β-Lactamase III of *Bacillus cereus* 569: Membrane Lipoprotein and Secreted Protein†

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ABSTRACT: A third β-lactamase in *Bacillus cereus* 569 has been identified and characterized. It corresponds to γ-penicillinase reported by Pollock [Pollock, M. R. (1956) *J. Gen. Microbiol. 15*, 154–169] but whose existence has been questioned since then. It will be called β-lactamase III. It resembles the class A β-lactamases but is immunologically distinct from the major class A secreted β-lactamase I of *B. cereus*. As with several other Gram-positive β-lactamases it occurs in two forms, membrane bound as a glyceride–cysteine lipoportein and as a hydrophilic secreted protein formed by cleavage on the carboxyl side of the modified cysteine that is the membrane attachment site. It is produced in all *B. cereus* 569 strains tested but is absent in *B. cereus* 5/B. Antibody to β-lactamase III interacts to varying degrees with all the known class A β-lactamases, most strongly with that of *B. licheniformis* 749/C.

The nature of what Pollock (1956) termed as γ-penicillinase in *Bacillus cereus* 569 has puzzled those interested in β-lactamases for 25 years. It was membrane activity not neutralizable by antibody to the major secreted form, now called *B. cereus* β-lactamase I [EC 3.5.2.6; Pollock (1963)]. The latter is a class A β-lactamase (Ambler, 1980), nearly completely released by cells in logarithmic growth unlike the class A β-lactamases of *B. licheniformis* strains 749 and 6346 and of *S. aureus*. We have reported (Nielsen & Lampen, 1982a) preliminary evidence that *B. cereus* 569 has a membrane-bound penicillinase which is a lipoprotein of the type well documented for the *B. licheniformis* 749/C and *S. aureus* β-lactamases and suggested that it might correspond to the β-lactamase I exoenzyme. Since then, the gene coding for β-lactamase I has been cloned and shown not to code for a glyceride–cysteine lipoprotein (P. S. F. Mezes, Y. Yang, M. Hussain, and J. O. Lampen, unpublished experiments). We therefore searched for a membrane-bound β-lactamase in *B. cereus* of different antigenic specificity from β-lactamase I; this is Pollock's description of γ-penicillinase. Unfortunately, the nomenclature of *B. cereus* β-lactamases has been confused by the adoption of the term γ for conformational variants of the β-lactamase I exoenzyme produced by physical treatments (Citri, 1958; Rudzik & Imsande, 1970). Pollock (1956) assigned the term α for the dominant exoenzyme and β for the loosely cell-bound material readily released by washing and apparently undistinguishable from α. The situation is further complicated by the existence in *B. cereus* 569 strains of a secreted β-lactamase belonging to class B, apparently unrelated to the class A β-lactamases (Ambler, 1980), differing from them in size, in amino acid composition, in substrate specificity, and in the fact that it is a sulfhydryl enzyme requiring zinc for activity. This enzyme is called β-lactamase II (EC 3.5.2.8; Kuwabara & Abraham, 1967), and no membrane-bound form can be detected (Abraham & Waley, 1979).

We now wish to confirm the existence of Pollock's γ-penicillinase, to be referred to as β-lactamase III. It is an antigenically distinct membrane β-lactamase in *B. cereus* 569 and is a lipoprotein, readily distinguishable from β-lactamase I by a variety of biochemical techniques. This lipoprotein is the membrane-bound partner of a secreted enzyme, not hitherto detected in the medium because of its relatively low specific activity compared with the highly active β-lactamase I exoenzyme and because it is sensitive to proteases secreted as cultures approach stationary phase. It appears to be a class A β-lactamase, as originally suggested by Pollock's substrate specificity measurements, and now by our finding that it interacts to varying extents with antisera to all the known class A β-lactamases.

Experimental Procedures

Bacterial Strains. *B. cereus* 569 (ATCC 27348) inducible for β-lactamase I and the constitutive mutants 569/H and 569/H/9 [a high producer selected by Davis et al. (1974)] were obtained from S. G. Waley, University of Oxford, Oxford England. *B. cereus* 569/H was also obtained from T. Viswanatha, University of Waterloo, Canada, and from N. Citri, Hebrew University, Jerusalem. No significant differences were found between strains from the three sources. *B. cereus* 5/B (ATCC 13061) (magnoconstitutive for a variant of β-lactamase I) was obtained from R. A. Day, University of Cincinnati.

Assays. Penicillinase activity was assayed as described by Sargent (1968). One unit is defined as the enzyme hydrolyzing 1 μmol of benzylpenicillin in 1 h at 30 °C. Protein was estimated by the method of Lowry as modified by Wang & Smith (1975) to prevent interference by Triton X-100.

Purification of β-Lactamase III. A 30-L batch of *B. cereus* 569/H was grown in 2% CH/S [2% Bacto acid hydrolyzed casamino acids, 20 mM KH_2PO_4, 10 mM $MgCl_2$, and 0.1% Pollock's salts, pH 6.5 (Pollock, 1963)] at 34 °C with vigorous aeration. The casamino acids were raised from 1% to 2% to repress proteases (Neumark & Citri, 1962). Significantly more extracellular β-lactamase III can thus be detected. The innoculum was 2% of an overnight culture. After 6 h the cells were harvested at a density of about A_{540} = 2, yielding 410 g of cell paste.

Extraction was performed in two batches from frozen cell paste essentially as described by Connolly & Waley (1983). Briefly, the cell paste was frozen and thawed 6–8 times in 20 mM sodium citrate, pH 6, to disrupt the cell wall and to remove the loosely bound β-lactamase I [Pollock's (1956) β form]. Membrane proteins were extracted by stirring the cells overnight at 4 °C with 500 mL of 20 mM sodium citrate, pH

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6, and 0.5% Triton X-100. The extract, after dialysis against 20 M sodium citrate, pH 5, and 0.5% Triton X-100, was clarified by centrifugation and fractionated on a column of Sephadex CM-50 (2.5 × 100 cm) with a 1-L gradient of NaCl from 0 to 0.6 M. β-Lactamase III was eluted at about 0.2 M NaCl and β-lactamase I at about 0.4 M. β-Lactamase III was further purified by chromatography on Sephadex DE-50 (2.0 × 50 cm) in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, and 0.1% Triton X-100 with a 0.5-L gradient of NaCl from 0 to 0.4 M. Finally the small amount (5–10%) of hydrophilic β-lactamase III arising during the isolation procedure by hydrolysis of the hydrophobic form was removed by gel filtration on Sephadex G-75 in 50 mM Tris-HCl, pH 7.5, and 0.1% Triton (Yamamoto & Lampen, 1976). It is retained while the hydrophobic material elutes in the void volume in the Triton micelle.

Labeling of Cultures. The *B. cereus* strains were labeled during growth in 2% CH/S. The use of acid-hydrolyzed casein, in which cysteine has been destroyed, allows excellent labeling with cysteine, and the elevation of casamino acids from 1 to 2% represses proteases and enhances palmitate labeling, which is relatively poor in 1% CH/S (Nielson & Lampen, 1982b).

Cultures (2.5 mL) were labeled for 2 h with 100 μCi/mL [^{35}S]cysteine (1200 Ci/mmol; New England Nuclear) and 5-mL cultures with 80 μCi/mL [9,10-^{3}H]palmitic acid (11.8 Ci/mmol; New England Nuclear) for 1.5 h; during this time there was no significant recycling of ^{3}H via C_2 units, which would be detectable by incorporation into nonlipoproteins. The harvesting of secreted and membrane proteins, antibody preparation, immunoprecipitation, sodium dodecyl sulfate (SDS) gel electrophoresis, and autoradiography were performed as described previously (Izui et al., 1980; Nielsen et al., 1981; Nielsen & Lampen, 1982a) except that excess *S. aureus* Cowan 1 strain cells were added to ensure complete immunoprecipitation. The cells were prepared by using extensive guanidinium chloride washing as described by Westhoff & Zetsche (1981). Even after extraction in boiling 2× electrophoresis buffer for 5 min, no *S. aureus* proteins were detectable by Coomassie blue staining while the antigen was fully solubilized. Diisopropyl fluorophosphate (1 mM) was added prior to all immunoprecipitations.

Purified β-lactamase III (20 mg) was coupled to 10 mL (settled volume) of Sepharose 4B (Pharmacia) activated with CNBr (Livingston, 1974). The γ-globulin fraction of crude anti-β-lactamase III antiserum obtained by $(NH_4)_2SO_4$ precipitation was applied in 50 mM soodium phosphate, pH 7.0. After extensive washing with this buffer, the β-lactamase III selected antibody was eluted with 0.1 M glycine, pH 2.0, and quickly neutralized. This preparation was used for all immunoprecipitations.

Gel systems used were those of Laemmli (1970) and Davies–Ornstein (Davies, 1964). Proteins were eluted from dried gels as previously described (Nielsen & Lampen, 1982a). V_8-protease (Miles Laboratories) was used as described by Cleveland et al. (1977). Gels were stained with Coomassie blue or silver stain (Merril et al., 1981) as noted. The isoelectric point of β-lactamase III was determined on a Pharmacia Chromatofocussing column (gradient pH 8.0–6.0) performed as described in the manufacturer's literature.

Materials. Purified *S. aureus* penicillinase (type A) was the gift of R. Sykes of the Squibb Institute for Medical Research and purified *B. cereus* 569/H β-lactamase I exoenzyme the gift of T. Viswanatha. The affinity columns for β-lactamase I (Clarke et al., 1980) and the *E. coli* ^{R}TEM β-lactamase were prepared by P. Mézes and E. Yeh, respectively, of this institute.

Table I: Characteristics of Gram-Positive β-Lactamases

	B. cereus β-lactamase III	*B. cereus* β-lactamase I	*B. licheniformis* 749/C
specific activity (μmol h^{-1} mg^{-1})	40–60[a]	340	340
isoelectric point	pH 6.3–6.8	pH 9.2	pH 5.0
effect of trypsin	sensitive	sensitive	resistant core
binding to affinity column	−	+	−

[a] Varied with different preparations. β-Lactamase III is gradually denatured in 0.1% Triton X-100 (adequate for stability of 749/C enzyme). *B. cereus* β-lactamase I is the purified exoenzyme. For *B. licheniformis* 749/C penicillinase, the parameters listed above are shared by the exoenzyme (Izui et al., 1980) and the membrane form (Nielsen et al., 1981).

Results

Isolation of β-Lactamase III. The purification scheme summarized under Experimental Procedures results in the isolation of a nearly homogeneous protein, 32K in size according to its mobility in SDS gels, and soluble only in the presence of detergent. The overall yield during purification was 25% of the activity remaining cell bound after repeated freeze–thawing. Approximately 50% of this cell-bound activity was β-lactamase I, which eluted as a broad peak later than β-lactamase III in the NaCl gradient on Sephadex CM-50. It was a mixture of hydrophilic and hydrophobic material and was identified by its precipitation with antibody to β-lactamase I exoenzyme and by its retention on an affinity column designed for that enzyme (Clarke et al., 1980); β-lactamase III was not precipitated by this antibody or retained by the affinity column at pH 6.5. Furthermore, β-lactamases I and III differ markedly in specific activity under the conditions of the Sargent assay with benzylpenicillin as suubstrate and in isoelectric point (Table I). The 749/C penicillinase is included in this table because of the cross-reaction exhibited between β-lactamase III and this enzyme (see later). The differing charges, resulting from differing numbers of acidic residues, lead to readily distinguishable V_8-protease fingerprints (Figure 1) (V_8 cleaves on the carboxyl side of glutamic acid residues).

We examined the effect of trypsin digestion on purified β-lactamase III because it has proved useful in elucidating the structure of *B. licheniformis* penicillinase (Nielsen et al., 1980) and other secretory lipoproteins (Nielsen & Lampen, 1982b). Trypsin removes from 749/C membrane penicillinase a small peptide bearing the N-terminal diacylglycerylcysteine modification, leaving a highly soluble trypsin resistant core. β-Lactamase III has no such trypsin resistant core, but characteristic peptides are detected after short digestion times (Figure 2). Part A shows results with a nondenaturing gel system. Untreated β-lactamase III (lane a) barely entered the running gel and precipitated as a blur near the top as the protein migrated away from the Triton X-100 in which it was applied. With increasing concentrations and times (lanes b–h) the precipitating blur was diminished and a hydrophilic protein entered the gel and ran with an R_f of about 0.3. Simultaneously, identically treated samples were run on an SDS gel (Figure 2B) where size rather than hydrophobicity is the major determinant of migration; the removal of a peptide can clearly be seen in lanes c–h. Further cleavage of the faster moving species resulted in loss of enzymatic activity. This change in solubility upon limited trypsin treatment is analogous to that

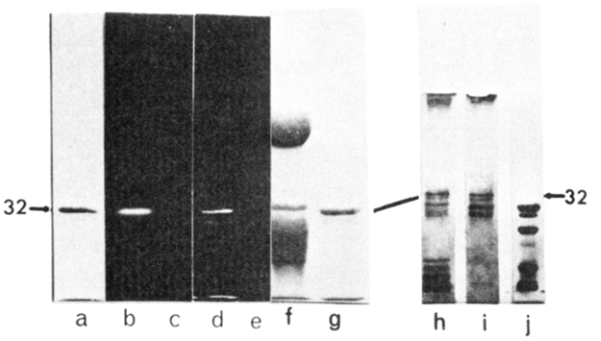

FIGURE 1: SDS gel electrophoretogram of membrane and secreted forms of β-lactamase III and the labeling of the membrane form by [^{3}H]palmitate and [^{35}S]cysteine. Lane a, membrane-bound β-lactamase III purified as described under Experimental Procedures, electrophoresed on a 10% Laemmli gel, and stained with Coomassie blue; lanes b and c, radioautograms of β-lactamase III isolated by antibody precipitation from *B. cereus* 569H membranes (lane b) and from the culture supernatant (lane c) after labeling with [^{3}H]palmitic acid; lanes d and e, similar radioautograms after [^{35}S]cysteine labeling (lane d, membrane form; lane e, secreted form); lane f, immunoprecipitated exo-β-lactamase III, stained with Coomassie blue, shown next to purified membrane β-lactamase III in lane g. V_8-protease patterns from β-lactamase III (lane h), *B. licheniformis* 749/C membrane penicillinase (lane i), and *B. cereus* β-lactamase I (exoenzyme) (lane j); 1 μg of each protein was digested with 0.2 μg of V_8-protease for 30 min at 37 °C (Cleveland et al., 1977), and the fragments were run on a 12% Laemmli gel, and visualized by silver staining.

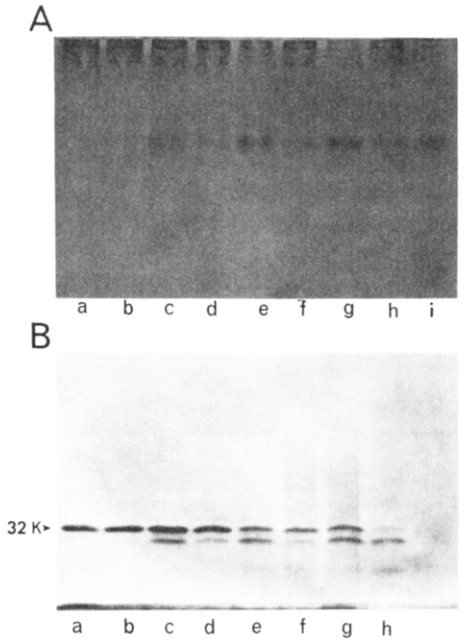

FIGURE 2: Loss of hydrophobic behavior of the membrane form of β-lactamase III after trypsin digestion. (A) Migration in a nondenaturing Davies–Ornstein gel (10%), stained with Coomassie blue; (B) mibration in a 10% SDS gel (stained with silver). Lane a, no trypsin; lane b, digestion by 2 μg/mL trypsin, 1 min; lane c, 10 μg/mL, 1 min; lane d, 2 μg/mL, 4 min; lane e, 10 μg/mL, 4 min; lane f, 2 μg/mL, 9 min; lane g, 10 μg/mL, 9 min; lane h, 2 μg/mL, 12 min; lane i, upper gel only 10 μg/mL, 12 min. The β-lactamase concentration was 400 μg/mL in 50 mM Tris-HCl, pH 7.5, 0.1 Triton X-100, and 2 mM $CaCl_2$. Digestion was at 37 °C and was stopped with 2 mM diisopropyl fluorophosphate.

of 749/C membrane penicillinase.

Labeling of β-Lactamase III. When purified β-lactamase III was obtained, we prepared antibody, and we used this to examine whether the cell-bound enzyme is a lipoprotein by isotopic labeling, as described previously for other class A β-lactamases. Membrane-bound β-lactamase III is very

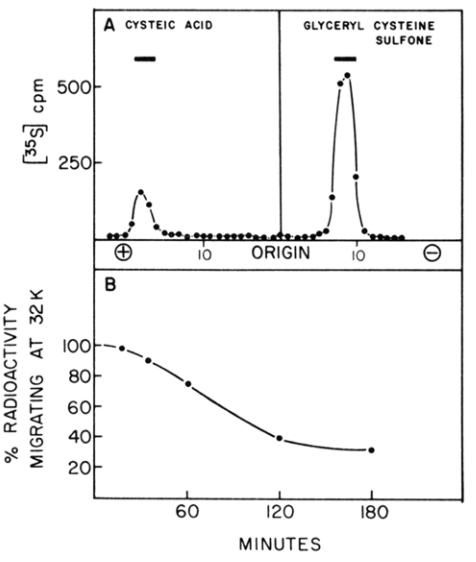

FIGURE 3: Membrane form of β-lactamase III is a glyceride–cysteine lipoprotein. (A) High-voltage electrophoresis of [^{35}S]cysteine-labeled β-lactamase III, eluted from the 32K band shown in Figure 1 (lane d), oxidized, and hydrolyzed. Migration is in centimeters. (B) [^{3}H]Palmitate label in membrane β-lactamase III is present in both alkali-sensitive and alkali-insensitive forms. The 32K [^{3}H]palmitate labeled band shown in Figure 1 (lane b) was eluted and exposed to 0.1 N NaOH at 37 °C. At the designated times samples were removed and neutralized prior to rerunning on an SDS gel. The percentage of label migrating with the 32K band was calculated from a scan of an autoradiograph of the enhanced dried gel.

readily labeled by [^{3}H]palmitate under conditions where no incorporation of label was detected in the secreted form (Figure 1, lanes b and c). Similar results were obtained with [^{35}S]-cysteine. The secreted form of β-lactamase III contains no cysteine residues [demonstrated also by Connolly & Waley (1983)] while the membrane-bound form can be labeled with [^{35}S]cysteine (Figure 1, lanes d and e). The cysteine-labeled band at 32K comigrates with intact β-lactamase III; the band apparent at about 29K is a breakdown product obscured in stained gels by the diffuse light band of γ-globulin. The band at 32K was eluted, oxidized, hydrolyzed, and subjected to high-voltage electrophoresis as previously described (Nielsen & Lampen, 1982b). Eighty percent of the ^{35}S was found at the mobility of glycerylcysteine sulfone and 20% at that of cysteic acid (Figure 3A). The glycerylcysteine sulfone is characteristic of the thioether lipoproteins found in other class A β-lactamases (Nielsen & Lampen, 1982a), and together with the fatty acid labeling data demonstrates conclusively that the cell-bound β-lactamase III is a thioether lipoprotein with at least 80% modification of the cysteine residue(s).

The cross-reaction reported below between β-lactamase I of *B. cereus* and antibody to β-lactamase III (7%) presents no experimental difficulty in the labeling experiments because we now know (P. S. F. Mêzes, Y. Yang, M. Hussain, and J. O. Lampen, unpublished results) that β-lactamase I has no lipoprotein form. The small amount of total activity that remains cell bound after washing is Pollock's β-form, indistinguishable from β-lactamase I exoenzyme.

The membrane-bound class A β-lactamases have blocked N-termini, presumably by a long-chain fatty acid in amide linkage to the α-amino group of the N-terminal cysteine. This structure was deduced from their resemblance to the N-terminus of the mature *E. coli* lipoprotein (Hantke & Braun, 1973; Nielsen et al., 1981). O-Linked fatty acids, modifying the glycerol moiety, are readily removed by 0.1 N NaOH at 37 °C (Hantke & Braun, 1973) while the N-linked fatty acid is resistant. [^{3}H]Palmitate-labeled β-lactamase III was eluted

Table II: Serological Cross-Reactions of β-Lactamase III with Antibodies to Class A β-Lactamases[a]

(A) Protection from Activity Loss (%)

	antibody					
	BcIII[b]	B1	Sa	Bc I	Ec	control or none
residual activity of β-lactamase III	90	80	85	50	30	10

(B) Precipitation by Antibody to β-Lactamase III (%)

	β-lactamase				
	BcIII	B1	Sa	Bc I	Ec
30 μg of antibody	90	55	36	0	−9[c]
60 μg of antibody	100	72	55	7	−24[c]

[a] In part A, 5 μg of β-lactamase III was incubated for 60 min at 37 °C in 50 mM Tris, pH 7.5, 150 mM NaCl, and 1% Triton X-100 in the presence of 200 μg of γ-globulin raised to each of the listed β-lactamases. In part B, 12 μg of each β-lactamase was incubated 60 min at 20 °C with 30 or 60 μg of anti-β-lactamase III γ-globulin purified by binding to β-lactamase III coupled to Sepharose 4B. The mixture was centrifuged at 12000*g* for 10 min and the residual activity in the supernatant assayed. [b] BcIII, *B. cereus* β-lactamase III; B1, *B. licheniformis* 749/C; Sa, *S. aureus* type A; Bc I, *B. cereus* β-lactamase I; Ec, *E. coli* [R]TEM. [c] Activity increased in supernatant.

from a gel and treated with 0.1 N NaOH at 37 °C for intervals up to 3 h. Samples were then neutralized and rerun on an SDS gel. The percentage of label remaining in the 32K band is shown in Figure 3B. The removal over this time period of about 70% of the label is consistent with the distribution of [^{3}H]palmitate between the two alkali-sensitive O linkages and one alkali-insensitive N linkage.

Cross-Reactivity with Class A β-Lactamases. We examined this question in two ways because we have both the purified exoproteins from most of the known class A β-lactamases and their antibodies (purified γ-globulin fractions, free of proteases, were used in all cases). β-Lactamase III incubated with or without control antiserum for 1 h at 37 °C became denatured and lost 90% of its activity. Antibody to β-lactamase III or to the class A β-lactamases prevented this loss to varying degrees (Table IIA). Interaction, and thus protection, takes place most strongly with homologous antibody; however, antibodies to *B. licheniformis* and *S. aureus* β-lactamases exerted a considerable protective effect with lesser effect by antibodies to β-lactamase I and least with that to *E. coli* [R]TEM antibody. Connolly & Waley (1983) report inhibition of β-lactamase III by the homologous antiserum. We did not observe this, but individual preparations of anti-β-lactamase do differ in their effects on enzyme activity. To minimize this variation, we have used stabilization and precipitation as measures of relatedness.

A very similar order of interaction was suggested by a converse experiment in which anti-β-lactamase III antibodies selected by binding to antigen column were used to precipitate the various purified β-lactamases, as shown in Table IIB. There was complete precipitation of the homologous antigen, about 70% with the *B. licheniformis* antigen, 55% with *S. aureus*, and very little with β-lactamase I or [R]TEM. [R]TEM β-lactamase appeared to undergo activation. *B. licheniformis* 6346 exoenzyme (to which we have not prepared antibody because it cross-reacts strongly with antibody to *B. licheniformis* 749/C penicillinase) was tested in the experiment shown in Table IIB. About 50% of the activity was precipitated, indicating considerable homology.

Secretion of β-Lactamase III Exoprotein. Pollock (1956) reported that β-lactamase III was undetectable in the medium of *B. cereus* cultures. Certainly its presence is difficult to detect by activity measurements because its specific activity is only about 15% that of the β-lactamase I. Furthermore, the latter is secreted in very large amounts (up to 25–30 μg/mL). With specific antibody available we reexamined this question. A secreted form of β-lactamase III, with a slightly slower mobility on SDS gels than that of the lipoprotein form (Figure 1), is readily detected by immunoprecipitation from logarithmic phase cultures of *B. cereus* 569 (induced) and 569/H and 569/H/9 (both magnoconstitutive for β-lactamase I), but not from *B. cereus* 5/B. The hydrophobic N-terminus of lipoproteins binds excess SDS and causes them to migrate anomalously fast in Laemli gels.

In 2% CH/S medium the maximum concentration of secreted β-lactamase III is 2–3 μg/mL at early stationary phase, a figure calculated from the intensity of the immunoprecipitated band compared with a standard of the purified protein. This is approximately the same as the amount of cell-bound β-lactamase III at this time, as seen both from staining of appropriate SDS gels and a calculation of yields in the purification (2 μg/mL culture).

The recovery of exo-β-lactamase III is affected by the medium. At a pH higher than 6.5 or in 1% CH/S medium, where proteases are more active than in 2% CH/S (see Experimental Procedures), distinctly less exo-β-lactamase III could be detected. Moreover, if sterile filtered 1% CH/S medium from a late logarithmic phase culture was added to the purified enzyme, about 50% degradation could be seen in 2 h at 30 °C. This degradation was inhibited by 1 mM diisopropyl fluorophosphate (results not shown). Much less degradation occurred with medium from early logarithmic cultures, or from late logarithmic cultures in 2% CH/S medium. Therefore, it appears likely that the degradation of exo-β-lactamase III is caused by a serine protease secreted in late growth phase.

Discussion

With the findings outlined above, we confirm Pollock's report of 1956 that *B. cereus* cells exhibit cell-bound β-lactamase activity that is antigenically distinct from the major secreted enzyme. We can, however, now supply a much more complete description of this activity, termed β-lactamase III. It has the properties of a class A β-lactamase and resembles several Gram-positive β-lactamases of this class by having both membrane-bound and secreted forms which differ only at their N-termini (Nielsen & Lampen, 1982a). The N-terminal cysteine residue of the bound form is modified with glycerol in thioether linkage esterified with two long-chain fatty acids, and its α-amino group is blocked with a further long-chain fatty acid. Proteolytic cleavage on the carboxyl side of the modified cysteine can occur, causing release of a cysteine-free hydrophilic β-lactamase III into the medium. In the *B. cereus* membrane, β-lactamase III, despite its low specific activity, constitutes about half the total β-lactamase activity, as Pollock reported, because only a small proportion of the total β-lactamase I remains cell bound; at the same time the secreted β-lactamase III constitutes a tiny proportion of the total activity in the medium, escaping detection without specific antibodies. In rich media, when proteases are relatively inactive, β-lactamase III is moderately stable in the medium but breakdown by a serine protease takes place in a lean medium. β-Lactamase III of *B. cereus* resembles *B. licheniformis* 749/C penicillinase in that approximately half is cell bound while the other half is in the medium at early stationary phase. This places it in contrast to β-lactamase I which is 2–5% cell bound and β-lactamase II (the sulfhydryl-containing cephalospori-

nase) which has no cell-bound activity.

Another way in which β-lactamase III more closely resembles the *B. licheniformis* 749/C penicillinase than *B. cereus* β-lactamase I is shown in an examination of cross-reactivities with the class A β-lactamases. Anti-β-lactamase III antibody interacts to some extent with all of them. The least interaction in both tests was, not surprisingly, that with the Gram-negative ^{R}TEM β-lactamase, which is the least related to the Gram-positive members of the class by amino acid homology (Ambler, 1980). β-Lactamase III appears more closely related by both tests to the *B. licheniformis* 749/C and *S. aureus* penicillinases than it is to β-lactamase I from *B. cereus*. *B. licheniformis* 749/C penicillinase precipitated by anti-β-lactamase III antibody and β-lactamase III precipitated by anti 749/C penicillinase can both readily be visualized on a gel (data not shown). Fortunately β-lactamase III and 749/C penicillinases can readily be distinguished by their sensitivies to trypsin and V_8-protease (Figure 1 and Table I). The weak interaction between β-lactamase III and antibody to β-lactamase I, and the reverse, is insufficient to precipitate the nonhomologous protein. This is fortunate, for both proteins are produced simultaneously in *B. cereus* strains. The cross-reactivities, however, add to the difficulties of examining the labeling and distribution of any minor membrane form of β-lactamase I in the presence of the related lipoprotein form of β-lactamase III.

β-Lactamase III is not produced in *B. cereus* 5/B. This strain produces a variant of the 569 β-lactamase I, slightly larger (Hall & Ogsten, 1956) but cross-reactive with antiserum to the 569 enzyme (Pollock, 1963), even more completely released into the medium; it also forms a secreted β-lactamase II. Thus, 5/B has very low levels of membrane-bound penicillinase activity. In light of this, it is not clear why *B. cereus* 569 strains have retained β-lactamase III in addition to highly active β-lactamases I and II.

Connolly & Waley (1983) have independently isolated and identified γ-penicillinase in *B. cereus* 569/H/9. Their findings and ours are in agreement.

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Registry No. β-Lactamase III, 86846-23-3.

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