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# Membrane-bound β-Lactamase Forms in *Escherichia coli**

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**Frameshift pseudo-revertants of *Escherichia coli* RTEM β-lactamase were obtained by a selection procedure, starting from frameshift mutants at the signal-processing site. These pseudo-revertant proteins, which have a totally altered COOH-terminal part of the signal sequence, are attached to the outer face of the inner membrane. The mutant proteins are enzymatically active *in vitro* and *in vivo*, and the membrane localization has no deleterious effect on cell growth. We conclude that initiation of transport across the membrane does not require the COOH-terminal part of the signal, but this part of the sequence determines whether the protein is released to the periplasm either with or without cleavage of the signal, or whether the protein remains anchored to the membrane. Mutants with two signals in series were used to show that a truncated signal is not refractory to transport *per se*. If neither signal contains a functional cleavage site, the protein is at least partially found on the outer face of the inner membrane. If both signals contain functional cleavage sites, both are removed and the protein is released to the periplasm. If only the first signal contains a cleavage site, a longer fusion protein is transported and released. The results presented here show that a pre-β-lactamase-like protein can fold properly even as a membrane-bound species.**

The mechanism of translocation out of the bacterial cytoplasm is still only poorly understood (reviews, Refs. 1–8). Rather little is known about the nature of the components of the transport apparatus, and the informational content of the signal sequences required for protein secretion is still not satisfactorily decoded (9). We are investigating these questions using the particularly well-characterized RTEM β-lactamase from *Escherichia coli* that is transported to the periplasm. Since β-lactamase can be expressed and translocated in both prokaryotes and eukaryotes (10–13), we hope to generate, using this system, tools for the direct comparison of the different sequence requirements and for the mechanistic dissection of the transport process in prokaryotes and in eukaryotes.

β-Lactamase is a water-soluble monomeric enzyme, the proper folding of which is signaled by its enzymatic activity. Furthermore, the secretion of active β-lactamase can be selected for by the enzyme's ability to confer resistance to β-lactam antibiotics on the host organism, *only* when the enzyme is in a periplasmic location where it may catalyze the hydrolysis of the β-lactam. It is this property that we have exploited in the generation of a diverse set of functional signal sequences.

We have used frameshift mutants (constructed *in vitro*) that contain a frameshift mutation at the signal-processing site, and therefore produce no functional enzyme, to select for functional second-site frameshift mutants created by random mutagenesis. Unexpectedly, some of the functional mutant β-lactamases thus selected are translocated but remain anchored to the outer face of the inner membrane. We also describe the construction of multiple signal sequences. We wished to elucidate whether a construct having two functional signal sequences would be processed to lose one or both of these signals. Cleavage after only the first functional signal peptidase cleavage site would provide a simple method for the preparation of the precursor protein in large amounts. We then constructed fusions in which a complete signal is followed by a truncated one lacking amino acids −1 to −11 of the second signal. Since such a truncated signal by itself does not direct the enzyme out of the cytoplasm, it was important to determine whether its presence inhibits transport, even when coupled to a complete functional signal.

To permit construction of the mutants described here, a derivative of pBR322 was used (14) that contains a unique *Eco*RI site at the beginning and a unique *Bst*EII site at the end of the signal codons of RTEM β-lactamase. Since the new plasmid (pTG2) encodes two mutations (Ser −22 to Arg, and Ala −1 to Gly) that are phenotypically silent (15), it is referred to as the wild type in this work.

It is now clear that nearly all secreted proteins are synthesized with a $NH_2$-terminal signal sequence that is necessary for protein translocation and that normally contains a positively charged $NH_2$ terminus, a hydrophobic region of 9–15 amino acids, and a polar COOH terminus having small amino acids at positions −1 and −3 and a larger one at −2 (9, 16–18). Early hopes that these facts were adequate for the construction of vectors for the expression and secretion of any foreign protein were dampened, however, by the observation that at least two precise fusions of the β-lactamase signal codons to the genes of heterologous nonsecreted proteins (chicken triosephosphate isomerase and human β-globin) did not result in protein transport whereas the inclusion of about 12 $NH_2$-terminal amino acids of the mature β-lactamase between the signal and the heterologous protein did permit translocation of the hybrid proteins.[1] These two normally cytoplasmic proteins are not, therefore, refractory to transport *per se*. Detailed studies by deletion analysis of the signal-joining region (19) suggested the possibility of a critical conformation of the signal peptidase recognition region around the cleavage site as well as specific charge requirements. The

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[1] R. Summers and J. R. Knowles, unpublished work.

mutants described in this paper address the question of specific sequence requirements for this region. It appears that the sequence around the cleavage site is not necessarily involved in translocation but may rather be critical in the release of the protein from the membrane into the aqueous periplasmic space, even when no signal cleavage occurs.

Mechanistic models of bacterial transport have been formulated by analogy with the transport of proteins into the endoplasmic reticulum in eukaryotes (1–8). They were based on the similarity of signal sequences between bacteria and eukaryotes and on the frequently successful secretion (of normally secreted proteins) in heterologous hosts. The mechanism for eukaryotes, as originally proposed (7), suggests that a signal recognition particle, a complex of RNA and proteins, binds to the signal peptide as it emerges from the ribosome. The whole complex diffuses to the membrane, where a docking protein displaces the signal recognition particle from the signal thus permitting continued peptide synthesis and translocation through the membrane, by an unknown mechanism. Recent results suggest that eukaryotic transport may, just like bacterial transport, be post-translational (2, 20–27). This finding poses the question of energy requirements in both systems (3, 26–33). The importance of a "transport-competent conformation" for proteins that are destined for transport has been stressed for bacteria (34, 35) and eukaryotes alike (36, 37). This evidently non-native conformation may be stabilized by the signal recognition particle and/or by other cellular components (2, 34–37). We demonstrate in this paper that a precursor-like protein can, at least after passage through a membrane, assume an enzymatically active conformation *in vivo*.

## EXPERIMENTAL PROCEDURES

*Enzymes and Chemicals*—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 P-L 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, United Kingdom).

*Bacterial Strains*—*E. coli* K12 strain DH1 (*F⁻endA1 thi-1 hsdR17*($r_K^-$, $m_K^+$) *supE44 recA gyrA96 (relA1)*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 supE44* λ⁻) was obtained from F. Ausubel (Harvard Medical School). *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*) was obtained from B. Bachman (*E. coli* genetic stock center). Phenotypes were determined in DH1, and HB101 was used for plasmid production for *in vitro* constructions.

*General Methods*—Bacterial growth was performed according to Miller (38). Recombinant DNA techniques were based on Maniatis *et al.* (39). Plasmid DNA was isolated as described by Ish-Horowicz and Burke (40) or Birnboim and Doly (41). Transformation of *E. coli* was carried out either by the method of Dagert and Ehrlich (42) or Hanahan (43). Polyacrylamide gel electrophoresis was as described by Laemmli (44). 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 (21).

*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 previously (19). The resulting deletions were analyzed by denaturing gel electrophoresis. Ligations were carried out as described previously (19) and transformants were sequenced directly.

*Frameshift Revertants*—In the sequencing of constructs of deletion mutants around the processing site, several frameshift deletions (and in the case of *Bal*31 catalyzed deletions, also several insertions) were identified. These mutants (deletions of 1, 2, 5 and 14 bases, insertions of 1, 2, 4, and 5 bases) were used as starting plasmids for random frameshift mutagenesis to produce revertants. A portion (200 μl) of an overnight culture of HB101 harboring each of these mutant plasmids (grown in 2YT Tc (20 μg/ml broth) was plated out on a 2YT plate containing Tc (25) and Amp (200) to maintain the plasmid and select for β-lactamase activity. A crystal of the frameshift-inducing acridine derivative ICR 191 (38) was placed in the middle of the culture plate to obtain, by diffusion, a concentration gradient of the mutagen. This technique ensures that a suitable concentration range (*i.e.* one between having no effect at all and being lethal) is covered in one experiment. The plates were handled and incubated in the dark. Colonies so obtained were replated on Tc (25) plates containing no ampicillin and were then screened for β-lactamase activity by both of the filter paper assays (see below). Colonies staining positive on the bromocresol purple indicator papers (indicating active enzyme, but not necessarily a transported one, see Ref. 19) were grown up in broth, the plasmids were isolated, and retransformed into DH1. The transformants were screened as before, and the plasmids from bromocresol purple positive colonies were once again isolated and retransformed. After a further cycle to insure homogeneous plasmid populations within one cell, the remaining candidate plasmids were sequenced. The revertants obtained from the plasmids initially containing an insertion or deletion of one base were all identical to the wild type. Second site frameshift revertants were only obtained starting from a plasmid containing either an insertion or a deletion of five bases, as described under "Results."

*Filter Paper Assays*—Whatman filter paper No. 2 was impregnated with nitrocefin solution or a solution of penicillin G and bromocresol purple as described previously (19).

*Determination of $LD_{50}$*—All $LD_{50}$ determinations were carried out with DH1 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 with nitrocefin as substrate (46) at 25 °C. Assay buffer A is 0.1 M potassium phosphate buffer, pH 7.0. Assay buffer B was used in cell fractionation experiments (see below) and consists of 100 mM HEPES[2] ($Na^+$), 0.25 mM EDTA, 240 mM sucrose, 20 mM $MgCl_2$, pH 7.3. Initial rates were calculated from the change in absorbance at 482 nm.

*Cell Fractionation: A, Immunoprecipitation*—The cell fractionation with $^{35}$S-labeled proteins, immunoprecipitation, and gel electrophoresis was carried out as described previously (19).

*Cell Fractionation: B, Enzymatic Activity*—The procedure used was based on the method of Witholt *et al.* (47). All operations were carried out at 4 °C. Early stationary phase cells (10 ml), grown in 2YT medium, 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)). The suspension was split into two portions of 100 μl, which were very gently pipetted into Eppendorf tubes. One portion was for the generation of "unlysed," the other for "lysed" spheroplasts. To either tube lysozyme (40 μl of a fresh solution of 10 mg of lysozyme/ml of spheroplast buffer) was added, immediately followed by 800 μl of half-concentrated spheroplast buffer (spheroplast buffer as above diluted 1:1 with $H_2O$). 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 serving as the unlysed fraction were gently resuspended with a toothpick in assay buffer B (the assay buffer is: 100 mM HEPES ($Na^+$), 0.25 mM EDTA, 240 mM sucrose, 20 mM $MgCl_2$, pH 7.3, 500 μl). The other portion (the lysed fraction) was resuspended with vigorous vortexing in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, 500 μl) and residual clumps of cells were dissolved by vigorous repipetting. Where indicated spheroplasts were lysed instead by TET buffer (TE buffer, containing 1% Triton X-100). Additionally, in control experiments, the spheroplasts or the lysed spheroplasts were subjected to French press lysis. Enzymatic activity was determined with nitrocefin in assay buffer B (see above), which contains $MgCl_2$ to stabilize unlysed spheroplasts during the assay. For the enzymatic assays, 5–50 μl of cell lysates or cell suspensions were used.

*Maxicells*—The maxicell technique was carried out essentially as described by Sancar *et al.* (48) and Roberts *et al.* (49). An overnight culture was diluted 1:200 in a supplemented M9 medium and then diluted 1:100 again into fresh medium to insure that all cells were in

[2] The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

the logarithmic phase. Uridine was added to the minimal medium to 1 mg/ml (15). Labeling was carried out with [$^{35}$S]methionine and gel electrophoresis was performed as described previously (19).

## RESULTS

### *Construction of Mutants*

*Selection for Revertants*—Frameshift mutants at the signal-processing site were obtained by sequencing clones isolated after exonuclease digestion from the *Bst*EII site (19), which showed no β-lactamase activity. When exonuclease *Bal*31 was used, small insertions were also occasionally recovered. Pseudo-revertants were selected for as described under "Experimental Procedures." The resulting DNA and protein sequences are explained in Fig. 1.

The only type of revertant isolated from a starting frameshift mutant containing a five-base deletion was one (R4a) in which another base had been lost (Fig. 1). In the case of the starting plasmid containing an insertion of five nucleotides, three different revertants were found (R3a, R8a, and R15a) in which another base was inserted in the mutagenesis. Note that in all cases the "correction" occurred in a stretch of five or six consecutive AT base pairs and that the addition or removal of only one base restored the reading frame. Apparently "slippage" of the DNA polymerase occurs most easily in short stretches of the same nucleotide (50–54). This imposes some restriction on the randomness of second site reversions of frameshifts. From other frameshifts as starting plasmids (see "Experimental Procedures"), no revertants, or only reversions to wild type were obtained.

*Construction of Multiple Signal Sequences*—In the construction of double signal sequences, the *Eco*RI site at the $NH_2$ terminus of the downstream signal was fused in frame to a *Bst*EII site (at the COOH terminus of the upstream signal) (14, 15), as shown in Fig. 2. The downstream signal was obtained either from wild type or from a deletion mutant missing amino acids −1 to −11 (19). The in-frame fusions were constructed by partial filling in with the Klenow fragment of *E. coli* polymerase in the presence of only the required

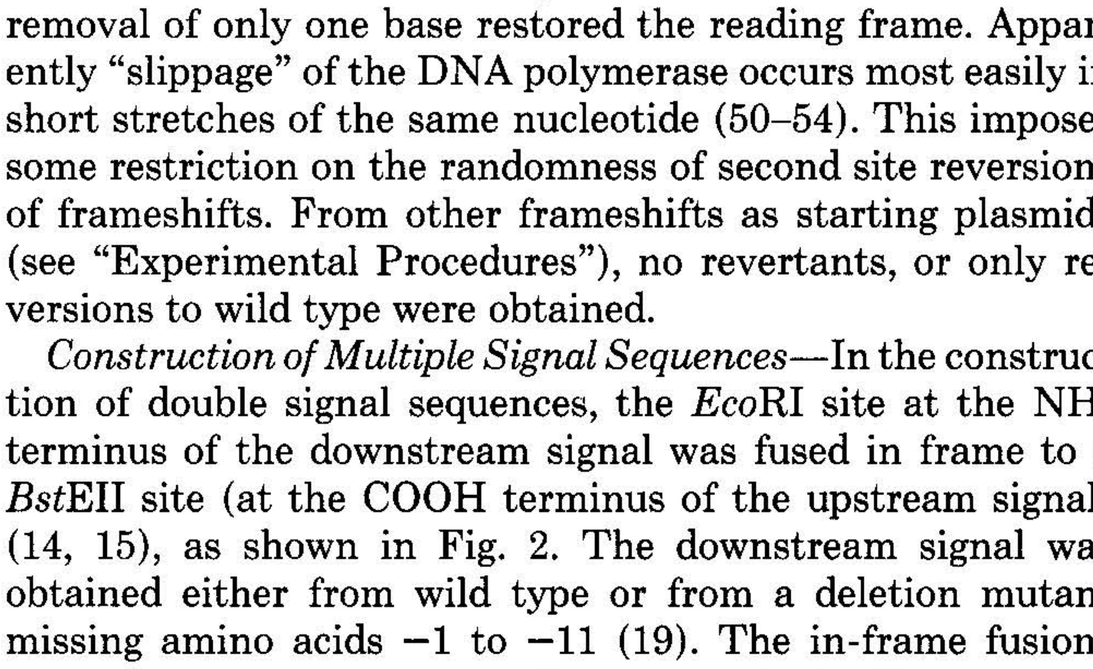


FIG. 1. **Frameshift revertants of β-lactamase.** The *top line* shows the wild-type sequence. The next three lines show the sequences of three revertants (*R3a, R8a,* and *R15a*) obtained from a frameshift containing an additional five bases at the cleavage site (*boxed nucleotides*). The frameshift was corrected *in vivo* by the insertion of an additional base indicated in *large bold capitals* over the beginning of the boxed amino acids. The amino acids encoded as a result are indicated in the *box*. The bottom mutant R4a was obtained from a deletion frameshift of five bases (indicated by *dashes in a box*). The *in vivo* selection led to the removal of one more base, indicated by a *bold dash*. The amino acids that were changed as a consequence are shown in the *box*.

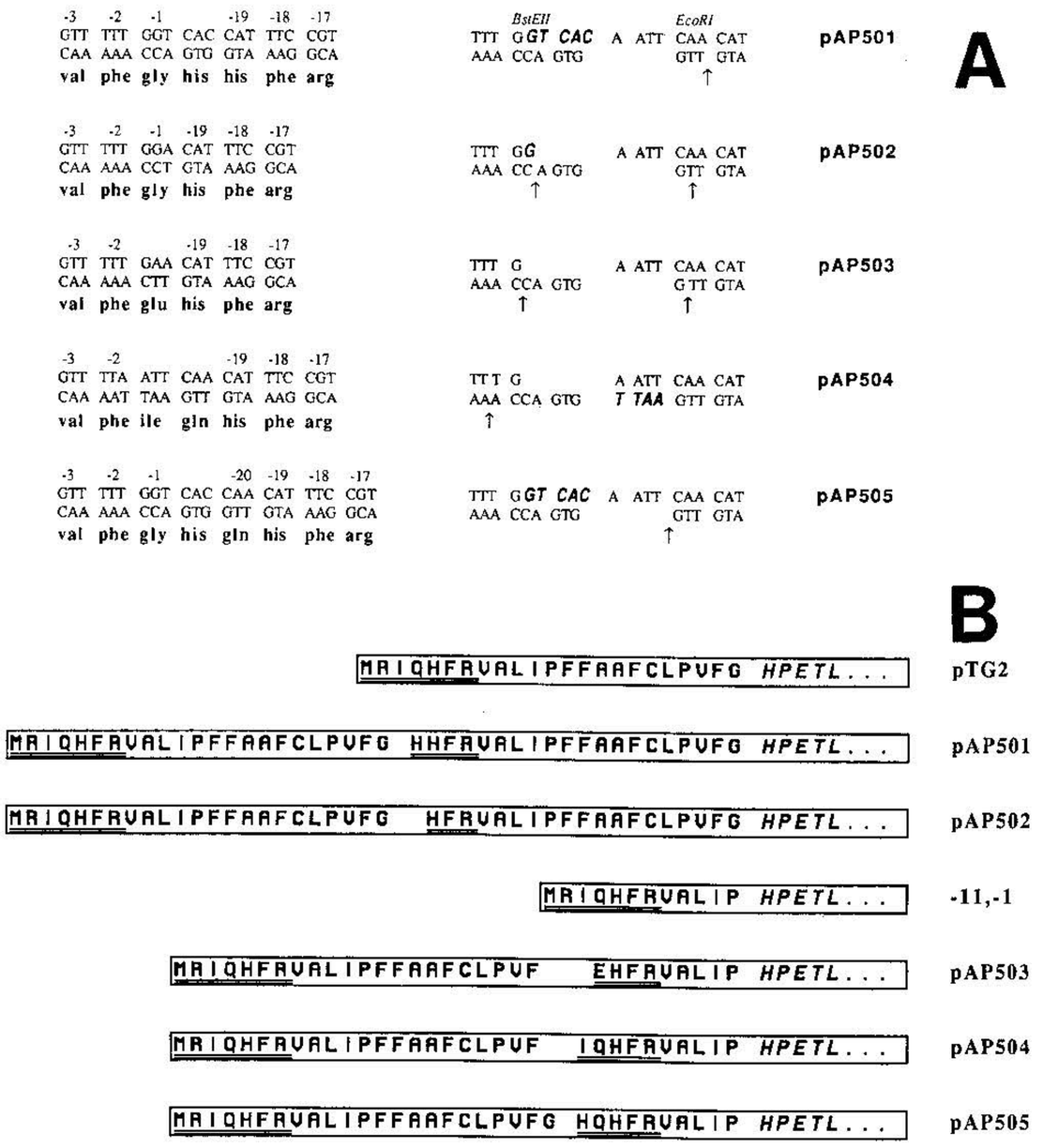


FIG. 2. **Construction of double signal mutants of β-lactamase.** *A*, on the left, the resulting sequence at the junction of the first signal with the second is shown, and on the right, the fragments from which it was obtained. pTG2 was digested with *Bst*EII and treated with Klenow polymerase in the presence of some or all dNTPs where indicated. The filled in nucleotides are shown in *large bold capitals*. In the case of partial or no fill in reaction, a nuclease S1 treatment was employed. The resulting cleavage is indicated by the *arrow*. Note that the enzyme did not cleave off the single strand overhang precisely in all cases, thus randomizing the junctions. Similarly, pTG2 or the deletion mutant −11,−1 (Ref. 19) was digested with *Eco*RI and either treated with nuclease S1 or with the Klenow fragment of polymerase in the presence of dATP and dTTP. The appropriate fragments were then ligated and sequenced. The numbering is that of the wild type (*w.t.*) signal. *B*, the resulting amino acid sequences of the double signal constructions. The polar $NH_2$-terminal regions are *underlined*, the mature protein is shown in *italics*.

nucleotides and subsequent digestion of the overhanging single strands with nuclease S1 as shown in Fig. 2*A*. Since the S1 reaction was carried out at 15 °C, attack of the double strand (resulting in removal of one to three bases) was also observed, thus randomizing the junction. Such overdigestion can often be minimized,[3] if desired, by carrying out this reaction at 4 °C. The different in-frame junctions between the two signals and how they were obtained are shown in Fig. 2.

### *Phenotypes of Mutants*

*Enzymatic Activities*—The frameshift revertants all result in transformants having very high enzymatic activity, although three of them have nothing resembling a signal peptidase recognition sequence. We conclude that in all cases active protein is made, albeit at a somewhat lower expression level and/or a somewhat lower specific activity.

All multiple signal sequence constructions also give transformants having substantial enzymatic activity, and it is evident that those which contain a potential signal peptidase cleavage site (pAP501, 502, and 505) have higher catalytic activity than those which do not (pAP503, 504). It must be noted that the catalytic activity produced by all mutants (relative to pTG2) is very dependent on the growth phase and

[3] A. Plückthun and J. R. Knowles, unpublished experiments.

the growth medium (19), presumably because of proteolysis. This proteolysis is more pronounced in the cytoplasm than in the periplasm, more pronounced in the stationary than in the logarithmic phase, and more pronounced in minimal than in rich medium. In late stationary phase, where cell fractionation is least plagued by cross-contamination because of labile spheroplasts, the total activity of pAP503 and 504 becomes too low for accurate determination. The *relative* activities of the mutants with respect to each other are, however, reproducible under defined conditions.

*$LD_{50}$ Values*—The $LD_{50}$ values of all mutants are listed in Table I, from which it can be seen that all mutant β-lactamases confer substantial resistance to ampicillin. This finding is only consistent with enzyme translocation across the inner membrane, since nontranslocated enzyme is known to confer no resistance to β-lactams whatsoever (14). A correlation between total catalytic activity and the $LD_{50}$ can be obtained (55) (data not shown) that is consistent with the proteins having been translocated across the inner membrane. We conclude from these *in vivo* data that a substantial fraction of each of the mutants must be transported to the outer face of the inner membrane or may be released into the periplasm.

*Cell Fractionations with Immunoprecipitation*—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 (19), were included as size markers and as controls for the quality of the fractionation procedure. Where appropriate, the deletion −11,−1 was also included as a size marker. Typical autoradiographs are shown in Fig. 3. The results of these fractionations are listed in Table I and can be summarized as follows. The revertant R4a behaves like the wild type. This sequence can be considered a deletion of the first two amino acids of the mature protein (which we constructed previously and determined to be translocated and cleaved (19)) with a simultaneous substitution of the COOH-terminal signal sequence residues from Phe-Gly to Leu-Ala. This substitution is conservative (16–18), and the sequence remains fully functional. Most interesting is the behavior of the other three revertants R3a, R8a, and R15a. Although we had observed a substantial *in vivo* resistance to ampicillin, the protein fractionates entirely with the spheroplasts and is not cleaved (Table I). These two findings are only consistent with a membrane location of these revertants such that the active site is capable of hydrolyzing periplasmic β-lactams. Anchoring by the uncleaved signal would be a likely way of achieving such a location.

The cell fractionation of the constructions with two complete signals in series (pAP501 and 502) shows a protein band of the size of the mature protein in the periplasmic fraction. This demonstrates that, although these constructions contain two cleavage sites, cleavage occurs quantitatively after the second, and folded precursor protein cannot be obtained this way. While this work was in progress, a similar observation was reported on another protein (56). Under the conditions of the labeling experiment, no intermediate band of the size of the precursor accumulates, leaving open the question of whether or not both signal peptidase sites are attacked sequentially.

In the case of a *truncated* downstream signal, the three constructions differ in the availability of a processing site after the first signal (Fig. 1). The plasmids pAP503 and 504 do not possess such a site, whereas pAP505 does. The fractionation results reflect this. A protein of the size of the processed deletion mutant −1,−11 is found in the periplasm for pAP505, indicating cleavage after the first signal and release into the periplasm of an active, soluble protein containing a truncated signal. In contrast, for pAP503 and 504, immunoreactive bands are observed in the cytoplasmic fraction and (for pAP503) in the membrane fraction as well, but only very little is found in the periplasm. The expression of pAP504 appears a little weaker so that only the main cytoplasmic band is visible. The distinction between "membranic" and "cytoplasmic" fraction from these experiments alone would be difficult since a weak association with a membrane may not survive the fractionation procedure. We deduce from the combination of resistance and fractionation data, however, that a large portion of the β-lactamase from pAP503 and 504 is membrane associated even though some of it may be lost from the membrane into the cytoplasmic fraction upon cell fractionation. The important point of these results is the correlation between the fractionation data and the *in vivo* measurements of ampicillin resistance. Both of these plasmids confer high resistance to β-lactams (Table I) indicating that the enzymes are active and their active sites are accessible to β-lactams in the periplasm. We conclude, therefore, that a large fraction of these mutant proteins encoded by pAP503 and 504, similar to the revertants R3a, R8a, and R15a described above, remains anchored to the outer face of the inner membrane in an enzymatically active state.

*Cell Fractionations with Activity Determinations*—To demonstrate the location of the gene products on the outer face of the inner membrane, protease accessibility experiments are traditionally used (57). The active, folded form of β-lactamase is, however, very protease resistant, and the catalytic activity was therefore localized as such. For this purpose cell fractionations were carried out, and enzymatic activity was determined in all cell fractions. A substantial activity in the unlysed spheroplasts, when compared with suitable controls, would

TABLE I
*Mutant phenotypes*

| Mutant[a] | Activity[b] | $LD_{50}$[c] | Growth rate[d] | Processing[e] | Location[f] |
|---|---|---|---|---|---|
| | % | μg/ml | | | |
| pTG2 | 100 | >4000 | + | + | p |
| −11,−1 | 57 | 5 | + | − | c |
| −20,−1 | 106 | 5 | + | − | c |
| pAP501 | 14 | 500 | + | + | p |
| pAP502 | 24 | 700 | + | + | p |
| pAP503 | 8 | 100 | + | − | c,m,(p) |
| pAP504 | 8 | 100 | + | − | c,m,(p) |
| pAP505 | 23 | 500 | + | + | p |
| R4a | 18 | 400 | + | + | p |
| R3a | 14 | 400 | + | − | c,m |
| R8a | 15 | 400 | + | − | c,m |
| R15a | 20 | 700 | + | − | c,m |

[a] Deletions are specified by the residue numbers of the first and last amino acids that are missing. The signal sequence is numbered from −23 (Met) to −1 (Gly), and the mature protein from +1 to +263. The other mutants are defined in Figs. 1 and 2. pTG2 specifies the wild type (Ref. 15).

[b] In French press lysates, compared to wild type (pTG2).

[c] Determined in broth, using ampicillin.

[d] The symbol + signifies normal growth within one standard deviation from wild type.

[e] Determined from the size of the β-lactamase species on denaturing gel electrophoresis.

[f] Summary from cell fractionation experiments (Fig. 3) and conclusions from experiments in Tables II and III. The symbol p signifies a periplasmic location, c, cytoplasmic, and m, membrane. A parenthesis signifies a minor portion at this location.

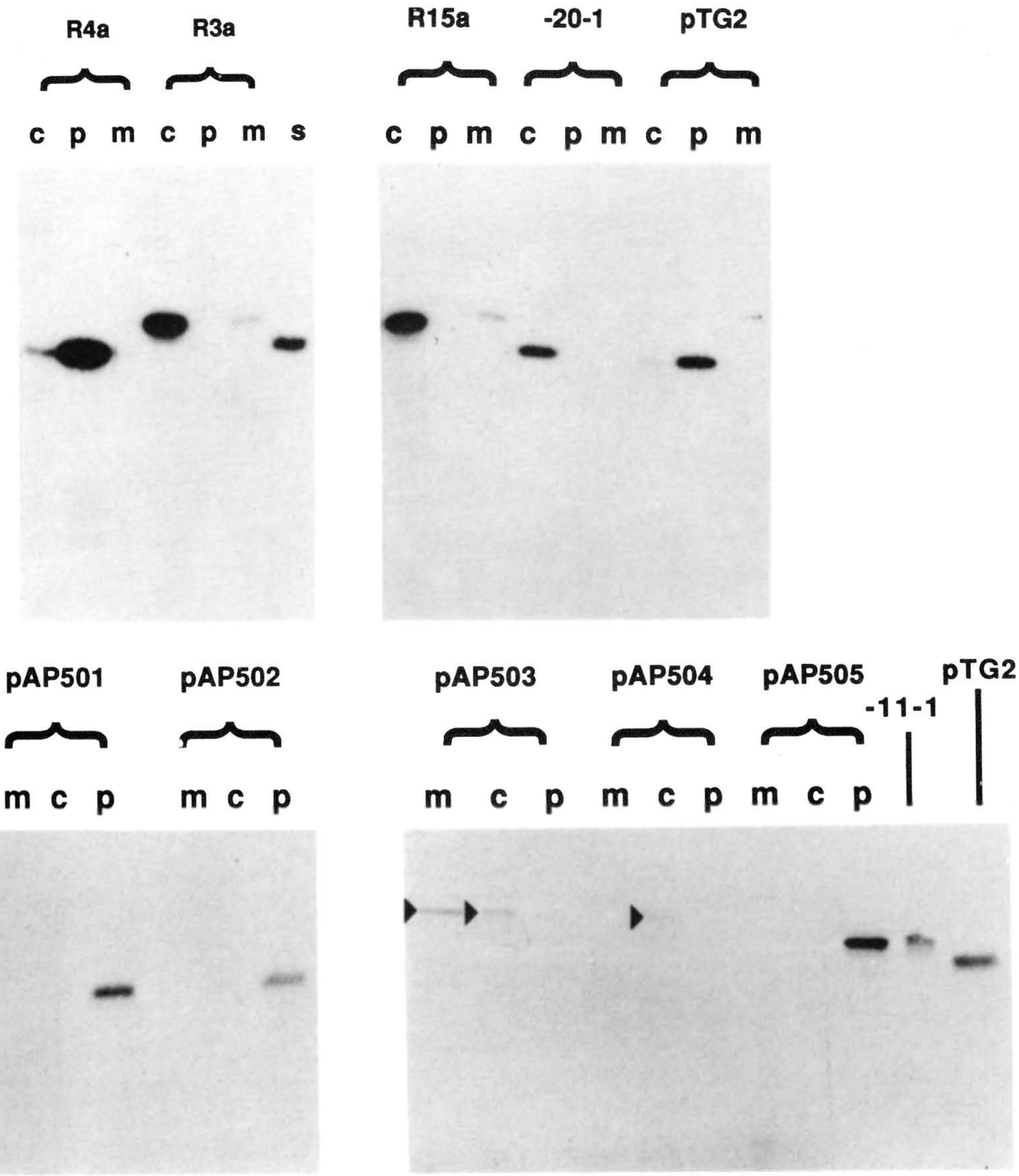


FIG. 3. **Cell fractionation of sample mutants.** For each mutant, three fractions were applied to the gel: *c* (cytoplasm), *p* (periplasm), and *m* (membrane). The conditions for the cell fractionation and the gel electrophoresis are described under "Experimental Procedures." The symbol *s* (standard) signifies wild-type β-lactamase (*pTG2*) as a size marker.

TABLE II

*β-Lactamase activities of revertants*

Relative activities of various fractions in cell fractionation experiments. The first number in each column indicates the β-lactamase activities related to the French press lysate of the wild type (Fraction of wild type, *Fr. of w.t.*). The French press lysate of the wildtype is set to 1.0. The second number in each column in parentheses relates each faction to the French press lysate of the particular mutant and is expressed in percent (percent of French press, *% of F.P.*).

| Mutant[a] | French press | Periplasm | Cytoplasm | Cytoplasm/ Triton | Unlysed spheroplasts |
|---|---|---|---|---|---|
| | *Fr. of w.t. (% of F.P.)* | *Fr. of w.t. (% of F.P.)* | *Fr. of w.t. (% of F.P.)* | *Fr. of w.t. (% of F.P.)* | *Fr. of w.t. (% of F.P.)* |
| pTG2[b] | 1.0 (100) | 0.90 (90) | 0.068 (7) | 0.047 (5) | 0.08 (8) |
| −20,−1 | 0.64 (100) | 0.015 (2) | 0.56 (88) | 0.663 (103) | 0.068 (11) |
| R3a | 0.21 (100) | 0.0023 (1) | 0.036 (17) | 0.072 (34) | 0.065 (31) |
| R8a | 0.23 (100) | 0.0026 (1) | 0.055 (24) | 0.083 (36) | 0.068 (30) |
| R15a | 0.29 (100) | 0.0027 (1) | 0.049 (17) | 0.102 (35) | 0.068 (24) |

[a] Mutants are specified as described in Table I and Figs. 1 and 2.
[b] pTG2 specifies the wild type.

directly demonstrate the location of the protein on the outer face of the inner membrane, *as well as its correct folding.* The results of these experiments are summarized in Tables II and III. Fractionation of the revertants R3a, R8a, and R15a all show very similar levels of activity in intact spheroplasts, in spheroplasts lysed by osmotic shock, or in Triton-solubilized

TABLE III

*β-Lactamase activities of double signal mutants*

Relative activities of various fractions in cell fractionation experiments. The first number in each column indicates the β-lactamase activities related to the French press lysate of the wild type (Fraction of wild type, *Fr. of w.t.*). The French press lysate of the wild type is set to 1.0. The second number in each column in parentheses relates each fraction to the French press lysate of the particular mutant and is expressed in percent (percent of French press, *% of F.P.*).

| Mutant[a] | French press | Periplasm | Cytoplasm | Cytoplasm/ Triton | Unlysed spheroplasts |
|---|---|---|---|---|---|
| | *Fr. of w.t. (% of F.P.)* | *Fr. of w.t. (% of F.P.)* | *Fr. of w.t. (% of F.P.)* | *Fr. of w.t. (% of F.P.)* | *Fr. of w.t. (% of F.P.)* |
| pTG2[b] | 1.0 (100) | 0.77 (77) | 0.057 (6) | 0.058 (6) | 0.069 (7) |
| −20,−1 | 0.55 (100) | 0.0096 (2) | 0.41 (74) | 0.44 (80) | 0.048 (9) |
| −11,−1 | 0.35 (100) | 0.0038 (1) | 0.15 (42) | 0.099 (28) | 0.031 (9) |
| pAP501 | 0.075 (100) | 0.058 (77) | 0.0095 (13) | 0.0077 (10) | 0.0094 (12) |
| pAP502 | 0.14 (100) | 0.10 (73) | 0.011 (8) | 0.0104 (7) | 0.012 (9) |
| pAP503 | 0.038 (100) | 0.0064 (17) | 0.0063 (17) | 0.0079 (21) | 0.021 (55) |
| pAP504 | 0.016 (100) | 0.0020 (13) | 0.0021 (13) | 0.0015 (9) | 0.013 (77) |
| pAP505 | 0.13 (100) | 0.096 (75) | 0.014 (11) | 0.012 (9) | 0.012 (9) |

[a] Mutants are specified as described in Table I and Figs. 1 and 2.
[b] pTG2 specifies the wild type.

spheroplasts. These results indicate that the active site of the enzyme is accessible to the outside of the spheroplasts. In the control experiments using the mutant −20,−1 in which most of the signal sequence has been deleted, only a small fraction of the total activity of the lysed spheroplasts is accessible in intact spheroplasts, indicating a cytoplasmic location of the enzyme. The wild type carrying pTG2 has, as expected, almost all of the β-lactamase activity in the periplasmic fraction.

The effectiveness of the fractionation protocol is limited by two problems: First, early log phase cells of the mutant strains all show higher relative catalytic activity with respect to pTG2 than do stationary phase cells. The spheroplasts are, however, rather labile, making these fractionations difficult. Late stationary phase cells can be fractionated more reproducibly, but the catalytic activities of the poorly expressed revertants are too low for accurate activity determinations, possibly due to protease degradation. As an acceptable compromise, we used cells harvested in early stationary phase.

Most (>97%) of the catalytic activity determined in a total French press lysate can be recovered in the appropriate fraction in the controls pTG2 and −20,−1. In the case of the pseudo-revertants, however, only about one-third of the total activity found in the lysate is recovered in the cell fractions of the same mutant, even in the presence of detergent. Several control experiments demonstrated that a lysis of whole cells under standard fractionation conditions (high concentration of cells, resuspended in hypertonic medium in the presence of excess EDTA) leads to decreased recoveries of activity for β-lactamase from *all* locations (periplasmic, cytoplasmic, and membrane). Similar results are obtained when spheroplasts are subjected to lysis in the French press. The loss of activity is less when $Mg^{2+}$ is added to the hypertonic solution and also if the French press lysis is carried out at lower cell densities. Neither of these modifications can be used in actual cell fractionations, however, since a minimum cell density is necessary to detect the low activity of some mutants, and incomplete release of cytoplasmic protein is observed in the presence of $Mg^{2+}$. The possibility that the spheroplast buffer itself is responsible for lower β-lactamase activities was eliminated with suitable controls. It seems likely that in hypertonic medium, some membrane aggregation or vesicle formation occurs at high cell densities, and this makes some of the enzyme inaccessible to substrate.

For the double signal sequence constructions, the results are summarized in Table III. For pAP501, 502, and 505, a fractionation similar to the wild type is observed with a similar percentage yield of activity. Again, the important cytoplasmic control −20,−1 shows only negligible leakage during cell fractionation. These results are then consistent with a periplasmic location of the gene products of the constructions pAP501, 502, and 505. In contrast pAP503 and 504 contain the greatest activity in the *unlysed* spheroplasts, even more than upon lysis by osmotic shock or by Triton treatment. These findings are consistent with a location of these mutant proteins predominantly on the outer face of the inner membrane, just as for the revertants R3a, R8a, and R15a. It is difficult to determine experimentally whether the small amount of activity found in the periplasm is released *in vivo* or is a consequence of the cell fractionation.

*Growth Rates*—Cell growth rates were determined for all transformants, in order to detect any effects of the mutant proteins on other cellular processes. Since the 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 explains a low growth rate for β-lactamase mutants (19). In the present case it was of particular interest to determine whether a membrane location of β-lactamase could impair other cellular functions and cause a decreased

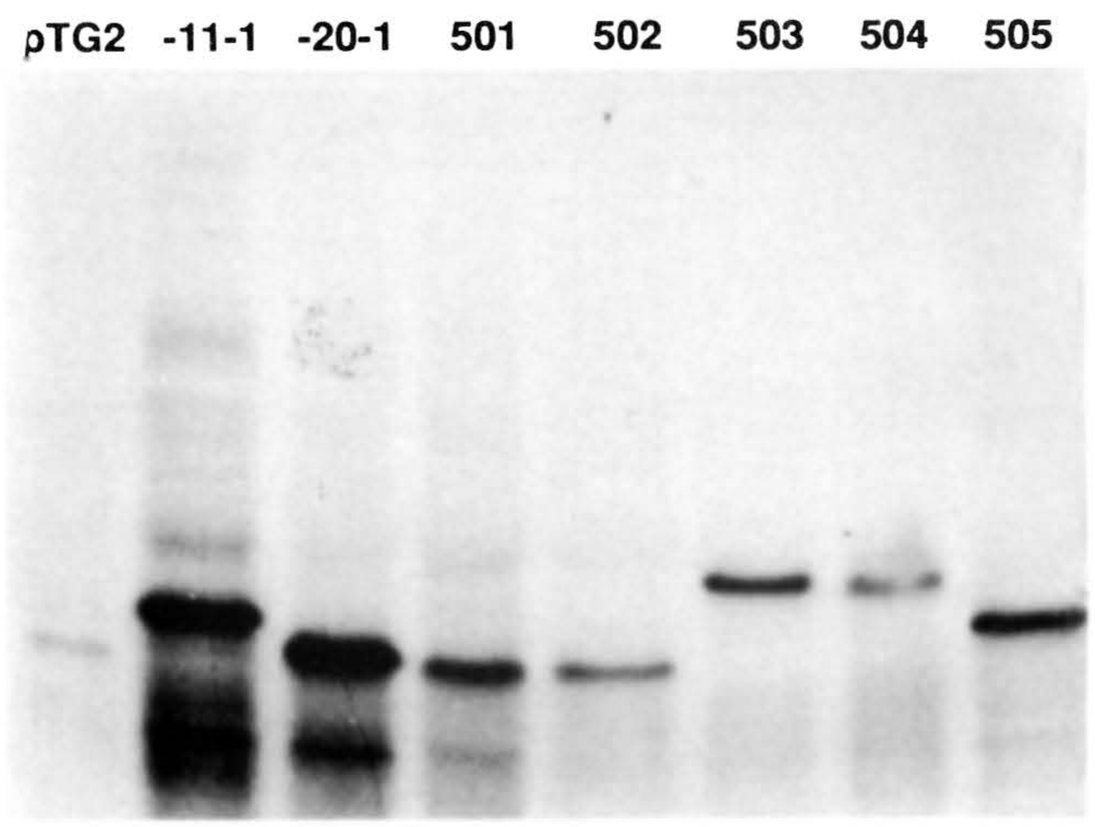


FIG. 4. **Analysis of sample mutants by the maxicell technique.** Irradiated cells were treated for 30 min with [$^{35}$S]methionine for 15 min at 37 °C, lysed, and applied to acrylamide electrophoresis as described under "Experimental Procedures."

growth rate. Under the conditions of the experiments (*E. coli* host strain DH1, rich medium 2YT, growth temperature 37 °C), no significant decrease in growth rate was observed for any of the plasmid constructions (see Table I). This indicates a surprising tolerance of *E. coli* toward extraneous protein on the outer face of its inner membrane.

*Maxicells*—To confirm the data on cleavage by the signal peptidase, the mutants pAP501–505 were also analyzed in maxicells (Fig. 4), especially since antibody precipitation of pAP503 and 504 resulted only in very weak bands. The results are entirely consistent with the other data. Signal processing occurs for pAP501, 502, and 505 but not for pAP503 and 504.

## DISCUSSION

We have obtained, both by construction and selection procedures, mutants of β-lactamase that remain anchored to the outer face of the inner membrane. There are two significant observations about these mutants. First, they are enzymatically active, and second, their expression does not cause any harmful effects to the cells that harbor them. The $NH_2$ terminus of β-lactamase may be extended without elimination of activity (19, 58). This finding is consistent with the recently published crystal structure (59). We show here that catalytic activity is still maintained even if the $NH_2$ terminus is membrane associated. On the one hand we obtained revertants in which a completely altered polar COOH-terminal portion of the signal, devoid of any signal peptidase recognition site, causes the signal to be uncleavable. On the other hand we specifically constructed transported proteins with truncated signals, with similar results. The completely unrelated signal sequence of the pseudo-revertants, whose only notable feature is a lack of charged amino acids, appears not to inhibit transport. We must conclude, therefore, that for the initiation of transport through the membrane only the $NH_2$-terminal charged part of the signal, and a sufficiently long hydrophobic portion is necessary. It is the sequences further downstream that determine the further fate of the protein. The results obtained here can be contrasted with our earlier findings (19, 55), in which deletions of more than seven amino acids from the COOH terminus of the signal were found to prevent translocation of the protein. We show here, by the fusion of another, complete signal $NH_2$-terminal to the truncated one, that the protein with the truncated signal is not itself refractory to transport. While both in the deletion mutants (with a single signal, Ref. 19) and in the revertants described here the signal peptidase recognition sequence is absent, the hydrophobic core sequence is much shorter in the deletion mutants tested previously, and this is the most likely cause of the different translocation behavior. Note that the COOH-terminal part of the signal sequence in the revertants does not contain any charged amino acid, and its amino acid composition is somewhat similar to that of natural signal sequences. In the deletion mutants of the COOH-terminal part of the signal, however, charged amino acids were introduced by the beginning of the mature protein. The amino acids following the hydrophobic core may thus determine the transport and release of the protein to the periplasm.

The question then arises whether release from the membrane into the periplasm is dependent upon a functional signal peptidase recognition site. We believe that this is not necessarily the case, since we have previously shown (55) that mutants appear to be released to the periplasm without cleavage of the signal. We cannot rigorously prove that the previously described mutants are found in the periplasmic fraction because of a true release *in vivo* since the protein might be released upon cell fractionation if the association to the membrane is very weak. It must be noted, however, that in those mutants (55) almost *all* of the activity was found in the periplasmic fraction whereas in the mutants described here, no activity (R3a, R8a, and R15a) or only a very small percentage (pAP503 and pAP504) appeared in the periplasmic fraction. This reproducible difference in fractionation behavior must be caused by a different property of the proteins. We suggest, therefore, that the previously described mutants (55) are largely released to the periplasm *in vivo* whereas only small fractions of the double signal constructions (pAP503 and pAP504) are released, and the revertant proteins (R3a, R8a, and R15a) are not found in the periplasm. It appears, therefore, that it is *not* necessarily the act of signal cleavage that permits release of the protein to the periplasm, but the signal "joining" sequence itself may permit the release of some preproteins but not others. Its structure may determine whether a preprotein can remain anchored to the membrane after the translocation step is completed.

None of the constructs described here have any measurable effect on the growth rate (which reflects, presumably, the effect on the transport of other, essential proteins) whereas some of our earlier mutants do have severe pleiotropic consequences (19, 55). Very small deletions (for example, of either residue +1 or −1) were found to have drastic effects on the growth rates (19). It is surprising that the expression of these new "membrane proteins" in *E. coli* has no ill effect on the cells. Since there must be a finite number of sites for protein secretion (possibly the SecY/SecA complex), the mutant β-lactamase molecules must be able to dissociate from these sites of translocation and become anchored in the lipid bilayer without great structural disturbance to the membrane as a whole.

We must then pose the question of why other uncleavable mutants of β-lactamase have such drastic consequences on cell growth (*e.g.* point mutants mp15, mp17, and mp63 (Ref. 55) and especially deletions −1 and +1 (Ref. 19)). Since these point mutants are close analogs of the wild type, their signal sequences may be competitive inhibitors of signal peptidase (19), whereas the constructs reported here may be so different at the signal peptidase recognition site that binding to the enzyme does not occur. This is plausible for R3a, R8a, and R15a and for the (absent) second cleavage site of pAP505. It is not clear, however, why pAP503 and pAP504 behave so different from the mutant −1, when a very similarly truncated first signal is present. The reconciliation of these differences must involve the sequence following the first signal, but we cannot yet provide a plausible molecular explanation. It is known (60) that the presence of signal peptidase is essential for growth, and inhibition of this enzyme may well interfere with normal essential cellular traffic. Alternatively, we cannot yet rule out that the protein conformation around the signal cleavage site may in some mutants slow down translocation, *e.g.* by preventing proper hairpin formation or proper recognition by a transport component.

Another deduction from these experiments is that a $NH_2$-terminal extension of the β-lactamase neither abolishes activity nor prevents protein folding. This does not imply that the folding kinetics and thermodynamics of the pre-β-lactamase are necessarily identical to the mature β-lactamase, but it shows that the protein can eventually reach an active, folded state even in the presence of various $NH_2$-terminal extensions. This finding stands in contrast to several observations in yeast, where no enzymatic activity of the β-lactamase precursor was found (11, 61, 62). In yeast the precursor has, however, presumably not traversed any membrane. The signal may thus influence the folding kinetics in the cytoplasm and/or

alter the folding pathway such that the native structure can only be achieved after passage through a membrane. Alternatively, a cytoplasmic factor may influence the folding of the precursor protein. It can be deduced from the experiments described here that cleavage of the signal is not necessary for the folding of the protein on the periplasmic face of the membrane. Rather, it appears that the membrane anchoring of the signal permits the rest of the protein fold into an enzymatically active state.

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