Processing of *Bacillus cereus* 569/H $\beta$-Lactamase I in *Escherichia coli* and *Bacillus subtilis*

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The gene for *Bacillus cereus* 569/H $\beta$-lactamase I, *penPC*, has recently been cloned and sequenced (Mézes, P. S. F., Yang, Y. Q., Hussain, M., and Lampen, J. O. (1983) FEBS Lett. 161, 195–200). A typical prokaryotic signal peptide but with no lipoprotein modification site, as present in the *Bacillus licheniformis* 749/C $\beta$-lactamase, was indicated by the DNA sequence for this secretory protein. We have here purified the $\beta$-lactamase I products found in *Escherichia coli* and *Bacillus subtilis* carrying *penPC* and have determined the first 20 NH$_2$-terminal amino acids of each of the forms. Processing of the $\beta$-lactamase I in *E. coli* occurs at a single site which is characteristic for cleavage by a signal peptidase. *B. subtilis* secreted two distinct products to the culture medium which were both smaller than the single product formed in *E. coli*. Sequencing of $[^{35}S]$Met-labeled pre-$\beta$-lactamase I from phenylethyl alcohol-treated cells of *B. cereus* 569/H indicated that UUG is being utilized as the initiation codon for *penPC*. The same result was obtained for the pre-$\beta$-lactamase I from similarly treated cells of the closely related *B. cereus* 5/B strain.

The penicillinases produced by *Staphylococcus aureus* PC1 (*blaZ*), *Bacillus licheniformis* (*penP*), *Escherichia coli* R6K (*bla*), and *Bacillus cereus* (*penPC*) are secretory enzymes belonging to the Class A $\beta$-lactamases (1). They were probably derived from the same ancestral gene, with $\sim$30–60% amino acid homology between the various pairs of this set (1). Moreover, a conserved Ser residue has been implicated in the catalytic mechanism for each enzyme (Ref. 2 and references therein). The signal sequences of all four penicillinases have now been determined, but unlike the homology seen in the mature forms of the enzymes, no apparent conservation is observed for the signal sequences (for a review see Ref. 3).

The lipoproteins of *penPC* have been described, where the lipophilic modification consists of a diacyl glycerol group in thioether linkage to an NH$_2$-terminal Cys residue and a long-chain fatty acid in amide linkage with the amino group of the Cys residue (4). This structure is analogous to the NH$_2$ terminus of the major outer membrane lipoprotein of *E. coli* (5). The signal peptidase cleavage sites for the translation product forms of these proteins all occur at the end of the hydrophobic stretch of amino acids in the signal sequences to render the Cys residues involved in membrane attachment NH$_2$-terminal (6, 7).

In *S. aureus* and *B. licheniformis* various proteases release shortened hydrophilic forms of penicillinase from the membrane lipoproteins, especially at the onset of stationary growth. Alternatively, the translation products may sometimes be processed directly to the extracellular forms by the proteases earlier in the growth cycle. These particular processing proteases do not appear to be present in *E. coli*. For example, the lipoprotein remains covalently attached to the inner and outer membranes of *E. coli* carrying the *penP* gene, and no periplasmic or extracellular forms are detected (8, 9). As such, a lipoprotein form, when it occurs as part of a membrane-bound secreted pair in Gram-positive organisms, represents an isolatable signal peptidase-processed intermediate. It is not known if the signal peptidase giving the lipoprotein form of a Gram-positive protein in *E. coli* is related to the enzyme giving the lipoprotein form in the *Bacillus*.

Recently, the gene for *B. cereus* 569/H $\beta$-lactamase I, *penPC*, was cloned, sequenced, and the signal peptide described (10, 11). We have also sequenced the very closely related *penPC* gene of *B. cereus* strain 5/B (12) and found $\sim$90% amino acid sequence homology between these two *penPC*$\beta$-lactamase I products. Unlike the other characterized Gram-positive $\beta$-lactamases of the class A set, no membrane-bound lipoprotein is formed, and both enzymes are practically entirely secreted to the culture medium as processed forms (3). In this way, $\beta$-lactamase I resembles the *bla* gene product of *E. coli*, which also has no modifiable Cys residue in its signal sequence and exists entirely in the periplasm as a soluble signal peptidase-processed form. In order to understand more fully the secretion process in the *Bacilli* we examine in this paper the processing of the *B. cereus* 569/H $\beta$-lactamase I products when *penPC* is expressed in *E. coli* and *B. subtilis*. In addition, we show that UUG is the translational initiation codon for both the 569/H and 5/B *penPC* pre-$\beta$-lactamase I products.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids**—*B. cereus* 569/H was provided by T. Viswanatha of the University of Waterloo, Ontario, while strain 5/B of *B. cereus* was a gift from R. Day of the University of Cincinnati. The plasmid vector for cloning *penPC* in *B. subtilis* BD170 (13) was pRMW3, a 4.25-kilobase pair pUB110 derivative (14) whose *EcoRI* site had been removed and an $\sim$250-base pair fragment from the *PvuII* to *BanHI* sites deleted. A 1.7-kilobase pair DNA fragment from pRYW22, containing the *penPC* gene, was inserted at the

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were used per reaction mixture containing 30 pCi of \([35S]\)Met (Amersham Corp., specific activity 1250 Ci/mmol). The reaction was carried out at 37 °C for 45 min at 37 °C before adding excess unlabeled Met and incubating 5 min longer. The translation products were immunoprecipitated in the presence of 5 μg of added carrier \(\beta\)-lactamase I purified from \(B.\) cereus 569/H.

Isolation of Pre-\(\beta\)-lactamase I from Phenylethyl Alcohol-treated \(B.\) cereus 569/H Cells—Phenylethyl alcohol (Sigma) treatment of \(B.\) cereus strain 569/H or 5/B and subsequent incubation of treated cells with \([35S]\)Met were performed essentially as described earlier for \(B.\) licheniformis (19). In a typical experiment, \(B.\) cereus cells were grown in 5.0 ml of minimal medium to \(A_{600}\) of 1.2, harvested, and resuspended in 0.5 ml of cold 10 mM Tris-HCl, pH 7.5, 50 mM NH₄Cl, and 10 mM MgCl₂. Phenylethyl alcohol was added to 0.7%, and the cells were kept for 10 min in ice. An equal volume of 2 × reaction mix (19) was added including 250 μCi of \([35S]\)Met. At the end of the incubation period and chase, the cells were immediately sonicated in an MSE sonicator for 15-s periods (amplitude, 12 μ peak to peak) with cooling for 1-min intervals for a total of 3 min. Cell debris was removed by microcentrifuging for 15 min. The pre-\(\beta\)-lactamase I in the supernatant was immunoprecipitated in the presence of 5 μg of added carrier, as above. The immunoprecipitate was solubilized in running buffer, as described below, and about 5% of the total sample was used for comparison with processed forms of \(\beta\)-lactamase I in a gradient polyacrylamide gel (6). The remainder of the sample was electrophoresed on a 10% polyacrylamide slab gel containing 0.1% SDS (20). The pre-\(\beta\)-lactamase I product was visualized by exposure of x-ray film to the untreated freeze-dried gel for 12 h at −70 °C and then cut out of this gel. The protein band was eluted by incubation in 1.0 ml of 50 mM NaHCO₃ and 0.1% SDS at 37 °C for 30 min. Ovalbumin (2 mg) was added as a carrier protein. The solution was finally dialyzed against water for 24 h and freeze-dried.

Purification of \(\beta\)-lactamase I from \(E.\) coli and \(B.\) subtilis—\(E.\) coli/pRWY22 cultures were grown to stationary phase and then centrifuged at 5,000 \(\times\) g. The cells were resuspended in 0.05 volume of 75 mM potassium phosphate buffer, pH 7.4, and the mixture was sonicated for 30-s intervals with periods of cooling in ice in between. Cell debris was removed by centrifugation at 20,000 \(\times\) g for 30 min. Cell pellets were precipitated in the presence of 5 μg of added carrier, as above. The immunoprecipitate was solubilized in running buffer, as described below, and about 5% of the total sample was used for comparison with processed forms of \(\beta\)-lactamase I in a gradient polyacrylamide gel (6). The remainder of the sample was electrophoresed on a 10% polyacrylamide slab gel containing 0.1% SDS (20). The pre-\(\beta\)-lactamase I product was visualized by exposure of x-ray film to the untreated freeze-dried gel for 12 h at −70 °C and then cut out of this gel. The protein band was eluted by incubation in 1.0 ml of 50 mM NaHCO₃ and 0.1% SDS at 37 °C for 30 min. Ovalbumin (2 mg) was added as a carrier protein. The solution was finally dialyzed against water for 24 h and freeze-dried.

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Amino Acid Sequence Analysis—The NH₂-terminal sequences of the purified processed forms of \(\beta\)-lactamase I from \(E.\) coli and \(B.\) subtilis were determined by stepwise Edman degradation of 1 mmol (≥30 μg) of sample using a gas phase sequencer (Applied Biosystems, model 470A) (22). The resulting phenylthiohydantoin amino acids were separated on an amino acid analyzer by high pressure liquid chromatography as described by Hawke et al. (23). Purified \([35S]\)Met-labeled pre-\(\beta\)-lactamase I from phenylethyl alcohol-treated cells of \(B.\) cereus was subjected to 20 cycles of Edman degradation. The fractions were dried, dissolved in Liqulilor and analyzed by scintillation counting.

Processing of \(B.\) cereus \(\beta\)-Lactamase I

RESULTS

Identification of Translational Initiation Site—A precursor form of the \(\beta\)-lactamase I was obtained in both the cell-free prokaryotic translation system using purified pRWY22 obtained from \(E.\) coli and phenylethyl alcohol-treated cells of \(B.\) cereus 569/H. Both immunoprecipitated products have the same apparent \(M_r\) of 33,500 daltons upon electrophoresis in a 15–22% gradient polyacrylamide gel containing 8 M urea and 0.1% SDS. These values are larger by 2,500–5,000 daltons when compared to the \(M_r\) of the various processed forms of \(\beta\)-lactamase I (Fig. 1) produced by \(B.\) subtilis (28,500 and 29,000 daltons) or \(E.\) coli carrying penPC (31,000 daltons).

The pre-\(\beta\)-lactamase I of phenylethyl alcohol-treated cells of \(B.\) cereus would not be expected to be blocked at the NH₂ terminus with a formyl group as the cell-free in vitro synthesized form would be (6). Thus, Edman degradation of \([35S]\)Met-labeled precursor should be possible and would give a unique pattern of radioactivity that would identify the NH₂-terminal position. We had shown previously that there were benzy1 penicillin (Lilly) in 1 h at 30 °C. Localization of penicillinase activities, antibody preparation, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis were performed as previously described (8, 25). Approximate protein concentrations for \(\beta\)-lactamase I were determined spectrophotometrically assuming \(A_{280} = 10.0 (26)\).

![FIG. 1. Comparison of \(B.\) cereus \(\beta\)-lactamase I precursor with processed forms.](image-url)
two possible ATG initiation codons in the first and second in-frame positions immediately following the Shine-Dalgarno sequence of penPC (10) and a TTG codon at the fourth position which could also be functioning as the initiation codon. The next ATG occurs at the ninth position. [35S]Met-labeled residues were found at cycles 1 and 6 for the pre-β-lactamase I obtained from phenylethyl alcohol-treated cells of *B. cereus* 569/H (Fig. 2A). It is extremely unlikely that one of the ATGs would have been the initiation codon and that a cleavage would then have occurred 2 or 3 amino acid residues from the NH₂ terminus. If this had happened the TTG would have coded for Leu, not Met, as observed here. From these data, it is apparent that TTG would have to be the initiation codon to align the labeled positions correctly (Fig. 3).

Furthermore, when cells of *B. cereus* 5/B were treated with phenylethyl alcohol the pre-β-lactamase I contained [35S]Met at positions 1, 6, and 11 (Fig. 2B). This penPC gene differs from that in strain 569/H in that the codon at the second position immediately after the ribosome binding site is ATA rather than ATG, and the fourteenth position is ATG in place of ATA (Fig. 3). Alignment of the labeled positions gives congruence only when TTG is the initiation codon for the strain 5/B penPC gene. Thus, the single base change of an A to a G in the codon at the fourteenth position, which codes for Met in the 5/B penPC gene, is substantiated by the appearance of radioactivity at cycle 11. Numbering of the amino acids for pre-β-lactamase I, therefore, begins with Met coded for by TTG at the fourth position after the Shine-Dalgarno site in both the 569/H and 5/B penPC products.

**Purification of Processed Forms of β-Lactamase I from E. coli and B. subtilis**—In order to identify processing sites of cloned β-lactamase I, the 569/H penPC products from *E. coli* and *B. subtilis* were purified. The total amount of enzyme produced by the parent, *B. cereus* 569/H, or by *B. subtilis/pRWM5 was approximately the same (7,000–10,000 units/ml) under the same culture conditions. In both cases, greater than 90% of the total penicillinase activity was present in the culture supernatant. An affinity support column of N-acetylpencilamine-Sepharose 4B was used to purify the β-lactamases (21). From *B. subtilis/pRWM5 two distinct secreted products were obtained that migrated differently from the three forms purified from *B. cereus* 569/H upon gel electrophoresis (Fig. 4, lanes 1 and 2). The immunoprecipitated β-lactamase I products from the culture fluid of *E. coli/pRWM5* (Fig. 1, lane 4) corresponded to the two affinity-purified forms mentioned above (results not shown).

Total penicillinase activity in *E. coli/pRWM22* (500 units/ml) was cell bound. We had shown previously that approximately 25% of the activity was found free in the periplasm (10), and the remainder was membrane associated. None of the β-lactamase I could be labeled with [3H]palmitate and it was readily released by sonication without detergent (10).

The purification of β-lactamase I from *E. coli* was modeled after the procedure for the *Bacillus* species. After sonication and removal of cell debris, water-soluble proteins at pH 4.5 were adsorbed on Celite. Immunoprecipitation of a small sample of sonicate (containing ~1,500 units of penicillin activity) and subsequent electrophoresis showed that there was a single form of cell-bound β-lactamase I (Fig. 4, lane 4, and Fig. 1, lane 3). This protein migrated more slowly than any of the processed forms secreted by *Bacillus* species (Fig. 4). Electrophoresis of a sample (containing ~1,500 units penicillin activity) from the pooled active fractions eluted from

![Fig. 2. Location of [35S]Met residues of pre-β-lactamase I.](image)

![Fig. 3. DNA and amino acid sequence of the NH2-terminal region of the two penPC genes.](image)

**Processing of *B. cereus* β-Lactamase I**
Celite showed that a larger number of E. coli proteins had been adsorbed to Celite (Fig. 4, lane 5). The major portion of these contaminants passed unhindered through the affinity column in the next step of the purification, but some impurities were still present in the fractions containing β-lactamase I (Fig. 4, lane 6). Homogeneous enzyme used for NH₂-terminal sequencing was obtained by introducing a gel filtration step after the affinity chromatography. The total yield of pure β-lactamase I from the Sephadex G-100 column was 0.36 mg for a 1.0-liter batch of original culture.

**NH₂-terminal Sequence of β-Lactamase I**

The NH₂-terminal sequence of β-lactamase I purified from B. subtilis/pRW5 and B. subtilis/pRWY22 are shown in Figure 5. The NH₂-terminal sequence of β-lactamase I purified from B. subtilis/pRW5 and B. subtilis/pRWY22 were sequenced as a single sample, and two distinct amino acid sequences were observed (A, B, C, and D).

**DISCUSSION**

The β-lactamase I of B. cereus 569/H is a secreted enzyme which may be purified in large quantities directly from the culture supernatant of late log cultures. We have shown here that the precursor of β-lactamase I formed in a cell-free prokaryotic transcription system using pRWY22 as a template is indistinguishable from the product formed in phenylthiohydantoin alcohol-treated cells of B. cereus on the basis of electrophoretic mobility and is larger than the processed forms (Fig. 1).

The apparent molecular weight of the pre-β-lactamase I (33,500 daltons) is in close agreement with the calculated molecular weight of the translation product (33,000 daltons) based on the complete nucleotide sequence of the penPC gene. The pattern of incorporation of [35S]Met among the first 20 residues of the pre-β-lactamase I from phenylthiohydantoin alcohol-treated cells of B. cereus 569/H or of the closely related 5/B strain indicates that UUG is being utilized as the translational initiation site in both cases (Figs. 2 and 3). In addition, we have determined the NH₂-terminal residues and the next 19 amino acids of each of the processed forms of β-lactamase I purified from E. coli and B. subtilis carrying the 569/H penPC gene on plasmids.

The UUG initiation codon for a wild-type Gram-positive protein was first observed for the S. aureus β-lactamase by McLaughlin et al. (28) and shown to be utilized by both B. subtilis and E. coli ribosomes in vitro (29). The utilization of UUG for the pre-β-lactamase I penPC products of B. cereus strains 569/H and 5/B adds to the list of genes having UUG as the initiation codon in both S. aureus (30, 31) and E. coli (32). The most recent additions include the B. licheniformis spoOH gene having a UUG initiation codon (32) and the B. subtilis and B. subtilis subtilisin genes having UUG initiation codons (33-35).

As far as we are aware, UUG as a translational initiation codon has been found for only a few E. coli wild-type proteins (36, 37), and out of 123 E. coli ribosome binding sites catalogued by Gold et al. (38) only four had a UUG initiation codon. With proteins from euukaryotic cells only AUG has been reported as the initiation codon, but it has recently been
shown that yeast can utilize UUG and AUA although at low efficiency (39). The very low level of expression of the 569/H penPC gene in E. coli (10) is possibly due to poor recognition of UUG as an initiation codon in this organism. However, further work is required to determine the relative efficiency of UUG versus AUG or GUG before any conclusions can be drawn.

The variation in initiating codons does not seem to affect translation in Bacillus species; for example the levels of penicillinase expressed in B. subtilis from the penPC gene are about equal to the level observed in the parent. The total amount of \( \beta \)-lactamase I produced in B. subtilis/pRW56 is still less than what would be expected for a multicopy plasmid. The factors affecting regulation of \( \beta \)-lactamase synthesis at this level are not known.

What makes the case of the penPC genes interesting is the demonstration that the spacing between the end of the Shine-Dalgarno sequence and the initiation codon cannot be 0 or 3 nucleotides. The AUG codons following the Shine-Dalgarno sequence are in the correct reading frame but are not being utilized. The UUG initiation codon is 9 nucleotides away (see Fig. 3). The spacing in known initiation sites recognized by B. subtilis ribosomes varies between 5 to 14 nucleotides (28, 31), the average being 7-8 nucleotides (28). Thus, the spatial orientation of the initiating codon to the ribosome is critical for effective translation and expression, and the spacing between the end of the Shine-Dalgarno sequence and the initiation codon should be larger than 3 nucleotides.

The form of \( \beta \)-lactamase I purified from E. coli R1/pRW22 has been processed at a single site after Ala 25 in the signal peptide, most likely by a signal peptidase. The processing site is typical when compared to a catalogue of \( \beta \)-lactamase processing sites occurring here.

This prediction fits in well with the observed proteolytic site in pRWY22 which has been processed at a single site after Ala 25 in the prolipoprotein to lipoprotein (42, 43). It remains to be seen if either of these signal peptidases is responsible for the processing of \( \beta \)-lactamase I in either B. cereus or B. subtilis, the initial signal peptidase product may be immediately accessible to the proteases. It remains to be determined if any sequences past the putative signal peptide site in \( \beta \)-lactamase I are necessary for correct processing or secretion.

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Processing of B. cereus β-Lactamase I