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Cloning and Sequencing of the *blaZ* Gene Encoding β-Lactamase III, a Lipoprotein of *Bacillus cereus* 569/H

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It has not been clear whether the membrane-bound β-lactamase III of *Bacillus cereus* 569 is a separate enzyme or a modified form of the secreted β-lactamase I. The membrane enzyme is an acyl-glyceride thioether-linked lipoprotein (J. B. K. Nielsen and J. O. Lampen, Biochemistry 22:4652–4656, 1983) and thus is probably a separate entity. We cloned the β-lactamase III gene (*blaZ*) on a 4.9-kilobase-pair *Cla*I fragment from mutant strain 569/H (constitutive for high-level production of β-lactamases I, II, and III), and the nucleotide sequence was determined. The structural gene was flanked by typical promoter, transcription termination, and translation initiation sequences. Expression of the cloned gene in *Escherichia coli* was low in exponential-phase cultures and increased only as the cultures reached the stationary phase. The deduced amino acid sequence indicates a pre-β-lactamase III of 316 amino acid residues (35,021 daltons), with a 29-residue signal peptide and a mature lipoprotein form of ~32,500 daltons. The 12 NH_2-terminal residues of a 21-kilodalton tryptic peptide from the *B. cereus* membrane enzyme were in agreement with the sequence deduced from the cloned gene. The amino acid sequence was highly homologous to the class A β-lactamases, especially that of *Bacillus licheniformis* 749. β-Lactamase III is a distinct class A enzyme and the product of a separate gene (*blaZ*).

Bacillus cereus 569/H is unusual in that it makes three different β-lactamases. β-Lactamase I is a serine enzyme and is grouped in class A (1). Its amino acid sequence shows good homology with the β-lactamases of *Bacillus licheniformis* 749/C and *Staphylococcus aureus* PC1 and with the *bla* gene product of pBR322. The structural gene, *blaY*, for β-lactamase I has been cloned and sequenced (21, 29). β-Lactamase II is a smaller protein with little sequence homology to the class A enzymes. It requires a thiol group and Zn^{2+} for activity and is grouped in class B. This gene, *blm*, has recently been cloned and sequenced (10). The history of β-lactamase III, on the other hand, has been complicated. Pollock (26) had identified it as a separate protein from β-lactamase I, but subsequently it was thought to be a membrane-bound form of β-lactamase I, which, along with β-lactamase II, is found mostly in the culture fluid. In 1983, Nielsen and Lampen (25) and Connolly and Waley (4) showed that β-lactamase III is probably separate from β-lactamase I. β-Lactamase III, purified from membranes of *B. cereus* 569/H, was a lipoprotein similar to the penicillinase of *B. licheniformis* (*blaP*) and to the major outer membrane lipoprotein of *Escherichia coli*, i.e., there is a diacylglyceride thioether-linked to the NH_2-terminal cysteine residue and a fatty acid amide-linked to the same cysteine (25). (We renamed the β-lactamase gene of *B. licheniformis* 749 *blaP* [instead of *penP*] to emphasize its close relationship to the other class A β-lactamases [31].) This hydrophobic moiety functions as the anchor of the protein in the membrane lipid bilayer. In fact, both the penicillinase of *B. licheniformis* and the β-lactamase III of *B. cereus* have membrane-bound and secreted forms. The membrane-bound forms contain a single cysteine residue (N-terminal), while the hydrophilic *exo*-forms lack cysteine (25).

The membrane lipoproteins are synthesized as precursors containing NH_2-terminal signal peptides which are subsequently modified by lipids (11, 12). In *E. coli* they are processed by a specific lipoprotein signal peptidase (30, 33), and bacilli appear to have enzymes with similar specificity (16). The prelipoproteins show a conserved sequence, Leu-Ala-Gly-Cys, around the site of lipid modification and processing (24, 32). This could be a signal for the enzymes engaged in lipid modification and for the specific lipoprotein signal peptidase.

Here we report the cloning and sequencing of the structural gene (*blaZ*) for β-lactamase III of *B. cereus* 569/H and show that the cloned gene encodes the β-lactamase III previously purified from *B. cereus* 569/H (4, 25). β-Lactamase III has a lipid-modifiable cysteine residue characteristic of lipoproteins, and its primary structure is homologous to that of the known class A β-lactamases.

MATERIALS AND METHODS

Bacteria and plasmids. *B. cereus* 569/H (21), constitutive for production of β-lactamase III, *E. coli* RR1 (F^- *hsdS20* [r^-_B m^-_B] *ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-) (2), and *E. coli* JM101 [Δ(*lac proA, B*) *supE thi* (r^+_K m^+_K)/F′ *traD36 proAB lacI*^{q}AΔM15] (18) were used. *B. cereus* 569/H was grown at 34°C in 2% CH/S medium (24), and the *E. coli* strains were grown at 37°C in LB broth or in M-9 supplemented with 19 amino acids without methionine (100 μg of each per ml), adenosine, guanosine, cytidine, and thymine (40 μg of each per ml), and thiamine (5 μg/ml) (22). β-Lactamase activity was assayed by the method of Sargent (28); 1 U is defined as the amount of enzyme hydrolyzing 1 μmol of benzyl penicillin in 1 h at 30°C. The plasmid vector for cloning was pRW33 (20), which carries Cm^r and Tc^r genes. Transformants were selected on LB agar containing chloramphenicol (30 μg/ml) or ampicillin (50 μg/ml). Plasmid pRWH61 contains a 4.9-kilobase-pair (kbp) fragment of *B.*

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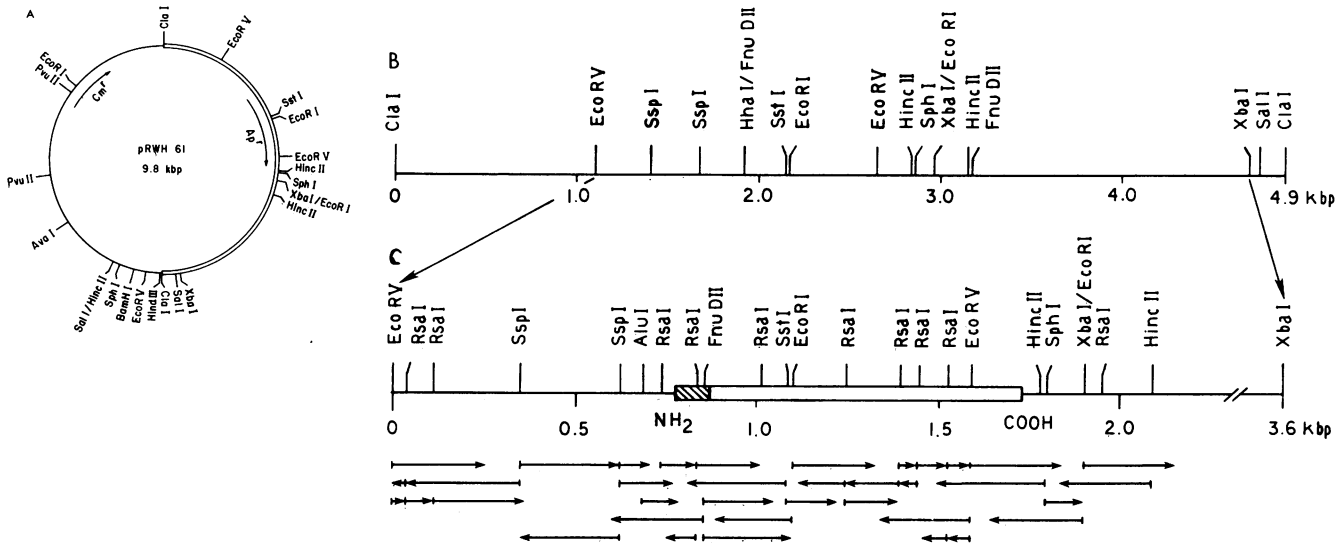


FIG. 1. Restriction map and sequencing strategy for the *B. cereus* 569/H β-lactamase III gene. (A) Map of plasmid pRWH61. The broad segment indicates *B. cereus* DNA inserted in the *Cla*I site of pRW33. The insert of 4.9 kbp is the same size as the vector. The locations of the genes for chloramphenicol resistance (Cm^r) on the vector and for ampicillin resistance (Ap^r) due to β-lactamase III on the insert are shown. (B) Map of the 4.9-kbp *Cla*I fragment from *B. cereus*. (C) Region of the 4.9-kbp *Cla*I fragment that was sequenced. The region corresponding to β-lactamase III is boxed, and the segment coding for the signal peptide at the NH_2-terminal end is hatched.

cereus 569/H DNA inserted in the *Cla*I site of pRW33 (Fig. 1A). Plasmids pRWP23 and pRWP30 are subclones of pRWH61. Plasmid pRWP23 contains the 1.6-kbp *Ssp*I-*Xba*I fragment (nucleotides 347 to 1897) inserted in the *Cla*I site of pRW33. Plasmid pRWP30 has the 1.3-kbp *Ssp*I-*Xba*I fragment (nucleotides 623 to 1897) in the *Cla*I site of the vector in the opposite orientation to pRWH61 or pRWP23 (Fig. 1B and C).

Preparation of DNA and sequencing. Chromosomal DNA from *B. cereus* 569/H was prepared by the method of Dubnau and Davidoff-Abelson (5). Small- and large-scale plasmid preparations from *E. coli* and all manipulations with DNA, e.g., enzyme digestion, ligation, transformation, etc., were performed by the methods of Maniatis et al. (17). For sequencing, DNA fragments were cloned in M13 mp18 or mp19 and used as templates to sequence by the method of Sanger et al. (27).

Radiolabeling and immunoprecipitation. *E. coli* RR1 (pRWH61) was grown in supplemented M-9 medium containing appropriate antibiotics and labeled with [^{35}S]methionine (62 μCi/50 pmol per ml) from the early logarithmic phase for two generations (about 90 min at 37°C) or with [^{35}S]methionine (10 μCi/10 pmol per ml) from late logarithmic phase for 1 h at 37°C. For the palmitate labeling, cultures were grown in 2% CH/S medium and labeled for 1 h with [9,10-^{3}H]palmitic acid (20 μCi/1.3 nmol per ml). Cells were harvested and disrupted by heating at 100°C in 2% sodium dodecyl sulfate (SDS)–1 mM phenylmethylsulfonyl fluoride (PMSF) for 5 min. The suspension was sedimented, and the supernatant was used for immunoprecipitation by the method of Nielsen and Lampen (25). *B. cereus* 569/H was grown at 37°C in supplemented M-9 medium or in 2% CH/S to mid-logarithmic phase and labeled for 1 h with [^{35}S]methionine or [^{3}H]palmitic acid under the same conditions as for *E. coli*. After sonication of the cells, the particulate fraction was isolated and extracted with hot SDS-PMSF. The solubilized proteins were immunoprecipitated with anti-β-lactamase III antibody. The DNA-directed transcription-translation system of Amersham Corp. (Arlington Heights, Ill.) was used for in vitro protein synthesis. [^{35}S]methionine (1.35 mCi/1.27 mmol per ml) was used for labeling. Proteins were separated on 12.5% acrylamide–SDS gels (15). Gels were treated with En^{3}Hance and exposed to Kodak X-Omat AR films.

Amino acid sequence analysis. β-Lactamase III was purified from *B. cereus* 569/H membranes and partially digested with trypsin as described by Nielsen and Lampen (25). The products were separated in a 10% acrylamide–SDS gel and stained with 0.008% Coomassie brilliant blue in 7% acetic acid. A 21-kilodalton (kDa) band was excised from the gel and extracted with 0.2% SDS–0.1% mercaptoethanol in 100 mM Tris hydrochloride (pH 8.4)–1 mM EDTA for 24 h at 37°C. The polypeptide was precipitated with 0.0165 M quinine. The sequence of amino acids was determined from the NH_2 terminus by stepwise Edman degradation with an Applied Biosystems sequencer (model 120A PTH Analyzer) (9).

Materials. Enzymes for DNA manipulation, DNA primer, and radiolabeled protein standards were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., or New England Biolabs, Inc., Beverly, Mass. [^{35}S]methionine (10 mCi or 10 nmol/ml), [9,10-^{3}H]palmitic acid (10 mCi or 0.65 μmol/ml), and En^{3}Hance were from New England Nuclear Corp., Boston, Mass. Trypsin was from Sigma Chemical Co., St. Louis, Mo. Purified signal peptidase (Lep) was a gift of William Wickner.

RESULTS

Cloning of β-lactamase III. The approach to cloning β-lactamase III was that used for the other two β-lactamases of *Bacillus cereus* 569/H (10, 21). Chromosomal DNA from *B. cereus* 569/H was digested with *Hin*dIII or *Cla*I, which cleave within the genes for β-lactamase I or II. These fragments were inserted in the corresponding sites of plasmid pRW33 (Ap^s Tc^r Cm^r) (20) and were shotgun-cloned in *E. coli* RR1 (Ap^s). Ap^r Tc^s colonies were selected, and the plasmids were isolated. *E. coli* RR1 cells were transformed

TABLE 1. β-Lactamase III activity in *E. coli* RR1 transformants[a]

Plasmid	Growth phase[b]	β-Lactamase activity (U/ml of culture)			
		Total	Membrane (% of total)	Cytoplasm-periplasm (% of total)	Culture fluid
pRWH61	Exponential	12	12 (100)	0	0
	Early stationary	153	135 (88)	18 (12)	0
pRWP23	Exponential	24	ND[c]	ND	ND
	Early stationary	149	ND	ND	ND
pRWP30	Exponential	7	ND	ND	ND
	Early stationary	38	ND	ND	ND

[a] *E. coli* RR1(pRWH61) cultures were centrifuged, and the cells were suspended in 100 mM Tris hydrochloride, pH 7.5 and sonicated three times for 20 s each on ice. The sonicates were centrifuged at 40,000 × *g* for 100 min, and the pellets were suspended in the same buffer. The supernatants, the resuspended pellets, and the corresponding culture fluids were made 1% in Triton X-100 and 150 mM NaCl, kept at room temperature for 15 min, and assayed for penicillinase activity (28). The cells from cultures of *E. coli* RR1(pRWP23) and *E. coli* RR1(pRWP30) were collected and sonicated as for *E. coli* RR1 (pRWH61). The sonicates were made 1% in Triton X-100 and 150 mM NaCl, and the penicillinase activity was determined.

[b] The cell density of the exponential-phase cultures was about 160 Klett units; that of cultures at early stationary phase was about 350 Klett units.

[c] ND, Not determined.

with these preparations to make sure that the Ap^r trait was borne on the plasmid. Next, the plasmids were digested with *Hin*dIII or *Pst*I-*Cla*I, subjected to gel electrophoresis, and screened for bands characteristic of β-lactamase I (0.6-kbp *Hin*dIII fragment) or β-lactamase II (240-bp *Pst*I-*Cla*I fragment). Five plasmids that did not yield these fragments were studied further. Only pRWH61 was found to contain the gene for β-lactamase III.

Identification of the β-lactamase III clone. pRWH61, a 9.8-kbp plasmid, has a 4.9-kbp fragment of *B. cereus* 569/H DNA inserted in the *Cla*I site of pRW33 (Fig. 1A). The following evidence indicated that pRWH61 contained the gene for β-lactamase III. (i) *E. coli* RR1(pRWH61) cultures, at early stationary phase, had β-lactamase activities of 150 to 200 U/ml. The active enzyme remained membrane bound after rupture of the cells by sonication; the soluble fraction (cytoplasm and periplasm) contained only 12% of the total activity, and there was no detectable enzyme in the culture medium (Table 1). (ii) *E. coli* RR1(pRWH61) cells in late exponential phase were labeled with [^{35}S]methionine for 1 h and extracted with hot 2% SDS. The soluble products were immunoprecipitated in the presence of 1 mM PMSF and analyzed by SDS-polyacrylamide gel electrophoresis. A protein band was observed that comigrated with β-lactamase III from *B. cereus* 569/H membranes (Fig. 2, lanes 3 and 4). Labeling at mid-exponential phase for two generations yielded no immunoprecipitable band. The protein synthesized in *E. coli* was also labeled with [^{3}H]palmitate (Fig. 2, lane 5), indicating that the cloned protein was lipid modified, as in *B. cereus* 569/H (Fig. 2, lane 6). (iii) Only pRWH61, among the plasmids purified from the five clones mentioned above, yielded an immunoprecipitable protein band in the in vitro protein synthesis system. This band was ~3,000 daltons larger than the membrane form of β-lactamase III from *B. cereus* (Fig. 2, lane 2). (iv) The lipoprotein form of β-lactamase III had an NH_2 terminus which was blocked to Edman degradation and could not readily be sequenced. To avoid this problem, we prepared a 21-kDa tryptic fragment from the lipoprotein form of *B. cereus* 569/H (25) and purified it as described in Materials and Methods. The fragment was then subjected to 12 cycles of Edman degradation. The amino acid sequence obtained was identical to that of a potential tryptic fragment of the protein deduced from the *blaZ* gene as cloned in *E. coli*(pRWH61). The corresponding amino acid residues (106 to 117) are underlined in Fig. 3. The peptide extended from residue 106 to the COOH terminus of the protein (residue 287). The deduced molecular weight of the fragment (20, 128) was in agreement with the apparent size (21 kDa) of the polypeptide sequenced.

Sequencing of the β-lactamase III gene (*blaZ*). A restriction map of the 4.9-kbp *Cla*I insert in pRWH61 is shown in Fig. 1B. We removed the 0.8-kbp *Eco*RI fragment and religated the plasmid. *E. coli* RR1(pRWH61-0.8), which contains the truncated plasmid, was Cm^r Ap^s, and the plasmid did not yield the putative pre-β-lactamase III when used as the template in the in vitro protein synthesis system (data not shown). These findings established the location of the Ap^r gene in pRWH61.

The sequencing strategy is shown in Fig. 1C. The 0.8-kbp *Eco*RI fragment was sequenced first. An open reading frame lacking cysteine residues was found which contained a number of the amino acid residues conserved in class A β-lactamases (1) (Fig. 4). The complete DNA sequence and the deduced amino acid sequence are shown in Fig. 3. The open reading frame started with ATG (nucleotides 784 to 786), had a length of 948 nucleotides coding for a protein of 316 amino acid residues (M_r, 35, 021), and terminated with TAA (nucleotides 1732 to 1734). A second termination codon (TAG) followed in frame after a gap of one triplet. Ten bases upstream from the translation initiation codon there was a putative ribosome-binding site. Further upstream were sequences which could be the −10 region (TAAATT [nucleotides 744 to 749] or AATATT [nucleotides 620 to 625]) and the corresponding −35 regions (TACACT [nucleotides 720 to 725] or TTGCAA [nucleotides 599 to 604]) of two putative promoters.

To define the role of regions outside the open reading

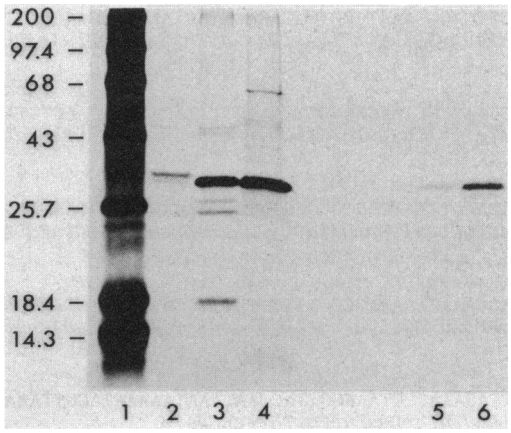
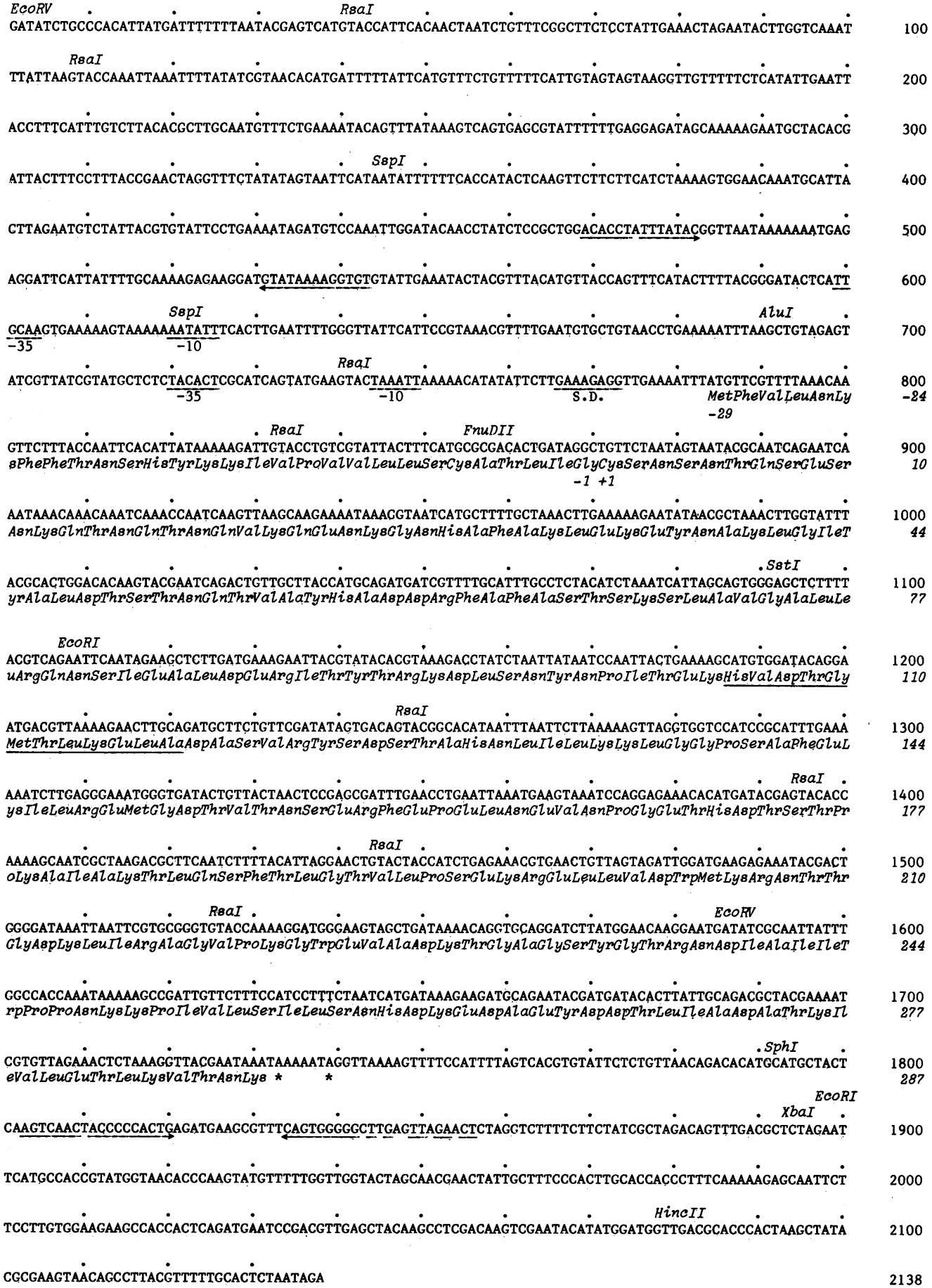


FIG. 2. Comparison of the in vitro and in vivo products of the *blaZ* gene. Autoradiogram of the SDS-polyacrylamide gel. Lane 2, Immunoprecipitate of the [^{35}S]methionine-labeled in vitro translation products of *blaZ* with pRWH61 as the template. Immunoprecipitate of the β-lactamase III obtained from the hot SDS extract of *E. coli* RR1(pRWH61) (labeled with [^{35}S]methionine [lane 3] or [^{3}H]palmitic acid [lane 5]). Immunoprecipitated product from the membrane fraction of *B. cereus* 569/H cells labeled with [^{35}S]methionine (lane 4) or [^{3}H]palmitic acid (lane 6). M_r standards (lane 1): myosin, H-chain (200,000); phosphorylase B (97,400), bovine serum albumin (68,000), ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), and lyzozyme (14,300).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RsaI
RsaI
SspI
SspI
AluI
-35
-10
RsaI
-35
-10
S.D.
MetPheValLeuAsnLy
-24
-29
RsaI
FnuDII
sPhePheThrAsnSerHisTyrLysLysIleValProValValLeuLeuSerCysAlaThrLeuIleGlyCysSerAsnSerAsnThrGlnSerGluSer
10
-1 +1
AsnLysGlnThrAsnGlnThrAsnGlnValLysGlnGluAsnLysGlyAsnHisAlaPheAlaLysLeuGluLysGluTyrAsnAlaLysLeuGlyIleT
44
SstI
yrAlaLeuAspThrSerThrAsnGlnThrValAlaTyrHisAlaAspAspArgPheAlaPheAlaSerThrSerLysSerLeuAlaValGlyAlaLeuLe
77
EcoRI
uArgGlnAsnSerIleGluAlaLeuAspGluArgIleThrTyrThrArgLysAspLeuSerAsnTyrAsnProIleThrGluLysHisValAspThrGly
110
RsaI
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RsaI
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177
RsaI
oLysAlaIleAlaLysThrLeuGlnSerPheThrLeuGlyThrValLeuProSerGluLysArgGluLeuLeuValAspTrpMetLysArgAsnThrThr
210
RsaI
EcoRV
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244
rpProProAsnLysLysProIleValLeuSerIleLeuSerAsnHisAspLysGluAspAlaGluTyrAspAspThrLeuIleAlaAspAlaThrLysIl
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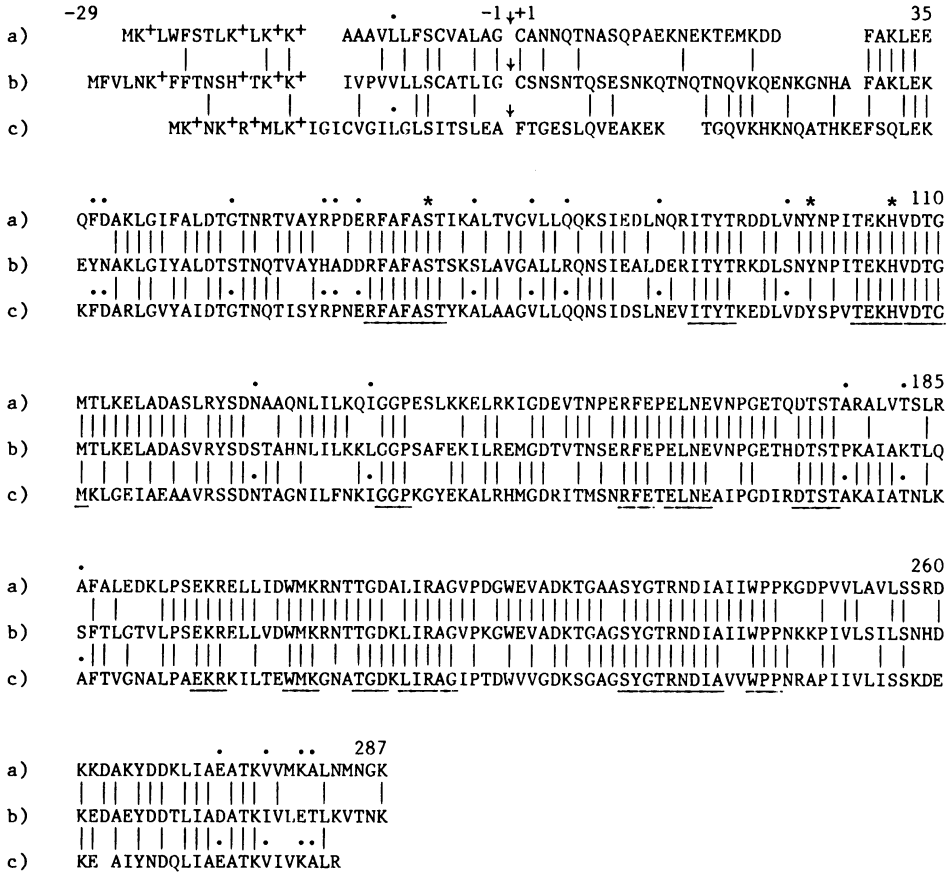

FIG. 4. Amino acid homologies in the primary structure of β-lactamase III and two other class A β-lactamases. For explanation, see Results. The numbering is that of pre-β-lactamase III, and the signal sequences are aligned at the end of the NH_2-terminal charged segments and at the probable sites of signal peptidase cleavage. (a) Pre-β-lactamase of *B. licheniformis* 749/C (BlaP); (b) pre-β-lactamase III of *B. cereus* 569/H (BlaZ); (c) pre-β-lactamase I of *B. cereus* 569/H (BlaY). The three residues Ser-66, Tyr-98, and His-106 which appear to be essential for catalytic activity are marked with an asterisk (*). The vertical lines indicate homologies between β-lactamase III and BlaP or BlaY. Dots above BlaP and BlaY sequences indicate homologies not shared with β-lactamase III. Stretches where identical residues occur in all three sequences are underlined. One-letter codes for amino acids: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

frame in regulating production of β-lactamase III, we constructed a number of smaller plasmids (two listed in Table 1) by deleting portions of DNA flanking the structural β-lactamase gene in pRWH61 and introduced them into *E. coli* RR1. All clones showed a pattern and level of β-lactamase formation similar to that of *E. coli* RR1(pRWH61). Moreover, pRWP30, the smallest construct, which included only one of the two putative promoters (the one closer to the structural gene), produced a low level of β-lactamase and showed similar timing of the production of the enzyme.

Signal sequence of β-lactamase III. The membrane form of β-lactamase III is a lipoprotein with an apparent size of about 32,000 daltons (25). The sequence upstream from the NH_2-terminal cysteine residue of the lipoprotein form (negatively numbered in Fig. 3) is the presumed signal peptide. The NH_2-terminal segment of the 29-amino-acid signal peptide (3,299 daltons) contained four basic residues. It is followed by a stretch of 14 hydrophobic or nonpolar residues. The deduced amino acid sequence contains two cysteine residues, one in the sequence Leu−9-Leu-Ser-Cys−6 and the other in Leu−3-Ile-Gly-Cys-+1. The second is closer to the conserved sequence, Leu-Ala-Gly-Cys, characteristic of lipoproteins (24, 32) and allows a hydrophobic stretch of 13 rather than 7 residues. Therefore, we have assumed that Cys-+1 undergoes lipid modification, although Cys−6 may also be modified to some extent (8). Presumably the processing site for lipoprotein signal peptidase (Lsp [30, 33]) is between Gly-1 and Cys-+1. The calculated molecular size of the resulting protein (287 amino acid residues with Cys-+1 as the NH_2 terminus) is 31,740 daltons. With the attached lipid (assuming C_{16} fatty acids), it is about 32,500 daltons, close to the 32,000 daltons estimated for the membrane form of β-lactamase III. The amino acid composition calculated from the deduced sequence of the processed

FIG. 3. DNA sequence and deduced amino acid sequence of *B. cereus* 569/H β-lactamase III (*blaZ*). The −35 and −10 regions of two promoterlike sequences upstream from the ribosome-binding site (S.D.) are underlined. The amino acids are marked with italic numbers and the termination codons with *. The stretch of residues (106 to 117) at the NH_2 terminus of the 21-kDa polypeptide that was sequenced by Edman degradation is underlined. Arrows indicate inverted repeats. The restriction sites used for determining the sequence are shown.

lipoprotein is also close to the published values (4) for β-lactamase III.

The Ala-−5-Thr-−4 linkage in the signal sequence of β-lactamase III might be a site for cleavage by the general signal peptidase (Lep) of *E. coli* (34). We synthesized the pre-β-lactamase III in vitro with pRWH61 as the template and subjected it to the action of purified Lep. As positive controls, pre-β-lactamases I and II were treated with Lep. The pre-β-lactamases I and II were processed to their respective mature forms, but pre-β-lactamase III was not cleaved (F. I. J. Pastor, M. Hussain, and J. O. Lampen, unpublished observation).

Comparison with class A enzymes. Figure 4 illustrates the homology in primary structure between the β-lactamase III and two class A enzymes, the penicillinase of *B. licheinformis* 749/C (23), and the β-lactamase I of *B. cereus* 569/H. (The sequence of β-lactamase I as corrected by M. J. Madonna, Y. F. Zhu, and J. O. Lampen [unpublished observations] has been used.) The relative alignment of the three proteins is basically similar to that presented in Ambler (1). No gap was allowed COOH-terminal to Phe-30 except in position 263 for β-lactamase I (Fig. 4, line c). The probable processing sites of the three preproteins in *E. coli* (13, 19) and the COOH termini of the charged segments of the signal peptides are aligned. A few gaps are allowed for the best homology.

Identical residues occur at a number of stretches along the length of the proteins in all three sequences. The membrane form of β-lactamase III is 66% homologous to the membrane form from *B. licheniformis* (b and a, Fig. 4). Two large segments (residues 37 to 119, which include the active center serine, and residues 186 to 260) are 75 and 77% homologous, respectively. The overall homology between β-lactamase III and the β-lactamase I of *B. cereus* is 56% (b and c, Fig. 4). The value is highest (64%) in the 37 to 110 region and gradually declines to an average of 58% for the following portion.

DISCUSSION

We have cloned in *E. coli* RR1 the gene (*blaZ*) for the β-lactamase III of *B. cereus* 569/H, a mutant which is constitutive for high-level production of the enzyme, and determined the nucleotide sequence. With *blaZ* in pRWH61, a high-copy-number plasmid, expression of the gene in terms of amount of protein and enzyme activity was low in the early to mid-exponential phase of growth, but increased about 10-fold in late exponential and stationary phase. Radiolabeling at late exponential phase and immnoprecipitation were successful, and we were able to identify a membrane-bound lipoprotein form of β-lactamase III in *E. coli* RR1(pRWH61). The amino acid sequence deduced from *blaZ* contains a 29-residue NH_2-terminal segment of the type that can undergo lipid modification. It should also undergo cleavage by the lipoprotein-specific signal peptidase (Lsp) in *E. coli*, and presumably in *Bacillus* species as well. The resulting lipoprotein form has a molecular size (~32,500 daltons) close to that of β-lactamase III purified from *B. cereus* 569/H membranes (32,000 daltons), and its amino acid composition closely resembles that of purified β-lactamase III (4). A hydrophilic extracellular form, similar in mobility on SDS gels to the membrane enzyme, has been detected in *B. cereus* 569/H cultures grown in medium repressive for protease formation (25). The long hydrophilic stretch following the lipid-modified Cys-+1 should offer a number of sites for proteolytic cleavages.

To complete the demonstration that β-lactamase III is encoded by *blaZ*, a 21-kDa tryptic peptide from a sample of β-lactamase III isolated from *B. cereus* 569/H membranes was subjected to stepwise Edman degradation. The 12 NH_2-terminal residues were identical to the corresponding sequence deduced from the cloned *blaZ* gene.

The β-lactamase III from *B. cereus* is precipitable by antibodies to *B. licheniformis* β-lactamase and, to a lesser extent, by antibodies to β-lactamase I (25). As expected, the protein from the cloned gene showed considerable sequence homology to both of these enzymes. Moreover, β-lactamase III had a Ser residue in the conserved region that contains the active-site Ser of the other class A enzymes. We conclude that the Ap^r gene cloned in pRWH61 is that of *B. cereus* β-lactamase III. From the extent of its homology with the two class A enzymes and its size, catalytic specificity, and serological relationship (4, 25) β-lactamase III belongs in class A.

β-Lactamase III as a lipoprotein. The β-lactamase III is present in *B. cereus* 569/H membranes as a glyceride-cysteine lipoprotein (25) and in *E. coli* as an apparently identical membrane-bound form that can be labeled with [^{3}H]palmitic acid (Fig. 2). Comparison of its precursor form with those of other lipoproteins (32) indicates that the Ile–2 residue replaces the Ala of the consensus sequence Leu-Ala-Gly-Cys. The importance of Ala is unknown, but in a few naturally occurring lipoproteins Ser is present in place of the Ala (32). Giam et al. (6) noted that the modifiable Cys residue is part of a β-turn. In pre-β-lactamase III, residue Ile–2 is just before the beginning of a β-turn. Highly hydrophobic residues such as Ile are often located just outside a β-turn (3) and are thought to provide stability. Prediction of the secondary structure of pre-β-lactamase III by the principles of Chou and Fasman (3) indicates that a six-member β-turn region can be formed, beginning with the Cys residue (Cys-1-Ser-Asn-Ser-Asn-Thr-6). The fact that lipid modification occurs efficiently even when the β-turn extends only downstream from the modifiable Cys is evidence for the importance of that region.

Regulatory regions for *blaZ*. The structural gene for β-lactamase III, *blaZ*, is flanked by apparent regulatory sequences. There are two inverted repeats: one ends 239 bp upstream from the start codon and has a ΔG of −13.8 kcal/mol; the other begins 67 bp downstream from the stop codon and has a ΔG of −20.5 kcal/mol (Fig. 3). The latter sequence may function as the trancriptional terminator for *blaZ*. Between the upstream inverted repeat and the translation initiation codon, there are two sets of promoterlike sequences. There is also a strong Shine-Dalgarno sequence, almost identical to that of *trpC* of *B. subtilis* (7), 10 bases upstream from the presumed initiation codon. The calculated free energy for the interaction of this Shine-Dalgarno sequence and the 3′ end of the *B. subtilis* 16S rRNA is about −15 kcal/mol. Consequently, *blaZ* appears to be an independent gene with adequate promoter, translation initiation, and transcription termination sequences. Despite this, there was poor expression of *blaZ* in the early to mid-exponential phase of growth even though the β-lactamase III protein was obtained in good amounts when pRWH61 was used as the template in an in vitro transcription-translation system with components derived from *E. coli* grown to exponential phase.

It was conceivable that the low expression of *blaZ* in pRWH61 was determined by negative regulatory elements encoded in the large flanking regions of the 4.9-kb *Cla*I insert. To test this possibility, we constructed pairs of clones

containing shortened fragments inserted in both orientations (two are shown in Table 1). Their patterns of β-lactamase production were basically the same as for pRWH61. We conclude that the large insert in pRWH61 does not carry a negatively acting regulatory element. Moreover, the positive results with pRWP30 (Table 1) indicate that *blaZ* probably can be expressed from the putative promoter combination TACACT (nucleotides 720 to 725) and TAAATT (nucleotides 744 to 749), which is the only one present in pRWP30 (this fragment still contains the major transcriptional terminator). The reason for the low expression of *blaZ* in *E. coli* and its differential expression during growth is not yet understood.

There is ~70% homology at the DNA level between the portions of *blaZ* and *blaP* coding for the mature proteins and 48% between the signal sequence regions. Upstream of the translation initiation codon and downstream of the termination codon, the degree of DNA homology declines abruptly, although there are short stretches with high homology. There is considerable evidence that the various β-lactamases and penicillin-binding proteins derive from a common ancestor (1, 14, 31). The fact that the *blaZ* gene of *B. cereus* 569/H is less homologous to the other class A β-lactamase of that strain than it is to the *blaP* gene of *B. licheniformis* makes it likely that *blaZ* did not evolve within *B. cereus*. It may well have been transferred relatively recently from *B. licheniformis* to *B. cereus*, in which some divergence from *blaP* (or a progenitor) occurred. If this is correct, only the structural gene and not the surrounding regions has been well conserved in *B. cereus*.

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