genetic selection or by oligonucleotide-directed site-specific mutagenesis. Each of these procedures, however, has certain limitations. Signal sequence mutants isolated by classical genetic techniques are necessarily phenotypically biased. Oligonucleotide-directed mutagenesis, on the other hand, gives only specific mutations, and since the critical functional elements of signal peptides have not yet been identified, it is impossible to know what mutations will be interesting and important. Random mutagenesis is preferred in such a situation where, because of insufficient understanding of the system under scrutiny, there is no obvious rationale for specific base substitutions.

We report here the generation of a series of mutations in the 23-amino acid signal sequence of the periplasmic TEM[2] β-lactamase and studies of the phenotypic consequence of these changes. First, a derivative of pBR322 was constructed that contains a unique *Eco*RI restriction site in the beginning and a unique *Bst*EII site at the end of the *bla* signal codons (7, 8). This new plasmid, pTG2 (which is defined to be the wild-type *bla* gene in this work), encodes a pre-β-lactamase that contains two phenotypically silent mutations in the signal sequence. (Changing Ser 2 to Arg simply creates a second cationic residue at the NH_2 terminus, and altering Ala 23 to Gly places an even smaller amino acid at the peptide cleavage site of maturation. The sequence of the mature β-lactamase is unchanged.) By using the *bla* gene from pTG2, many mutant *bla* alleles, which are identical to the wild-type *bla* gene except for random GC to AT transition mutations within the signal codons, have been generated by mutagenesis with methoxylamine (9). In the present work, 15 β-lactamase signal sequence mutants have been characterized.

EXPERIMENTAL PROCEDURES

All restriction endonucleases, T4 DNA ligase, and *E. coli* DNA polymerase I (large fragment) were purchased from New England Biolabs (Beverly, MA). Deoxyadenosine 5′-[α-^{32}P]triphosphate (800 Ci/mmol) and L-[^{35}S]methionine (1000 Ci/mmol) were obtained from Amersham Corp. Nitrocefin was purchased from BBL Microbiology Systems (Cockeysville, MD). IgGsorb was obtained from The Enzyme Center (Malden, MA). Culture media were prepared from the products of Difco. The oligonucleotide 5′-AATACATTCAAATATGTAT was synthesized by Michael Edge (Imperial Chemical Industries, Runcorn, U.K.). All other biochemicals were from Sigma.

Bacterial and Phage Strains—*E. coli* K12 strain RB971 [W3110 *lacI*^{q}*L8*] (10) was obtained from Roger Brent (Department of Biochemistry, Harvard University). *E. coli* K12 strain JA221 (F$^-$ *hsdR* Δ*trpE5 recA1 leuB6 lacY*] was from Andrew Charles (Imperial Chemical Industries, Runcorn, U.K.). *E. coli* K12 strain JM101 [Δ(*lac-pro*) *supE thi*/F′ *traD36 proAB lacI*^{q}*Z*Δ*M15*] (11) was from Robin Wharton (Department of Biochemistry, Harvard University). *E. coli* K12 strain CSR603 [F$^-$ *thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uvrA6 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 supE44 gyrA48*] (12, 13)

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[1] The abbreviation used is: SRP, signal recognition particle.

[2] TEM specifies the source of the β-lactamase (57).

was from Barbara Bachmann (Yale University). *E. coli* K12 strain SE6004 [F⁻ *araD139 Δ(lac)U169 rpsL relA thi lamBS60 prlA4*] (14) was from John Schultz (Frederick Cancer Research Center, MD). M13ss1 and its derivatives are described elsewhere (9).

General Methods—Bacterial growth was performed according to Miller (15). Recombinant DNA techniques were carried out essentially as described by Maniatis *et al.* (16). DNA sequencing was performed by the chain-termination method of Sanger *et al.* (17) using M13 virion DNA as the template and the synthetic oligonucleotide 5′-AATACATTCAAATATGTAT (which is complementary to a region upstream of the *bla* initiation codon) as the primer. Plasmid DNA was isolated by the method of Birnboim and Doly (18) as modified by Ish-Horowicz and Burke (19). Transformation of *E. coli* with plasmid DNA was carried out by the method of Dagert and Ehrlich (20). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed by the procedure of Laemmli (21). Estimation of the intensities of bands in autoradiograms was carried out by scanning densitometry. Wild-type and mutant pre-β-lactamases were synthesized *in vitro* from plasmid DNA essentially as described by Chen and Zubay (22) by courtesy of Toni Gautier (Biogen S. A.).

Determination of LD_{50}—Overnight cultures of RB791 derivatives (grown in YT medium (15) containing tetracycline (20 μg/ml)) were diluted 10^5-fold in fresh YT medium. Samples (50 μl) were added to YT medium (2 ml) containing varying amounts of ampicillin (Na^+ salt). These cultures were incubated at 37 °C with shaking until the cells had reached the log phase (A_{550nm} = 0.3–0.5) (incubations containing no ampicillin reached an A_{550nm} of roughly 0.4 in 7 h). The values of A_{550nm} were plotted against ampicillin concentration, and the LD_{50} was determined (as the concentration of ampicillin that inhibits the growth of the culture by 50%) by interpolation.

Measurement of β-Lactamase Activity—Assays of β-lactamase activity were performed in 100 mM potassium phosphate buffer, pH 7.1, using Nitrocefin (compound 87/312) as substrate and monitoring the rate of increase in the absorbance at 482 nm (23).

Cell Fractionation—Spheroplasts were prepared from stationary phase bacteria (transformed RB791) by slight modification of the method of Witholt *et al.* (24) as follows. All operations were performed at 4 °C. An overnight culture (0.5 ml) was centrifuged at 12,800 × *g* for 45 s, and the supernatant was discarded. The cells were suspended in 100 mM Tris-HCl buffer, pH 8.0, containing 0.5 mM EDTA and 0.5 M sucrose (0.25 ml). A freshly prepared solution (10 μl) of lysozyme (1.5 mg/ml in H_2O) was added, and the cells were mixed gently. Water (0.24 ml) was then immediately added. The cells were gently mixed and then allowed to stand for 20 min. The spheroplasts were pelleted at 12,800 × *g* for 30 s, and the supernatant was saved as the periplasmic fraction. The pellet was vigorously suspended in 10 mM Tris-HCl buffer, pH 8, containing 1 mM EDTA (0.5 ml) to lyse the spheroplasts. This suspension was the lysed spheroplast fraction. Strain RB791 (pTG2*del*1), the β-lactamase of which is entirely cytoplasmic (8), and RB791 (pTG2), the β-lactamase of which is entirely periplasmic, were used as controls.

Maxicell Technique—The maxicell procedure of Sancar *et al.* (13) (as modified by Roberts *et al.* (25)) was followed with the exception that 1 mg/ml uridine was included in the M9 casamino acids growth medium. The addition of uridine to the culture medium results in an enhancement of the ^{35}S-labeled plasmid-encoded proteins relative to the ^{35}S-labeled chromosomal proteins (8).

Immunoprecipitation—β-Lactamase was purified from ^{35}S-labeled cultures by immunoprecipitation using a slight modification of the procedure of Oliver and Beckwith (26). Unless indicated otherwise, all operations were performed at 4°C. Cells (1 ml of a late log culture grown in M9 medium containing glycerol (0.4%, v/v), adjusted to a density of 1 A_{550nm} unit/ml) were treated with L-[^{35}S]methionine (100 μCi/ml of culture) for 30 min at 37 °C. The cells were then pelleted at 12,800 × *g* for 2 min, and the supernatant was discarded. The cells were washed with 10 mM Tris-HCl buffer, pH 8.0, and then suspended in 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 1% (w/v) sodium dodecyl sulfate (50 μl). This mixture was heated to 95 °C for 2 min and subsequently placed on ice. A solution (1 ml) of 50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 1 mM EDTA, and 2% (v/v) Triton X-100 was added to lysed cells. Phenylmethylsulfonyl fluoride (10 μl of a freshly prepared 100 mM solution in EtOH) was then added to the mixture, and the solution was mixed by vortexing. Cell debris was removed by centrifugation at 12,800 × *g* for 15 min followed by transfer of the supernatant to a fresh test tube. Anti-β-lactamase antiserum (5 μl) was then added, and the resulting solution was mixed and allowed to stand overnight. A 10% (w/v) suspension of IgGsorb (formalin-fixed and heat-treated *Staphylococcus aureus*) was then added to the solution, and the mixture was incubated on ice for 30 min. The *S. aureus* cells were pelleted by centrifugation at 12,800 × *g* for 2 min, and the supernatant was discarded. The cells were washed twice with 50 mM Tris-HCl buffer, pH 7.6, containing 500 mM NaCl and 1% (v/v) Triton X-100 (0.6 ml); twice with 50 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl and 1% (v/v) Triton X-100 (0.6 ml); and then suspended in 10 mM Tris-HCl buffer, pH 8.0 (0.6 ml). This mixture was transferred to a fresh test tube. The cells were pelleted by centrifugation at 12,800 × *g* for 2 min, and the supernatant was discarded. The pellet was suspended in gel electrophoresis buffer (100 μl of 62.5 mM Tris-HCl buffer, pH 6.8, containing 3% (w/v) sodium dodecyl sulfate, 5% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol). The resulting mixture was then heated to 95 °C for 5 min, chilled on ice, and centrifuged at 12,800 × *g* for 5 min. A portion (typically, 10 μl) of the supernatant was used for gel electrophoresis.

RESULTS

Construction of pTG2 Derivatives That Contain Mutations in the bla Signal Codons—The nucleotide substitutions in the *bla* signal codons were generated in M13ss1, a M13-derived phage that contains the *bla* gene from pTG2 (9). The β-lactamase signal sequence codons (from Arg 2 to His 24) were then transferred from the mutated M13ss1 derivatives into pTG2, as shown in Fig. 1, and the nucleotide substitutions in the resulting pTG2 derivatives were confirmed by sequencing. Both strands of the signal codons of each mutant *bla* allele were thus sequenced. The transfer of the signal codons ensures that any phenotypic differences between the strains carrying pTG2 and the mutant plasmids derive *only* from substitutions in the *bla* signal sequence. The nucleotide and amino acid substitutions in the mutant *bla* signal sequences are listed in Table I.

Relative β-Lactamase Activities in Lysates of Strains Containing Wild-type or Mutant bla Alleles—The β-lactamase activity per A_{550nm} unit of culture was determined for French press lysates of bacteria in late log phase and compared with the enzyme activity in lysates of cells harboring the wild-type *bla* gene. These results are presented in Table II. The level of expression of the mutant *bla* alleles is the same whether SE6004 (a *prlA4* strain) or RB791 (a $PrlA^+$ strain) is the host (see Table II). Strains containing the m15, m17, and m63 alleles accumulate significant amounts of pre-β-lactamase, but since pre-β-lactamase possesses about 30% of the specific catalytic activity of mature β-lactamase (see below), the results in Table II indicate that the total number of β-lactamase molecules is much lower than the wild type in several of the mutants. Furthermore, this decrease in the expression of β-lactamase is unaffected by the use of a host strain containing the *prlA4* mutation, which has been shown to suppress the mutant phenotypes of altered *lamB*, *malE*, and *phoA* signal sequences (14, 27, 28). The LD_{50} values for ampicillin for cells containing either wild-type or mutant plasmids are also given in Table II. Comparison of these LD_{50} values with the relative β-lactamase activities per A_{550nm} unit of culture (Fig. 2) reveals that the LD_{50} values reflect the total amount of β-lactamase activity produced by the cell.

In Vitro Synthesis of Mutant Pre-β-Lactamases—To see if any of the mutations affects the transcription and translation of the *bla* gene *in vitro*, the pre-β-lactamases encoded by the wild-type, m14, m15, m17, m18, and m63 *bla* alleles were synthesized by using the supernatant from a 30,000 × *g* centrifugation of an *E. coli* lysate. The results are summarized in Table II. There is no significant difference in the amount of pre-β-lactamase obtained from pTG2, pM14, pM15, pM17, or pM18 plasmid DNA. Evidently the steady state concentra-

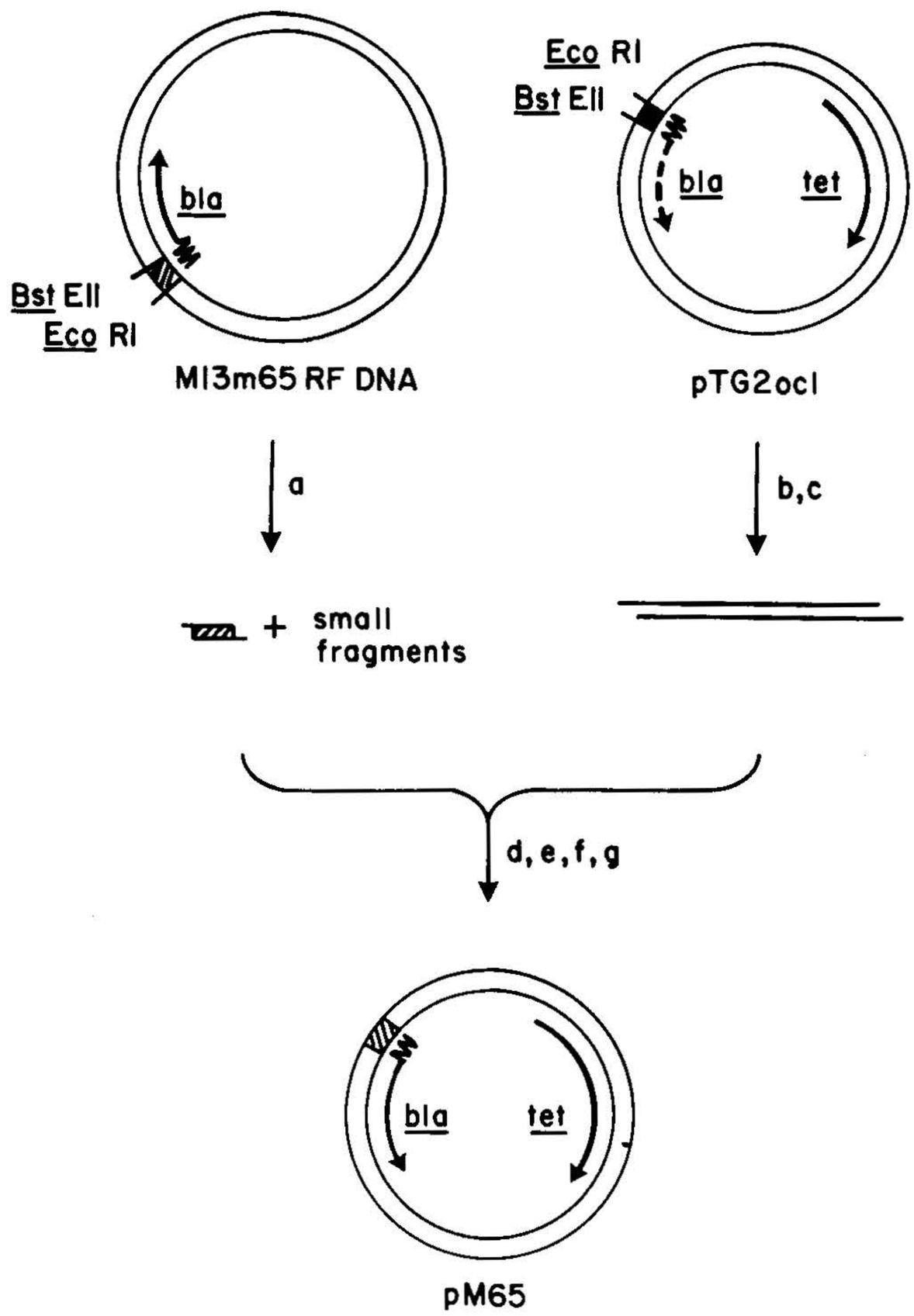

FIG. 1. **Transfer of mutations in the *bla* signal codons from derivatives of M13ss1 to pTG2.** *a*, *Eco*RI, *Bst*EII, and *Hae*III; *b*, *Eco*RI and *Bst*EII; *c*, gel purification of large fragment; *d*, T4 DNA ligase and ATP; *e*, transformation of *E. coli* K12 strain JA221 and selection for Tet^R; *f*, isolation of crude plasmid DNA, transformation of *E. coli* K12 strain RB791, and selection for Tet^R; *g*, DNA sequencing of region containing *bla* signal codons. As an example, the transfer of the m65 mutation from M13m65 (a derivative of M13ss1 that contains the m65 mutation) to pTG2 is shown. The resulting recombinant plasmid is named pM65. The plasmid pTG2oc1 is identical to pTG2 except for a TTT (Phe 17) to TAA (ochre) mutation in the *bla* signal codons. The small *Eco*RI-*Bst*EII fragment that is transferred from M13 replicative form DNA to pTG2 ranges from the Arg 2 codon to the Pro 25 codon of the *bla* gene.

tion of β-lactamase from pM14, pM15, and pM17 is lowered *in vivo* but there is no difference in the synthesis *in vitro*.

Cellular Localization of β-Lactamase Activity—Bacteria were grown to stationary phase before spheroplasting and separation of the intact spheroplasts from the periplasmic fraction. The β-lactamase activity in the lysed spheroplast fraction and in the periplasmic fraction was then measured. More than 96% of the total enzyme activity was present in the periplasmic fraction, with the exception of strains carrying pM15, pM17, pM20, and pM63, for which 5–25% of the enzyme activity appeared in the lysed spheroplast fraction. Since the specific catalytic activity of pre-β-lactamase is about 30% of that of the mature β-lactamase (see below), the location of both processed and unprocessed forms of β-lactamase is detected in these experiments. These cell fractionation results must be interpreted with caution, however, since such data can be misleading, especially for strains bearing unnatural proteins (29).

Processing of Mutant Pre-β-lactamases—The proteolytic processing of the pre-β-lactamases encoded by the mutant *bla* alleles was analyzed by using the maxicell technique, as shown in Fig. 3. It is evident that the processing of the wild-type β-lactamase from pTG2 is highly efficient (>99%) in both RB791 and the irradiated maxicell strain (CSR603). Those signal sequence mutants that were difficult to visualize (due to low expression) or that accumulated largely in the precursor form were analyzed by immunoprecipitation of the ^{35}S-labeled proteins. The results are shown in Fig. 4, from which it is clear that the β-lactamase precursors derived from the m17 and m63 alleles are only partially processed (70 and 40%, respectively) and the pre-β-lactamase encoded by the m15 allele is barely, if at all, cleaved (<5%).

Specific Activity of Pre-β-lactamase—Since a significant amount of the β-lactamase precursor is observed in several of the mutants, the specific catalytic activity of pre-β-lactamase was estimated. Previous attempts to compare the catalytic activity of pre-β-lactamase relative to that of mature β-lactamase have resulted in estimates ranging from 60% (30) to 5% or less (31). Examination of Fig. 4 shows that the amount of pre-β-lactamase in RB791(pM15), which contains only pre-β-lactamase, is roughly the same as the amount of mature β-lactamase in a similar number of cells carrying pM53, which contain only mature β-lactamase. (The mutant plasmid pM53 has four base changes in the signal sequence (which is why it is not included in Tables I and II): Ala 9 to Val (GCC to GTC), Pro 12 to Leu (CCC to CTC), Val 21 to Ile (GTT to ATT), and Phe 6 to Phe (TTC to TTT).) The β-lactamases were labeled with [^{35}S]methionine, and since pre-β-lactamase contains 10 Met residues and mature β-lactamase contains 9 Met residues, the similar intensities of the β-lactamase bands produced by pM15 and pM53 indicate a roughly similar number of β-lactamase molecules. Assuming that the pre-β-lactamase encoded by pM15 has the same specific catalytic activity as the wild-type pre-β-lactamase and that the mature β-lactamase from pM53 has been correctly processed, the specific catalytic activity of pre-β-lactamase relative to the specific catalytic activity of mature β-lactamase can be estimated by comparison of the total enzyme activities in lysates of similar amounts of RB791(pM15) and RB791(pM53). The β-lactamase activity per A_{550nm} unit of RB791(pM15) was 33% of the activity per A_{550nm} unit of RB791(pM53). We may thus conclude that the specific catalytic activity of pre-β-lactamase in the periplasm is about 30% that of the mature enzyme. This result does not, of course, provide any information about the catalytic activity of pre-enzyme or of mature enzyme, in the cytoplasm.

Growth Defects—Strains that contain the alleles m3, m8, m9, m14, m15, m17, m20, m52, or m63 grow more slowly (in YT medium, 37 °C) than strains carrying the wild-type *bla* gene (see Table II). The most severe growth defects were observed in bacteria containing pM17 or pM63, and stationary phase cultures of RB791 carrying these plasmids have less than half the cell density of the wild type RB791(pTG2).

DISCUSSION

The 15 plasmids listed in Tables I and II that encode a complete but mutated signal sequence fall into three groups. First, the changes in m7, m13, m16, m18, m61, and m65 do not significantly affect the host cell growth rate, and the expression of the mature periplasmic β-lactamase is of the same order as (that is, at least 25% of) that of the wild type. Although the consequential changes in the amino acid sequence of the signal peptide in these mutants are often functionally conservative (His 5 to Tyr, Val 8 to Ile, Ala 9 to Val, Leu 10 to Phe, Pro 12 to Leu or Ser, Ala 15 to Val, Ala

TABLE I

Signal sequence mutants of the bla gene

The amino acid sequence of the wild-type *bla* signal sequence is as follows: Met 1-Arg 2-Ile 3-Gln 4-His 5-Phe 6-Arg 7-Val 8-Ala 9-Leu 10-Ile 11-Pro 12-Phe 13-Phe 14-Ala 15-Ala 16-Phe 17-Cys 18-Leu 19-Pro 20-Val 21-Phe 22-Gly 23-.

Plasmid[a]	Allele	Amino acid substitutions[b]	Other nucleotide[c] substitutions
pTG2	Wild-type		
pTG2oc1[d]	oc1	Phe 17 to ochre (TTT to TAA)	
pM3	m3	Ala 9 to Thr 9 (GCC to ACC)	
pM7	m7	Val 8 to Ile 8 (GTC to ATC)	
pM8	m8	Arg 7 to His 7 (CGT to CAT)	
pM9	m9	Ala 15 to Thr 15 (GCG to ACG)	
pM13	m13	Ala 9 to Val 9 (GCC to GTC)	TTC to TTT (Phe 6)
		Leu 10 to Phe 10 (CTT to TTT)	
		Pro 12 to Leu 12 (CCC to CTT)	
		Ala 15 to Val 15 (GCG to GTG)	
pM14	m14	Val 21 to Ile 21 (GTT to ATT)	
pM15	m15	Pro 12 to Ser 12 (CCC to TCC)	
		Pro 20 to Phe 20 (CCT to TTT)	
pM16	m16	Ala 16 to Thr 16 (GCA to ACA)	
pM17[e]	m17	Pro 20 to Ser 20 (CCT to TCT)	
pM18	m18	Pro 12 to Ser 12 (CCC to TCC)	
		Leu 19 to Phe 19 (CTT to TTT)	
pM20	m20	Arg 7 to His 7 (CGT to CAT)	
		Val 21 to Ile 21 (GTT to ATT)	
pM52	m52	Val 8 to Ile 8 (GTC to ATC)	
		Val 21 to Ile 21 (GTT to ATT)	
pM61	m61	His 5 to Tyr 5 (CAT to TAT)	TTC to TTT (Phe 6)
pM63	m63	Arg 7 to His 7 (CGT to CAT)	
		Cys 18 to Tyr 18 (TGC to TAC)	
pM65	m65	Leu 10 to Phe 10 (CTT to TTT)	GCC to GCT (Ala 9)
			CCC to CCT (Pro 12)

[a] All plasmids are identical to pTG2 except for the indicated nucleotide substitutions. Both strands of the region containing the *bla* signal codons were sequenced.

[b] The codons corresponding to the amino acid substitutions are given in parentheses.

[c] The codons of the nucleotide substitutions that do not result in amino acid substitutions are listed. The unchanged amino acid residues are given in parentheses.

[d] The plasmid pTG2oc1 is identical to pTG2 except for a TTT (Phe 17) to TAA (ochre) mutation in the *bla* signal codons. Strains harboring pTG2oc1 were used as a Bla⁻ control.

[e] A similar CCT (Pro 20) to TCT (Ser 20) mutation in the signal codons of the *bla* gene of pBR322 has been isolated by Koshland *et al.* (58).

16 to Thr, and Leu 19 to Phe), this group emphasizes what has become evident from the diversity of signal sequences: that the "signal" is not a very precise one. Even pM13, which encodes a signal peptide having four amino acid differences from pTG2, does not generate a notably different phenotype.

Second, there is a group of signal codon mutations that result in slower growth of the host cell and a higher (m9), normal (m3, m8), or lower (m14, m20, m52) level of β-lactamase expression. Changes in the amount of β-lactamase per cell can, of course, have a number of causes that are unrelated to the secretion of the enzyme. First, a decrease in the efficiency of translation initiation by a direct interaction of the message with the ribosome could affect expression, but such effects are only likely for changes in the first dozen or so bases of the message (32), and none of these mutants contain changes so early in the translated region. Second, low expression could be the consequence of introducing a rare codon. This is unlikely: none of the new codons created by our mutations is uncommon in the coding sequences of *E. coli* proteins (33). Indeed, one of the least common is AGA, which is the Arg 2 codon of pTG2, from which β-lactamase is expressed at levels undetectably different from that produced by the parental plasmid pBR322. Third, changes in β-lactamase expression could be due to alterations in the lifetime of the mRNA or of the mutant unprocessed pre-enzyme molecules. (As a check on the latter possibility, the production of β-lactamase from the wild-type plasmid pTG2 and from the mutant pM14 was investigated at 37 °C using a short (30 s) pulse of [^{35}S]methionine followed by a chase of unlabeled methionine for 10, 60, 90, and 180 s. The intensities of the β-lactamase bands were entirely consistent with the measured steady state levels of enzyme activity (Table II), which eliminates the possibility that the lower expression of enzyme in strains carrying pM14 is due to a combination of a secretion defect and an unstable mutant pre-β-lactamase.) Fourth, the synthesis of β-lactamase could be affected by the stability of some mRNA secondary structure that affects the efficiency of translation. Certainly, for several of the mutants whose expression of β-lactamase is most severely affected (*e.g.* m15, m17), an increase in the predicted thermodynamic stability of a possible mRNA loop structure (34) can qualitatively account for the lower levels of enzyme. The difficulty is, of course, that the relationship between predicted loop stabilities in mRNA and the expression level of the translated product is not known, and only in the most extreme cases can one be confident that such a relationship is important. In general, it is not possible to relate changes in the level of expression of an exported protein to the phenomenon of secretion. Indeed, the fact that mutations that cause a severe reduction in the *in vivo* expression of the β-lactamase (*e.g.* m14, m15, m17) still generate normal levels of the pre-enzyme in an *in vitro* cell-free system does not require that translation and secretion are coupled events. The absence of any clear correlation between the expression levels even of *non*-secreted proteins

TABLE II

β-Lactamase production by cells carrying wild-type or mutant plasmids

Plasmid[a]	Activity[b]	Activity[c]	LD_{50} for ampicillin[d]	*In vitro* synthesis of β-lactamase[e]	Growth rate[f]
	%	%	*mg/ml*	%	
pTG2	(100)	(100)	4	(100)	+
pTG2ocl	0.15	0.14	0.01		−
pM3	95		4		−
pM7	94		4		+
pM8	74		3		−
pM9	169		6		−
pM13	39		1.5		+
pM14	13	10	0.5	100	−
pM15	0.24	0.13	0.05	100	−
pM16	95		4		+
pM17	6.8	7.8	0.5	100	=
pM18	76		3	100	+
pM20	7.2	5.9	0.5		−
pM52	15	10	0.5		−
pM61	35		1.5		+
pM63	3.1	2.1	0.1	40	=
pM65	28		1.5		+

[a] The *bla* signal sequence mutations in the plasmids are listed in Table I.

[b] Bacteria were grown in YT medium to the late log phage ($A_{550\ nm} \cong 0.8$). The host strain was RB791. Per cent β-lactamase activity is relative to RB791(pTG2). Each value is the average of two or three independent measurements. These values have been corrected for the small amount (~0.15% of RB791(pTG2)) of β-lactamase activity arising from the chromosomal AmpC enzyme.

[c] The host strain was SE6004, which contains the *prlA4* mutation (14). All of the SE6004 derivatives were checked for the *prlA4* allele by testing for phage λ sensitivity on maltose minimal agar plates (27). The per cent β-lactamase activities are given relative to SE6004(pTG2). Each value is the average of two independent measurements.

[d] The LD_{50} for ampicillin (Na^+ salt) was estimated for RB791 containing either pTG2 or a mutant derivative of pTG2. The values were obtained from two independent measurements. The LD_{50} is defined as the concentration of ampicillin that causes 50% inhibition in the growth of the strain.

[e] The intensities of the pre-β-lactamase bands in the autoradiograms (obtained by Dr. Toni Gautier) were determined by scanning densitometry.

[f] The host strain was RB791. Bacteria were grown at 37 °C with shaking in YT medium. The growth rates of the strains were measured using RB791(pTG2) as a reference. +, normal growth; −, slow growth (*i.e.* more than two standard deviations slower than wild type); =, very slow growth.

in vivo and *in vitro* precludes any conclusion from differences in the *in vivo* and *in vitro* levels of synthesis. The expression level notwithstanding, this second group of mutants all show growth defects compared with cells containing the wild-type *bla* gene. Strains that synthesize proteins defective in secretion commonly display abnormal growth behavior (see, for example, Refs. 27 and 35–38), and it is unreasonable to suppose that merely reducing the synthesis of a nonessential enzyme such as β-lactamase by some modulation of transcription or translation of the *bla* gene could reduce the cell's growth rate. It is more likely that the alterations in the signal codons interfere with the normal secretion of the β-lactamase and cause a pleiotropic block in the secretion of other, essential, exported proteins. A mutation in the signal sequence of β-lactamase may result in the preoccupation of one or more elements of the secretory machinery and a reduction in the growth rate of the cell. The fact that the expression level of β-lactamase is often concomitantly lowered is consistent with a coupling of translation and secretion (39, 40), but there are (as discussed above) several other possible causes for altered

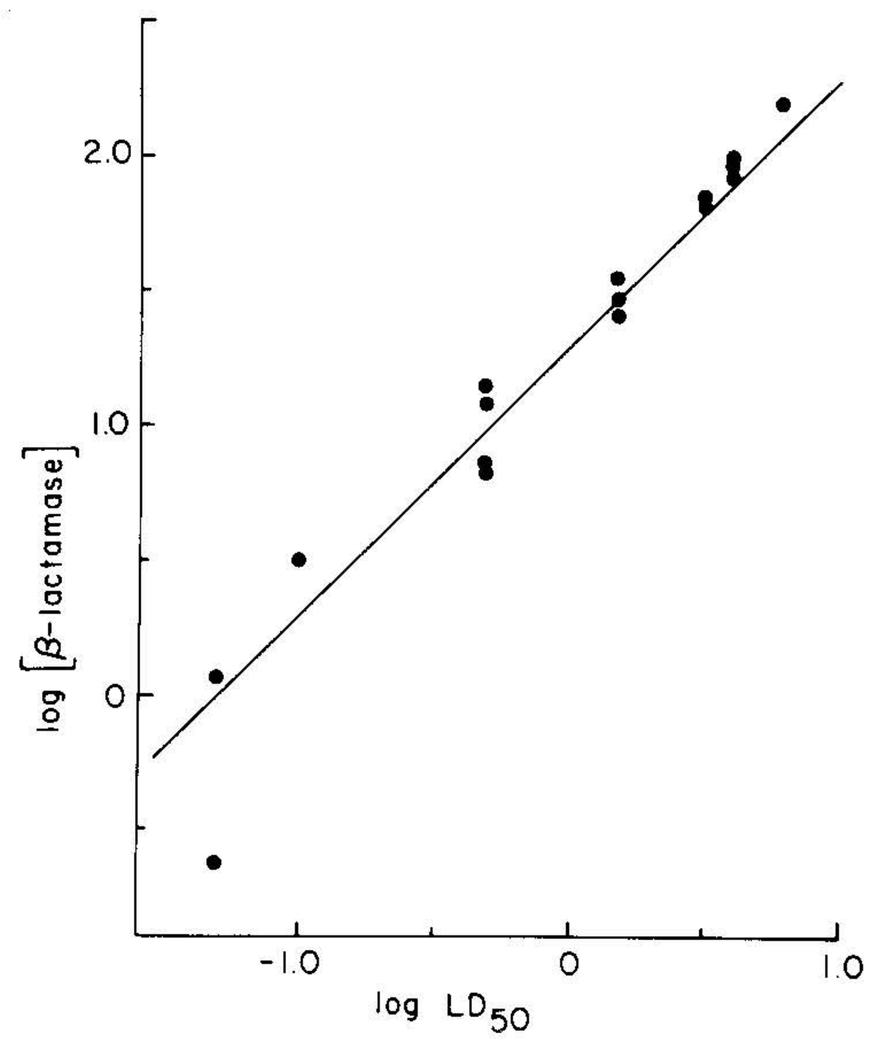

FIG. 2. **Correlation between the total β-lactamase activity and the LD_{50} for transformants carrying the plasmids listed in Table I.**

expression levels. Indeed, we have found examples of lowered β-lactamase expression without effects on growth (*e.g.* pM65) and of effects on growth with no change in expression levels (*e.g.* pM3).

The third group of signal sequence mutants (m15, m17, and m63) shows the most interesting phenotype, in that, as well as causing slower cell growth and a lower level of β-lactamase, these mutants make abnormal amounts (~30 to <95%) of the *pre*-enzyme (Fig. 4). Each of these mutants has a nonconservative amino acid change near the processing site (Pro 20 to the Phe or Ser, Cys 18 to Tyr) and can be compared with other less drastic changes in this region (Ala 15 to Thr or Val, Ala 16 to Thr, Leu 19 to Phe, Val 21 to Ile) that have no detectable effect on the processing of the pre-enzyme. In strains carrying pM15, *no* mature β-lactamase can be observed (Fig. 4), and this processing defect is evidently due to the change of Pro 20 to Phe (the second alteration, of Pro 12 to Ser, is unimportant, see m18). Now, it appears both from the cell fractionation experiments and (more securely) from the correlation shown in Fig. 2 that the β-lactamase activity is all periplasmic. That is, since only periplasmic β-lactamase can protect a cell against the antibiotic action of a β-lactam (cytoplasmic β-lactamase is entirely ineffective: see Ref. 8), the correlation between the total amount of β-lactamase activity and the LD_{50} (Fig. 2) means that all the β-lactamase activity produced by all the mutants is available for the cell's defense. So even the pre-enzyme produced by m15 (Fig. 4) is active in the periplasm. We must therefore conclude that signal sequence cleavage is *not* necessary for translocation. This view is in agreement with the recent findings of Müller and Blobel (41) who have found that some precursor species can be translocated into inverted plasma membrane vesicles from *E. coli* in a cell-free system, and with earlier observations of signal sequence mutants that affect processing but not transport (3).

The phenotypic behavior of the three classes of mutant obtained in this work is nicely accommodated by a secretory mechanism for bacteria that is the functional analogue of that defined in eucaryotes. Recent results from Blobel's laboratory (41, 42) suggest the existence of a soluble activity that is required for the export of secreted proteins from *E. coli*, and Beckwith and co-workers (39, 43) have implicated several

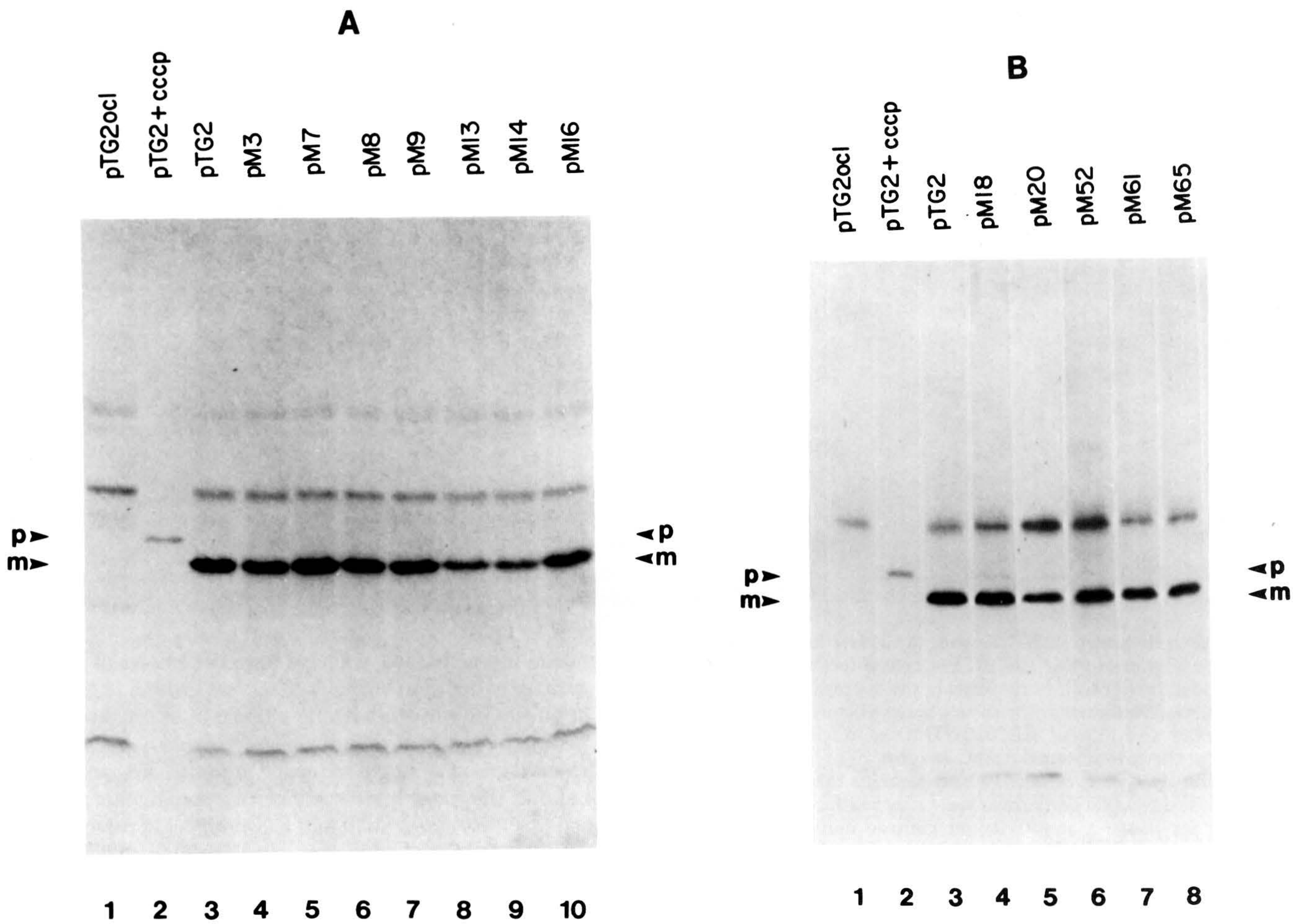

FIG. 3. **Analysis of β-lactamase signal sequence mutants by the maxicell technique.** Irradiated cells (CSR603, containing the plasmids specified below) were treated with L-[^{35}S]methionine (8 μCi/ml of culture) for 1 h at 37 °C, lysed, and subsequently electrophoresed on 12.5% polyacrylamide gels in the presence of sodium dodecyl sulfate, as described under "Experimental Procedures." *A*: *lane 1*, pTG2oc1 (Bla$^-$ control); *lane 2*, pTG2 (^{35}S-labeled for 20 min at 22 °C in the presence of 0.2 mM carbonyl cyanide *m*-chlorophenylhydrazone (*cccp*) to yield pre-β-lactamase (59, 60)); *lane 3*, pTG2 (wild-type *bla* control); *lane 4*, pM3; *lane 5*, pM7; *lane 6*, pM8; *lane 7*, pM9; *lane 8*, pM13; *lane 9*, pM14; *lane 10*, pM16. *B*: *lane 1*, pTG2oc1 (Bla$^-$ control); *lane 2*, pTG2 (as *lane 2* of *A*); *lane 3*, pTG2; *lane 4*, pM18; *lane 5*, pM20; *lane 6*, pM52; *lane 7*, pM61; *lane 8*, pM65. The exposure time of *lanes 5* and *6* is about twice that of the other *lanes*. The migration positions of pre-β-lactamase (*p*) and mature β-lactamase (*m*) are indicated by *arrowheads*.

gene products that may be components of a procaryotic secretory apparatus. A similar mechanism for protein translocation across the *E. coli* inner membrane and the endoplasmic reticulum of eucaryotic cells is also supported by studies on the export of TEM β-lactamase in eucaryotic systems. For example, TEM β-lactamase is secreted and processed in both *Xenopus laevis* oocytes (44) and *Saccharomyces cerevisiae* (45, 46) and is translocated into dog pancreas microsomes by a SRP-dependent mechanism (47). One additional feature is needed for procaryotic secretion, however, to account for the fact that some proteins are translocated post-translationally (29, 48–50), whereas others appear to be secreted cotranslationally (51–54). These findings can be explained if the pre-protein can be synthesized in a secretion-competent form on the cytoplasmic side of the inner membrane, this secretion-competent position being inaccessible to a soluble pre-protein in the cytoplasm. Whether a secreted protein appears to be translocated co- or post-translationally would then depend upon the relative rates of translation (into the secretion-competent location) and of translocation (from it, across the membrane).

According to this model of protein secretion, signal sequence recognition is required both by the soluble SRP-like entity and by the processing peptidase. If, by analogy with the eucaryotic model, the binding of the SRP-like entity to the emerging signal peptide causes a translation arrest, mutations in the signal sequence that interfere with this binding will prevent the arrest and result in the accumulation of pre-protein in the cytoplasm. Several such mutants have been obtained (see, for example, Refs. 27, 35, 55, and 56), and they commonly involve the introduction of a charged amino acid residue in the hydrophobic core of the signal. The signal sequence must also be recognized by the processing enzyme, and we may expect to find signal sequence mutations that are permissive in terms of recognition by the SRP-like entity and of the translocation apparatus, but that block or slow down maturation by the signal peptidase. Such mutations would allow the secretion of pre-enzyme into the periplasm (although it may remain anchored by the uncleaved signal to the outer face of the inner membrane). The proteins of m15, m17, and m63 appear to be of this type. Finally, there may exist a class of mutants for which the translation arrest is not re-

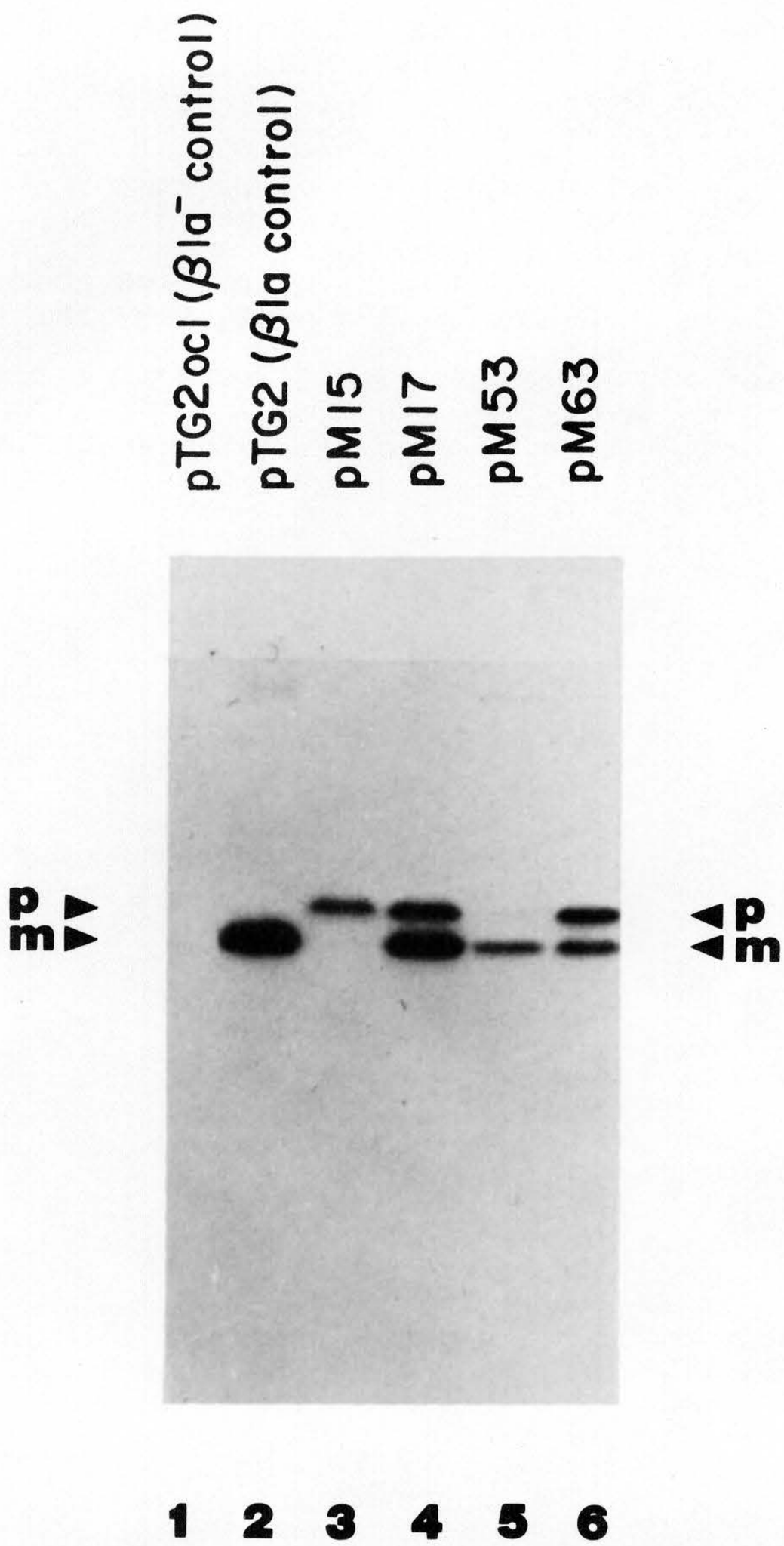

FIG. 4. **Immunoprecipitation of β-lactamase signal sequence mutants.** Labeled cell lysates were immunoprecipitated with anti-β-lactamase antiserum as described under "Experimental Procedures." The resulting ^{35}S-labeled β-lactamase samples were electrophoresed on a 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate. Strain RB791 was the host, carrying the plasmids specified: *lane 1*, pTG2oc1 (Bla$^-$ control); *lane 2*, pTG2 (wild-type *bla* control); *lane 3*, pM15; *lane 4*, pM17; *lane 5*, pM53; *lane 6*, pM63. The migration positions of pre-β-lactamase (*p*) and mature β-lactamase (*m*) are indicated by *arrowheads*.

leased, or is only released slowly. Such mutations would presumably result in low expression of the secreted protein, possibly coupled with a lowered growth rate of the host cell. Several of our mutants (*e.g.* m14, m20, m52) show this behavior.

In summary, the behavior of 15 signal sequence mutants of the *bla* gene is consistent with a mechanism for protein export that is analogous to that proposed for eucaryotic systems. Translation and translocation (or the achievement of a secretion-competent location) may be coupled, whereas the processing of the pre-enzyme is not necessarily linked to its passage across the membrane.

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