JOURNAL OF BACTERIOLOGY, Feb. 1994, p. 563–568
0021-9193/94/$04.00+0

Vol. 176, No. 3

Selection of Functional Signal Peptide Cleavage Sites from a Library of Random Sequences

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Received 21 September 1993/Accepted 19 November 1993

The export of proteins to the periplasmic compartment of bacterial cells is mediated by an amino-terminal signal peptide. After transport, the signal peptide is cleaved by a processing enzyme, signal peptidase I. A comparison of the cleavage sites of many exported proteins has identified a conserved feature of small, uncharged amino acids at positions −1 and −3 relative to the cleavage site. To determine experimentally the sequences required for efficient signal peptide cleavage, we simultaneously randomized the amino acid residues from positions −4 to +2 of the TEM-1 β-lactamase enzyme to form a library of random sequences. Mutants that provide wild-type levels of ampicillin resistance were then selected from the random-sequence library. The sequences of 15 mutants indicated a bias towards small amino acids. The N-terminal amino acid sequence of the mature enzyme was determined for nine of the mutants to assign the new −1 and −3 residues. Alanine was present in the −1 position for all nine of these mutants, strongly supporting the importance of alanine at the −1 position. The amino acids at the −3 position were much less conserved but were consistent with the −3 rules derived from sequence comparisons. Compared with the wild type, two of the nine mutants have an altered cleavage position, suggesting that sequence is more important than position for processing of the signal peptide.

Many secreted proteins in prokaryotes and eukaryotes are synthesized with an amino-terminal extension referred to as the signal peptide. The signal peptide directs protein translocation across membranes and is removed by a membrane-bound signal peptidase enzyme after transit through the membrane.

The signal peptide consists of three regions: a 1- to 5-residue amino-terminal positively charged segment, a 10- to 15-residue central hydrophobic core, and a 3- to 7-residue hydrophilic carboxy-terminal section (28). The carboxy-terminal domain is the site of signal peptide cleavage by the signal peptidase. There are two *Escherichia coli* signal peptidases. Signal peptidase II processes lipoproteins (10), and signal peptidase I processes other secretory proteins (4). Sequence comparisons of many cleavage sites indicate that there is no strong sequence homology. However, the sites have a common pattern of small amino acids at the −1 (Ala, Gly, and Ser) and −3 (Ala, Gly, Ser, Val, and Ile) residue positions (20, 25). Results from site-directed mutagenesis of the −1 and −3 positions support the −1 and −3 sequence requirements inferred from the sequence comparisons (7, 24).

In addition to the sequence requirements at −1 and −3, the transition from the central hydrophobic region to the cleavage region (−6 to −4) often contains a helix-breaking residue, such as Pro, Ser, or Gly. Studies that support the importance of a helix-breaking residue include mutagenesis experiments in which amino acid substitutions of the −4 proline of β-lactamase and the −6 proline of the M13 procoat were shown to result in inefficient signal peptide processing (11, 24). In contrast, signal peptides that include a proline at the +1 position are not cleaved and have been shown to act as competitive inhibitors of signal peptidase I (1).

TEM-1 β-lactamase is a periplasmic protein of gram-negative bacteria that confers upon these organisms resistance to β-lactam antibiotics, such as ampicillin (5). Previously, we used random-replacement mutagenesis to identify regions of TEM-1 β-lactamase that are important for the structure and function of the enzyme (18, 19). The strategy involves randomizing three to six contiguous codons to form a library containing all or nearly all possible substitutions for the region randomized. The percentage of random substitutions that result in a functional protein is then assessed by an in vivo assay. To experimentally determine the sequences required for efficient cleavage, we simultaneously randomized the amino acid residues from positions −4 to +2 of TEM-1 β-lactamase to form a library of random sequences. Randomizing the entire region has the advantage of minimizing the context effects of residues in this region that are unique to β-lactamase. Mutants that provide wild-type levels of ampicillin resistance were then selected from the random-sequence library and sequenced. The sequences of several mutants indicated a bias towards small amino acids, especially alanine, in the region from −4 to +2. The N-terminal amino acid sequence of the mature β-lactamase for nine mutants indicated that the sequences of the mutants are broadly consistent with the −3 and −1 sequence requirements and that proline is often the preferred residue at the −4 position.

MATERIALS AND METHODS

Materials. All enzymes were purchased from New England Biolabs, except for T7 DNA polymerase, which was purchased from United States Biochemical Corp. The antibiotic ampicillin was purchased from Sigma Chemical Co. Anti-β-lactamase polyclonal antibody was a gift from Jim Swartz of Genentech, Inc. Radiolabeled [^{35}S]dATP and [^{35}S]methionine were purchased from Amersham, Inc.

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Plasmid pBG66 is the parent plasmid of all random-sequence library constructions (17, 18). Plasmid pBG66 is a 4.8-kb derivative of pBR322 and pBR325 that contains the TEM-1 *bla* gene, as well as the *cat* gene, which encodes chloramphenicol acetyltransferase. The plasmid also contains the ColE1 and f1 origins of DNA replication.

Bacterial strains. *E. coli* HB101 (K-12 *hsdS20* [r_B^- m_B^-] *recA13 ara-14 proA2 lacY1 galK2 rpsL20* [Sm^r] *xyl-5 mtl-1 supE44* λ^-/F^-) (2) was used for the random-sequence library constructions. *E. coli* TG1 [K-12 Δ(*lac-pro*) *supE thi hsdDS*/F' *traD36 proA*$^+$*B*$^+$ *lacI lacZM15*] was used for all other experiments.

Construction of the random-sequence library. The −4 to +2 random-sequence library was constructed by random-replacement mutagenesis as described by Palzkill and Botstein (17, 18).

Selection of active mutants. To select for functional mutants, plasmid DNA from the random-sequence library was used to transform *E. coli* TG1 by the $CaCl_2$ method (21). The transformation mixture was plated on Luria-Bertani plates containing 1 mg of ampicillin, 500 μg of ampicillin, or 12.5 μg of chloramphenicol per ml.

Plasmid isolation and DNA sequencing. Single-stranded plasmid DNA was prepared for DNA sequencing as described by Sambrook et al. (21). DNA sequencing was performed by the dideoxynucleotide chain termination method (22). Custom-synthesized oligonucleotides were used to prime synthesis from defined sites within the TEM-1 *bla* gene. Plasmid DNA was prepared from *E. coli* by the alkaline lysis procedure (21).

Immunoblots of β-lactamases. Steady-state levels of precursor and mature β-lactamases were examined by immunoblotting. Cultures were grown in LB broth supplemented with 12.5 μg of chloramphenicol per ml to mid-log phase (A_{600} = 0.35). A 1.5-ml portion of each culture was pelleted in a microcentrifuge for 30 s. The pellets were resuspended in 0.1 ml of 50 mM Tris-Cl (pH 6.8)–100 mM dithiothreitol–2% sodium dodecyl sulfate (SDS)–0.1% bromophenol blue–10% glycerol. The cells were placed at 95°C for 10 min. A 20-μl portion of each lysate was resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (9% acrylamide). The proteins were transferred to nitrocellulose by electroblotting, and the blot was treated with anti-β-lactamase polyclonal antibody. The bands were visualized with anti-rabbit antibodies labeled with horseradish peroxidase by use of the Amersham ECL Western blot (immunoblot) system. The ratio of precursor to mature β-lactamases was quantitated by laser densitometry with an LKB 2222-010 UltroScan-XL laser densitometer.

Antibiotic susceptibility testing. MIC determinations were performed by agar dilution on LB agar containing serial twofold dilutions of ampicillin with an inoculum of 10^4 CFU per spot. Spotting of cells was done with a multipronged replicaplating device.

Cellular localization of β-lactamase. To localize β-lactamase in the signal peptide cleavage mutants, cultures were grown in LB broth supplemented with 12.5 μg of chloramphenicol per ml to mid-log phase (A_{600} = 0.35). A 1-ml quantity of cells was placed in duplicate in 1.5-ml Eppendorf tubes. Cells in each tube were pelleted for 30 s in a microcentrifuge. One pellet (whole-cell fraction) was resuspended in 0.2 ml of 10 mM Tris-Cl (pH 7.5), and the other pellet was resuspended in 0.2 ml of spheroplast buffer (30 mM Tris-Cl [pH 8.0], 1 mM EDTA, 20% sucrose). The cells in spheroplast buffer were pelleted and resuspended in 0.2 ml of ice-cold H_2O. The cells were again pelleted, and the supernatant (periplasmic fraction) was placed in a separate tube. The pellet (cytoplasmic and membrane fraction) was resuspended in 0.2 ml of 10 mM Tris-Cl (pH 7.5). A 25-μl quantity of each fraction was added to 25 μl of 100 mM Tris-Cl (pH 6.8)–200 mM dithiothreitol–4% SDS–0.2% bromophenol blue–20% glycerol, and the fractions were resolved by SDS-PAGE. β-Lactamase was visualized by Western blotting with anti-β-lactamase polyclonal antibody as described above.

β-Lactamase activity assay. Enzyme activity was assayed with 0.5 μg of each of the periplasmic extracts obtained by osmotic shock for the wild type and mutants 5-4, 5-14, and 7-4 (see Fig. 2). The hydrolysis of ampicillin was monitored spectrophotometrically at 30°C with 0.05 M phosphate buffer (pH 7.0) containing 100 μM ampicillin on a Beckman model DU 640 spectrophotometer. The spectral parameters used for ampicillin were 235 nm and $\Delta\varepsilon$ = 900 M^{-1} cm^{-1}. The unit used for the expression of β-lactamase activity was the amount of enzyme needed to hydrolyze 1 μM ampicillin per min at pH 7 and 30°C. The specific activity of each extract (see Table 2) is therefore the number of β-lactamase units per milligram of periplasmic extract protein.

Purification and amino-terminal sequence analysis of mature β-lactamase mutants. All β-lactamases were purified for N-terminal sequencing as follows. *E. coli* TG1 producing the altered β-lactamase enzymes was grown to late log phase in LB broth containing 12.5 μg of chloramphenicol per ml (21). β-Lactamase and other periplasmic proteins were isolated by an osmotic shock procedure (15). The resulting material was dialyzed overnight against 20 mM triethanolamine–0.5 M NaCl (pH 7.0). The β-lactamase activity was then eluted from a type B aminophenylboronic acid-agarose column that had been equilibrated with the same buffer as that used for dialysis (3). Fractions containing β-lactamase activity were identified by nitrocefin hydrolysis and SDS-PAGE (16). For amino-terminal sequencing, protein from fractions with the highest purity (80 to 90% pure) was fractionated by SDS-PAGE and electroblotted to a polyvinylidene difluoride membrane. The membrane was stained with Coomassie blue, and the band corresponding to β-lactamase was sliced from the membrane. The amino terminus of the immobilized β-lactamase was then sequenced with an Applied Biosystems 477A protein sequencer.

Radiolabeling of β-lactamase, immunoprecipitation, SDS-PAGE, and autoradiography. Cultures were grown to mid-log phase (A_{600} = 0.35) in M9 minimal medium (21) supplemented with 12.5 μg of chloramphenicol per ml. Cells were labeled with [^{35}S]methionine at a concentration of 30 μCi/ml. The chase was begun by adding unlabeled methionine to a concentration of 0.25%. After 10, 30, 60, and 300 s, 0.8 ml of the culture was removed and the β-lactamase was immunoprecipitated by a procedure described previously for maltose-binding protein (6). Immunoprecipitates were resolved by 9% SDS-PAGE. The SDS-polyacrylamide gel was placed against blotting paper and dried in a gel dryer. The dried gel was used for autoradiography.

RESULTS

Signal peptide cleavage site random-substitution library. To identify amino acid sequences that are effective substrates for signal peptidase, the codons for residues −4 to +2 were replaced with DNA of random sequences to form a library of random amino acid sequences for this region (Fig. 1). The nucleotides were randomized by the random-replacement mutagenesis protocol previously described (17, 18). In brief, the technique makes use of the type IIs restriction endonuclease *Bsp*MI, which recognizes a 6-nucleotide sequence and cleaves the DNA 4 nucleotides 3′ of the sequence. A short linker containing *Bsp*MI sites at each end was inserted into the gene

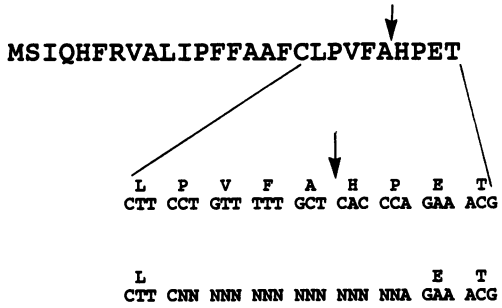

FIG. 1. Outline of the signal peptide cleavage site randomization experiment. At the top is the amino acid sequence of the β-lactamase signal peptide. The arrow shows the position at which the sequence is cleaved by signal peptidase. At the bottom are the amino acid sequence and the nucleotide sequence of the signal peptide cleavage site region. The nucleotides that were simultaneously randomized to create the random-sequence library are represented by N.

WT	C	L	P	V	F	A	H	P	E	T
						-1	+1			
1 mg/ml ampicillin										
3-1	C	L	**Q**	**S**	**A**	**D**	**A**	**K**	E	T
5-4	C	L	**P**	**A**	**S**	**A**	**H**	**E**	E	T
5-11	C	L	**P**	**A**	**L**	**A**	**R**	**T**	E	T
5-14	C	L	**P**	**A**	**L**	**E**	**A**	**K**	E	T
5-16	C	L	**P**	**T**	**A**	**A**	**R**	**E**	E	T
5-19	C	L	**P**	**A**	**C**	**G**	**A**	**R**	E	T
5-21	C	L	**P**	**C**	**L**	**A**	**A**	**R**	E	T
5-27	C	L	**P**	**A**	**W**	**A**	**S**	**G**	E	T
5-31	C	L	**P**	**R**	**S**	**A**	**H**	**E**	E	T
5-35	C	L	**P**	**A**	**Q**	**A**	**K**	**E**	E	T
6-6	C	L	**P**	**F**	**A**	**E**	**S**	**K**	E	T
500 μg/ml ampicillin										
1-26	C	L	**P**	**S**	**F**	**S**	**A**	**R**	E	T
2-17	C	L	**Q**	**A**	**R**	**M**	**T**	**S**	E	T
3-22	C	L	**P**	**H**	**A**	**L**	**A**	**G**	E	T
4-17	C	L	**P**	**L**	**Y**	**A**	**H**	**R**	E	T
No ampicillin selection										
7-2	C	L	**L**	**K**	**F**	**C**	**N**	**R**	E	T
7-4	C	L	**H**	**R**	**A**	**T**	**D**	**R**	E	T

FIG. 2. Amino acid sequences of mutants selected for function from the random-sequence library. The amino acids that were randomized are shown in boldface type. WT, wild type.

at the position of the codons to be randomized. Release of the linker by *Bsp*MI digestion created a defined deletion in the gene (14). A library was formed by use of a second linker to replace the deleted TEM-1 *bla* nucleotides with random nucleotides. A total of 6.0×10^5 colonies were pooled to create the library, and DNA sequencing was used to show that 90% of the colonies pooled contained a plasmid with a random DNA substitution.

Because the entire cleavage site was simultaneously randomized, the bias that residues unique to β-lactamase may have for the types of amino acids that can serve as a cleavage site was reduced. However, the possibility that residues in the N terminus of the signal peptide or the N terminus of the mature β-lactamase influence the types of cleavage site sequences selected cannot be eliminated.

To select for signal peptide cleavage site sequences that function efficiently, plasmid DNA from the random-sequence library was transformed into *E. coli*, and the transformants were spread on three sets of selective agar plates: (i) 1 mg of ampicillin per ml, which is the maximal concentration of ampicillin on which *E. coli* containing a wild-type copy of the TEM-1 *bla* gene will grow (therefore, this concentration selected for wild-type levels of β-lactamase activity); (ii) 500 μg of ampicillin per ml, which was used to select for partial function; and (iii) 12.5 μg of chloramphenicol per ml, which was used to select for the presence of the plasmid without selecting for β-lactamase activity. The use of different types of selection allowed the mutants to be placed into phenotypic classes.

The DNA sequence of the region coding for the signal peptide cleavage site was determined, and the corresponding amino acid sequence was inferred for mutants from each of the classes (Fig. 2). All of the mutants contained replacements equal in length to the wild-type DNA sequence, and no two mutants had the same DNA sequence. These results suggest that the library contains many different potential signal peptide cleavage sites. Among the mutants selected at 1 mg of ampicillin per ml, there is a bias for alanine residues, especially at the positions corresponding to the wild-type −1 and −3 positions. In addition, proline at position −4 is conserved among the functional mutants. It should be noted that only 2 nucleotides were randomized for the −4 codon, so only Leu, Pro, His, Gln, and Arg are represented in the random-sequence library at position −4. The sequences of mutants selected at 500 μg of ampicillin per ml have the same pattern of substitutions as those of mutants from the 1-mg/ml selection. Proline is not found at position −4 for either of the two mutants that were not selected for ampicillin resistance, and mutant 7-2 is the only mutant in the entire set that does not have an alanine in the region from −4 to +2.

Steady-state levels of precursor and mature β-lactamases. To determine whether there is a correlation between the ampicillin resistance phenotype and the level of signal peptide processing, cells were grown to mid-log phase and whole-cell lysates were resolved by SDS-PAGE. Precursor and mature β-lactamases were detected by immunoblotting with anti-β-lactamase polyclonal antibody (Fig. 3). Signal peptide processing was quantitated by determining the relative intensities of the bands corresponding to precursor and mature β-lactamases on the exposed film (Table 1). With the exception of mutant 5-11, there is very little accumulation of precursor β-lactamase among the mutants selected at 1 mg of ampicillin per ml. The large amount of mature β-lactamase that is also prsent for mutant 5-11 may explain why it was also selected at the high ampicillin concentration. Among the mutants selected at 500 μg of ampicillin per ml, mutant 4-17 accumulates precursor protein, while mutant 2-17 appears to be processed efficiently. Mutants 7-2 and 7-4, which were not selected for function, are essentially not processed. Although the mutant 7-2 and 7-4 enzymes are not processed, *E. coli* containing the enzymes has significant ampicillin resistance; the MIC is 256 μg/ml (the MIC for *E. coli* lacking β-lactamase is 1 μg/ml).

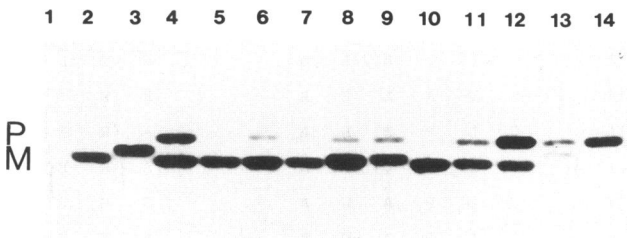

FIG. 3. Steady-state levels of precursor β-lactamase (P) and mature β-lactamase (M) in total cell lysates. The proteins were immunoblotted with polyclonal antibody against TEM-1 β-lactamase. Lanes: 1, *E. coli* TG1; 2, wild-type β-lactamase; 3 to 14, mutants 5-4, 5-11, 5-14, 5-16, 5-21, 5-27, 5-35, 3-1, 2-17, 4-17, 7-2, and 7-4, respectively.

TABLE 1. In vivo processing of random-sequence signal peptide cleavage site mutants

β-Lactamase	MIC of ampicillin (μg/ml)	% Processed
None	1	
Wild type	1,024	98
5-4	1,024	98
5-11	1,024	66
5-14	1,024	99
5-16	1,024	95
5-21	1,024	98
5-27	1,024	95
5-35	1,024	92
3-1	1,024	99
2-17	1,024	88
4-17	512	25
7-2	256	2
7-4	256	2

Nevertheless, the results indicate that there is a correlation between the ampicillin resistance phenotype and the level of signal peptide processing.

Amino-terminal amino acid sequencing of functional mutants. Because 6 amino acid residues were simultaneously randomized to create new cleavage sites, it is possible that the sites of cleavage for the mutant enzymes are different from that for the wild-type enzyme. Therefore, amino-terminal protein sequencing was performed on nine of the mutants that were selected for function at 1 mg of ampicillin per ml. The cleavage site for each mutant, based on the protein sequencing results, is shown in Fig. 4. It is clear that alanine is very important at position −1. Alanine also predominates at position −3, although other residues are also tolerated. In addition, the position of the cleavage site has shifted for mutants 3-1 and 5-14. Thus, the exact spacing of the cleavage site relative to the amino terminus and hydrophobic core of the signal peptide is not essential for efficient cleavage. For mutant 3-1, the shift in cleavage site places a helix-breaking serine residue at position −4. However, the shift in cleavage site for mutant 5-4 places a non-helix-breaking residue, alanine, at position −4 and shifts the proline to position −5. All of the other mutants sequenced have proline at position −4. There are no conserved residues at position −2. The residues at positions +1 and +2 are biased for the basic residues Arg, Lys, and His in that for all the mutants except 5-27 there is at least one basic residue at +1 or +2. Proline is never found at position +1, consistent with the previous finding that proline is not tolerated at position +1 (1).

					−3		−1				
wt		C	L	P	**V**	F	**A**		H	P	E	T
3-1	C	L	Q	S	**A**	D	**A**		K	E	T	
4-17		C	L	P	**L**	Y	**A**		H	R	E	T
5-4		C	L	P	**A**	S	**A**		H	E	E	T
5-11		C	L	P	**A**	L	**A**		R	T	E	T
5-14	C	L	P	A	**L**	E	**A**		K	E	T	
5-16		C	L	P	**T**	L	**A**		A	R	E	T
5-21		C	L	P	**C**	L	**A**		A	R	E	T
5-27		C	L	P	**A**	W	**A**		S	G	E	T
5-35		C	L	P	**A**	Q	**A**		K	E	E	T

FIG. 4. Results of amino-terminal protein sequencing. The amino-terminal amino acid sequence of mature β-lactamase was determined for nine mutant enzymes. The sites of signal peptide cleavage, inferred from the sequencing results, are shown. The amino acids at positions −1 and −3 are shown in boldface type. wt, wild type.

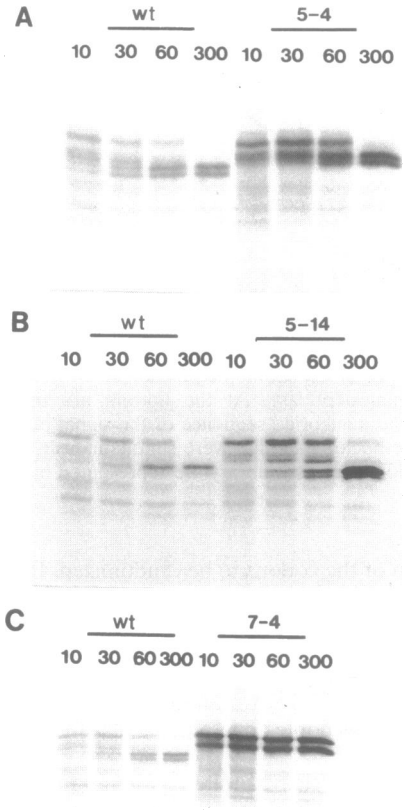

FIG. 5. Signal peptide processing kinetics. Cells were pulse-labeled for 30 s at 25°C with [^{35}S]methionine and chased in the presence of excess cold methionine. The chase was terminated at the times indicated above each set of lanes (time in seconds). (A) Wild-type (wt) TEM-1 versus mutant 5-4. (B) Wild-type TEM-1 versus mutant 5-14. (C) Wild-type TEM-1 versus mutant 7-4.

Further characterization of signal peptide cleavage site mutants. To confirm that the mutants selected from the library are processed efficiently, the signal peptide processing kinetics of mutants 5-4, 5-14, and 7-4 were examined (Fig. 5). Cells grown in minimal medium were pulse-labeled with [^{35}S]methionine and chased in the presence of excess cold methionine. β-Lactamase was immunoprecipitated from solubilized whole cells and analyzed by SDS-PAGE and autoradiography. The processing kinetics for mutants 5-4 and 5-14 were similar to those for the wild type, indicating that the mutant signal peptide cleavage sites function at efficiencies comparable to that of the wild type. The pulse-chase results indicate that mutant 5-4 is processed. This result, together with the N-terminal sequencing of the 5-4 mature protein, indicates that the mature protein migrates anomalously on the gel. Electrophoretic mobility changes caused by certain types of mutations have been reported elsewhere (12). Consistent with the steady-state levels of precursor and mature β-lactamases, the pulse-chase results show that mutant 7-4 is not processed. The β-lactamase doublet that is most prominent for mutant 7-4 is due to a disulfide bond in the mature enzyme. The oxidized and reduced forms of the disulfide have been shown elsewhere to result in different SDS-PAGE migration patterns (9, 11).

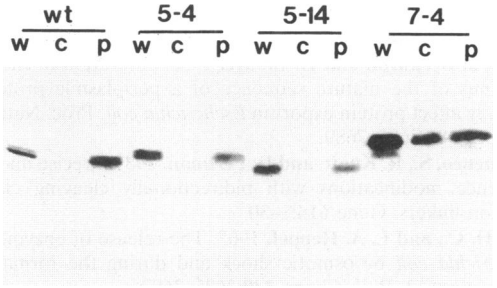

FIG. 6. Osmotic shock localization of β-lactamase signal peptide cleavage site mutants. β-Lactamase was obtained by SDS-PAGE from solubilized whole cells (w), shocked cells (i.e., cytoplasm and membranes) (c), and the periplasmic fraction from the osmotic shock (p). The proteins were immunoblotted with polyclonal antibody against TEM-1 β-lactamase. wt, wild type.

Finally, the cellular location of β-lactamase was determined for the wild type and mutants 5-4, 5-14, and 7-4. β-Lactamase was obtained by SDS-PAGE from solubilized whole cells, osmotically shocked cells (i.e., cytoplasm and membranes), and the periplasmic fraction from the osmotic shock (Fig. 6). β-Lactamase was detected by immunoblotting with anti-β-lactamase polyclonal antibody. Only mature β-lactamase was detected for the wild type and mutants 5-4 and 5-14, and it was only detected in the whole-cell and periplasmic fractions. This result is consistent with the pulse-chase data and indicates that these mutants are efficiently processed and transported to the periplasm. β-Lactamase from mutant 7-4 was found in all three fractions and was not processed, further demonstrating that this mutant is defective in signal peptide cleavage. β-Lactamase from mutant 7-4 was presumably found in the periplasm because cleavage site mutants are transported to the periplasmic face of the cell membrane (8, 11). β-Lactamase detected in the periplasm may have been released from the cell membrane by the osmotic shock procedure. A portion of the periplasmic extract that was used for immunoblotting was also assayed for β-lactamase activity with ampicillin as a substrate. Equal amounts of periplasmic protein were used for each assay. As shown in Table 2, the periplasmic extracts from the wild type and mutants 5-4 and 5-14 had similar levels of activity. The periplasmic extract from mutant 7-4 also exhibited activity, although it was 10-fold lower than that of the wild type. In combination with the immunoblotting results, this result suggests that the specific activity of the precursor β-lactamase from mutant 7-4 is significantly lower than that of the wild-type mature enzyme. Thus, the presence of the signal peptide sequence appears to have an adverse effect on the catalytic activity of β-lactamase.

DISCUSSION

In this study, we randomized the signal peptide cleavage site from positions −4 to +2 for TEM-1 β-lactamase and selected random-substitution mutant enzymes that function phenotypically like the wild-type enzyme. DNA sequencing and amino-terminal protein sequencing were used to identify the types of amino acids that are preferred at the signal peptide cleavage site. On the basis of the results, the most important requirement for cleavage of the β-lactamase signal peptide is the presence of an alanine residue at the −1 position. All nine of the substituted β-lactamases examined by amino-terminal protein sequencing have an alanine at the −1 position. Alanine is the most frequently encountered residue at −1 among prokaryotic signal peptides, although serine, glycine, and threonine have also been reported (26). These results do not eliminate the possibility that other amino acids could function at −1, but they do indicate that alanine is preferred. The narrow specificity observed at −1 for β-lactamase is similar to the processing of thylakoid signal peptides, in which only alanine at −1 provides efficient cleavage (23).

TABLE 2. Enzyme activity of periplasmic extracts

β-Lactamase extract	Activity, U/mg[a] (%)
Wild type	156 (100)
5-4	170 (109)
5-14	145 (93)
7-4	16 (10)

[a] Activity was determined with crude periplasmic extracts (see Materials and Methods for details).

The sequence requirements for efficient β-lactamase signal peptide cleavage are less stringent at the −3 position than at the −1 position. Alanine, leucine, threonine, and cysteine are represented among the nine efficiently processed mutants whose cleavage site was defined. Among prokaryotic signal peptides, glycine, alanine, serine, threonine, leucine, and valine have been found at the −3 position (26). Thus, of the amino acids found at −3 in this study, all but cysteine are consistent with previously observed signal peptide cleavage sites. However, mutagenesis studies of the maltose-binding protein have shown that cysteine at −3 is consistent with efficient signal peptide processing (7).

A helix-breaking residue, such as proline, glycine, or serine, is often found in the region from positions −4 to −6 of signal peptides (26). TEM-1 β-lactamase has a proline at the −4 position. It was previously shown that substitution of the −4 proline with leucine or serine inhibits processing (11). Among the mutants examined in this study, proline is most often found at −4. Only mutants 3-1 and 5-14, in which the cleavage site is shifted (Fig. 4), have a residue other than proline at −4. Interestingly, because of the shift in the cleavage site, mutant 3-1 has a serine at −4, despite the fact that the single amino acid substitution of serine at −4 diminishes the processing of the wild-type enzyme (11). Thus, the context of neighboring amino acids is important in determining what residue is required at the −4 position. Context effects may explain why a helix-breaking residue, such as proline, is not always found in the region from positions −4 to −6 of functional signal peptides (12).

The presence of a net positive charge at the amino terminus of the mature protein has been shown to inhibit the translocation to the cytoplasm of *E. coli* alkaline phosphatase (13). In addition, a statistical analysis of prokaryotic signal sequences indicates that there is a net charge of zero or less for the region including the signal peptide cleavage site and the first 5 residues of the mature protein (27). The results presented here clearly show that a positively charged residue at the +1 position does not interfere with β-lactamase transport and processing. The positively charged residue may be allowed at +1 because it is balanced by a glutamate residue within the first 5 residues of the mature protein. Thus, consistent with the previous studies, the net charge of the amino terminus of mutants with a functional signal peptide is zero or less.

Finally, this study demonstrates that the position of the β-lactamase signal peptide cleavage site can be shifted relative to the position of the cleavage site in the wild-type enzyme and still allow wild-type levels of function to be retained. This result indicates that the −1 and −3 sequence requirements are more

important than is the precise spacing of the cleavage site relative to other elements of the signal peptide sequence.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grants AI32956 (to T.P.) and GM46406 (to D.B.) from the National Institutes of Health and a Beckman Center Directors Research Grant funded by SmithKline-Beecham.

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