Bacillus cereus 569/H β-lactamase I: Cloning in *Escherichia coli* and signal sequence determination

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Received 23 June 1983

The gene, *penPC*, for β-lactamase I of *Bacillus cereus* 569/H has been cloned and its expression studied in *Escherichia coli*. The protein product from the in vitro translation of *penPC* was shown by gel electrophoresis to have an M_r of 36000 which is larger than the in vivo products found in *B. cereus* and *E. coli*. The DNA sequence of the signal region was determined. It revealed that the smallest known mature form present in *B. cereus* culture fluids is preceded by 45–48 amino acids in pre-β-lactamase I, considering that there are 3 initiation codons in the same reading frame, one or more of which might be initiating translation. Unlike the *Bacillus licheniformis* 749/C β-lactamase, which has a membrane-bound thioether lipoprotein form, the single Cys residue in the *B. cereus* β-lactamase I signal sequence is unmodified and a single processed form of the enzyme is present in *E. coli* cells carrying *penPC*.

Bacillus cereus *β-Lactamase I* Escherichia coli *Secretion* *Prepenicillinase* *Signal sequence*

1. INTRODUCTION

Of the well-characterized Class A β-lactamases, which are related by amino acid homologies ranging from 20–60% and by an average M_r of about 30000, the penicillinase from *Bacillus licheniformis* 749/C (ATCC 25972) is the most homologous to *Bacillus cereus* 569/H β-lactamase I (EC 3.5.2.5) [1]. Both are chromosomally-encoded proteins secreted to the growth medium as multiple-processed forms [2, 3]. *B. licheniformis* as well as *Staphylococcus aureus* PC1 retain a portion of their total penicillinase as membrane-bound thioether lipoproteins. *B. cereus*, on the other hand, releases virtually all of its β-lactamase I; only traces of a membrane-bound activity can be detected. A modifiable Cys residue in a highly conserved region spanning the end of the hydrophobic stretch of amino acids in the signal sequence is a requirement for lipoprotein formation [4]. We recently showed that the membrane-bound γ-penicillinase of *B. cereus* 569 strains [5] is a lipoprotein, part of which is processed to a secreted form that is rapidly degraded. This membrane-bound-secreted pair (now called β-lactamase III) has a lower specific activity and is distinct from β-lactamase I [6].

The signal sequence of *B. cereus* β-lactamase I when compared with those already known for the Gram-positive Class A β-lactamases should indicate why this penicillinase is almost completely secreted. To this end, we have isolated the structural gene which we denoted as *penPC* and sequenced the signal region. In addition, expression has been obtained in *Escherichia coli* and *Bacillus subtilis* which are potential hosts for secreting genetically-engineered proteins linked to a β-lactamase promoter-signal sequence.

Abbreviations: kb, kilobasepairs; bp, basepairs; SDS, sodium dodecyl sulfate

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

Bacillus cereus 569/H was provided by Dr T. Viswanatha of the University of Waterloo (Waterloo, Ontario) and was maintained on nutrient agar slants. Chromosomal DNA was prepared as in [7]. *E. coli* RR1 [8] and *B. subtilis* DB170 [9] were used as recipient strains for transformation by recombinant plasmids as in [7,10]. For cell-free protein synthesis, *E. coli* MRE600 (RNase I^-) [11] extract was used. Plasmids were isolated from *E. coli* RR1 grown in L-broth [12] after amplification with spectinomycin (200 μg/ml) and purified by CsCl density gradient centrifugation [13]; pRW33, a pBR325 derivative whose *bla* gene was previously inactivated, served as a general vector (4.6 kb) for cloning in *E. coli* [14]. The *penP* gene of *B. licheniformis* 749/C was present on a 1.4 kb *Hin*dIII–*Bam*HI DNA fragment inserted in the corresponding sites of pRW33 to give pRW83 [14]. A hybrid plasmid pRW103, provided by W. Wang of this laboratory, was constructed by linking pUB110 [15] with a 1.15 kb fragment from pBR322 containing the origin of replication, and an inactive 0.8 kb fragment of the *B. licheniformis* penicillinase gene. This 6.5 kb plasmid, with multiple cloning sites, serves as a shuttle vector between *B. subtilis* and *E. coli*. Restriction enzymes, DNA-modifying enzymes and linkers were purchased from New England Biolabs and Bethesda Research Laboratories and used according to the manufacturer's directions. Plasmids were prepared for rapid restriction enzyme analysis (2.0-ml cultures) by the boiling method in [16].

2.2. *Penicillinase activity*

One unit is the amount of enzyme hydrolyzing 1 μmol benzyl penicillin in 1 h at 30°C. The method in [17] was used for the assay. The soluble periplasmic proteins of *E. coli* were isolated by the osmotic shock procedure [18] as modified for use with small quantities of cells [19]. Membrane-associated or soluble proteins in *E. coli* were released by sonication. Diisopropylfluorophosphate (1 mM) was present during the fractionation steps to inhibit proteases. Total penicillinase activity was determined by assay of sonicated cell suspensions.

2.3. *Cell-free protein synthesis*

The DNA directed transcription–translation system was employed [20]. S30 was made from *E. coli* MRE600 and [^{35}S]methionine (1.35 mCi . 1.27 nmol^{-1} . ml^{-1}) was used for labeling. Reaction was stopped by addition of unlabeled methionine and precipitation of protein with chilled 10% trichloroacetic acid. The precipitate was washed with ether, dried and solubilized in gel sample buffer. A portion of the sample was immunoprecipitated with antibody against β-lactamase I as described below.

2.4. *Other methods*

Preparation of anti-β-lactamase I antibody, immunoprecipitation, DNA sequencing and labeling with [^{3}H]palmitate were as in [2,4,14,21]. The secreted forms of β-lactamase I from *B. cereus* 569/H were purified by affinity chromatography using *N*-acetyl-penicillamine Sepharose 4B gels [22]. *E. coli* cells producing β-lactamase I were boiled with 2% SDS for 5 min, centrifuged (12 000 $\times$ *g*), and the supernatant immunoprecipitated. Alkaline phosphatase and glucose-6-phosphate dehydrogenase were assayed as in [14].

3. RESULTS AND DISCUSSION

3.1. *Cloning of* penPC *in* E. coli

The *B. cereus* 569/H β-lactamase I structural gene, *penPC*, was initially obtained as a 4.3 kb *Eco*RI fragment in pRW33 after shot-gun cloning. A single clone which was tetracycline and ampicillin-resistant and chloramphenicol-sensitive was obtained. The DNA containing the *penPC* gene was isolated and shortened by treatment with *Bal*31. A 1.8 kb fragment (fig. 1) was cloned into the *Cla*I site of pRW33, with the aid of linkers, to give pRWY22 (6.4 kb) which has ampicillin, tetracycline and chloramphenicol resistance markers. Expression of *penPC* was obtained in *E. coli* RR1 at a level of 500 units/mg protein. In one clone, containing pRWY23, two copies of the gene were introduced on the vector, resulting in a concomitant doubling of the penicillinase activity.

3.2. *Immunoprecipitation of* penPC *products*

Antibody prepared against purified *B. cereus* β-lactamase I immunoprecipitated the product of

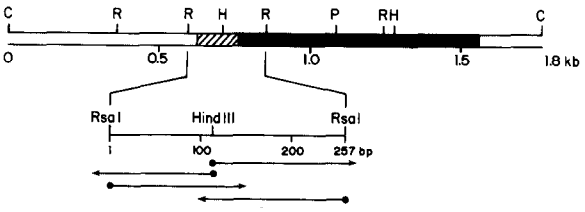

Fig.1. Physical map and DNA sequencing of *B. cereus* 569/H β-lactamase I gene. The region corresponding to the mature forms of penicillinase in *B. cereus* is shaded in, while the area coding for the signal sequence at the NH_2 terminus is hatched. A 257 bp *Rsa*I fragment containing the signal sequence region is enlarged to show overlapping areas sequenced. DNA sequencing from the *Hin*dIII site within this *Rsa*I fragment was performed on 0.56 kb *Hin*dIII–*Hin*dIII and 0.71 *Hin*dIII–*Cla*I recombinant clones of M13mp8. The abbreviations used for restriction enzymes are: C, Cla, H, *Hin*dIII; P, *Pst*I; R, *Rsa*I.

penPC formed in *E. coli* carrying pRWY22 (fig.2, 3). It is larger than the secreted form present in *B. cereus* cultures (lane 2) by about 1.3 kDa. In the cell-free protein synthesizing system using pRWY22, an even larger form was obtained (fig.2 (5)). This putative pre-β-lactamase I could be precipitated with antibody against β-lactamase I of *B. cereus* (fig.2 (4), shown with an arrowhead). The two darker bands below the pre-β-lactamase I were due to the non-specific binding of [^{35}S]methionine, used in cell-free synthesis, to the unlabeled exo-forms used to assist precipitation of the in vitro product. The pre-β-lactamase I migrates more slowly than the prepenicillinase of *B. licheniformis* 749/C (cf. 4,5 with 6). From fig.2 it is evident that the *penPC* product in *E. coli* has been processed to a single form.

3.3. *Localization and [^{3}H]palmitate labeling*

When the periplasmic contents of *E. coli* carrying *penPC* were assayed for penicillinase, about 25% of the total activity was present. The remainder of the enzyme was loosely bound to membrane, as it could be released by sonication without the use of detergent, and was available to substrate in shocked cells. These results indicate that β-lactamase I in *E. coli* has a relatively hydrophilic form, but still is hydrophobic enough to allow weak membrane association. No penicillinase activity was found in the culture supernatant. Alkaline phosphatase was used as a marker enzyme for periplasmic proteins. It was present in the shockates from cells grown in low phosphate medium [24] but not in cells cultures in L-broth. Glucose-6-phosphate dehydrogenase was found only in cytoplasmic fractions; none could be detected in the shockates. These results indicate

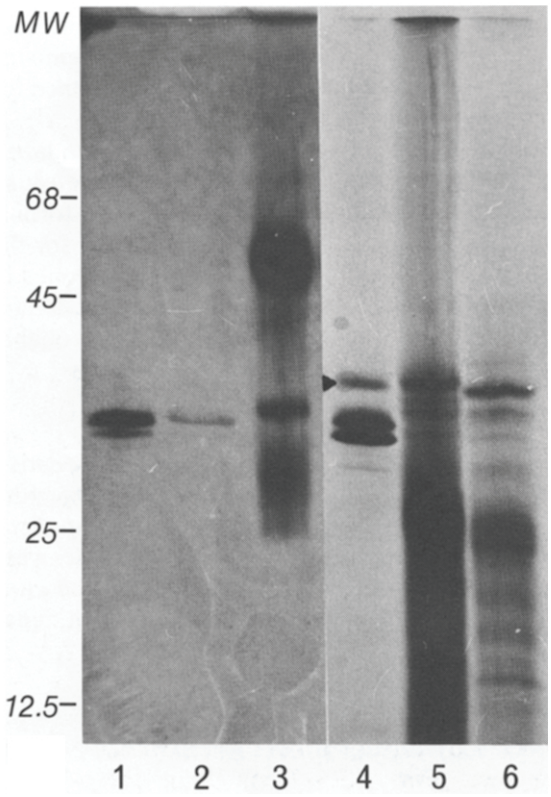

Fig.2. Comparison of the in vitro translation product of *penPC* with the in vivo products found in *B. cereus* and *E. coli*. Protein samples were analyzed in a polyacrylamide gradient gel. In vivo products in lanes 1–3 were stained with silver [34], while lanes 4–6 represent in vitro labeled products radioautographed for 16 h: (1) Partially purified membrane penicillinase of *B. licheniformis* 749/C (M_r 32 000); (2) purified β-lactamase I from *B. cereus* 569/H; (3) immunoprecipitate using anti-β-lactamase I antibody of the total extract of *E. coli* producing β-lactamase I of *B. cereus*; (5,6) cell-free translation products using pRWY22 (*penPC*) and pRW83 (*penP*), respectively; (4) immunoprecipitation of a portion of the sample as in 5, with anti-β-lactamase I antibody; the arrowhead shows the immunoprecipitated in vitro product. M_r (MW) standards were: bovine serum albumin (68 000), hen ovalbumin (45 000), chymotrypsinogen A (25 000) and cytochrome *c* (12 500).

that the periplasmic fraction containing the processed *penPC* penicillinase was not contaminated by cytoplasmic proteins. We have no evidence for any penicillinase in the cytoplasm itself.

In cultures labeled with [^{3}H]palmitate, no labeled penicillinase could be detected (not shown); so it is unlikely that the membrane-associated form is a membrane lipoprotein like that observed for the *B. licheniformis* 749/C penicillinase gene cloned in *E. coli* [21, 25]. The lipoprotein penicillinase observed in *B. cereus* 569/H [4] is probably restricted to β-lactamase III.

3.4. β-Lactamase I signal sequence

The amino acid sequence of the signal peptide from *B. cereus* β-lactamase I was deduced from the DNA sequence. Both strands of a 257 bp *Rsa*I fragment, cloned in M13mp9 at the *Sma*I site, were sequenced from the *Rsa*I sites and from the *Hin*dIII site within this fragment containing the signal region (see fig.1). The known N-termini of the mature charge variants of β-lactamase I present in the culture fluid of *B. cereus* 569/H, Lys/His/Lys/Asn, were conformed from the DNA sequence, as well as the next 28 amino acids previously determined by protein sequencing [3] (fig.3). Because of the high degree of amino acid homology between the mature forms of *B. licheniformis* penicillinase and *B. cereus* β-lactamase I and their probable origin from a common ancestral gene [1], one would expect some similarities in their signal sequences. Although there are no highly conserved stretches of amino acids, the overall length and breakdown of the signal sequence regions (42–48 residues to the smallest processed form) are similar. Each has 4 positively charged residues in a stretch of 8–12 amino acids at the NH_2 terminus and 15 amino acids in the hydrophobic stretch. In the hydrophobic stretch of *B. cereus* pre-enzyme there

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                                                                                    RBS
5'- AA  TTTGGTTCGG  TGATTGTCTA  TTTATGTGTAC  GTTATAAAAAG  GTGCTAAAAA  TTTGGAAGGA
3'- TT  AAACCAAGCC  ACTAACAGAT  AATACACATG  CATATTTTTC  CACGATTTTT  AAACCTTCCT
      -60                          Rsa I -30                              -1

                5                   10                  15                  20
Met Met Ile Leu Lys Asn Lys Arg Met Leu Lys Ile Gly Ile Cys Val Gly Ile Leu Gly
ATG ATG ATT TTG AAA AAT AAG AGG ATG CTA AAA ATA GGA ATA TGC GTT GGT ATA TTA GGT
TAC TAC TAA AAC TTT TTA TTC TCC TAC GAT TTT TAT CCT TAT ACG CAA CCA TAT AAT CCA
                                    30                                      60

                25                  30                  35                  40
Leu Ser Ile Thr Ser Leu Glu Ala Phe Thr Gly Glu Ser Leu Gln Val Glu Ala Lys Glu
TTA AGT ATT ACA AGC CTA GAA GCT TTT ACA GGA GAG TCA CTG CAA GTT GAA GCG AAA GAA
AAT TCA TAA TGT TCG GAT CTT CGA AAA TGT CCT CTC AGT GAC GTT CAA CTT CGC TTT CTT
                        Hind III    90                                      120

                45                  50                  55                  60
Lys Thr Gly Gln Val/Lys/His/Lys/Asn Gln Ala Thr His Lys Glu Phe Ser Gln Leu Glu
AAG ACT GGA CAA GTG AAA CAT AAA AAT CAA GCA ACG CAT AAA GAG TTC TCT CAA CTT GAG
TTC TGA CCT GTT CAC TTT GTA TTT TTA GTT CGT TGC GTA TTT CTC AAG AGA GTT GAA CTC
                                    150                                     180

                65                  70                  75
Lys Lys Phe Asp Ala Arg Leu Gly Val Tyr Ala Ile Asp Thr Gly Thr
AAA AAA TTT GAT GCT CGA TTA GGT GTA TAT GCG ATT GAT ACT GGT ACA -3'
TTT TTT AAA CTA CGA GCT AAT CCA CAT ATA CGC TAA CTA TGA CCA TGT -5'
                                    210                 Rsa I
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Fig. 3. DNA and deduced amino acid sequences of the signal region of *B. cereus* 569/H β-lactamase I (*penPC*). The NH_2 termini of the known β-lactamase I exo-forms [3] are indicated by diagonal lines. Possible initiation codons are underlined following the ribosome binding site (RBS); (----) Two possible '-10' promoter sequences.

is a Cys residue which is not in the consensus arrangement observed in *B. licheniformis* penicillinase and other membrane lipoproteins [4]. The single Cys residue in the signal sequence of the *E. coli* R6K β-lactamase [26], the only member of the class A β-lactamases derived from a Gram-negative organism, has also been shown to be unmodified [21]. In general, the modifiable Cys residue of various precursor lipoproteins occurs at the end of the hydrophobic segment in the signal sequence and upon processing becomes the NH_2 terminus [27–29]. In the case of *B. cereus* and *E. coli* the Cys residue in the hydrophobic region of the signal sequence of the pre-β-lactamases is more centrally located and may be unavailable for lipid modification. In this respect the *B. cereus* β-lactamase I signal sequence is more like the *E. coli* R6K signal sequence. The polar regions containing the proteolytic processing sites following the hydrophobic stretches are considerably longer in the two *Bacillus* species than in the corresponding segments of the *S. aureus* PC1 and *E. coli* R6K β-lactamases. A relatively lengthy sequence of 19 amino acids is present befor the first known processing site in *B. cereus* is encountered (fig.3). The processing site in E. coli possibly lies between Ala^{28} and Lys^{39} of the precursor sequence shown in fig.3, based on calculation from the app. M_r-values (fig.2) of the *penPC* in vitro product (M_r 36 000) and the in vivo products in *E. coli* (M_r 32 500) and *B. cereus* (M_r 31 200).

The NH_2 terminus of the primary translation product is not yet known. There are 2 ATG initiation codons immediately following the most likely Shine–Dalgarno sequence (fig.3). In addition, there is a TTG at the fourth position which is possibly functioning as the initiation codon. It has been shown that UUG initiates protein synthesis in *S. aureus* PC1 β-lactamase [30], and that is it utilized efficiently in vitro by both *B. subtilis* and *E. coli* ribosomes [31]. The spacing between the first and second initiation codons and the Shine–Dalgarno complementarity sequence in the *penPC* gene may be too small and may in fact be spatially unfavorable for initiation of translation when the mRNA is bound to 16 S rRNA. The average spacing for 11 known initiation sites utilized by *B. subtilis* ribosomes is 7–8 nucleotides [32, 33], a value also observed for 123 *E. coli* ribosome binding sites [33]. It does not seem unlikely then, that UUG may effect translational initiation with the *B. cereus penPC* mRNA as well.

We have successfully obtained expression of the *penPC* gene in *B. subtilis* BD170, where the product is totally secreted as a hydrophilic form. Investigations are in progress to determine the NH_2 termini of the β-lactamase I products in *B. subtilis* and *E. coli*, as well as of the in vitro translation product. The absence of a lipoprotein form in *B. cereus* β-lactamase I, combined with its high levels of production and secretion, make it an attractive vehicle for secretion of genetically-engineered proteins.

ACKNOWLEDGEMENTS

The gifts of benzyl penicillin from Eli Lilly and Co. and spectinomycin from The Upjohn Co. are appreciated. We thank Julia Sohm for expert technical assistance. This work was supported by grants from The National Science Foundation (PCM81-16888), Miles Laboratories, and the Charles and Johanna Busch Fund. P.S.F.M. was the recipient of a Waskman-Merck Postdoctoral Fellowship.

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