

Diverse Effects of Mutations in the Signal Sequence on the Secretion of β -Lactamase in *Salmonella typhimurium*

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Summary

Mutations in the β -lactamase structural gene that alter the signal peptide were used to study secretion into the periplasm of *Salmonella typhimurium*. Processing and cellular location of mutant gene products were followed by pulse-chase and cell-fractionation experiments and by trypsin accessibility in intact and lysed spheroplasts. The precursor proteins examined never appear as a free species in the periplasm. Two of the signal-sequence mutants accumulate a precursor form that is trypsin-accessible in intact spheroplasts; the precursors synthesized by the remaining mutants resemble wild-type in that they remain trypsin-inaccessible. One of the latter mutants does produce mature protein, but at a very reduced rate. It thus appears that signal-sequence mutations can affect more than one step in the secretion process, and that processing of the signal peptide is not required for the protein to be translocated (at least partially) across the inner membrane.

Introduction

Most secreted proteins in procaryotes and eucaryotes are synthesized with an extra amino-terminal peptide, which is referred to as the signal peptide. Signal peptides from different secreted proteins have similar structures (Michaelis and Beckwith, 1982). They contain a positively charged amino acid residue near the amino terminus of the peptide, followed by a "core" of 10-15 hydrophobic amino acid residues.

The role of signal peptides in the secretion of procaryotic proteins has been partially elucidated. Bassford and Beckwith (1979) have isolated mutations that alter the signal peptide of the maltose-binding protein (a periplasmic protein) and cause the accumulation of a precursor form of this protein in the cytoplasm. These mutations substitute a charged amino acid for one of the residues of the hydrophobic core (Bedouelle et al., 1980). These results suggest that signal peptides function to initiate secretion (that is, to interact with the cytoplasmic side of the inner membrane), and that the integrity of the hydrophobic core is important to that function.

TEM β -lactamase is a periplasmic protein of gram-negative bacteria that confers upon these organisms resistance to high levels of ampicillin. The β -lactamase encoded by the *bla* gene is synthesized initially in a precursor form, with an amino-terminal "signal" pep-

tide (Sutcliffe, 1978; Ambler and Scott, 1978). This signal peptide is cleaved from the amino terminus after the synthesis of β -lactamase is complete (Koshland and Botstein, 1980; Josefsson and Randall, 1981). The translocation of β -lactamase and across the inner membrane can also occur posttranslationally (Koshland and Botstein, 1982). β -Lactamase molecules altered at the carboxyl terminus are apparently translocated but not released into the periplasm (Koshland and Botstein, 1980, 1982). Thus the carboxyl end is required to finish the secretion process—that is, to become a free periplasmic protein (Koshland and Botstein, 1980, 1982).

We have characterized the synthesis and cellular localization of the β -lactamases produced by six mutants that contain alterations of the nucleotide sequence encoding the signal peptide. These mutations were isolated previously by localized in vitro mutagenesis (Shortle et al., 1980). The details of their history and the direct determination of their nucleotide sequences will be presented elsewhere (D. Koshland, D. Shortle, P. Grisafi, K. Talmadge and D. Botstein, manuscript in preparation). Pulse-chase, cell-fractionation and trypsin-accessibility experiments with these mutants suggest that changing the signal-peptide sequence can cause a wide variety of defects in secretion, including: the elimination of the translocation of β -lactamase across the inner membrane, a reduction in the rate of translocation of β -lactamase across the inner membrane and the accumulation of the precursor form of β -lactamase, which has translocated across the inner membrane but remains associated with a membrane in the periplasm.

Results

Signal-Sequence Mutations of *bla*

Figure 1 shows the nucleotide sequence of the region of the *bla* gene encoding the signal peptide, as well as the alterations in six mutants bearing lesions affecting only this region of the gene. The mutants were obtained by a combination of localized in vitro mutagenesis (Shortle et al., 1980) and in vivo methods: details of the origins of these mutants, the mapping of the mutations and the determination of their nucleotide sequences are being published separately (D. Koshland et al., manuscript in preparation).

Identification of the Protein Products of the Signal-Sequence Alleles

To identify the products of the *bla* gene harboring these signal-sequence mutations, we examined autoradiographs of SDS-polyacrylamide gels on which were displayed the labeled proteins synthesized in irradiated cells after infection with the appropriate P22*bla* phage (see Koshland and Botstein, 1982). In addition, we examined the products made by two frameshift mutants (B501 and B510) from which five

products of these revertants that comigrate with the wild-type mature species (*fs*(7,9) [Figure 2, lane 1], *pro*₂₀ → *ser* [Figure 2, lane n] and *ala*₉ → *val* [data not shown]) are identified as the mature form of β-lactamase. Rigorous proof that these putative mature forms have amino termini identical to that of the wild-type mature form will require sequencing of their amino termini. Products of these alleles that comigrate with the wild-type precursor (*fs*(14,21) [Figure 2, lane f]; *ala*₉ → *val* [data not shown]) or that migrate faster than the wild-type mature species (*pro*₂₀ → *leu* [Figure 2, lane h], *fs*(18,21) [Figure 2, lane j], *pro*₂₀ → *ser* [Figure 2, lane n] and *fs*(7,9) [Figure 2, lane l]) are identified as precursor forms of β-lactamase.

To establish that these putative precursors are the product of the *bla* gene and that they migrate more slowly than the wild-type mature species because of the presence of a signal sequence at each of their amino termini, we determined partial amino-terminal sequences (see Experimental Procedures). Cells infected with the appropriate P22*bla* phage were labeled with ³H-isoleucine and ³H-phenylalanine, and the labeled proteins were subjected to SDS-polyacrylamide gel electrophoresis. The labeled *bla*-encoded mutant proteins were purified from the gel and were subjected to automated Edman degradations.

Figure 3 summarizes the protein sequencing data. The peaks of radioactivity for the different precursor species appear in the expected proteins, given the amino acid sequence of the precursor form deduced from the DNA sequence of the gene (Sutcliffe, 1978), the labeled amino acids used in these experiments and the assumption that the amino-terminal methionine is removed. These data support our previous identification of the products of the *pro*₂₀ → *leu*, *fs*(14,21) and wild-type alleles that migrate more slowly than the mature form of β-lactamase as precursor forms of β-lactamase in which the signal peptide is still attached.

Rates of Processing

We performed pulse-chase experiments with the different signal-sequence mutants to examine the rates at which the mutant precursors of β-lactamase are matured. Irradiated cells were infected with the appropriate P22*bla* phage and subjected to a ³⁵S-methionine pulse, followed immediately by the addition of unlabeled methionine or chloramphenicol. Samples were added directly to sample buffer at 95°C immediately after the addition of unlabeled methionine (or chloramphenicol) and at intervals thereafter.

The results from these experiments are shown in Figure 4. When the infection was performed with phages carrying the *bla* alleles *fs*(14,21), *fs*(18,21) or *pro*₂₀ → *leu*, the intensity of the band at the precursor position remained constant over the length of the chase (as long as 10 min) and no band appeared at the position of the mature species (Figure 4D).

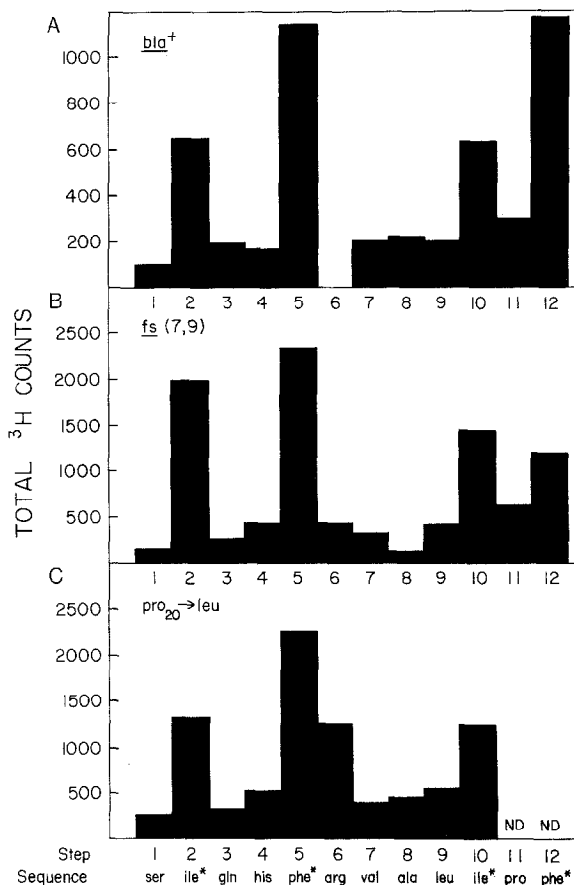


Figure 3. Partial Amino Acid Sequence of the Amino Termini of Precursor Forms of β-Lactamase

A culture of DB4381 was irradiated and infected with P22*bla* phages carrying the wild-type, *pro*₂₀ → *leu* or *fs*(7,9) *bla* allele at an moi of 10. The irradiated and infected cells were labeled with ³H-isoleucine and ³H-phenylalanine. The labeled *bla*-encoded proteins were purified and subjected to automated Edman degradation (see Experimental Procedures). The total ³H counts detected in each step of Edman degradation are given on the ordinate. The amino acid sequence of wild-type β-lactamase is listed below the step numbers. Asterisks: amino acid residues that should contain label.

When the infection was performed with phages carrying the *bla* alleles *fs*(7,9) or *pro*₂₀ → *ser*, a result was obtained that differed from the results with *fs*(14,21), *fs*(18,21), *pro*₂₀ → *leu* or the wild-type. In the insets of Figure 4, the autoradiographs from a pulse-chase experiment for *fs*(7,9) and *pro*₂₀ → *ser* are shown. The radioactivity in the precursor and product bands was quantitated for each lane of these gels and plotted as the percentage of total label present in *bla*-encoded species. The rate of transfer from the precursor to the mature species for these two *bla* mutants is much slower than the rate observed for the wild-type (compare Figures 3A, 3B and 3C). In addition, the percentage of total label in *bla*-encoded proteins that is in precursor form even after an extensive chase for *pro*₂₀ → *ser* is greater than that seen for the wild-type. For *ala*₉ → *val*, the rate of transfer of label from precursor to the mature species and extent

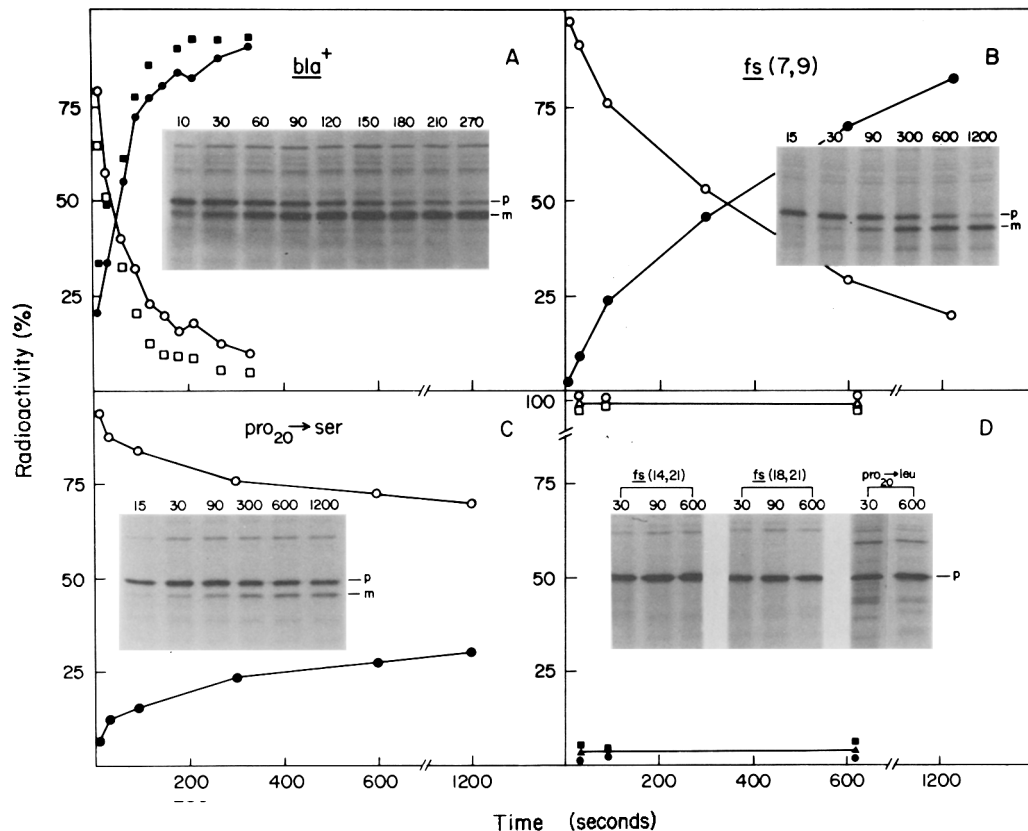


Figure 4. Pulse-Chase Analysis of the Gene Products of Signal-Sequence Alleles

Pulse-chase experiments for the *bla* alleles *tsH41*, *fs(7,9)*, *pro₂₀ → ser*, *fs(14,21)*, *fs(18,21)* and *pro₂₀ → leu* were performed as described by Koshland and Botstein (1980). Irradiated cells were infected with the appropriate *bla* phage and labeled with ³⁵S-methionine for 30 sec. The time of sampling during the chase varied for different experiments. Chloramphenicol instead of unlabeled methionine was used to stop the incorporation of label in the pulse-chase experiments with the alleles *fs(7,9)* and *tsH41*. (Insets) the autoradiographs for these pulse-chase experiments. p: precursor form. m: mature form. The autoradiograph of *tsH41* is not shown. The percentage of the total radioactivity in *bla*-encoded proteins in either precursor or mature form is plotted on the ordinates, and the length of the chase in seconds is plotted on the abscissas.

(A) The results from a pulse-chase experiment for the wild-type that was published previously (Koshland and Botstein, 1980) is presented for comparison. (○—○) *bla*⁺ precursor form; (●—●) *bla*⁺ mature form; (□—□) *tsH41* precursor form; (■—■) *tsH41* mature form. (B) (○—○) *fs(7,9)* precursor form; (●—●) *fs(7,9)* mature form, (C) (○—○) *pro₂₀ → ser* precursor form; (●—●) *pro₂₀ → ser* mature form. (D) (○—○) *fs(14,21)* precursor form; (●—●) *fs(14,21)* mature form; (□—□) *fs(18,21)* precursor form; (■—■) *fs(18,21)* mature form; (△—△) *pro₂₀ → leu* precursor form; (▲—▲) *pro₂₀ → leu* mature form.

of processing are indistinguishable from the wild-type (data not shown).

From these results, we draw the following conclusions. The alleles *fs(14,21)*, *fs(18,21)* and *pro₂₀ → leu* alter the amino acid sequence of the signal sequence of β-lactamase such that less than 5% (resolution of the autoradiographs) of these proteins is processed to give the mature species. The alleles *fs(7,9)* and *pro₂₀ → ser* alter the signal sequence such that the precursor is processed to the mature species, but at a much lower rate than the precursor containing the wild-type signal sequence.

Pulse-Chase Cell Fractionation

We investigated the cellular location of the precursor products of the wild-type and mutant *bla* genes by cell-fractionation experiments (see Koshland and Botstein, 1982). The procedures were designed to detect the possible change in cellular location of these pre-

cursors as a function of time. Irradiated cells were infected with the appropriate P22*bla* phage and labeled with ³⁵S-methionine for 30 sec, after which time unlabeled methionine was added. Samples of the labeled cells were removed at various times after the addition of the chase and immediately chilled to 0°C. The cells in each sample were separated into three subcellular fractions (soluble cytoplasmic, membrane-bound and periplasmic) as described by Koshland and Botstein (1982).

The results from these experiments are presented in Table 1, and are compared with data for the wild-type taken from Koshland and Botstein (1982). The wild-type and mutant precursors are recovered predominantly in the membrane and cytoplasmic fractions. The small amount of these precursors in the periplasmic fraction can be accounted for by contamination of this fraction with the cytoplasmic fraction. In control experiments, essentially no precursors were

Table 1. Pulse-Chase Membrane Fractionation of Wild-Type and Signal-Sequence Mutant Alleles of *bla*

Allele	Chase (sec)	Band	% Total Label in <i>bla</i> -Encoded Proteins		
			Periplasm	Soluble Cytoplasm	Membrane
<i>bla</i> ⁺	15	Mature	31	0	0
		Precursor	5	53	10
	30	Mature	49	0	0
		Precursor	1	41	9
	60	Mature	55	0	0
		Precursor	0	38	7
300	Mature	81	0	0	
	Precursor	0	11	8	
<i>fs</i> (7,9)	15	Mature	10	0	0
		Precursor	7	60	24
	90	Mature	22	0	0
		Precursor	3	44	31
	600	Mature	40	0	0
		Precursor	1	18	41
1200	Mature	42	0	0	
	Precursor	0	11	45	
<i>pro</i> ₂₀ → <i>ser</i> ^a	15	Mature	30	0	0
		Precursor	6	35	29
	90	Mature	41	0	0
		Precursor	3	21	35
	600	Mature	41	0	0
		Precursor	2	8	49
1200	Mature	51	0	0	
	Precursor	1	7	41	
<i>fs</i> (14,21)	15	Precursor	3	67	30
	90	Precursor	5	59	36
	180	Precursor	3	37	60
	390	Precursor	6	34	60
<i>fs</i> (18,21)	15	Precursor	3	43	57
	90	Precursor	2	42	55
	180	Precursor	0	27	73
	390	Precursor	0	26	74
<i>pro</i> ₂₀ → <i>leu</i>	15	Precursor	14	36	50
	90	Precursor	5	28	67
	180	Precursor	9	23	68
	390	Precursor	4	23	73

A culture of DB4381 was irradiated and infected with P22*bla* phages carrying the different signal-sequence mutant alleles of *bla*. The cells were labeled for 30 sec with ³⁵S-methionine, and then excess unlabeled methionine was added. At the indicated intervals after the addition of the chase, samples were removed and chilled to 0°C. Each sample was fractionated into three fractions: periplasmic, soluble cytoplasmic and membrane-bound (see Koshland and Botstein, 1982). Twenty microliters from each fractionation was subjected to SDS-polyacrylamide gel electrophoresis. The autoradiographs were exposed for 20–40 hr on preflashed film and scanned as described by Koshland and Botstein (1980). In all fractionations the apparent contamination of the periplasmic fraction with membrane and cytoplasmic fractions is less than or equal to 14%.

^a Unlike experiments with the wild-type *bla* allele, chilling of cells infected with *pro*₂₀ → *ser* did not inhibit processing of the precursor form to the mature form (see Koshland and Botstein, 1982).

recovered in the periplasmic fraction when this fraction was separated from the rest of the cell by osmotic shock (data not shown). The distribution of the mutant precursors among the cytoplasmic and membrane fractions is similar to that of the wild-type for all alleles except *fs*(18,21) and *pro*₂₀ → *leu*, whose precursors show a significantly greater association with the membrane at earlier time points in the chase.

From these experiments we conclude that the precursors of β -lactamase (independent of the allele) are never soluble periplasmic proteins. Since the precursor forms of these alleles differ from the mature β -

lactamase only by the presence of a signal peptide, processing of the signal peptide apparently is required for β -lactamase to become a soluble periplasmic protein.

Pulse-Chase Trypsin-Accessibility Experiments

To characterize further the cellular location of the *bla* signal-sequence mutants, we performed pulse-chase trypsin-accessibility experiments. Proteins that are localized in the periplasm or on the periplasmic side of the inner membrane should be digested by proteases when cells are converted to spheroplasts as well as

when the spheroplasts are lysed. In contrast, proteins that are localized in the cytoplasm should be protected from the degradative action of proteases when the cells are converted to spheroplasts, but not when the spheroplasts are lysed. Experimental details are given by Koshland and Botstein (1982).

The results of such experiments (Figure 5) indicate that the *bla* signal-sequence mutants are divided into two classes. The precursors of one class [*fs*(14,21) and *fs*(7,9)] behave identically to the precursors containing wild-type signal sequences (wild-type, *amH46*, *fsI7*, *tsH41* and *tsH1*) described by Koshland and Botstein (1982). These precursors are digested by trypsin only after lysis of the spheroplasts (Figure 5, panels 2a–2b, 3a–3b). For *fs*(14,21) the total amount of label in the precursor remains constant over the length of the chase, as expected, since this mutation abolishes processing of the signal sequence (see above). In the case of *fs*(7,9), 70% of the precursor that is trypsin-inaccessible in spheroplasts is converted to the secreted mature species during the chase (data not shown). These experiments suggest that essentially all of the precursor molecules of *fs*(7,9) and *fs*(14,21) are sequestered in the cytoplasm.

The precursors of the second class [*pro*₂₀ → leu,

*pro*₂₀ → ser and *fs*(18,21)] behave differently from the precursors described above. Sixty to seventy percent of the label in the precursors of *fs*(18,21) and *pro*₂₀ → leu after 20 sec of chase disappears when intact spheroplasts are exposed to trypsin (Figure 5, panels 4a and 6a). The remaining 30%–40% is digested if the spheroplasts are lysed (panels 4a and 6a). At the end of the chase more than 90% of the total label in these precursors disappears when intact spheroplasts are treated with trypsin (panels 4b and 6b). The precursor of *pro*₂₀ → ser behaves similarly to the precursor of *fs*(18,21) and *pro*₂₀ → leu in these experiments (panels 5a and 5b). However, like the cell-fractionation experiments (see legend to Table 1), the chilling of the samples apparently did not inhibit completely the processing and/or secretion of this precursor. As a result we are unable to determine whether the processed molecules that accumulate during the chase come from precursor that is inaccessible or accessible to trypsin in spheroplasts.

These data suggest that after a short chase the precursor molecules of *fs*(18,21), *pro*₂₀ → leu and *pro*₂₀ → ser are located in two cellular compartments. The fraction of precursor molecules that are trypsin-inaccessible is sequestered by the inner membrane, while the fraction of molecules that are trypsin-accessible

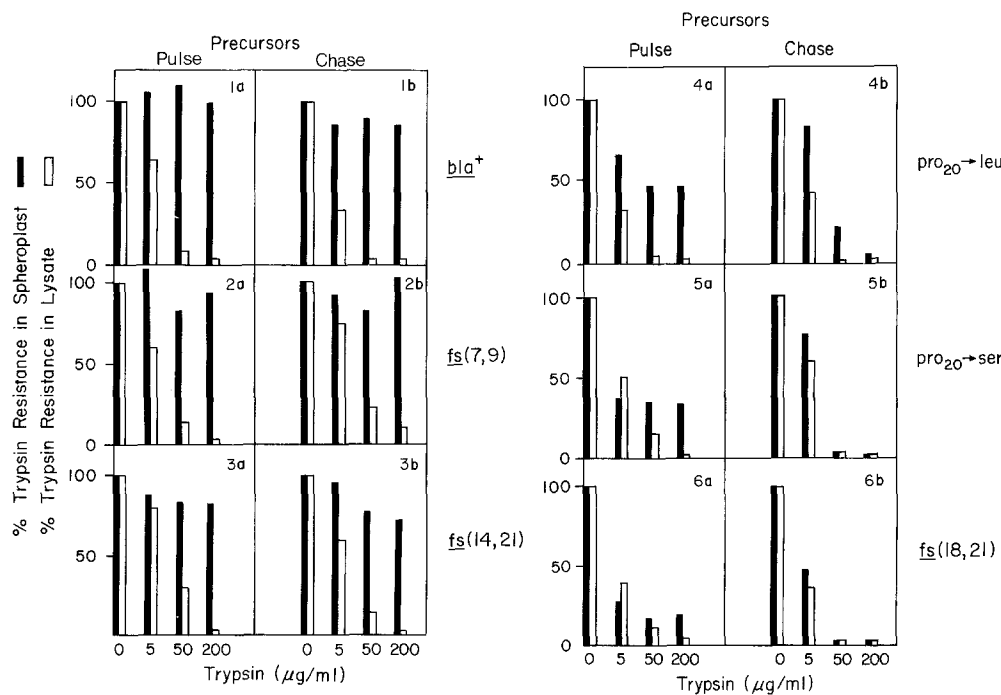


Figure 5. Pulse-Chase Trypsin-Accessibility Analysis of Precursors of *bla*-Encoded Proteins

A culture of DB4381 was infected with P22*bla* phages carrying the allele *fs*(7,9), *fs*(14,21), *fs*(18,21), *pro*₂₀ → ser, *pro*₂₀ → leu or wild-type at an moi of 10. The infected cells were labeled with ³⁵S-methionine and chased in the presence of unlabeled methionine. Samples were removed immediately after the addition of the chase and 10 min later, and chilled to 0°C. The samples were converted to spheroplasts and then split in half. The spheroplasts in one half were lysed osmotically. Aliquots of the intact and lysed spheroplasts were incubated with increasing amounts of trypsin for 10 min. Twenty microliters of each sample was subjected to SDS-polyacrylamide gel electrophoresis. The autoradiographs were exposed for 20–40 hr. Then for each of the alleles, the intensities of the bands due to *bla*-encoded proteins were quantitated. The results of a trypsin-accessibility experiment for the wild-type were taken from Koshland and Botstein (1982). Solid bars: percentage of total label in precursor or mature form that remains intact spheroplasts are treated with trypsin. Open bars: percentage of total label in precursor or mature form that remains after lysed spheroplasts are treated with trypsin.

sible is translocated at least partially across the inner membrane. After a long chase, apparently all of the precursors of these alleles are translocated at least partially across the inner membrane. We conclude that the processing of the signal peptide of β -lactamase is not required for β -lactamase to be translocated (at least partially) across the inner membrane.

The fraction of molecules that have translocated across the inner membrane can be estimated, in the case of these mutants, by the trypsin accessibility of the precursor form. For the wild-type, since apparently none of the precursor molecules is trypsin-sensitive in intact spheroplasts, the fraction of molecules that have translocated across the inner membrane after 20 sec of chase is equal to the fraction of label in the mature form (35%; data not shown). For the alleles $pro_{20} \rightarrow leu$ and $fs(18,21)$, which are defective for processing, the fraction of molecules that have translocated across the inner membrane after 20 sec is equal to the fraction of precursor molecules that are trypsin-accessible in intact spheroplasts—that is, 55% and 75%, respectively (Figure 5, panels 4a and 6a). These data suggest that the translocation across the inner membrane of the products of the alleles $fs(18,21)$ and $pro_{20} \rightarrow leu$ is either more rapid than the product of the wild-type allele, or more likely, less inhibited by chilling to 0°C.

The Doublet Phenomenon

When samples containing labeled proteins from irradiated cells infected with bla^+ phages were prepared for SDS-polyacrylamide gel electrophoresis by mixing them with sample buffer containing β -mercaptoethanol and heating them to 60°–90°C for 2 min, the mature species of β -lactamase migrated as a doublet on the gel (Figure 2, lane c). Subsequently, we determined that if the same samples are heated for an additional 3 min at 95°C, the mature species migrates as a single band that comigrates with the slower-migrating band of the doublet (Figure 2, lanes c and d). Under conditions where the mature species produces a doublet, the wild-type precursor does not (Figure 2, lane c).

Though we do not understand the cause of the doublet, it provides another property besides molecular weight and cellular location that differs between the wild-type precursor and mature species. Therefore, we investigated the ability of the proteins encoded by the signal-sequence alleles of bla to form doublets, to see if this phenotype correlates strictly with the processed state of the protein or with its cellular location.

In these experiments bla -encoded proteins for each signal-sequence allele were labeled (see Experimental Procedures), and sample buffer containing β -mercaptoethanol was added. The samples were split in two and either heated to 60°C for 2 min (doublet-forming conditions) or to 95°C for 5 min (non-doublet-forming conditions).

The results of this experiment are presented in Figure 2. They show that the ability to form a doublet correlates with the cellular location of the bla protein and not with the processed state of this protein. Precursors that accumulate on the cytoplasmic side of the inner membrane fail to form doublets under doublet-forming conditions. The precursor of $fs(14,21)$ behaves like wild-type precursor and fails to form doublets under doublet-forming conditions (Figure 2, lane e). The precursor of $fs(7,9)$ behaves mostly like wild-type, though a small amount of the faster-migrating species is apparent (Figure 2, lane l). However, in all cases in which we obtained evidence that a product (precursor or mature) of the bla allele is located on the periplasmic side of the inner membrane, that product formed a doublet. When the mature species is present, it forms a doublet under doublet-forming conditions regardless of the signal-sequence allele (Figure 2: $pro_{20} \rightarrow ser$, lane m; $fs(7,9)$, lane k; bla^+ , lane c). In addition, the precursors encoded by the alleles $fs(18,21)$, $pro_{20} \rightarrow leu$ and $pro_{20} \rightarrow ser$, which apparently accumulate precursor on the periplasmic side of the inner membrane, behave similarly to the wild-type mature species in two ways. They form easily visible doublets under the appropriate conditions, and all the label in precursor runs at the slower of the two precursor positions under non-doublet-forming conditions (Figure 2, lanes g, i and m). We conclude from these experiments that the ability of precursor or mature species to form a doublet is an intrinsic property of that protein that correlates strongly with its apparent cellular location.

Phenethyl Alcohol and β -Lactamase Secretion

Phenethyl alcohol (PEA), a molecule that affects membrane permeability (Silver and Wendt, 1967) and fluidity (Halegoua and Inouye, 1979), inhibits the processing of β -lactamase (Daniels et al., 1981). However, the inhibition of processing by PEA can be attributed either to its ability to inhibit directly the processing enzyme or to its ability to inhibit the secretion process such that the precursor fails to reach the processing enzyme.

We used PEA to inhibit the processing and/or secretion of β -lactamase (see Experimental Procedures). The amount of PEA required to inhibit processing of β -lactamase was identical to the amount previously determined in *Escherichia coli* (D. Oxender, personal communication). The precursors of $pro_{20} \rightarrow leu$ and wild-type that accumulated after exposure to PEA were tested for their ability to form a doublet. For both alleles, the precursor that accumulates in the presence of PEA fails to form a doublet. This result is particularly striking for $pro_{20} \rightarrow leu$, whose precursor forms a clear doublet in the absence of PEA (see Figure 7, compare lanes n and r with lanes a and f). Since a strong correlation exists between the ability of the bla -encoded protein to form a doublet and its cellular location, these results suggest that the pre-

cursors that accumulate in the presence of PEA are on the cytoplasmic side of the inner membrane.

To test this hypothesis, we performed trypsin-accessibility experiments in which half of the irradiated and infected cells were labeled and chased in the presence of PEA (see Experimental Procedures). The other half was labeled and chased in the absence of PEA, and then PEA was added. Standard trypsin-accessibility experiments were performed with these samples (see Experimental Procedures).

The results of these experiments for cells infected with the phages carrying the wild-type and $pro_{20} \rightarrow leu$ alleles are shown in Figures 6 and 7. For the wild-type infection, the precursor that accumulates as a result of PEA is digested with trypsin only in lysed, and not intact, spheroplasts (Figure 6). For $pro_{20} \rightarrow leu$, the precursor that is present after the cells are treated with PEA differs from the precursor when no PEA is present. In the absence of PEA, the precursor that accumulates after a 5 min chase is digested by trypsin in intact spheroplasts (Figure 7, lanes b, c and d). In the presence of PEA, the precursor of $pro_{20} \rightarrow leu$ is trypsin-insensitive in intact (Figure 7, lanes n, o and p) but not lysed (lanes r, s and t) spheroplasts. In all cases the same concentration of PEA was present in each digestion reaction, indicating that this drug does not inhibit directly the proteolytic activity of trypsin. We conclude that the effect of PEA on both mutant and wild-type is to cause the accumulation of precursor that by our criteria is on the cytoplasmic side of the inner membrane.

Discussion

In this study the products of the different signal-sequence alleles of *bla* have been characterized with respect to four criteria; their ability to be processed, their ability to form doublets under partial denaturing

conditions, their behavior in cell-fractionation experiments and their accessibility to trypsin in intact spheroplasts. These results can be summarized as follows. First, the signal-sequence alleles *fs*(14,21) and *fs*(7,9) apparently eliminate and slow down, respectively, the

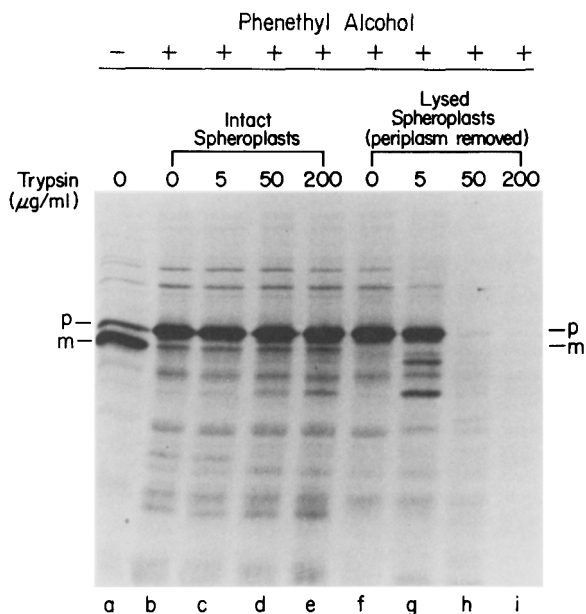


Figure 6. Trypsin-Accessibility Analysis of Proteins Produced by Cells Treated with Phenethyl Alcohol after Infection with P22*bla*⁺ Phage

A culture of DB4381 was irradiated and infected with P22*bla*⁺ phages at an moi of 10. The infected cells were labeled with ³⁵S-methionine for 30 sec in the presence (lanes b-i) or absence (lane a) of PEA and then chased with excess unlabeled methionine (see Experimental Procedures). The cellular locations of *bla*-encoded proteins in cells treated with PEA were analyzed by trypsin-accessibility experiments (Koshland and Botstein, 1982; see legend to Figure 4). Aliquots of 20 µl were loaded in each lane. Electrophoresis was carried out on a 12.5% gel, and the autoradiograph was exposed for 24 hr. p: precursor form. m: mature form.

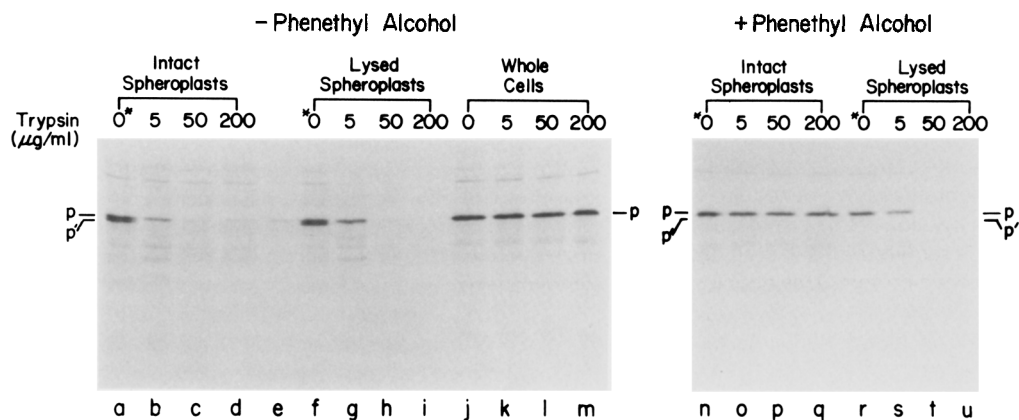


Figure 7. Trypsin-Accessibility Analysis of Proteins Produced by Cells Treated with Phenethyl Alcohol after Infection with P22*bla* Phages Carrying the $pro_{20} \rightarrow leu$ Allele

This experiment was performed exactly as described in the legend to Figure 6. The cellular locations of *bla*-encoded proteins labeled in the presence or absence of PEA were analyzed by trypsin-accessibility experiments (Koshland and Botstein, 1982; see legend to Figure 4). p: precursor form. p': precursor form present only under doublet-forming conditions. Asterisks: samples treated under doublet-forming conditions.

translocation of β -lactamase across the inner membrane. Second, the alleles $\text{pro}_{20} \rightarrow \text{leu}$, $\text{pro}_{20} \rightarrow \text{ser}$ and $\text{fs}(18,21)$ do not inhibit translocation of β -lactamase across the inner membrane, but do inhibit the processing of the signal peptide from the precursor form. The precursor forms of these alleles, though translocated across the inner membrane, remain associated with a membrane in the periplasm. Third, the products of certain *bla* alleles migrate as doublets on SDS-polyacrylamide gels under special conditions. The ability of *bla*-encoded proteins to migrate as doublets correlates with the cellular location of that protein and not with that of its processed state. Finally, PEA apparently blocks the translocation of β -lactamase across the inner membrane.

Novel Phenotypes

The products of the signal-sequence mutants of *bla*, except $\text{fs}(14,21)$, have properties not observed before in studies of mutations affecting signal sequences of secreted proteins. There are two obvious considerations that help to account for the differences between our results and those reported by others. First, the behavior of the wild-type precursor of β -lactamase is different from the precursors of other secreted proteins (see below). Second, signal-sequence mutations have often been selected by methods that favor the isolation of mutations that prevent specifically the initiation of secretion and leave the mutant protein free in the cytoplasm (Bassford and Beckwith, 1979). In contrast, the mutations that alter the signal sequence of β -lactamase were isolated by selection for the restoration of some *bla* function (that is, frameshift revertants) or by screening for the loss of *bla* function. Neither of these methods contains an obvious bias that would favor a class of mutations with a particular defect in secretion. Therefore, we might expect to isolate mutations in *bla* that affect initial, intermediate or terminal steps in the secretion of β -lactamase.

Processing of the Signal Peptide and Secretion

The properties of the precursors encoded by the alleles $\text{pro}_{20} \rightarrow \text{leu}$, $\text{fs}(18,21)$ and $\text{pro}_{20} \rightarrow \text{ser}$ (trypsin accessibility in intact spheroplasts and failure to fractionate as periplasmic) suggest that the processing of the signal peptide of β -lactamase is not required for translocation of at least a fraction of this protein across the inner membrane but is required for β -lactamase to become a soluble periplasmic protein. This conclusion resembles that drawn in studies of the f1 coat protein (Russel and Model, 1981). A model such as the one proposed by Inouye (DiRienzo et al., 1978), in which the signal sequence interacts with the cytoplasmic side of the inner membrane as a loop, accounts nicely for the requirement of the removal of the signal sequence prior to release of a translocated protein from the surface of a membrane.

At the moment, the nature of the defect in the

precursors encoded by $\text{fs}(18,21)$, $\text{pro}_{20} \rightarrow \text{ser}$ and $\text{pro}_{20} \rightarrow \text{leu}$ that results in the inhibition of processing is not understood. The amino acid substitutions in the signal peptide that are caused by these mutations are near the cleavage site. These amino acid substitutions may alter the cleavage site that is recognized by the protein responsible for removing the signal peptide from the precursor. Alternatively, one could hypothesize that the amino acid substitutions in the signal peptide divert these proteins from the normal pathway for the secretion of β -lactamase, and as a result divert them from the processing enzyme for β -lactamase.

From our results it is not possible to be certain that the precursor forms of the alleles $\text{pro}_{20} \rightarrow \text{leu}$, $\text{pro}_{20} \rightarrow \text{ser}$ and $\text{fs}(18,21)$, all of which are trypsin-accessible in intact spheroplasts, represent intermediates to the formation of the mature form. However, the translocation of these mutant precursor forms and the translocation of wild-type β -lactamase across the inner membrane apparently share at least one common step. The precursors of $\text{pro}_{20} \rightarrow \text{leu}$ and the wild-type mature form are both translocated across the inner membrane in a way that is blocked by PEA.

Translocation across the Inner Membrane

We reported (Koshland and Botstein, 1982) evidence that the translocation of β -lactamase molecules containing wild-type signal sequences across the inner membrane can occur posttranslationally. The properties of β -lactamase molecules containing signal-sequence mutations are consistent with this view of the translocation step. For all alleles of *bla* except $\text{fs}(14,21)$ we detected an increase in the fraction of molecules (either in the precursor or mature form) that had translocated across the inner membrane during the chase. The remaining allele, $\text{fs}(14,21)$, apparently is completely defective in translocation, and resembles signal-sequence mutations that have been isolated in another periplasmic protein, the maltose-binding protein (Bassford and Beckwith, 1979).

The fraction of β -lactamase molecules that have translocated across the inner membrane after a short chase may be significantly greater for the alleles $\text{fs}(18,21)$ and $\text{pro}_{20} \rightarrow \text{leu}$ than for the wild-type allele. The altered signal peptides of these alleles might increase the rate of translocation across the inner membrane or might improve the ability of β -lactamase molecules to translocate across the membrane at low temperatures.

The Doublet Phenomenon

Proteins encoded by *bla* that are localized to the cytoplasmic side of the inner membrane fail to migrate as a doublet on SDS-polyacrylamide gels, while *bla*-encoded proteins localized to the periplasmic side of the inner membrane to migrate as a doublet. This result suggests that the structure of β -lactamase is altered during or after its translocation across the

inner membrane. The formation of doublets cannot be accounted for by proteolysis at either the amino or carboxyl ends. However, other structural alterations are possible. Specific modifications of extracytoplasmic proteins have been reported (Hussain et al., 1980). Alternatively, β -lactamase may undergo some conformational change after translocation across the inner membrane. Since the formation of a doublet depends on the presence of β -mercaptoethanol and heat, the formation of a doublet may reflect the presence of a disulfide bond in the translocated *bla* protein that is absent while it is on the cytoplasmic side of the inner membrane. In support of this latter model, two cysteine residues exist in the mature polypeptide, though it is unknown whether these two amino acids form a disulfide bond.

A Working Model for the Secretion of β -Lactamase

We present a working model for the secretion of β -lactamase that is derived from the properties of the products of the wild-type, temperature-sensitive, chain-terminating and signal-sequence alleles of *bla* in pulse-chase cell-fractionation and trypsin-accessibility experiments. This model accounts simply for all the properties of the products of the different alleles of *bla*; it therefore seems helpful for assimilating these properties by organizing them into a useful framework. Second, it introduces a novel idea that the secretion of β -lactamase into the periplasm may be broken down through mutants into relatively defined steps like the morphogenesis of a phage.

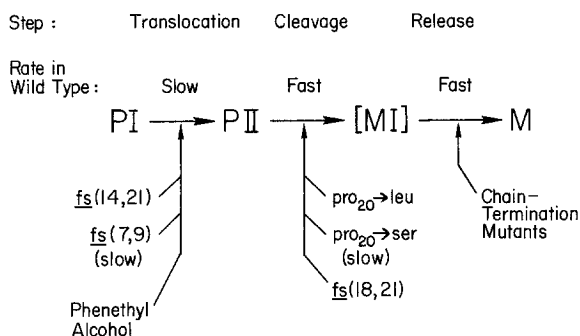


Figure 8. A Working Model for the Secretion of β -Lactamase

During the synthesis of wild-type β -lactamase the signal peptide may or may not interact with the inner membrane. Once the synthesis of the precursor is completed (PI precursor), it translocates across the inner membrane (the translocation step) but remains associated with the periplasmic surface of the inner membrane (PII precursor). After processing of the signal peptide, the mature form (MI) assumes a conformation that frees the β -lactamase from the inner membrane to give the secreted, mature form (M). For the secretion of the wild-type protein we postulate that the translocation step is the rate-determining step in this pathway, and therefore the only intermediate form of β -lactamase that accumulates is PI precursor. Mutations in this protein can change the rate of any one of these three steps and cause the accumulation of any of the three intermediates, PI precursor, PII precursor or MI mature form. The steps at which particular *bla* mutations or PEA exert their effects are indicated.

that the secretion of β -lactamase can be described as a simple pathway in which the precursor is synthesized on the cytoplasmic side of the inner membrane. The resulting precursor species, PI, is then at least partially translocated across the inner membrane to give the PII precursor. The signal sequence of the PII precursor is then removed. Finally, if the mature form that results from processing (MI) can assume its proper configuration, it is released from the membrane to give the secreted form, M.

In this model, wild-type precursor accumulates in the PI form and not the PII or MI forms because the translocation of PI across the inner membrane is the rate-determining step. The suggested slowness of this step could be due to the slow insertion of PI into the membrane or to the failure of the signal peptide, after it has inserted into the membrane, to be recognized rapidly by some component of a cellular-secretion machinery. It should be remembered that the *bla* gene only recently has been introduced into *Salmonella typhimurium* and *E. coli* as the result of transposition and studies in recombinant DNA (Heffron et al., 1975). The suggested slow function of the *bla* signal sequence in *S. typhimurium* relative to that of other secreted proteins of *S. typhimurium* and *E. coli* may result from the fact that the organism in which the *bla* gene evolved and in which the *bla* signal sequence was selected to function is taxonomically quite distant from *S. typhimurium*.

The model supposes that β -lactamase accumulates in the intermediate forms, PII and MI, only as the result of mutations (Figure 8) that reduce the rate of the steps involved with processing or release from the membrane. However, it should be emphasized that no direct evidence exists that shows that PII precursor forms of MI mature forms are normal intermediates to the formation of the secreted mature form, M. Furthermore, the order of the steps defined by the PII precursor and MI mature forms is uncertain; all we know is that the execution of both steps is required for the complete release of the protein from the membrane.

How a particular mutation or reagent inhibits the execution of a putative step in this model is unknown. However, it is interesting that the translocation of β -lactamase across the inner membrane is inhibited by mutations [*fs*(7,9) and *fs*(14,21)] that alter the length of the hydrophobic core of the signal peptide at low temperature and in the presence of PEA. Similar observations have been made in the study of other secreted proteins (Bedouelle et al., 1980; Emr et al., 1980; Pages et al., 1978; Lazdunski et al., 1979). In addition, processing of the signal peptide from β -lactamase apparently is not inhibited by low temperature (see properties of *pro*₂₀ \rightarrow *ser*). Russel and Model (1981) have reported a similar observation for the processing of the f1 coat protein. These similarities between β -lactamase and other secreted proteins

suggest that our observations on the secretion of β -lactamase should prove useful in understanding secretion in *E. coli* and *S. typhimurium*, and that our working model for the secretion of β -lactamase may prove useful in the study of other secreted proteins.

Experimental Procedures

Strains and Materials

Construction of the P22 specialized transducing derivative, P22Ap31pfr1 (referred to as P22*bla*), which carries the structural gene for β -lactamase (*bla*) is described by Winston and Botstein (1981). The origin of *bla*⁻ mutants and the genetic and physical mapping of the *bla* gene are described elsewhere (D. Koshland et al., manuscript in preparation). Mutants isolated by the mutagens hydroxylamine, ICR-191, ultraviolet light and bisulfite are designated by the letters H, U, U and B. Mutations known to be amber, ochre or frameshift on the basis of their response to host tRNA suppressors are marked *am*, *oc* and *fs*. Revertants of bisulfite-induced *bla* mutations are designated by the amino acid change(s) that result from the lesion, as determined by its nucleotide sequence. L-2,3-³H-alanine, L-2,6-³H-phenylalanine, L-4,5-³H-isoleucine, L-3,4(n)-³H-valine and ³⁵S-methionine were purchased from Amersham/Searle.

General Methods

Labeling of phage-encoded proteins after infection, autoradiography, SDS-polyacrylamide gel electrophoresis, sample buffer and quantitation of autoradiographs have been described previously (Koshland and Botstein, 1980). Pulse-chase experiments were also performed as described by Koshland and Botstein (1980), except that in some experiments incorporation of ³⁵S-methionine into protein was stopped by the addition of chloramphenicol (200 μ g/ml final concentration). Pulse-chase cell-fractionation experiments and pulse-chase trypsin-accessibility experiments are described by Koshland and Botstein (1982).

Trypsin-Accessibility Experiments of Cells Treated with PEA

These experiments were carried out exactly as described for the pulse-chase trypsin-accessibility experiments, with the following modifications. At 20 min after the infection began, 0.5 ml of the irradiated and infected cells were transferred to a tube containing 15 μ l of a solution that contained 10% PEA and 20% ethanol. Fifteen microliters of 20% ethanol was added to 0.5 ml culture as a control. Thirty minutes after infection, the cells in the two cultures were labeled for 30 sec, and then unlabeled methionine was added as described above. Five minutes after the addition of the chase, the tubes were removed to ice and a standard trypsin-accessibility experiment was performed.

Partial Amino Acid Sequence of the Amino Terminus of *bla*-Encoded Proteins

Cells were irradiated and infected as described above. Thirty minutes after infection, 1.0 ml of these cells was labeled for 3 min with either 100 μ Ci L-3,4(n)-³H-valine and 100 μ Ci L-2,3-³H-alanine (to determine the partial amino acid sequence of the mature species); 100 μ Ci L-2,6-³H-phenylalanine and 100 μ Ci L-4,5-³H-isoleucine (to determine the partial amino acid sequence of the precursor species); or 100 μ Ci ³⁵S-methionine (to act as a marker; see below). Cells were centrifuged in a microfuge, and the supernatant was discarded. The pellet was resuspended in 50 μ l sample buffer and heated to 95°C for 5 min.

To purify the ³H-labeled *bla*-encoded proteins the following steps were taken. Twenty microliters of the sample that contained the ³H-labeled proteins was loaded onto a lane of an SDS-polyacrylamide gel. The lanes flanking this lane were loaded with 10 μ l of cells treated identically to the ³H-labeled cells but labeled with ³⁵S-methionine instead. The stacking gel and running gel were poured 24 hr in advance. After electrophoresis, the gel was soaked in 10% acetic acid for 5 hr and then dried down under vacuum (see above). The dried gel was marked with ¹⁴C-labeled radioactive ink and then used

to make a 5–10 hr exposure of an autoradiograph. The gel and the autoradiograph were aligned by the ink spots. ³H-labeled *bla*-encoded proteins were not visible in the autoradiograph, but the ³⁵S-methionine-labeled *bla*-encoded proteins were. The bands from the latter proteins were used to determine the positions of the ³H-labeled *bla*-encoded proteins in the gel. A slice of the gel containing the ³H-labeled *bla*-encoded proteins was removed from the gel with a razor. The gel slice was soaked overnight at 37°C in 1.0 ml of a solution containing 1% triethylamine, 100 μ g/ml bovine serum albumin and 0.1% SDS. The supernatant containing the eluted protein was frozen at –20°C.

To determine the partial amino acid sequence of the gel-purified proteins, we subjected the ³H-labeled *bla*-encoded proteins to automated Edman degradation in a Beckman 890C sequencer using the 0.1 M Quadrol program described by Brauer et al. (1975). Aliquots of the products of the first 10–20 cycles were resuspended in Aquasol and were counted in an LS-230 Beckman liquid scintillation counter. In some cases the labeled phenylthiohydantoin amino acid derivatives were identified by high-performance liquid chromatography.

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