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Exocellular β-Lactamases of *Streptomyces albus* G and Strains R39 and K11

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The β-lactamases excreted by the highly benzylpenicillin-susceptible *Streptomyces* strain R39 and the highly benzylpenicillin-resistant *Streptomyces albus* G were isolated and purified. Neither β-lactamase exhibited DD-carboxypeptidase activity. Both were anionic at *p*H 8.3, did not require metal ions, and were not sensitive to iodine, but were inhibited by Cu^{2+} and readily inactivated by heat. *p*-Chloromercuribenzoate, iodoacetate, *p*-aminobenzoate, and substrates and inhibitors of DD-carboxypeptidase had no effect on β-lactamase activity. The K_m and V_{max} values for β-lactamase activity were studied with 6-aminopenicillanic acid and with various penicillins and cephalosporins. The β-lactamase from the related strain K11 of *Streptomyces*, which is intermediate in its susceptibility to benzylpenicillin, was partially purified, and its activity was compared on the various substrates.

The killing target of penicillin in bacteria is the membrane-bound transpeptidase which catalyzes peptide cross-linking in the nascent peptidoglycan (21, 23). In an attempt to elucidate the mechanism of the transpeptidation reaction and of its inhibition by penicillin, DD-carboxypeptidases-transpeptidases that are spontaneously excreted by strains R39, R61, and K11 of *Streptomyces* during growth were isolated and studied (8, 10, 17; Ghuysen et al., Proc. Symp. Molecular Mechanisms of Antibiotic Action on Protein Biosynthesis and Membranes, Granada, p. 51-52, 1971). These enzymes act on peptides ending in an L-R_3-D-alanyl-D-alanine sequence. When water is the only nucleophile present in the reaction mixture, they hydrolyze the C-terminal D-alanyl-D-alanine peptide bond (carboxypeptidase activity). In the presence of a suitable NH_2-R nucleophile, they catalyze the synthesis of a D-alanyl-R peptide bond with the concomitant release of the C-terminal D-alanine residue of the peptide donor (transpeptidase activity; 17). The substrate requirements of these *Streptomyces* DD-carboxypeptidases-transpeptidases and their sensitivity to very low doses of penicillin, made it clear that they could be the exocellular forms of the enzymes that effect the closure of the bridges between the peptide units of the nascent peptidoglycan in vivo.

β-Lactamases (i.e., β-lactam hydrolases; EC 3.5.2.6) and DD-carboxypeptidases-transpeptidases have in common the ability to recognize the benzylpenicillin molecule. β-Lactamases, however, hydrolyze the β-lactam ring of most penicillins and cephalosporins to form biologically inactive products (for recent reviews, see 1, 2), whereas DD-carboxypeptidases-transpeptidases from *Streptomyces* R61, K11, and R39 reversibly bind the antibiotic but do not hydrolyze it to penicilloic acid (10; *unpublished data*).

Experiments were undertaken to study any relationship that may exist between DD-carboxypeptidases-transpeptidases and β-lactamases and their involvement in penicillin susceptibility in vivo and peptidoglycan biosynthesis. This paper describes the isolation and substrate requirements of the exocellular β-lactamases excreted by three strains of *Streptomyces* sp., the benzylpenicillin-susceptible strains R39 and K11 and the more benzylpenicillin-resistant strain *S. albus* G. The properties of the DD-carboxypeptidase excreted by *S. albus* G have also been previously described (4, 7, 9). This enzyme exhibits a high degree of resistance to inhibition by benzylpenicillin but has not yet been shown to be able to catalyze transpeptidation reactions (17).

MATERIALS AND METHODS

Streptomyces strains. The organisms used belong to the laboratory collection. They are soil isolates which were identified as being of the genus *Streptomyces* but were not further classified as to species. In the collection, they were assigned the

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strain designations: *S. albus* G, *Streptomyces* R39, and *Streptomyces* K11. *S. albus* G and strain R39 were grown in peptone-Oxoid medium (8) and K11 was grown in glycerol-casein medium (8). In all cases, incubation was aerobic (shaking at 28 C).

Susceptibility of Streptomyces strains to benzylpenicillin. The LD_{50} values of benzylpenicillin for single-cell colonies were as follows: for R39, 0.7×10^{-6} M; for K11, 3.9×10^{-6} M; and for *S. albus* G, 32×10^{-6} M. The LD_{50} values of other penicillins and cephalosporins for strains R39 and K11 are reported in an accompanying paper (3).

Antibiotics. Structures of the antibiotics used are shown in Fig. 1. Benzylpenicillin was purchased from Rhône-Poulenc, Paris, France. Ampicillin and oxacillin were obtained from Bristol Benelux, S.A., Brussels, Belgium. 6-Aminopenicillanic acid was purchased from Sigma Chemicals Ltd. Carbenicillin, cloxacillin, and methicillin were obtained from Beecham Research Laboratories, Brentford, England. Penicillin V was a gift of Imperial Chemical Industries Ltd., Macclesfield, England. Cephalothin, cephalosporin C, cephaloglycin, and cephalexin were gifts of Eli Lilly & Co., Indianapolis, Ind.

Analytical techniques. One unit of β-lactamase is defined as the amount which catalyzes the degradation of 1 μmole of benzylpenicillin per hr at 30 C and *p*H 7.0 (2). β-Lactamase activity on benzylpenicillin and on the other antibiotics listed in Fig. 1 was estimated by using a modification (10) of the iodometric technique of Perret (16) and Novick (15).

Separation of benzylpenicillin, benzylpenicilloic acid or 6-aminopenicillanic acid was carried out by chromatography on thin-layer plates of Stahl's silica gel G (Merck), with the solvent 1-butanol-water-ethanol-acetic acid (10:4:3:3, v/v; 22). The plates were heated at 110 C for 20 min, cooled, and sprayed with the starch-iodine solution reagent previously described (10), whereupon the three aforementioned compounds appeared as white spots on a mauve background. R_F values were 0.85 for benzylpenicillin, 0.65 for benzylpenicilloic acid, and 0.57 for 6-aminopenicillanic acid.

DD-Carboxypeptidase activity was estimated by using N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine as substrate. Liberated D-alanine was estimated as previously described (5).

Protein was estimated by the Lowry modification of the Folin-phenol technique (11) or by the fluorodinitrobenzene technique (5), with bovine serum albumin as standard.

Intracellular and cell-bound β-lactamase from Streptomyces R39 and S. albus G. Mycelia harvested after 48 hr of growth, i.e., the time of maximal exocellular β-lactamase production, were treated as previously described (13) for the isolation of the membrane fraction from *S. albus* G. The β-lactamase activity of the first supernatant, the SN″ supernatant, the membrane, and the last pellet fractions (13) was estimated by using the iodometric technique with benzylpenicillin as substrate.

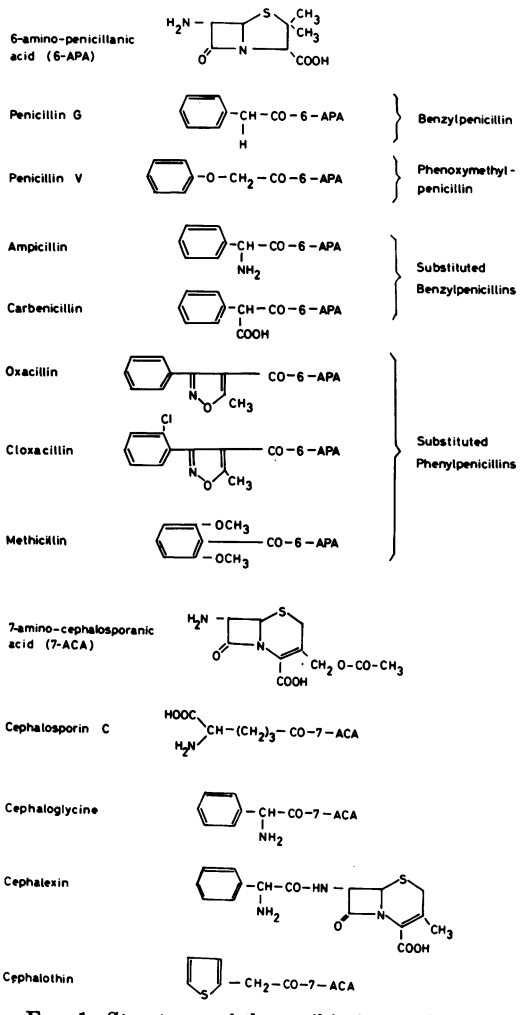

FIG. 1. *Structures of the antibiotics used.*

DD-Carboxypeptidase substrates and inhibitors. The following DD-carboxypeptidase substrates were used: N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine, N^{α}-acetyl-L-lysyl-D-alanyl-D-alanine (14), and the disaccharide pentapeptide-pentaglycine (N^{α}-[β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl]-N^{ϵ}-[pentaglycyl]-L-lysyl-D-alanyl-D-alanine; 12). The following DD-carboxypeptidase inhibitors were used: acetyl-D-alanyl-D-glutamic acid, N^{α},N^{ϵ}-disuccinyl-L-lysyl-D-alanyl-D-glutamic acid, and L-lysyl-D-glutamyl-D-alanine, which inhibit the *S. albus* G DD-carboxypeptidase but have no inhibitory effect on the R39 DD-carboxypeptidase-transpeptidase (Nieto et al., Biochem. J., *in press*).

Electrophoresis. Analytical and preparative polyacrylamide gel electrophoreses were carried out with an Acrylophor apparatus (Pleuger, Antwerp, Belgium) and a Shandon apparatus (no. SAE-2782), respectively, under exactly the same conditions as those previously described (10).

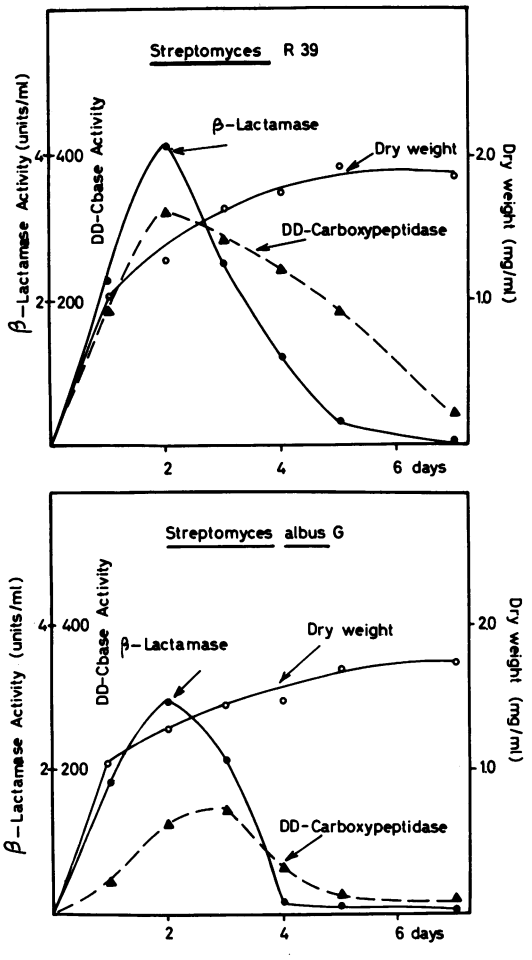

FIG. 2. *Excretion of β-lactamase and* DD*-carboxypeptidase during growth of Streptomyces R39 and S. albus G. One-liter flasks containing 500 ml of peptone-Oxoid medium were inoculated with 1% (v/v) of 48-hr precultures of each organism. The cultures were grown with shaking at 28 C, and duplicate 20-ml samples were withdrawn at the indicated times for estimation of dry weight and content of β-lactamase and* DD*-carboxypeptidase activities. Portions (5 to 15 μliters) of the culture filtrates were incubated at 30 C with 30 nmoles of benzylpenicillin in 30 μliters (final volume) of 0.03 M sodium phosphate buffer, pH 7.0. Results are expressed as micromoles of benzylpenicillin degraded per hour per milliliter of culture.* DD*-Carboxypeptidase activity was estimated by determining the amount of* D*-alanine liberated when 20-μliter samples of culture filtrate were incubated at 37 C for 2 hr with 50 nmoles of N^{α},N^{ϵ}-diacetyl-*L*-lysyl-*D*-alanyl-*D*-alanine in a total volume of 35 μliters of 0.014 M Tris-hydrochloride buffer, pH 7.5. Results are expressed as nanomoles of* D*-alanine liberated per hour per milliliter of cultured The mycelia were washed three times with distilled water. Washed pellets were then dehydrated in previously tared vessels for 24 hr at 110 C and dry weight was determined by difference. Results are expressed as milligrams (dry weight) per milliliter of culture.*

Preparative liquid curtain electrophoresis was carried out with an Elphor Vap 2 apparatus (Drs. Bender and Hobein, Munich, Germany).

RESULTS

Excretion of β-lactamase and DD**-carboxypeptidase.** Excretion of β-lactamase and DD-carboxypeptidase during growth of *Streptomyces* R39 and *S. albus* G is shown in Fig. 2A and B. Maximal production of β-lactamase by both strains (with benzylpenicillin as substrate) occurred at 48 hr. Strain R39 produced more enzyme than *S. albus* G during the entire course of the experiment. In both cases, β-lactamase production decreased sharply between 3 and 4 days of growth. As evidenced by the increase in dry weight, the bacteria were still growing actively during this phase. During growth of strain R39, DD-carboxypeptidase and β-lactamase production were maximal at 48 hr; however, the former activity decreased at a significantly slower rate than the latter. With *S. albus* G, maximal DD-carboxypeptidase production followed maximal β-lactamase production, and both activities declined to low levels more rapidly than in strain R39.

Characterization of β-lactamase activity during growth of S. albus G and Streptomyces R39. Samples (20 μliters) of 48-hr culture filtrates were incubated at 30 C for 1 hr with benzylpenicillin (1 mM) in 30 μliters (final volume) of 0.033 M sodium phosphate, *p*H 7.0. Each reaction mixture (10-μliter samples) was submitted to chromatography on thin-layer plates (Materials and Methods). Detection with the starch-iodine reagent revealed the presence of benzylpenicilloic acid. Traces of 6-aminopenicillanic acid were not detected. After breakage of the mycelia (Materials and Methods), very little β-lactamase activity was found associated with the various cellular fractions. The excreted β-lactamase activity represented about 90% in the case of strain R39 and 99% in the case of *S. albus* G of the total β-lactamase activity of the corresponding cultures (after 48 hr of growth).

Isolation and purification of exocellular β-lactamase from Streptomyces R39. The organism was grown with shaking at 28 C in 1-liter flasks containing 500 ml of peptone-Oxoid medium for 48 hr, the mycelia were removed by centrifugation, and the culture supernatant fluid was passed through Whatman no. 3 filter paper. All ensuing operations were performed at 4 C.

Step 1. The enzyme in the culture filtrate (9 liters was adsorbed on 140 g of moist diethylaminoethyl (DEAE)-cellulose previously equilibrated against 0.05 M sodium phosphate buffer, *p*H 7.0. The resin-adsorbed complex was suspended in 0.05 M sodium phosphate buffer (*p*H 7.0) containing 1 M NaCl, the resin was removed by filtration, and the desorption was repeated. Pooled extracts (2 liters) were concentrated to 80 ml by dialysis against dry Carbowax 20,000 and were subsequently dialyzed against four 1-liter changes of 0.05 M sodium phosphate buffer, *p*H 7.0.

Step 2. The solution, after step 1, was applied in amounts of 40 ml to a column of Sephadex G-75 (V_0 = 265 ml; V_t = 965 ml) previously equilibrated against 0.05 M sodium phosphate buffer, *p*H 7.0, and eluted with the same buffer. At this stage, β-lactamase (elution volume, 280 to 680 ml) and DD-carboxypeptidase (elution volume, 250 to 530 ml) overlapped. Fractions containing β-lactamase (360 to 540 ml) were pooled, concentrated to 35 ml by ultrafiltration through a UM-10 membrane on an Amicon apparatus, and dialyzed against 0.05 M sodium phosphate buffer, *p*H 7.0, for 24 hr. The solution was refiltered on the same Sephadex G-75 column. The β-lactamase fractions were pooled, concentrated, and dialyzed as above. The final volume was 30 ml.

Step 3. After step 2, the enzyme solution was applied to a 400-ml column of DEAE-cellulose previously equilibrated against 0.05 M sodium phosphate buffer, *p*H 7.0. After the column was washed with the same buffer until only traces of material absorbing at 280 nm remained, enzyme was eluted from the resin with an increasing gradient of sodium phosphate-NaCl buffer, *p*H 7.0 (mixing flask, 500 ml of 0.05 M sodium phosphate buffer, *p*H 7.0; solution added, 0.05 M sodium phosphate buffer, *p*H 7.0, containing 1 M NaCl; Fig. 3). The active fractions (290 to 480 ml) were partially separated from a black inklike pigment. They were pooled as described in step 2. Finally, to remove residual pigment, the enzyme solution was filtered through a 400-ml column of Biogel P-60 previously equilibrated against 0.05 M sodium phosphate buffer, *p*H 7.0 (Fig. 4). Active fractions (130 to 220 ml) were pooled, concentrated by ultrafiltration, dialyzed as before, and stored in 200-μliter portions at −20 C. The preparation contained no detectable DD-carboxypeptidase activity after periods of up to 24 hr of incubation at 37 C with N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine. Filtration of the purified enzyme on Sephadex G-75 in 0.05 M sodium phosphate buffer, *p*H 7.0, gave rise to a series of fractions exhibiting almost the same specific activity (range of variation, 10%).

In some cases, residual pigment was removed by continuous curtain electrophoresis on an Elphor V2P apparatus with sodium acetate buffer (*p*H 4.9, ionic strength 0.033) as electrolyte (200 ma, 1,800 v, 4 hr). Samples were equilibrated against the buffer by several hours of dialysis at 4 C. Injection into the electrophoresis

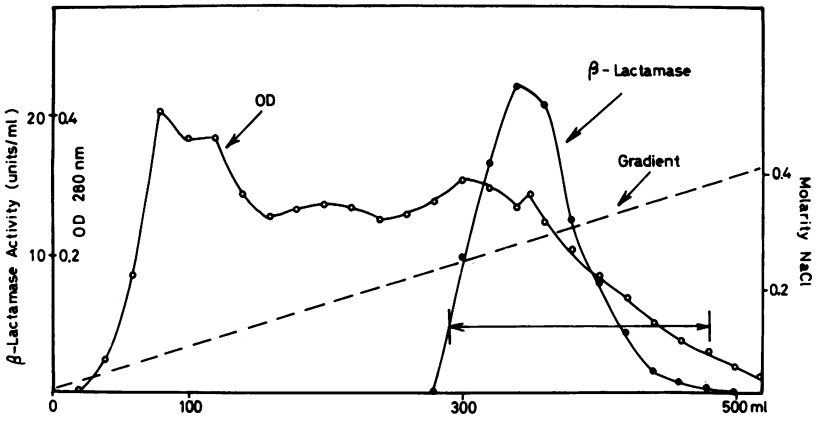

FIG. 3. *Purification of β-lactamase of Streptomyces R39. Elution from DEAE-cellulose with an increasing gradient of NaCl in 0.05 M sodium phosphate buffer, pH 7.0. For conditions of chromatography, see text. Samples (5 μliters) of fractions were incubated at 30 C for 3 to 10 min (depending upon the samples) with 30 nmoles of benzylpenicillin in 30 μliters (final volume) of 0.03 M sodium phosphate buffer, pH 7.0. Results are expressed as micromoles of benzylpenicillin degraded per hour per milliliter of sample. For DD-carboxypeptidase activity, samples (15 μliters) were incubated at 37 C, for 1 hr, with 50 nmoles of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine, in 35 μliters (final volume) of 0.02 M Tris-hydrochloride buffer, pH 7.5. Results are expressed as micromoles of D-alanine released per hour per milliliter of sample. OD, Optical density. Fractions pooled for β-lactamase activity are indicated by the arrows, ↔.*

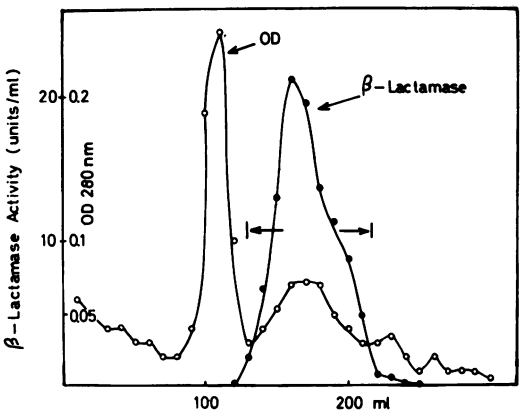

FIG. 4. *Purification of β-lactamase of Streptomyces R39. Elution from Biogel P-60. For conditions of filtration, see text. Results are expressed as micromoles of benzylpenicillin degraded per hour per milliliter of sample. Fractions pooled for β-lactamase activity are indicated by the arrows, ↔.*

chamber was at a rate of 1 ml/hr. All manipulations were carried out at 4 C.

Table 1 gives the recoveries and specific activities with sodium benzylpenicillin as substrate. The final enzyme preparation contained 235 units of β-lactamase per mg of protein.

Isolation and purification of exocellular β-lactamase from S. albus G. *S. albus* G was grown with shaking in 1-liter flasks containing 500 ml of peptone-Oxoid medium for 48 hr, at which time the mycelia were removed by centrifugation, and the supernatant fluid was filtered through Whatman no. 3 filter paper. All subsequent manipulations were performed at 4 C.

Step 1. The enzyme was adsorbed from the culture filtrate (12 liters) on 240 of g Amberlite CG-50 by adjusting the *p*H to 4.0 with acetic acid. The Amberlite-adsorbed enzyme complex was suspended in 0.15 M K_2HPO_4, and the *p*H of the suspension was adjusted to 8.0 by the addition of concentrated ammonia. Resin was removed by filtration, the desorption was repeated with fresh 0.15 M K_2HPO_4 (final *p*H 8.0), the filtrates were pooled (1-liter final volume), and 226 g of solid $(NH_4)_2SO_4$ was added. The precipitate was collected by centrifugation and discarded; the supernatant fluid was adjusted to 75% satuation by the addition of solid $(NH_4)_2SO_4$ (222 g/liter). The precipitate was collected by centrifugation, resuspended in 0.05 M sodium phosphate, *p*H 7.0, and dialyzed against four 1-liter changes of 0.05 M sodium phosphate, *p*H 7.0. The final volume was 50 ml.

Step 2. After step 1, the enzyme solution was filtered through a column of Sephadex G-75 (1,850 ml) previously equilibrated against 0.05 M sodium phosphate buffer, *p*H 7.0. At this stage, β-lactamase (elution volume, 800 to 1,300 ml) and DD-carboxypeptidase (1,100 to 1,300 ml) overlapped. Fractions containing β-lactamase (800 to 1,200 ml) were pooled, concentrated by ultrafiltration through a UM-10 membrane on an Amicon apparatus, and dialyzed for 24 hr against 0.05 M sodium phosphate buffer, *p*H 7.0.

Step 3. After step 2, the enzyme solution was applied to a column of DEAE-cellulose (200 ml) previously equilibrated against 0.05 M sodium phosphate buffer, *p*H 7.0, and the column was washed until only traces of material absorbing at 280 nm remained. β-Lactamase was eluted from the resin with an increasing NaCl gradient from 0 to 0.25 M. Approximately 75% of the enzyme

TABLE 1. *Isolation and purification of β-lactamases from Streptomyces R39 and S. albus G*

Strain	Step	Specific activity[a]	Total activity	Recovery (%)	Total protein (mg)	Purification
R39	Culture filtrate	0.38	12,730	100	33,630	—
	1	16.2	14,560	114	900	42.5
	2	137.5	10,040	82	73	362
	3	235	1,170	9	5	620
S. albus G	Culture filtrate	0.74	28,560	100	39,840	—
	1	31	21,000	73.5	580	42
	2	74	15,550	55	210	100
	3	555	8,700	30	16	750
	4	1,630	1,465	5	0.9	2,200

[a] Expressed as micromoles of benzylpenicillin degraded per hour per milligram of protein. Benzylpenicillin (final concentration, 1 mM) was incubated with the enzyme preparation, at 30 C, in a 30-μliter final volume of 0.03 M sodium phosphate, *p*H 7.0. Proteins were estimated by measuring the amount of total amino groups available to fluorodinitrobenzene after 6 N HCl hydrolysis (100 C, 17 hr), with bovine serum albumin as standard. In the case of the R39 enzyme, for steps 1 to 3, proteins were estimated by Lowry's technique.

was eluted as a major peak (between 0 and 0.1 M NaCl). Fractions containing the residual activity (between 0.1 and 0.2 M NaCl) were pooled, concentrated, dialyzed as described in step 2, and subsequently rechromatographed on a DEAE-cellulose column (200 ml) previously equilibrated against 0.05 M sodium phosphate buffer, *p*H 7.0. Elution with an NaCl gradient from 0 to 0.15 M permitted separation of this residual β-lactamase activity from contaminant material absorbing at 280 nm. The active fractions of these two chromatographic separations were pooled, concentrated, and dialyzed as in step 2. The enzyme solution was rechromatographed on a column of DEAE-cellulose (200 ml) previously equilibrated against 0.05 M sodium phosphate buffer, *p*H 6.5. β-Lactamase was eluted with an increasing NaCl gradient from 0 to 0.15 M (Fig. 5). Active fractions (300 to 600 ml) were pooled, concentrated, and dialyzed as in step 2.

Step 4. After step 3, the enzyme was filtered through a column of Sephadex G-75 (400 ml) previously equilibrated against 0.05 M sodium phosphate buffer, *p*H 7.0. Active fractions (600 to 800 ml) were pooled, concentrated by ultrafiltration, and dialyzed against 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 7.0. To remove residual pigment, the enzyme solution was finally subjected to preparative electrophoresis on polyacrylamide gel in a Shandon electrophoresis apparatus (Fig. 6). Active fractions were pooled, concentrated, dialyzed for 24 hr against 0.05 M sodium phosphate buffer, *p*H 7.0, and stored in 100-μliter samples at −20 C. Glycerol (10%, v/v) was sometimes added to enhance the stability of the enzyme during storage. DD-Carboxypeptidase activity was not detectable after incubation at 37 C for up to 24 hr with N^{α}, N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine. Filtration of the purified enzyme on Sephadex G-50 in 0.05 M sodium phosphate buffer, *p*H 7.0, containing 10% (v/v) glycerol gave rise to a series of fractions exhibiting almost the same specific activity (range of variation, 6%).

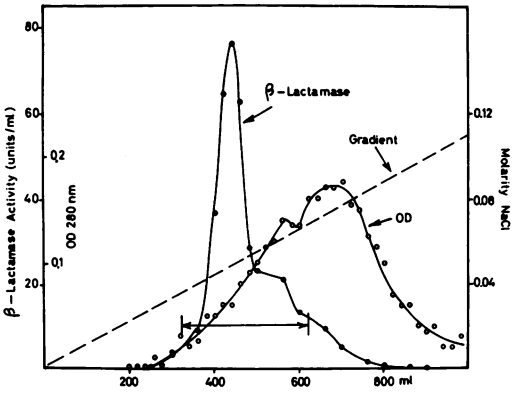

FIG. 5. *Purification of β-lactamase of Streptomyces albus G. Rechromatography on DEAE-cellulose with an increasing gradient of NaCl in 0.05 M sodium phosphate buffer, pH 6.5. Assay conditions were as described in Fig. 2. Results are expressed as micromoles of benzylpenicillin degraded per hour per milliliter of sample. Fractions pooled for β-lactamase activity are indicated by the arrows, ↔.*

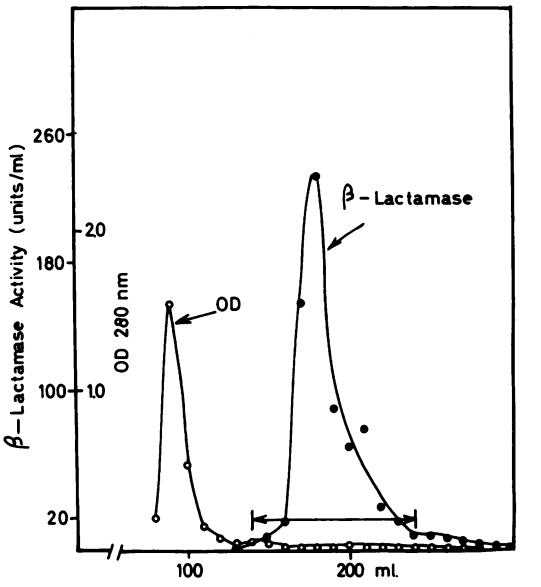

FIG. 6. *Purification of β-lactamase of Streptomyces albus G. Elution after preparative polyacrylamide gel electrophoresis in 0.042 M (in Tris) Tris-acetate buffer, pH 8.3 (40 ma; 320 v; 2 hr; height of the column, 3 cm). Results are expressed as micromoles of benzylpenicillin degraded per hour per milliliter of sample. Fractions pooled for β-lactamase activity are indicated by the arrows, ↔.*

Table 1 gives the recoveries and improvement in specific activities. The final enzyme preparation contained 1,630 units per mg of protein as determined with benzylpenicillin as substrate.

Partial purification of exocellular β-lactamase from Streptomyces K11. *Streptomyces* K11 was grown with shaking in 1-liter flasks containing 500 ml of glycerol-casein medium for 48 hr. After centrifugation, the culture supernatant fluid was passed through Whatman no. 3 filter paper. All ensuing operations were performed at 4 C.

The enzyme in the culture supernatant fluid was adsorbed onto DEAE-cellulose (25 g of wet weight per liter) which had been previously equilibrated against 0.05 M phosphate buffer, *p*H 7.0.

Over 95% of the activity was adsorbed and was then eluted from the resin by 1 M NaCl in 0.05 M phosphate buffer, *p*H 7. Stepwise addition of solid $(NH_4)_2SO_4$ resulted in precipitation of the activity in the 20 to 40% saturation fraction. The precipitate was resuspended in 0.05 M sodium phosphate buffer, *p*H 7.0, and dialyzed against the same buffer. The enzyme solution was applied to a column of DEAE-cellulose previously equilibrated against 0.05 M sodium phosphate, *p*H 7, and β-lactamase activity was eluted from the resin with an increasing NaCl gradient, in 0.05 M phosphate, *p*H 7, from 0 to 1 M. The active fractions were concentrated against Carbowax 4000 and dialyzed against 0.2 M NaCl in 0.05 M phosphate buffer, *p*H 7.0. The solution was applied to a column of DEAE-cellulose previously equilibrated against this NaCl-phosphate buffer, and the activity was eluted with a 0.2 to 0.8 M gradient of NaCl in the above phosphate buffer. This last chromatography on DEAE-cellulose was repeated twice. At this stage, the enzyme preparation, with a specific activity of 6.6 units per mg of protein, represented about a 100-fold purification (total recovery, 75%) but still retained most of the black pigmentation. Rechromatography on DEAE-cellulose and gel filtration on Sephadex G-25 did not further separate the pigment from β-lactamase activity. Since the K11 β-lactamase was to be used only for purposes of comparison with the β-lactamases from the strains that exhibit the extremes of susceptibility to β-lactam antibiotics, namely, R39 and *S. albus* G, further purification attempts were abandoned.

Polyacrylamide gel electrophoreses. Samples of the R39 and *S. albus* G enzymes, adjusted to 12% (w/v) with sucrose, were placed on polyacrylamide gels at *p*H 8.4 and subjected to electrophoresis for 2 hr at 80 v (4 to 5 ma/tube; 10). As indicated in Fig. 7, both preparations were anionic at *p*H 8.4, exhibited equivalent electrophoretic mobilities, and contained only one band of protein. Parallel gels not stained for protein were cut into 2-mm slices from the anode end, each slice was incubated overnight at 4 C in 0.03 M sodium phosphate buffer, *p*H 7.0, and samples of the eluates were tested for β-lactamase activity with benzylpenicillin as substrate. In each case, β-lactamase activity was associated only with the protein band detected with Coomassie blue in the corresponding gel.

Properties of the β-lactamases. The *p*H optima for all three β-lactamases were between 6 and 8 in 0.03 M sodium phosphate buffer. The *S. albus* G enzyme was considerably more sensitive to *p*H changes either below 6 or above 8 than was the R39 enzyme. In all cases, activity was lower in Tris-hydrochloride buffer at *p*H 8 or 9 than in sodium phosphate buffer, *p*H 8, or glycine-NaOH buffer, *p*H 9.

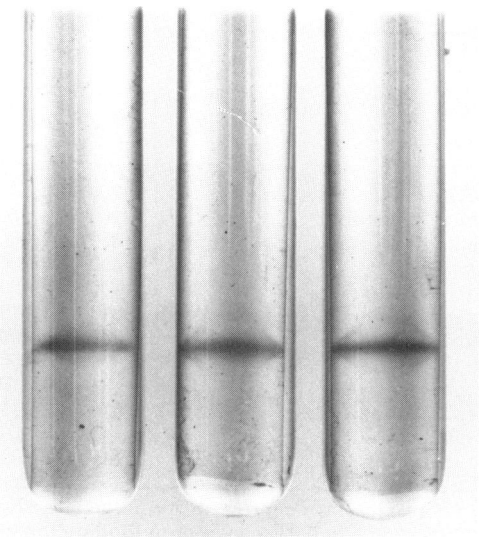

FIG. 7. *Analytical polyacrylamide gel electrophoresis of β-lactamase from Streptomyces R39 and S. albus G. Samples, i.e., each of the two purified enzymes either separately or mixed together, were applied directly to the tops of the gels. A potential of 80 v was applied for 2 hr. Prints show half-gels stained with Coomassie blue. Both enzymes run towards the anode at pH 8.3. In all cases, β-lactamase activity was found associated with the protein bands. From left to right, enzyme R39, enzyme S. albus G, and a mixture of two enzymes. Anode is at the bottom of the tubes.*

Optimal ionic strength for the R39 β-lactamase was between 0.01 and 0.03 M sodium phosphate buffer, *p*H 7.0. In the complete absence of buffer (i.e., a preparation dialyzed against water), the R39 β-lactamase retained high levels of activity.

In contrast to the R39 enzyme, the purified *S. albus* G enzyme was dilution sensitive and required at least 10% glycerol to retain maximal activity. Thus, unless otherwise stated, the *S. albus* G enzyme was assayed in the presence of 10% glycerol (final concentration) in 0.03 M phosphate buffer, *p*H 7.

There was no evidence of a metal ion requirement for activity with either the R39 or the *S. albus* G β-lactamase preparation. Sodium ethylenediaminetetraacetate and Mg^{2+}, Mn^{2+}, Ca^{2+}, and Zn^{2+}, each cation being used as its chloride at a final concentration of 2 mM, had virtually no effect. The only divalent cation producing a significant effect was Cu^{2+}; 2 mM Cu^{2+} was sufficient to produce about 50% inhibition of the R39 enzyme and approximately 90% inhibition of the *S. albus* G enzyme.

TABLE 2. *Efficiency of hydrolysis of the β-lactamases from Streptomyces R39, S. albus G, and Streptomyces K11*[a]

Substrate[b]	R39				*S. albus* G				K11 relative efficiency[c]
	K_m	V_{max}	Efficiency	Relative efficiency	K_m	V_{max}	Efficiency	Relative efficiency	
6-APA	72	200	2,750	70	600	1,500	2,500	93	111
Benzylpenicillin	65	260	3,950	100	740	2,000	2,700	100	100
Penicillin V	285	590	2,060	52	625	1,600	2,600	95	116
Ampicillin	575	1,350	2,300	59	900	4,160	4,600	170	82
Carbenicillin	260	100	420	11	1,000	200	200	7	27
Oxacillin	540	840	1,540	40	330	370	1,100	41	0
Cloxacillin	420	80	190	5	250	35	140	5	0
Methicillin	290	70	250	6	770	30	38	1.4	0
Cephalosporin C	1,050	33	32	0.8	3,330	110	33	1.3	5
Cephaloglycin	2,860	500	175	4	3,840	150	39	1.4	88
Cephalexin	910	45	50	1.3	4,550	23	5	0.2	0
Cephalothin	123	145	1,200	30	1,330	50	38	1.4	116

[a] K_m values are expressed in micromoles; V_{max} values, in micromoles per milligram of enzyme per hour (at 30 C, in 0.03 M sodium phosphate buffer, pH 7.0). For the *S. albus* G β-lactamase data, glycerol was added at a final concentration of 10%; efficiency is expressed (2) as the V_{max} value (micromoles per microgram of enzyme per hour) divided by the K_m value (molarity). Relative efficiency:efficiency of benzylpenicillin = 100.

[b] Formulas are given in Fig. 1. 6-APA, 6-aminopenicillanic acid; penicillin V, phenoxymethylpenicillin.

[c] See text. The K_m value for benzylpenicillin was 500 μM.

p-Chloromercuribenzoate, iodoacetate, or *p*-aminobenzoic acid, at concentrations of 1 mM, had no effect on the activities of either the R39 or the *S. albus* G enzyme preparation. Unlike the β-lactamase activities of other bacteria (19, 20), neither the R39 nor the *S. albus* G β-lactamase was inactivated by 2.5 mM iodine, even after prolonged treatment (30 min at 30 C). All three β-lactamases were sensitive to inactivation by heat. Preincubation for 5 min at 60 C was sufficient to abolish activity completely. The curves obtained for thermal inactivation of the R39 and *S. albus* G β-lactamases were virtually superimposable.

Substrate profiles. For the R39 and *S. albus* G enzymes, typical Michaelis-Menten kinetics were observed over a wide range of antibiotic concentrations (Table 2). Michaelis constants (K_m) and maximal velocity (V_{max}) were obtained at 30 C in 0.03 M sodium phosphate buffer, pH 7.0, on the basis of initial velocity measurements. Enzyme efficiency (V_{max}/K_m), as introduced by Pollock (18), was also calculated.

For the K11 enzyme, the kinetics of hydrolysis of sodium benzylpenicillin were obtained in an identical manner, and the relative efficiency on the other antibiotics was tested with three concentrations of antibiotic (1.66×10^{-4}, 1×10^{-4}, and 3.3×10^{-5} M), by calculating an average rate and comparing it with the rate on benzylpenicillin (set as 100) run in each case simultaneously with the test antibiotic (Table 2).

Effect of DD-carboxypeptidase substrates and inhibitors. The peptides listed in Materials and Methods were tested for their effect on the activity of both R39 and *S. albus* G β-lactamases. Concentrations of benzylpenicillin (i.e., 0.125 mM for the R39 enzyme and 1 mM for the *S. albus* G enzyme) were near the K_m values of the respective β-lactamases. Concentrations of peptides were between 1.1 and 1.7 mM. Neither inhibition nor activation of the β-lactamases was observed.

DISCUSSION

The β-lactamases from *Streptomyces* R39 and *S. albus* G were purified until apparent homogeneity by gel electrophoresis. They have properties typical of other β-lactamases (1, 2). Lack of a cation requirement for activity is not surprising in that, to date, only the Zn^{2+}-requiring β-lactamase II of *Bacillus cereus* exhibits such a property (6). Both R39 and *S. albus* G β-lactamases, however, were found to be inhibited (or inactivated) by Cu^{2+}. Their insensitivity to inactivation by thiol reagents, such as *p*-chloromercuribenzoate or iodoacetate, is a property shared with the majority of β-lactamases studied. This is consistent with the ob-

servation that β-lactamases do not generally contain cysteine. The *S. albus* G β-lactamase shows a sharp decline of activity at *p*H values higher than 9, but the R39 β-lactamase retains 80% of its activity at *p*H 10. The *Streptomyces* enzymes thus exhibit slightly broader *p*H optima than several other β-lactamases. The R39 and *S. albus* G β-lactamases are anionic. At *p*H 8.4, their electrophoretic mobility is identical and very similar to that of the R39 DD-carboxypeptidase-transpeptidase (10). At that *p*H, the *S. albus* G DD-carboxypeptidase was found to be cationic (4). β-Lactamases from other bacteria show wide variations in their electrical charge. Finally, the only peculiar property of the R39 and *S. albus* G β-lactamases is their remarkable resistance to iodine/KI solution.

The K_m values of both *S. albus* G and R39 β-lactamases for the various penicillins and cephalosporins (Table 2) are well within the norm of other β-lactamases (2). Nevertheless, the physiological efficiencies of the *Streptomyces* enzymes (2×10^3 to 4×10^3 for the best substrates; Table 2) are low compared with other β-lactamases (2×10^4 to 7×10^6 for the best substrates; 2). Sephadex chromatography and polyacrylamide gel electrophoresis, however, strongly suggest that both *Streptomyces* enzymes were highly purified by the reported procedure.

Both R39 and *S. albus* G β-lactamases hydrolyze 6-aminopenicillanic acid and benzylpenicillin with virtually the same efficiency (assuming that both enzyme preparations were equally pure; Table 2). However, this efficiency reflects a low K_m value (65 to 72 μM) combined with a low V_{max} value (200 to 260 μmoles per mg per hr) in the case of the R39 enzyme and a high K_m value (600 to 740 μM) combined with a high V_{max} value (1,500 to 2,000 μmoles per mg per hr) in the case of the *S. albus* G enzyme. Compared with 6-aminopenicillanic acid and benzylpenicillin, phenoxymethylpenicillin (penicillin V) is also a good substrate despite a fourfold increase of the K_m value with the R39 enzyme.

α-Substitution of benzylpenicillin with an amino group (ampicillin) increases the V_{max} value more in the case of the R39 enzyme than in the case of the *S. albus* G enzyme, has no effect on the K_m value with the *S. albus* G enzyme, and increases this latter parameter by a factor of 9 with the R39 enzyme (Table 2). As a result, ampicillin is hydrolyzed at a high rate by both β-lactamases. In marked contrast, α-substitution of benzylpenicillin with a carboxyl group drastically decreases the V_{max} values so that carbenicillin is hydrolyzed at a very low rate by both enzymes (Table 2).

The replacement of the CH_2 group of benzylpenicillin by a methyl-isoxazolyl ring (oxacillin) does not greatly affect the enzyme efficiency. Compared with benzylpenicillin, however, both K_m and V_{max} values for oxacillin are increased in the case of the R39 enzyme and decreased in the case of the *S. albus* G enzyme. Strikingly, the introduction of a Cl atom in the phenyl ring of oxacillin (i.e., cloxacillin) drastically decreased the V_{max} values with both β-lactamases. Very low V_{max} values were also observed with methicillin.

Both *Streptomyces* R39 and *S. albus* G β-lactamases bind the cephalosporin-type antibiotics. As shown by the K_m values reported in Table 2, the affinity varies greatly depending upon the structure of both the nucleus and the side chain. The enzyme efficiency on cephalosporin C, cephaloglycin, and cephalexin is only 1 to 4% of the enzyme efficiency on benzylpenicillin, although the two latter cephalosporins have a side chain which is identical to that of ampicillin. This would suggest that these *Streptomyces* β-lactamases function primarily as penicillinases rather than cephalosporinases. The occurrence of a thiophene-2-acetamido side chain in cephalothin, however, considerably increases the efficiency of the R39 enzyme by decreasing the K_m value (Table 2).

The β-lactamase from *Streptomyces* K11 was not highly purified. However, the comparison between the relative efficiency values on penicillins and cephalosporins (Table 2) showed that the K11 enzyme was different from the R39 and *S. albus* G enzymes. The main differences resided in the fact that the K11 enzyme exhibited no detectable activity on oxacillin, and, conversely, its efficiency on cephalothin and to a lesser extent on cephaloglycine was very high.

From the foregoing, it follows that all three *Streptomyces* β-lactamases differ from one another and from other systems previously described (1, 2).

With regard to the possible relationship between β-lactamase and DD-carboxypeptidase-transpeptidase (see introduction), the present work shows that the β-lactamases isolated from *Streptomyces* R39 and *S. albus* G do not have any DD-carboxypeptidase activity and are not affected by DD-carboxypeptidase substrates and inhibitors. Since, as shown in previous studies, the DD-carboxypeptidases of the same *Streptomyces* strains have no β-lactamase activity, it follows that DD-carboxypeptidases and β-lactamases are distinct enzymes characterized by specific differences in their relationships between binding and catalytically active sites.

With regard to the possible involvement of β-

lactamase in susceptibility (or resistance) of *Streptomyces* to β-lactam antibiotics, the present work facilitated a study of the possible correlation between the in vivo susceptibility of *Streptomyces* to a series of antibiotics and the catalytic efficiency and specificity profile of the corresponding β-lactamases for the same antibiotics. The results obtained with *Streptomyces* R39 and K11 are presented and discussed separately (3).

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