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The Consequences of Stepwise Deletions from the Signal-processing Site of β-Lactamase*

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Andreas Plückthun‡ and Jeremy R. Knowles§
From the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

Amino acids have been deleted from the processing site of pre-β-lactamase, either into the signal sequence or into the mature protein. Whereas the loss of more than 2 amino acid residues from the *C*-terminal end of the signal sequence prevents the translocation of the protein into the periplasm, the removal of two or more amino acids from the beginning of the mature protein has no effect on the translocation of the truncated protein. The insertion of an additional one to three amino acids at the processing site has no detectable phenotypic consequence either. It appears that many sequences for the first few residues of the mature protein allow successful translocation and processing. In sharp contrast, the removal of one (but not both) of the amino acids that flank the processing site results in a severe growth defect in the host cell and very low expression of the protein. Yet removal of *two* amino acids from either side of the processing site, or removal of *both* the flanking residues of the processing site, results in normal secretion and signal cleavage. These results illustrate the limits on the amino acid sequence around the processing junction and suggest that interference with the signal cleavage step can lead not only to aborted secretion but also to pleiotropic consequences for the growth of the host organism.

The nature of the information that causes bacterial proteins to be directed out of the cytoplasm is still as poorly understood as the mechanism by which this translocation occurs. While it is clear that nearly all secreted proteins are synthesized with an N-terminal signal sequence that is necessary for transport (1–5), the diversity of functional sequences of the signal, and of the transported protein remains remarkable. If the receptor were as promiscuous as the diversity of signal sequences and of the proteins transported with them suggests, one would expect that any signal sequence that contained the classical elements (*i.e.* a positively charged N terminus, a hydrophobic region of 9–15 amino acids, and a polar C terminus with small amino acid residues at positions −1 and −3 and a larger one at −2 (6, 7)) should result in successful translocation. We now know, however, that things are not so simple. For example, when the β-lactamase signal codons are fused precisely to the gene for triosephosphate isomerase from chicken, the protein is not directed to the periplasm (8).[1] Rat proinsulin is not transported into the periplasm by the β-lactamase signal sequence, but is transported by its own signal,[2] even though both these signals are apparently built according to the same rules (6, 7). We have therefore undertaken an investigation of the information content of the signal and the mature protein as well as of the fate of the protein, by generating a set of deletion mutants at the processing junction. These mutants contain either truncated signals fused to the complete β-lactamase or complete signals fused to the truncated β-lactamase. Fortuitously isolated insertions at the processing site are also described.

The choice of the β-lactamase system was governed by the following considerations: (*a*) β-lactamase is one of the very few well-characterized *enzymes* that are transported into the periplasm of *Escherichia coli*. The proper folding of the protein can, therefore, be examined by enzymatic activity assays. (*b*) The expression and transport of this enzyme can be easily screened, and its transport can be selected for since only periplasmic β-lactamase confers resistance to β-lactam antibiotics. (*c*) Assays can be devised that test for translocation without disruption of the cells. This is an important criterion that avoids the pitfalls associated with cell fractionation especially when unnatural proteins are expressed that may make the membrane unstable (9, 10). (*d*) The β-lactamase is a globular, water soluble, monomeric protein. (*e*) The enzyme is expressed constitutively. (*f*) The β-lactamase can be expressed in eucaryotic cells as well as procaryotes (11–14), thus permitting comparison of the effects of mutations on different transport systems.

To permit construction of the deletion mutants described here, a derivative of pBR322 was first produced (8, 15) in which a unique *Eco*RI site was introduced at the beginning and a unique *Bst*EII site was introduced at the end of the signal codons of RTEM β-lactamase (8). Since the new plasmid (called pTG2) encodes two mutations (Ser^{-22} to Arg, and Ala^{-1} to Gly) that are phenotypically silent (8, 15), it is referred to as the wild-type in this work.

The transport of proteins into the endoplasmic reticulum in eucaryotic cells is dependent on signal sequences that have many features in common with those of bacterial signals (16). In eucaryotic systems, it is believed (17, 18) that the signal recognition particle (SRP),[3] a complex of RNA and proteins, binds to the signal peptide as it emerges from the ribosome and causes an arrest of translation (but see Ref. 19). The whole complex then migrates to the membrane and binds to a docking protein. This interaction releases the arrested translation, and the nascent polypeptide may then be cotranslationally extruded through the membrane. While the similarity

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‡ Present address: Gen-Zentrum der Universität München im Max-Planck-Institut für Biochemie, Am Klopferspitz, D-8033 Martinsried, Germany.

§ To whom correspondence should be addressed.

[1] R. Summers, unpublished work.
[2] A. Gautier, unpublished experiments.
[3] The abbreviation used is: SRP, signal recognition particle.

of signal sequences in procaryotes and eucaryotes and their heterologous recognition (11–14) make it tempting to suggest an analogy in translocation mechanisms as well, there are few facts that support a parallelism with the eucaryotic SRP-dependent mechanism as originally proposed. First, transport in bacteria can occur post-translationally *in vitro* (20) and *in vivo* (9, 21, 22), even for proteins that are translocated cotranslationally *in vivo* (20), implying a stochastic process of synthesis and translocation. This is not easily reconciled with the view of an obligatory SRP-dependent mechanism for higher cells that was proposed earlier. Interestingly, though, an incomplete SRP that has been reconstituted from only some of its subunits can still promote transport into membrane vesicles but does not cause a translation arrest (19, 23). Furthermore, it has now been shown that translocation can occur in eucaryotic systems rather late in translation, at least for some proteins, after much (or all) of the protein synthesis has been completed (24, 25). Second, experiments with bacterial systems have suggested the existence of soluble factor(s) that may be required for translocation (26, 27), but it is not clear whether their role is analogous to that of the SRP in eucaryotes. The correlation of such factors with genetically defined lesions in *E. coli* (1–3) still remains poorly characterized. Third, transmembrane protein transport in bacteria is dependent upon an energy source other than that provided by translation (28–32), whereas transport across the endoplasmic reticulum membrane in eucaryotes is believed not to be. Exceptions to this belief have been found in yeast (24, 25, 33) and it is not clear how common these will be. Whatever the outcome, it is clear that the original SRP-dependent mechanism will need modification to accommodate all the data now available on eucaryotic secretion, and that the relationship between the secretory mechanisms in eucaryotes and procaryotes remains to be defined.

By generating families of signal sequence mutants, we hope to facilitate the mechanistic dissection of protein transport *in vitro*, both in procaryotes and in eucaryotes. As a first step, we must know the behavior of these mutant constructions *in vivo*, and this is reported here.

EXPERIMENTAL PROCEDURES

Restriction endonucleases, *E. coli* DNA polymerase (Klenow fragment), exonuclease III, and exonuclease *Bal*31 were from New England Biolabs or Bethesda Research Laboratories. S1 nuclease was from PL Biochemicals. ^{32}P-Labeled nucleotides and [^{35}S]methionine were from Amersham Corp. Nitrocefin was from BBL Microbiology Systems (Cockeysville, MD). Components of culture media were from Difco. Other biochemicals were from Sigma or Aldrich and were the highest grades available. Antibody against β-lactamase was a generous gift of Dr. Andrew Charles (I.C.I., Runcorn, U.K.). Oligonucleotides were kindly synthesized by Dr. Michael Edge (I.C.I., Runcorn, U.K.).

Bacterial Strains—*E. coli* K12 strain DH-1 (F^- *endA1 thi-1 hsdR17* (r_K-, m_K+) *sup-E44 recA1 gyrA96* (*relAI*)λ^-) was obtained from D. Hanahan (Harvard University). *E. coli* strain HB101 (F^- *hsdS20* (r_B −, m_B −) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 sup-E44* λ^-) was obtained from F. Ausubel (Massachusetts General Hospital). Phenotypes were determined in DH-1, and HB-101 was used for plasmid production for *in vitro* constructions.

General Methods—Bacterial growth was performed according to Miller (34). Recombinant DNA techniques were based on Maniatis *et al.* (35). Plasmid DNA was isolated as described by Ish-Horowicz and Burke (36) or Birnboim and Doly (37). Transformation of *E. coli* was carried out either by the method of Dagert and Ehrlich (38) or Hanahan (39). Polyacrylamide gel electrophoresis was as described by Laemmli (40). Plasmids were maintained in all experiments by growth on tetracycline (20 μg/ml in broth, 25 μg/ml on plates).

Sequencing—Sequencing of DNA was carried out by the method of Maxam and Gilbert (41) or by a variation of the chain-termination method of Sanger *et al.* (42), using double-stranded plasmid as the template and a labeled oligonucleotide as the primer. For sequencing, plasmid DNA (2–10 μg in 200 μl), from a standard miniprep by alkaline lysis (37), was digested to completion remote from the site of sequencing (with *Pst*I, *Bam*HI, or *Sal*I). DNase-free RNase was then added to the reaction mixture, and after 10 min at 37 °C, an equal volume of 1 M NaOH was added. After 30 min at 37 °C, the solution was chilled, and neutralized with 5 M NaOAc buffer (300 μl, pH 5.6). The DNA was precipitated with isopropyl alcohol and the pellet carefully rinsed with ethanol. The pellet was taken up in TE buffer (200 μl, 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA) and polyethylene glycol (80 μl, 30% w/v containing 1.8 M NaCl) was added. After 1–4 h at 4 °C, the DNA was pelleted, and the pellet dissolved in TE buffer (300 μl), phenol-extracted, and ethanol-precipitated. The primer oligonucleotide was end-labeled with polynucleotide kinase and 2 eq of [α-^{32}P]ATP of the highest specific radioactivity available. The reaction was terminated by boiling (2 min) and the product separated from residual [^{32}P]ATP by chromatography on Sephadex G-50 superfine. The chain extension reactions were carried out with a two-fold molar excess of primer over template as described (42) at 31–37 °C, except that no radioactive nucleotide was used. This procedure eliminates the need for a chase, and gives very consistent results. The primers were complementary to base pairs 4010–4024 and 4231–4212, of pBR322.

Deletions—Deletions were constructed either using exonuclease III followed by S1 nuclease, or using *Bal*31. Reactions were carried out for a fixed amount of time as described below. The resulting deletions were analyzed by denaturing gel electrophoresis, and deletions of similar lengths from both enzyme systems were pooled into four groups of deletions of various extents. Ligations were carried out as described below and transformants were either sequenced directly, screened by the filter paper assays described below, or screened with labeled oligonucleotides.

Exonuclease III/S1 Digestion—Samples of DNA (approximately 10 μg) in buffer (120 μl, 66 mM Tris-HCl, pH 8.0, containing NaCl (0.125 M), $MgCl_2$ (5 mM) and dithioerythritol (10 mM)) were incubated with exonuclease III (16 units) at room temperature for times between 1 min and 15 min. After the required time, buffer (13.5 μl, 40 mM NaOAc, pH 4.0, containing NaCl (50 mM) and $ZnSO_4$ (6 mM)) was added, followed by S1 nuclease (1000 units). After 15 min on ice, the reaction was quenched with EDTA and phenol-extracted. The DNA was precipitated with ethanol and then cut with an appropriate restriction enzyme.

Bal31 Digestion—In preliminary experiments it was found that to obtain a reasonably uniform series of short deletions, a large amount of enzyme under suboptimal reaction conditions is needed. Accordingly, the buffer used for the exonuclease III digestions described above (supplemented with 10 mM $CaCl_2$) and similar reaction conditions (10 μg DNA, 1.5 units *Bal*31, 0.5–15 min, room temperature) were employed. The mixture was phenol-extracted, ethanol-precipitated, and treated with DNA polymerase (Klenow fragment) in the presence of all four deoxynucleotides to flush off possible overhanging ends. The extent of the deletions was determined by cutting with *Eco*RI and labeling with [α-^{32}P]dATP, dTTP, and DNA polymerase (Klenow fragment). Portions were then run out on a sequencing gel and compared to an undigested standard.

Determination of LD_{50}—All LD_{50} determinations were carried out with DH-1 as host in 2YT medium containing tetracycline (20 μg/ml) at 37 °C in 5 ml cultures. The antibiotic was the sodium salt of ampicillin.

Enzymatic Activity—β-Lactamase was assayed at 25 °C in 0.1 M potassium phosphate buffer, pH 7.0, with Nitrocefin as substrate (43). Initial rates were calculated from the change in absorbance at 482 nm.

Cell Fractionation—The procedure used was based on the method of Witholt *et al.* (44). All operations were carried out at 4 °C. M9 minimal medium (5 ml) supplemented with casamino acids was inoculated from a fresh overnight culture. The cells were grown to early stationary phase and then pelleted. The cells were washed twice with minimal medium (not containing casamino acids or sulfate) and resuspended in M9 minimal medium (10 ml) not containing sulfate and supplemented with the 18 nonsulfur amino acids. The suspension was shaken at 37 °C for 15 min, and [^{35}S]methionine (0.1 mCi, 800 Ci/mmol) was then added. After 10 min, the tube was transferred to ice water for 15 min. Labeled cells were collected by centrifugation and gently resuspended in spheroplast buffer (200 μl, of 200 mM Tris-HCl, pH 8.0, containing sucrose (0.5 M) and EDTA (0.5 mM)), and the suspension was very gently pipetted into an Eppendorf tube. Lysozyme (20 μl of a fresh solution of 10 mg of lysozyme/ml of spheroplast buffer) was added, immediately followed by spheroplast buffer (400 μl) in H_2O (400 μl). Mixing was achieved by gentle rocking

of the tube. The tubes were left for 30–40 min at 4 °C. Spheroplasts were collected by centrifugation for 1 min. The supernatant served as the periplasmic fraction.

The spheroplasts were resuspended in spheroplast buffer (50 μl) by vigorous vortexing and EDTA (1 ml, 5 mM, pH 8.0) was added. Residual clumps of cells were dissolved by vigorous repipetting. The clear solution (0.9 ml) was transferred to an ultracentrifuge tube and centrifuged at 40,000 rpm for 1 h at 4 °C. The supernatant (0.8 ml) was transferred to an Eppendorf tube and defined as the cytoplasmic fraction. The pellet was resuspended in TE buffer (0.9 ml, of 10 mM Tris-HCl, pH 8.0, containing EDTA (1 mM)) and centrifuged again at 40,000 rpm for 1 h. The supernatant was discarded. The pellet was then resuspended in TET buffer (0.9 ml, of TE buffer containing 1% (v/v) Triton X-100) and centrifuged again for 1 h at 50,000 rpm. This supernatant (0.8 ml) served as the membrane fraction.

Immunoprecipitation—To the cytoplasmic fraction and the membrane fraction, buffer (200 μl, of 250 mM Tris-HCl, pH 8.0, containing sucrose (1.5 M)) was added. To the periplasmic and the cytoplasmic fractions, 100 μl of precipitation medium (Triton X-100 (20% v/v) containing NaCl (1.5 M)) and to the membrane fraction, 50 μl of this medium, was added. Anti-β-lactamase serum (1 μl) was then added to all tubes, which were left at 4 °C overnight. A suspension of protein A bound to Sepharose (100 μl, from Sigma, 0.145 g in 5 ml of 50 mM potassium phosphate buffer, pH 7.5) was then added to each tube, and the samples were left to stand at 4 °C for 15 min. The mixtures were centrifuged and the pellets washed twice with high-salt solution (50 mM Tris-HCl, pH 7.0, containing NaCl (0.5 M) and Triton X-100 (0.25% v/v)) and then twice with low-salt solution (as above, except 150 mM in NaCl). The resulting pellets were boiled for 10 min with Laemmli gel sample buffer (100 μl) (40), and 10- to 20-μl samples were used for gel electrophoresis (40).

Filter Paper Assays—Circles (8.25-cm diameter) of Whatman filter paper No. 2 were impregnated with the following solutions and then air-dried for about 1 h. Nitrocefin papers: Nitrocefin (25 mg) was dissolved in a mixture of ethanol (50 ml) and 0.1 M potassium phosphate buffer (50 ml), pH 7.0. Bromocresol-purple papers: penicillin G (potassium salt, 18 g) was dissolved in H_2O (90 ml) and added to a solution (10 ml) of bromocresol purple (0.5 mg/ml). This solution was titrated to a deep blue color with 1 M KOH. The dried papers were stored in desiccators over Drierite.

For assays, colonies were gridded on plates containing either tetracycline (25 μg/ml) or ampicillin (100 μg/ml). The filter paper was placed on top of the agar and left on the plate until completely wet (20–30 s). With Nitrocefin papers, only colonies carrying *periplasmic* β-lactamase show an intense red-purple coloration with a halo after a few seconds. Transport-deficient mutants show a weaker red coloration with no halo, after about 60 s. With bromocresol-purple papers, all colonies that carry an active β-lactamase, whether transported or not, develop a bright yellow color on blue-purple background. This assay allows a clear identification of frameshift mutants. From the combination of these two simple tests, preliminary screening for transport mutants is greatly facilitated.

Protein Sequencing—DH-1 containing the plasmids encoding the wild-type β-lactamase, or the mutants containing the deletions −2−1 or −1+1, were grown to an A_{550nm} of 0.05 in M9 minimal medium (500 ml). The cells were isolated by centrifugation, resuspended in M9 minimal medium (2 ml), shaken at 37 °C for 10 min, and then supplemented with [2,3-^{3}H]valine (Amersham Corp., 0.4 mCi, 48 Ci/mmol) and grown for 1 h. Cell fractionation was performed to enrich the β-lactamase since it had already been established that the mutant proteins are, as the wild-type, all periplasmic. The periplasmic fraction was treated with anti β-lactamase antibody as described above. The washed, immobilized protein A fractions were then treated with aqueous formic acid (10%, v/v). The protein in the eluate was lyophilized, then taken up in loading buffer and subjected to polyacrylamide gel electrophoresis. High pH electroblotting, essentially according to Aebersold *et al.* (45) was then performed using a semidry electroblotter and GF/C glass fiber sheets derivatized with *N*-trimethoxysilylpropyl-*N*,*N*,*N*-trimethylammonium chloride. Some minor modifications in the acid etching of the glass fiber sheets, the blotting buffer composition, and the staining procedure were performed.[4] The glass fiber sheets were used directly for gas-phase sequencing on an Applied Biosystems 470A sequenator. The appearance of tritium-labeled material was determined by scintillation counting of the phenylthiohydantoin derivatives. The wild-type gave the expected pattern with negligible background, but the expression of the mutants is low enough that the background becomes somewhat more significant in these cases.

[4] C. Eckerskorn and F. Lottspeich, manuscript in preparation.

RESULTS

Construction of Deletions

The stepwise deletions of amino acids from the *Bst*EII site was carried out as illustrated in Fig. 1. Reconstruction of the complete gene for the mature enzyme so as to give a precise junction on religation was achieved by partially filling in the *Bst*EII overhang in the presence of only the required nucleotides, followed by S1 digestion. The deletions into the signal codons were obtained by digestion with *Bal*31 or with exonuclease III followed by S1. After fragment purification and ligation, in-frame constructs were found either by direct sequencing of transformants (which does not make any assumption about expression or transport and only presumes that the construction is not lethal) or after preliminary screening using the filter-paper method as described under "Experimental Procedures." Deletions into the signal codons from the *Eco*RI site and into the gene of the mature protein were obtained analogously. All candidate mutant plasmids were sequenced across the new junction and the in-frame deletions that were obtained from this work are shown in Fig. 2.

Since the signal deletions −3−1; −4−1; −5−1; and −6−1 (the pair of numbers specifies the residues at each end of the deleted segment) were not found this way although the pieces used for ligation showed abundant bands at these lengths on sequencing gels, oligonucleotides were synthesized to probe for these deletions with colony hybridization (46). The desired deletions were not found, however. Instead, only frameshift mutants differing by one base from the desired deletion were detected. It may be noted that all deletions from the *Bst*EII site including the most severe one (−20−1) retain the *Eco*RI site, thus permitting a convenient transfer to other vectors and promoters.

Phenotypes of Deletion Mutants

Enzymatic Activities—The total β-lactamase activities in French press lysates are summarized in Table I. Except for the +1 and −1 constructions that lack a single amino acid at the processing site, it is evident that the addition even of 15 or so amino acids onto the N terminus does not abolish the enzymatic activity of β-lactamase, whereas the removal of only a few amino acids from the normal N terminus of the mature β-lactamase drastically reduces the catalytic activity. Interestingly, in cases where large deletions into both protein and signal were constructed (which results in an uncleavable partial signal and, therefore, a protein of similar length as mature β-lactamase but with a modified N-terminal sequence), significant levels of enzymatic activity are detected. The total enzymatic activity per cell is, of course, a function both of the expression and of the specific activity of the mutant protein. We can therefore conclude that both the expression and the specific activity of enzyme having large deletions in the signal must be similar to that of the wild-type. (The expression of, for example, the cytoplasmic −20−1 mutant relative to the periplasmic wild-type was found to depend, however, on both the host strain and the growth conditions. Such variability probably relates to differences in the arsenal of proteases and the metabolic regulation of protein degradation. The numbers reported here refer, therefore, only to the specific conditions indicated.)

From the intensity of the bands on gel electrophoresis, we know that (except the +1 construct) the β-lactamase levels are roughly similar among the deletions into the mature enzyme as well. We can, therefore, attribute the lowering of

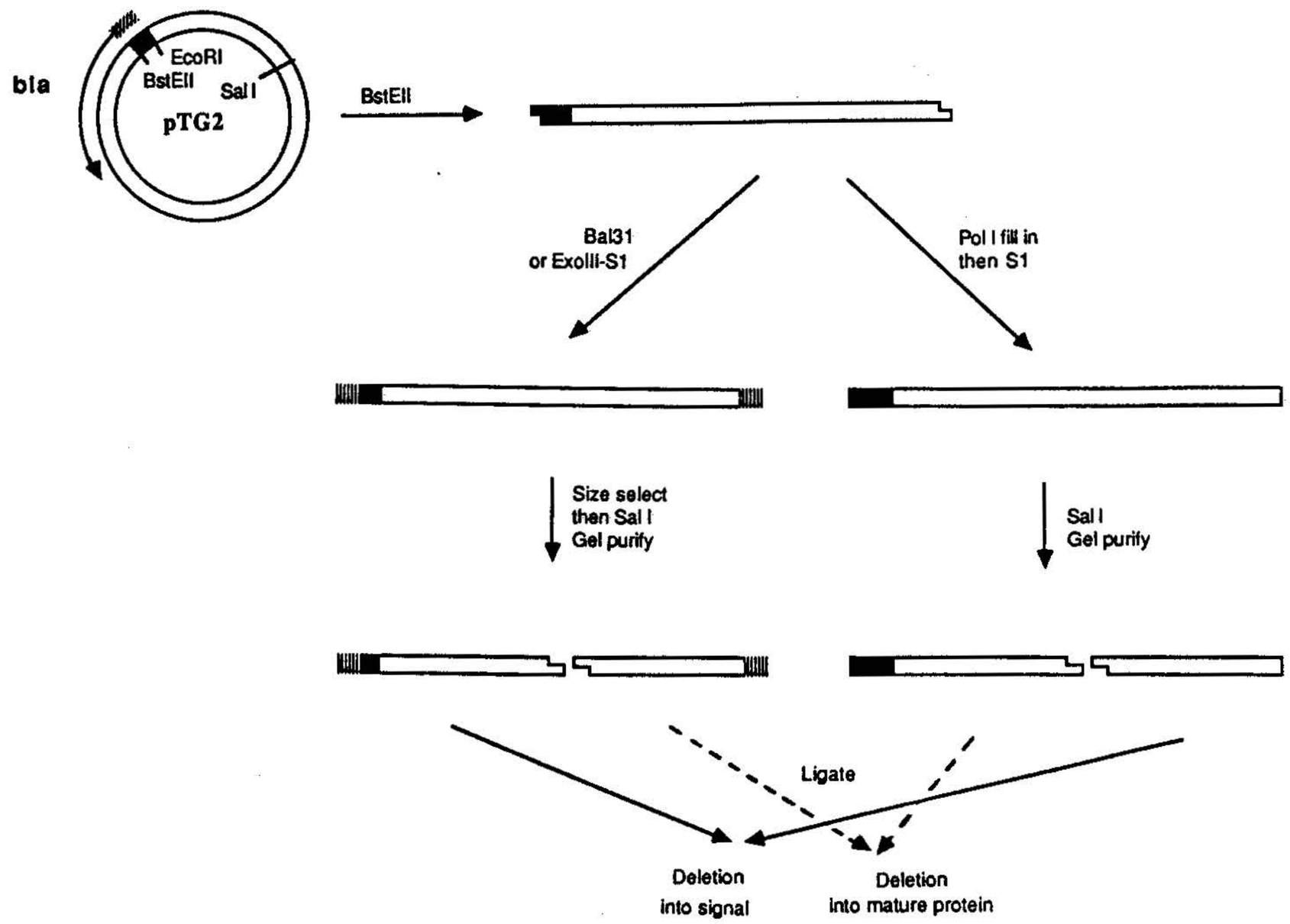

FIG. 1. **Construction of mutants containing deletions into the signal sequence from the processing site, or into the mature protein from the processing site.** DNA fragments were gel-purified before ligation and candidate plasmids purified by retransformation before sequencing. The construction of other sets of deletion mutants was carried out analogously.

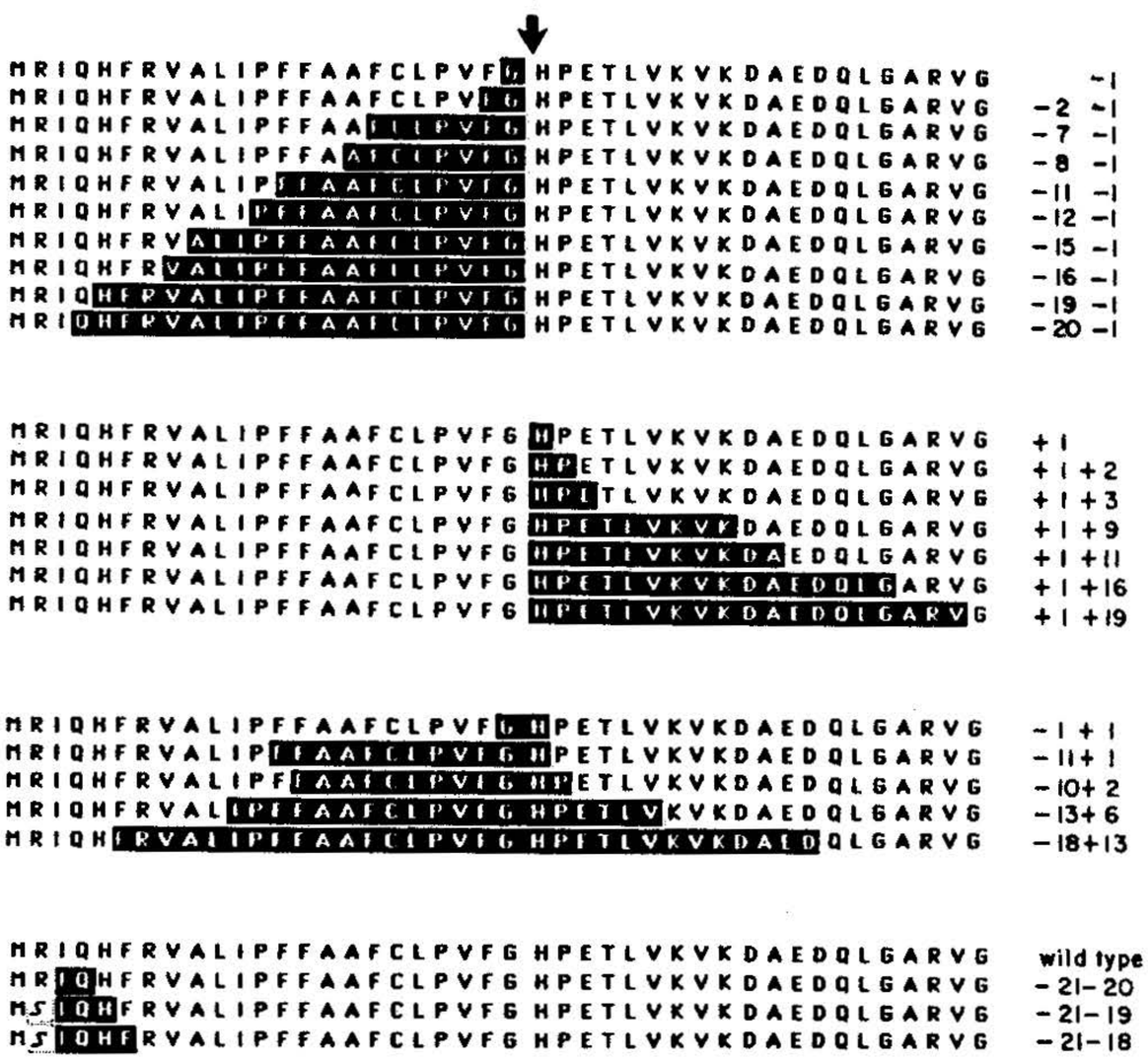

FIG. 2. **Deletion mutants of β-lactamase.** The deleted residues are printed *white-on-black*. The code for each mutant is shown on the *right*.

the total activity per cell when the N terminus of the mature enzyme is deleted mainly to a loss of specific enzymatic activity. Removal of only a few N-terminal amino acids has deleterious consequences catalytically, whereas the addition of extra residues onto the natural N terminus has little effect on the enzyme's activity. As we have pointed out previously (47), the interpretation of protein expression levels is fraught with many problems and requires very carefully designed experiments to delineate that portion that is really due to an effect of impaired translocation on the synthesis of the protein. At present, we cannot conclusively attribute observed changes in expression to causes rooted in translocation.

The insertion of additional residues (at least of the type reported here: see Table I) at the processing site has little or no effect on the catalytic activity per cell, presumably because of similar expression levels and similar specific activities of the proteins produced.

A very dramatic effect on all phenotypic parameters is displayed by the deletion of either the last amino acid of the signal (Gly) or the first amino acid of the mature protein (His). In each of these cases, no enzymatic activity over background was detectable, and only an extremely weak protein band could be seen on denaturing gels. This is in sharp contrast to the deletion of *pairs* of amino acids around the cleavage site (−2−1, −1+1, +1+2), where catalytic activities around 20% of the wild-type are observed (Table I). The deletion of the amino acid either preceding or immediately following the cleavage site has, therefore, a larger effect on expression than that seen with any other mutant of the β-lactamase hitherto examined.

LD_{50} Values—It can be seen from Table I that the deletion of more than six amino acids from the C-terminal part of the signal results in the complete loss of resistance to ampicillin, which falls to the same level as when the cell carries no plasmid. Since the enzyme activity in cell lysates is comparable to wild-type, we can conclude that these enzymes do not reach a location where they have access to the antibiotic. These enzymes appear in the cytoplasmic fraction and are either soluble in the cytoplasm, or possibly weakly bound to the inner face of the inner membrane. When the mature enzyme is truncated from the N terminus and the signal is left intact, the proteins appear to be translocated, processed, and located in the periplasm. The only exception is the construct that lacks the single N-terminal His residue of the mature sequence (the +1 construct in Table I). In this group of mutants, a rough correlation is found, as expected (47), between the total catalytic activity in lysates, and the LD_{50}. This correlation is consistent with the complete transport of mutant proteins that have a lower specific catalytic activity or are expressed at levels lower than the wild-type. Aside from the −1+1 construct, substantial deletions across the processing junction lead unsurprisingly to unprocessed species in the cytoplasmic fraction. In the fourth group of mutants in Table I, we see that deletions from the charged N terminus of the signal lower both the LD_{50} and the total activity in lysates, suggesting that these deletions have no effect on transport (since the proteins are properly transported and processed to the wild-type mature enzyme). Finally, insertions of some

TABLE I
Mutant Phenotypes

Deletion[a]	Activity[b]	LD_{50}[c]	Growth rate	Processing[d]	Location[e]
from, to	%	*μg/ml*			
None[f]	100	>4000	+[h]	+	p
−1	0.1	5	−−	−[i]	c
−2−1	22	350	+	+	p
−7−1	20	5	+	−	c
−8−1	19	5	+	−	c
−11−1	40	5	+	−	c
−12−1	37	5	+	−	c
−15−1	98	5	+	−	c
−16−1	120	5	+	−	c
−19−1	130	5	+	−	c
−20−1	88	5	+	−	c
+1	0.1	5	−−	−[i]	c
+1+2	14	500	+	+	p
+1+3	4.5	50	+	+	p
+1+9	0.5	5	+	+	p
+1+11	0.3	5	+	+	p
+1+16	0.1	5	+	+	p
+1+19	0.1	5	+	+	p
+1+75	0.1	5	+−		
−1+1	16	200	+	+	p
−11+1	40	5	+	−	c
−10+2	12	5	+	−	c
−13+6	20	5	+	−	c
−18+13	3	5	+	−	c
−21−20	9	300	+	+	p
−21−19	9	350	+	+	p
−21−18	1.5	90	+	+	p
∇R[g]	92	4000	+	+	p
∇H[g]	92	>4000	+	+	p
∇HIP[g]	66	3000	+	+	p

[a] The deletion is specified by the residue numbers of the first and last amino acids that are missing. The signal sequence runs from −23 to −1, and the mature protein from +1 to +263.
[b] In French press lysates, compared to wild-type (pTG2).
[c] Determined in broth, using ampicillin.
[d] Determined from the size of the β-lactamase species on denaturing gel electrophoresis.
[e] From cell fractionation experiments (see, *e.g.* Fig. 3).
[f] The wild-type is specified by pTG2.
[g] ∇ implies the *insertion* of the amino acids specified, precisely at the processing site.
[h] The symbol + signifies normal growth. The symbol −− signifies very slow growth.
[i] Partial processing or proteolytic degradation is observed.

amino acids at the processing site have little effect on the LD_{50} or total catalytic activity (Table I), suggesting only minor effects on expression or secretion in these cases.

The effects on enzymatic activity observed for mutants having small deletions around the cleavage site (−2−1, −1+1, +1+2, −1, and +1) are fully reflected in the LD_{50} values toward ampicillin. The three mutants lacking two amino acids around the cleavage site give LD_{50} values consistent with complete transport of somewhat low levels of β-lactamase (47), whereas the two mutants that lack a single amino acid at the cleavage site produce extremely low levels of enzyme, and the cells show very low resistance to ampicillin.

Cell Fractionation and Protein Processing—Cells were fractionated into three "compartments": periplasm, cytoplasm, and membrane, as described under "Experimental Procedures." Each fraction was analyzed by immunoprecipitation and gel electrophoresis. In each set of fractionations, the wild-type and the (−20−1) mutant from which the signal has been almost completely deleted, were included as size markers and as controls for the quality of the fractionation procedure. Typical results both for signal deletions (the products from which are cytoplasmic) and for mature protein deletions (the products from which are periplasmic), are shown in Fig. 3. The location of the gene products are listed in Table I and can be summarized as follows. C-terminal truncation of the signal sequence by six or more amino acids prevents translocation and processing, and the precursor proteins appear in the cytoplasmic fraction. In sharp contrast, all mutants having deletions of two or more amino acids from the mature protein are translocated into the periplasm and are processed. The same is true of the three mutants that contain extra amino acids at the processing site (see Table I). While the precise site of processing has not yet been determined for all mutants, the sizes of these species are consistent with processing very close to the normal site.

For all mutants that lack some part of the C-terminal end of the signal, proteins of the expected size for the *uncleaved* construction are observed. In contrast, mutants that lack some of the N-terminal end of the mature protein give bands of slightly smaller molecular weight than the wild-type, implying cleavage of the complete signal peptide. Deletion of part of the positively charged N-terminal region of the signal appears to have no effect on processing, either. In summary, all constructs that have a complete wild-type signal sequence appear to be processed correctly, though if either of the amino acids that flank the cleavage site is deleted, export is prevented and no processing is seen. Strikingly, if *two* amino acids are removed from either side of the processing site (−2−1, +1+2, and −1+1 in Table I) normal export and processing is restored. Protein sequencing of the periplasmic products of the deletion mutants −2−1 and −1+1 shows that cleavage occurs in the "normal" position, between the 23rd and 24th residue. The peptidase cleaves the −1+1 and −2−1 mutants at -Val-Phe-Pro↓Glu-Thr-Leu- and -Val-His-Pro↓Glu-Thr-Leu-, respectively. While each of the upstream amino acids is precedented (*i.e.* Val at −3 (6, 7), Phe or His at −2 (6, 7), and even Pro at −1 (48)), these cleavages do not fall easily into the consensus patterns of von Heijne (6, 7).

Growth Rates—Growth rates were determined for all transformants to detect any effects of the mutant proteins on other cellular processes. Since β-lactamase is not essential for cell growth or survival in the absence of β-lactam antibiotics, only the impaired transport of other, essential, proteins could explain a decreased growth rate.

Since relative growth rates for the various β-lactamase mutant strains depend on the growth conditions and hosts used, we only report here results from a single set of conditions: rich medium (2 YT), normal growth temperature (37 °C), and a standard host (DH-1). Under these conditions, most of the plasmids reported in Table I cause no decrease in growth rate greater than one standard deviation (about ±6%). In the case of the mutants lacking one amino acid at the processing site, however (−1, and +1, Table I), we see severe effects on growth: the generation time for these transformants is about *twice* that of wild-type host cells.

DISCUSSION

The mutations that we have constructed here fall into three groups. Members of the first group produce β-lactamase that is translocated normally, cleaved, and provides *in vivo* protection against β-lactam antibiotics (as long as essential pieces of the mature enzyme have not been deleted away). Members of the second group make β-lactamase that is cytoplasmic, soluble (or at most weakly membrane-associated), from which

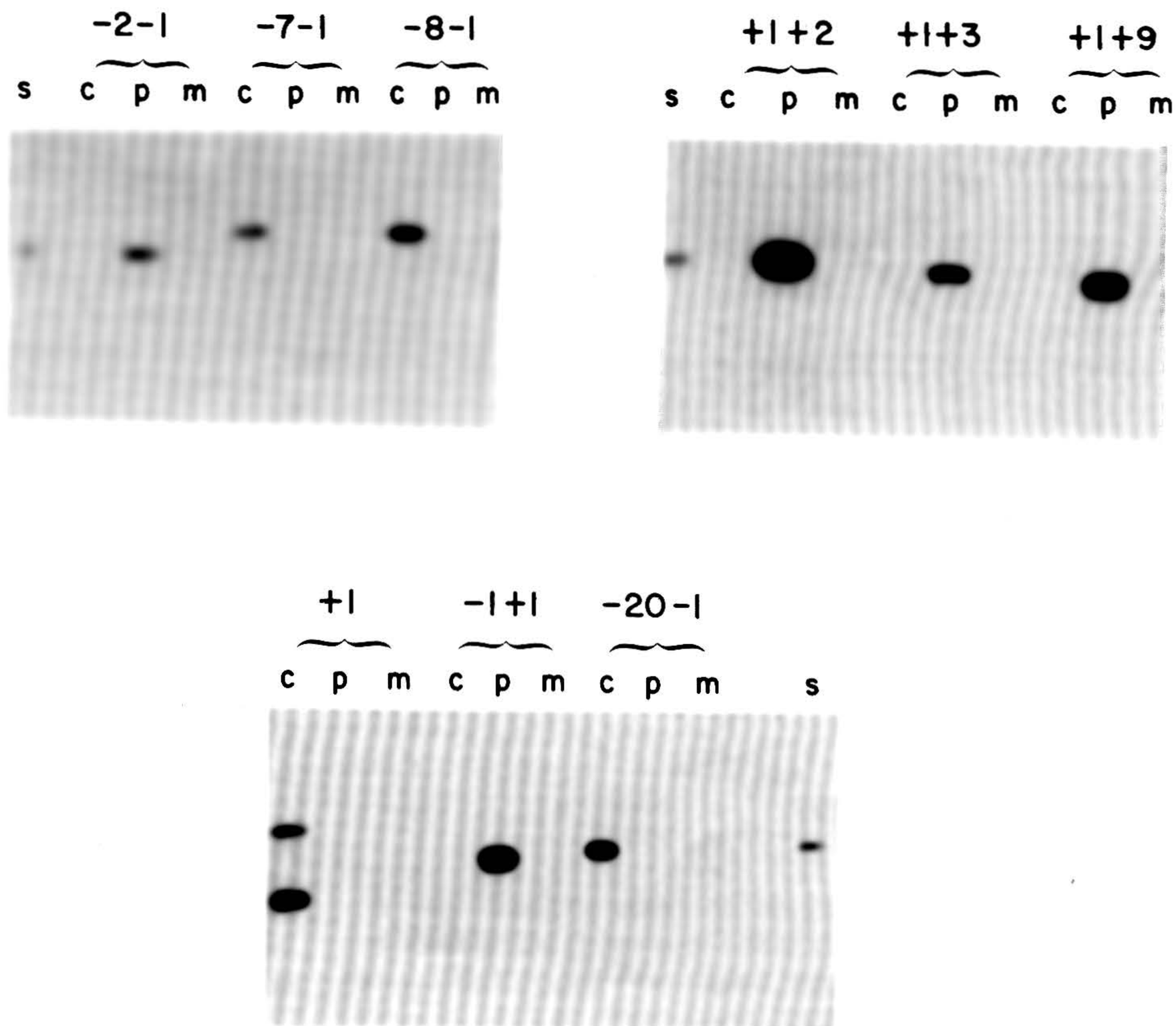

FIG. 3. **Cell fractionation of sample deletion mutants.** For each mutant, three fractions were applied to the gel: *c* (cytoplasm), *p* (periplasm), *m* (membrane). *s* is the standard mature enzyme. Conditions for cell fractionation and gel electrophoresis are described under "Experimental Procedures."

the signal is not cleaved and which (because of its location) confers no resistance to β-lactam antibiotics despite the existence of high levels of catalytically active enzyme. Members of the third group have severe growth defects and make very low levels of β-lactamase that is not detectably translocated into the periplasm. From these sets of mutations we have obtained, therefore, a clear distinction between changes that prevent translocation and those that do not. We also see from the third group of mutants that the deletion of a single amino acid from a nonessential plasmid-encoded protein can cause severe changes in the growth characteristics of the host cell.

The deletions that we have made of amino acids from the beginning of the mature protein have no discernible effect on translocation or processing. From this observation, we can deduce either that the mature β-lactamase contains no information that is required for protein translocation, or that such information lies further downstream in the sequence of the mature enzyme, or that all our constructs happen to have sequences immediately following the signal that are "acceptable" to the transport apparatus. While there may be an effect on the kinetics of secretion that our labeling conditions will not illuminate, it is clear that there is no qualitative consequence of these deletions. The amino acids immediately downstream from the cleavage site that result from the deletions from the beginning of the mature protein that we have made are of many types, and it is difficult to see how an acceptable pattern has been reconstructed in each case. The simpler deduction is that the sequence following the cleavage site either does not contain essential information for transport or that the range of acceptable sequences is large. In contrast to this conclusion, it has been proposed for the lamB protein (49, 50) that information important for protein translocation lies between residues +27 and +39. This hypothesis was arrived at from the study of translocation of fusion proteins between lamB and lacZ. In the present case, we have not found any sequence homologies, patterns of similar charge or hydrophobicity, or secondary structural similarities, in the region of these fusions. To try to illuminate this problem, we constructed a gene having a very large deletion (equivalent to the first 75 amino acids of the mature β-lactamase), but this gene product has so far been undetectable in fractionation experiments of whole cells and in the protein products from maxicells. From these results, we cannot exclude the possibility of some signal information being contained in the mature protein. Indeed, when the signal codons of the β-lactamase are fused *precisely* to the gene of a cytoplasmic protein (that encoding chicken triosephosphate isomerase), no secretion is observed. Yet when DNA encoding several N-terminal amino acids of the mature β-lactamase is inserted between the β-lactamase signal and the isomerase genes, transport and processing *are* seen.[1] In this case at least, therefore, we must conclude that there are some constraints on what sequence of amino acids can follow the signal if secretion is to be achieved.

Deletion of more than 2 residues from the C terminus of the signal sequence eliminates translocation. The resulting proteins fractionate in the cytoplasmic fraction, and the cell experiences no measurable ill effects from these constructions (as evidenced by the normal growth rates and normal expression levels of these mutant proteins: see Table I). Proteins having major deletions of residues across the processing junction fail to be translocated or processed, as expected. Surprisingly, these proteins retain some enzymatic activity, in contrast to those constructions where the wild-type signal is cleaved from a mature protein that lacks some portion of its N terminus. It appears that *any* amino acids at the N terminus are better than none for enzymatic activity.

Remarkably, the removal of either one of the amino acids that flank the cleavage site results in an extreme growth defect of the cell. It is conceivable that we have constructed inhibitors of the signal peptidase. In such a case, signal peptidase would be bound by the inhibitor and unable to process proteins essential for the growth of the cell. In the +1

and the −1 mutants, if the peptidase were to recognize and bind as if to cleave between residues 23 and 24, the leaving group would be the imino acid proline. Most peptidases are unable to cleave such a bond, and proline has never been observed in the +1 position of a secreted protein (51). In contrast, peptidase-catalyzed cleavage of the bond C-terminal to proline residues is not uncommon, and there is, indeed, precedent for proline in the −1 position (of a eucaryotic preprotein: (48)). Whether our observations relate more to the sequence selectivity of the signal peptidase, or whether there are as yet undefined limitations on which peptide bond is presented to the leader peptidase for cleavage, remains to be seen. It is certainly curious that the position of processing (between residues 23 and 24) remains exactly the same, despite the fact that the sequences that are cleaved are quite different from the wild-type, and do not readily fit the patterns discerned by von Heijne for this region (6, 7). The simplest interpretation of our results that accounts both for the pleiotropic consequences of the +1 and −1 mutants and for the cleavage patterns of the −2−1 and −1+1 mutants is that a given peptide bond (in the case of the β-lactamase, that between residues 23 and 24) is presented to the signal peptidase, which then cleaves the bond with greater or lesser ease (or, in the case of Pro at +1, not at all). What determines the bond that is presented to the signal peptidase we do not know, but from the diversity of signal sequences it is clearly not a function of the number of residues from the start of the signal, nor of the distance from the start of the hydrophobic core region.

What must a signal sequence and the mature protein look like to effect protein transport into the periplasm? The immense variety of signal sequences and of transported proteins makes any exclusively linear comparisons of sequences a rather naive approach. Conserved secondary structures might play a role, but such features are simply not yet adequately predictable (52, 53), even if the milieu in which folding occurs (that is, aqueous or membranous) were known. On the one hand we require a library of data on the *in vivo* behavior of a range of modified signals and proteins and as much information as possible about the conformations of signal sequences in various environments. The goal of such studies must be to discern the features that nonfunctional signals do not share with functional ones. On the other hand, we require a much more detailed picture of the species with which the signal sequence and the transported proteins interact, to obtain a tighter definition of what is necessary, and what is sufficient, to guide a procaryotic protein across the bacterial membrane.

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