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 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$^*$), 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 conglomrate (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$^*$ β-lactamase and studies of the phenotypic consequence of these changes. First, a derivative of pBR322 was constructed that contains a unique EcoRI restriction site in the beginning and a unique BstEI 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 methotrexamine (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’-[β-32P]triphosphate (800 Ci/mmOL) and 1-[35S]methionine (1000 Ci/mmOL) were obtained from Amersham Corp. Nitrocefin was purchased from BBL Microbiology Systems (Cockeysville, MD). IgG sorb was obtained from The Enzyme Center (Malden, MA). Culture media were prepared from the products of Difco. The oligonucleotide 5’-AACTACATTCAAATATGTAT 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 lac*P*L8] (10) was obtained from Roger Brent (Department of Biochemistry, Harvard University). *E. coli* K12 strain JA221 (F+ hsdR ΔtrpE5 recA1 leuB6 lacY) was from Andrew Charles (Imperial Chemical Industries, Runcorn, U.K.). *E. coli* K12 strain JM101 (lac-pro supE thi-1 TraD36 proA8 lacZΔM15) (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 mit-1 rpsL31 tsx-33 supE44 gyrA48] (12, 13) TEM specifies the source of the β-lactamase (57).
Lactamase was purified from 35S-labeled Lactamase Activities in Lysates of Strains carried out by scanning densitometry. Wild-type and mutant gDNA was isolated by the method of Birnboim and Doly (18) as with plasmid DNA was carried out by the method of Dagert and using M13 virion DNA as the template and the synthetic oligonucleotide (a region upstream of the bia 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 Dagenet 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-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 LD₀—**Overnight cultures of RB791 derivatives (grown in YT medium (15) containing tetracycline (20 μg/ml)) were diluted 105-fold in fresh YT medium. Samples (50 μl) were added to YT medium containing varying concentrations of ampicillin (sodium salt). These cultures were incubated at 37°C with shaking until the cells had reached the log phase (A500nm = 0.3–0.5) (incubations containing no ampicillin reached an A500nm of roughly 0.4 in 7 h). The values of A500nm were plotted against ampicillin concentration, and the LD₀ 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 carried out by the method of Witholt et al. (26). Unless indicated otherwise, any of the mutations affects the transcription and synthesis of the bla gene, which has been shown to suppress the mutant phenotype of altered lamB, malE, and phoA signal sequences (14, 27, 28). The LD₀ values for ampicillin for cells containing either wild-type or mutant plasmids are also given in Table II. Comparison of these LD₀ values with the relative β-lactamase activities per A₅₀₀ₙₙ unit of culture (Fig. 2) reveals that the LD₀ values reflect the total amount of β-lactamase activity produced by the cell.

**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 A₅₀₀ₙₙ 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 PriA⁺ 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 pre-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 LD₀ values for ampicillin for cells containing either wild-type or mutant plasmids are also given in Table II. Comparison of these LD₀ values with the relative β-lactamase activities per A₅₀₀ₙₙ unit of culture (Fig. 2) reveals that the LD₀ 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-
The m65 mutation from M13m65 (a derivative of M13ssl that contains plasmid is named pM65. The plasmid pTG2ocl is identical to pTG2.

The small EcoRI-BstEII fragment that is transferred from M13 replicative form DNA to pTG2 ranges from the Arg 2 codon to 25 codon of the bla gene. Since the specific catalytic activity of pre-blacmase is lowered from pM14, pM15, and pM17 is lowered but there is no difference in the synthesis in vitro.

**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 35S-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 35S-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 A500nm unit of RB791(pM15) was 33% of the activity per A500nm unit of RB791(pM53). We may thus conclude that the specific catalytic activity of pre-β-lactamase in the periplasm is about 30% of 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...
Signal Sequence Mutants of $\beta$-Lactamase

The amino acid sequence of the wild-type $\beta$-lactamase 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.

**TABLE I**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Allele</th>
<th>Amino acid substitutions</th>
<th>Other nucleotide substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTG2</td>
<td>Wild-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTG2oc1</td>
<td>oc1</td>
<td>Phe 17 to ochre (TTT to TAA)</td>
<td>TTC to TTT (Phe 6)</td>
</tr>
<tr>
<td>pM5</td>
<td>m5</td>
<td>Ala 9 to Thr 9 (GCC to ACC)</td>
<td></td>
</tr>
<tr>
<td>pM7</td>
<td>m7</td>
<td>Val 8 to Ile 8 (GTC to ATC)</td>
<td></td>
</tr>
<tr>
<td>pM8</td>
<td>m8</td>
<td>Arg 7 to His 7 (CGT to CAT)</td>
<td></td>
</tr>
<tr>
<td>pM9</td>
<td>m9</td>
<td>Ala 15 to Thr 15 (GCG to ACG)</td>
<td></td>
</tr>
<tr>
<td>pM13</td>
<td>m13</td>
<td>Ala 9 to Val 9 (GCC to GTC)</td>
<td></td>
</tr>
<tr>
<td>pM14</td>
<td>m14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM15</td>
<td>m15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM16</td>
<td>m16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM17</td>
<td>m17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM18</td>
<td>m18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM20</td>
<td>m20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM52</td>
<td>m52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM61</td>
<td>m61</td>
<td>His 5 to Tyr 5 (CAT to TAT)</td>
<td>TTC to TTT (Phe 6)</td>
</tr>
<tr>
<td>pM63</td>
<td>m63</td>
<td>Arg 7 to His 7 (CGT to CAT)</td>
<td>GCC to GCT (Ala 9)</td>
</tr>
<tr>
<td>pM65</td>
<td>m65</td>
<td>Cys 18 to Tyr 18 (TGC to TAC)</td>
<td>CCC to CCT (Pro 12)</td>
</tr>
</tbody>
</table>

* All plasmids are identical to pTG2 except for the indicated nucleotide substitutions. Both strands of the region containing the $\beta$-lactamase signal codons were sequenced.

* The codons corresponding to the amino acid substitutions are given in parentheses. The unchanged amino acid residues are given in parentheses.

* The plasmid pTG2oc1 is identical to pTG2 except for a TTT (Phe 17) to TAA (ochre) mutation in the $\beta$-lactamase gene. Strains harboring pTG2oc1 were used as a Bla" control.

* A similar CCT (Pro 20) to TCT (Ser 20) mutation in the signal codons of the $\beta$-lactamase 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, m52), normal (m3, m8), or lower (m14, m20, m62) level of $\beta$-lactamase expression. Changes in the amount of $\beta$-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 $\beta$-lactamase is expressed at levels undetectably different from that produced by the parental plasmid pBR322. Third, changes in $\beta$-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 $\beta$-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 $\beta$-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-$\beta$-lactamase.) Fourth, the synthesis of $\beta$-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 $\beta$-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 $\beta$-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
6-lactamase is often concomitantly lowered is consistent with 6-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 6-lactamase in the growth of the strain.

This view is in agreement with the recent findings of Miiller et al. (41, 42) suggest the existence of a soluble activity that is not detectable effect on the processing of the pre-enzyme. In strains carrying pm15, no mature 6-lactamase can be observed (Fig. 4), and this processing defect is evidently due to the change of Pro 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 6-lactamase activity is all periplasmic. That is, since only periplasmic 6-lactamase can protect a cell against the antibiotic action of a 6-lactam (cytoplasmic 6-lactamase is entirely ineffective; see Ref. 8), the correlation between the total amount of 6-lactamase activity and the LD50 (Fig. 2) means that all the 6-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

**Table II**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Activity</th>
<th>Activity</th>
<th>LD50 for ampicillin</th>
<th>In vitro synthesis of 6-lactamase</th>
<th>Growth rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTG2</td>
<td>(100)</td>
<td>(100)</td>
<td>4</td>
<td>(100)</td>
<td>+</td>
</tr>
<tr>
<td>pTG2ocl</td>
<td>0.15</td>
<td>0.14</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM5</td>
<td>95</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM7</td>
<td>94</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM8</td>
<td>74</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM9</td>
<td>169</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM13</td>
<td>39</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM14</td>
<td>13</td>
<td>10</td>
<td>0.5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>pM15</td>
<td>0.24</td>
<td>0.13</td>
<td>0.05</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>pM16</td>
<td>93</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM17</td>
<td>6.8</td>
<td>7.8</td>
<td>0.5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>pM18</td>
<td>76</td>
<td>3</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>pM20</td>
<td>7.2</td>
<td>5.9</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM52</td>
<td>15</td>
<td>10</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM61</td>
<td>35</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM63</td>
<td>3.1</td>
<td>2.1</td>
<td>0.1</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>pM65</td>
<td>28</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The bla signal sequence mutations in the plasmids are listed in Table 1.
* Bacteria were grown in YT medium to the late log phase (A600nm = 0.8). The host strain was RB791. Per cent 6-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 6-lactamase activity arising from the chromosomal AmpC enzyme.
* 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 sensitivities on maltose minimal agar plates (27). The per cent 6-lactamase activities are given relative to SE6004(pTG2). Each value is the average of two independent measurements.
* The LD50 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 LD50 is defined as the concentration of ampicillin that causes 50% inhibition in the growth of the strain.
* The intensities of the pre-6-lactamase bands in the autoradiograms (obtained by Dr. Toni Gautier) were determined by scanning densitometry.
* 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.

**Fig. 2. Correlation between the total 6-lactamase activity and the LD50 for transformants carrying the plasmids listed in Table 1.**

**Expression levels.** Indeed, we have found examples of lowered 6-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 6-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 6-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 6-lactamase activity is all periplasmic. That is, since only periplasmic 6-lactamase can protect a cell against the antibiotic action of a 6-lactam (cytoplasmic 6-lactamase is entirely ineffective; see Ref. 8), the correlation between the total amount of 6-lactamase activity and the LD50 (Fig. 2) means that all the 6-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
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 \( \beta \)-lactamase in eucaryotic systems. For example, TEM \( \beta \)-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-
leaked, 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 \( \beta \)-lactamase 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-envelope is not necessarily linked to its passage across the membrane.

REFERENCES


