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Production of β-Lactamase by Non-*Streptomyces Actinomycetales*

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Supernatants and whole cells from fermentation broths of *Micromonospora, Nocardia, Oerskovia,* and other genera of the *Actinomycetales* were examined for the presence of β-lactamase activity by using the chromogenic cephalosporin 87/312. Nearly 60% of the 250 isolates examined produced detectable levels of β-lactamase. All enzyme preparations were active over a range of pH values from 6.5 to 8.2, with maximum activity occurring between 7.0 and 7.8. The preparations varied in their stability at 60°C. An examination of selected enzyme preparations revealed a similarity between substrate specificities of the non-*Streptomyces Actinomycetales* and gram-negative-bacterial β-lactamases.

The nature and occurrence of β-lactamases from bacteria have been extensively studied for many years (1, 2, 7, 8). Although recent reports have shown that *Streptomyces* produces β-lactamase (3, 5, 6), little information is available about β-lactamase production in other genera of the *Actinomycetales.*

This report examines the production of β-lactamases by 250 strains of the *Actinomycetales* from genera other than *Streptomyces.* For the sake of brevity, these strains will be referred to as non-*Streptomyces Actinomycetales.* The substrate specificities of selected enzyme preparations and their similarities to the bacterial β-lactamases are described.

MATERIALS AND METHODS

Actinomycete strains. The strains of *Micromonospora, Nocardia, Microbispora, Oerskovia,* and *Actinoplanes* were soil isolates unclassified as to species. Type strains of 13 genera were also obtained for testing from Mary LeChevalier, Waksman Institute, Rutgers University New Brunswick, New Jersey.

Media. The following media were prepared, using distilled water: (A) 3% toasted Nutrisoy flour (Archer Daniel Midland Co., Minneapolis, Minn.), 0.25% Yeastamine (Vico Products Co., Chicago, Ill.), 0.7% $CaCo_3$, 5.0% glucose; (B) 1.5% toasted Nutrisoy flour, 1.5% soluble starch (J. T. Baker, Phillipsburg, N.J.), 0.0005% $CoCl_2 \cdot 6H_2O$, 5% glucose; (C) 1% yeast extract (Difco Laboratories, Detroit, Mich.) 0.5% casein hydrolysate (Nutritional Biochemicals Corp., Cleveland, Ohio), 2% soluble starch, 0.4% $CaCO_3$, 0.0005% $CoCl_2 \cdot 6H_2O$, 1% glucose; (D) 0.15% beef extract (Difco), 0.3% yeast extract, 0.6% peptone (Amber Laboratories, Juneau, Wis.), 0.1% glucose; (E) 2% soybean meal (Archer Daniel Midland Co.), 0.5% yeast extract, 1.0% $Na_2HPO_4 \cdot 7H_2O$, 2.1% KH_2PO_4, 0.08% $MgCl_2 \cdot 6H_2O$, 3% glycerol; (F) 2% oatmeal (Gerber Products Co., Fremont, Mich.), 2% tomato paste (Contadina Foods, Los Angeles, Calif.), 1.5% agar (Difco) (pH adjusted to 7.0 before autoclaving); (G) 3.05% seed agar (Baltimore Biological Laboratory [BBL], 0.5% NaCl.

Chemicals and enzymes. The chromogenic cephalosporin, 87/312 (4), was a gift from Glaxo Research Ltd., Greenford, Middlesex, England. Class Ia β-lactamase (7), isolated from a strain of *Enterobacter cloacae,* was purchased from Miles Laboratories, Inc., Elkhart, Ind.; class IVc β-lactamase (7) was a partially purified preparation isolated from a suspension of *Klebsiella aerogenes* 1082E (SC 10436) by sonic disruption of the cells followed by ultracentrifugation for 2 h at 105,000 × *g.*

Carbenicillin and cloxacillin were obtained from Beecham Pharmaceuticals, Bristol, Tenn. Cephaloridine and cephalexin were obtained from Eli Lilly & Co., Indianapolis, Ind. The other antibiotics were from the stock of the Squibb Institute for Medical Research (Princeton, N.J.).

β-Lactamase detection. (i) Method A. A reaction mixture consisting of 1 ml of centrifuged fermentation broth, 2 ml of 0.05 M phosphate buffer (pH 7.0), and 0.2 ml of compound 87/312 (500 μg/ml) was incubated at 37°C for 2 h. An increase in optical density at 482 nm greater than 20% as detected on a Spectronic 20 spectrophotometer indicated the presence of β-lactamase.

(ii) Method B. A reaction mixture consisting of 0.5 ml of fermentation supernatant or centrifuged cells and 0.05 ml of compound 87/312 (500 μg/ml) was incubated at 37°C for 1 h in porcelain wells. The development of a distinct red color during incubation indicated the presence of β-lactamase.

Primary screening for β-lactamase production. Table 1 summarizes the media used for growing the various *Actinomycetales* genera. All isolates were inoculated from agar slants (medium F) into 250-ml Erlenmeyer flasks containing 50-ml amounts of germination media, and the flasks were incubated on a

rotary shaker (280 strokes per min) at 26°C. After 3 days of incubation, 5% transfers of the germination media were made to fermentation media, and the newly inoculated flasks were incubated for 7 days at the conditions described above. Samples were removed from the fermentation flasks during the incubation period and assayed for β-lactamase activity.

β-Lactamase preparations. Intracellular β-lactamases were isolated from cells grown in fermentation media (see Table 1) for 48 h at 26°C on a rotary shaker. The cells were disrupted by sonic treatment at 20,000 Hz for 5 min. The suspensions were centrifuged at 15,000 × *g* for 10 min, and the supernatants were retained as β-lactamase sources. Extracellular β-lactamase preparations were obtained by passing the broth supernatant from a fermentation possessing β-lactamase activity through a membrane filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.).

Substrate specificity. Substrate specificities were determined for β-lactamase preparations isolated from five strains of *Nocardia* sp., two strains of *Micromonospora* sp., one *Microbispora* sp., one *Streptomyces* sp., and two bacteria. A reaction mixture (2.0 ml) consisting of 0.05 M phosphate buffer (pH 7.0), β-lactam antibiotic (1 mg/ml), and β-lactamase was incubated at 37°C for 6 h. At various times, 30-μl amounts were removed from the incubation tubes, absorbed in 0.25-inch (ca. 0.63-cm) paper disks (Schleicher & Scheull Co., Keene, N.H.), and assayed for bioactivity by the plate agar diffusion method. Inoculum was prepared from a turbid culture of *Micrococcus luteus* SC 2495, grown for 24 h in antibiotic assay broth (BBL). A 1-ml amount of the inoculum was added to 350 ml of melted agar (medium G) cooled to 50°C. The assay disks were placed on the solidified agar, and the plates were incubated overnight at 37°C.

The zone sizes obtained on diffusion assay were converted to antibiotic concentrations (in micrograms per milliliter) by referring to the previously prepared standard curve (zone size versus concentration) for each antibiotic. These curves were obtained by using agar diffusion plates as previously described. Hydrolysis rates were then calculated.

pH optimum and heat stability. β-Lactamases from the eight strains of non-*Streptomyces Actinomycetales* were incubated with 0.1 μmol of compound 87/312 in 0.1 M phosphate buffer at various pH values. The rate of development of red color indicated β-lactamase activity at a particular pH. Samples of the same β-lactamase preparations were also incubated at 60°C in 0.05 M phosphate buffer, pH 7.0, and then assayed for activity with compound 87/312.

RESULTS

β-Lactamase production. Table 1 summarizes the results of screening 250 strains of non-*Streptomyces Actinomycetales* for β-lactamase production. Nearly 60% of all isolates tested produced detectable levels of β-lactamase.

The β-lactamases produced by the *Micromonospora* strains were predominantly intracellular. In contrast, β-lactamase activity with *Nocardia* was detected both inside and outside the cells. The apparent amounts of enzyme varied from strain to strain in both genera. Either intracellular or extracellular β-lactamase production was observed in all remaining genera.

Substrate specificity. The substrate specificities of 11 β-lactamases are summarized in Table 2. The intracellular *Nocardia* enzymes and the class IVc bacterial β-lactamase hydrolyzed all the β-lactam substrates tested. The remaining enzyme preparations were more specific in their substrate profiles. The class Ia bacterial enzyme functioned as a cephalosporinase, whereas the *Streptomyces* β-lactamase was primarily a penicillinase. The two *Micromonospora* enzymes hydrolyzed only benzylpenicillin, ampicillin, and cephaloridine.

No investigation was made as to whether these enzyme preparations were mixtures of different β-lactamases.

pH optimum and heat stability. All enzymes were active over a range of pH values from 6.5 to 8.2, with maximum activity occurring between pH 7.0 and 7.8.

The *Microbispora* β-lactamase and *Nocardia* extracellular enzyme (Table 2, e) retained 30 and 50% of their initial activities, respectively, after 30 min at 60°C. The other enzyme preparations retained less than 5% of their initial activities after 10 min of exposure.

DISCUSSION

The production of β-lactamase by non-*Streptomyces Actinomycetales* is widespread throughout the various genera. Nearly 60% of the strains tested produced detectable levels of β-lactamase, and it is quite possible that with

TABLE 1. *Media for growth and production of β-lactamase by non-Streptomyces Actinomycetales*

Genus	Germination media	Fermentation media	No. screened	Positive	
				No.	%
Micromonospora	A, C	A, B, C, D	150	88	58
Nocardia	A, D	A, B, D, E	81	47	58
Oerskovia	D	A, B, C, D	4	4	100
Other[a]	A	A, B, C, D, E	15	9	60

[a] Other genera include *Actinoplanes* (2 positive/4 screened), *Actinomadura* (1/1), *Streptoverticillium* (1/1), *Pseudonocardia* (1/1), *Microellobosporia* (1/1), *Microtetraspora* (1/1), *Microbispora* (1/2), *Mycoplana* (0/1), *Ampullariella* (0/1), *Dactylosporangium* (0/1), and *Micropolyspora* (1/1).

TABLE 2. *β-Lactamase substrate specificity—relative hydrolysis rates*

Enzyme source	Hydrolysis rate (%)[a] of:						
	Benzyl-penicillin	Ampicil-lin	Carbeni-cillin	Cloxacil-lin	Cephalori-dine	Ceph-alexin	Cephra-dine
Nocardia strains							
(a) Intracellular	100	21	28	36	76	16	<10
(b) Intracellular	100	15	37	48	110	13	<10
(c) Intracellular	100	330	230	60	330	110	14
(d) Extracellular	100	400	260	0	4,800	0	0
(e) Extracellular	100	330	260	100	320	0	0
Micromonospora strains							
(a) Intracellular	100	190	0	0	800	0	0
(b) Intracellular	100	120	0	0	400	0	0
Microbispora	100	690	200	0	1,700	0	0
Streptomyces sp[b]	100	80	50	20	<10	0	0
Class Ia	100	0	0	0	1,200	600	400
Class IVc	100	110	70	40	80	10	<10

[a] Percentage of the hydrolysis rate of each substrate against that of benzylpenicillin.
[b] Extracellular enzyme isolated from a *Streptomyces* strain.

more vigorous fermentation studies this percentage would be even higher. β-Lactamase activity was detected in 58% of the strains of *Nocardia* and *Micromonospora* tested and in more than 68% of the strains in the remaining 12 genera. Ogawara and co-workers, by comparison, found that detectable levels of β-lactamase were produced by at least 50% of the strains of *Streptomyces* tested in one study (5) and 75% of the *Streptomyces* strains in another (6). Our results, combined with the numerous other reports describing β-lactamase production, further confirm the ubiquity of β-lactamase production in a wide segment of the microbial population.

The substrate specificities of the two gram-negative-bacterial β-lactamases assayed agree with the Richmond and Sykes classification (7). The results indicating that the *Streptomyces* β-lactamase functions primarily as a penicillinase agree with those obtained by Ogawara and co-workers (5, 6). The agreement of our substrate specificities with those previously reported confirms the validity of our assay system.

The substrate specificities of the β-lactamase preparations examined from eight non-*Streptomyces Actinomycetales* are similar to the specificities of some of the classes of gram-negative-bacterial β-lactamases described by Richmond and Sykes (7). For example, the three intracellular *Nocardia* enzymes and one of the two extracellular *Nocardia* enzyme preparations (Table 2, e) hydrolyze cephaloridine and the penicillin substrates, as do class IV bacterial β-lactamases. The other extracellular *Nocardia* enzyme (Table 2, d) and the *Microbispora* β-lactamase have similar hydrolysis patterns, except they do not hydrolyze cloxacillin. This pattern of hydrolysis matches that of class III bacterial β-lactamases. Similarly, the pattern of hydrolysis exhibited by the two *Micromonospora* enzymes resembles that of class I bacterial β-lactamases. They hydrolyze cephaloridine more rapidly than either ampicillin or benzylpenicillin and have no effect on cloxacillin and carbenicillin.

Though the substrate profiles are similar, a direct comparison between β-lactamases from non-*Streptomyces Actinomycetales* and gram-negative bacteria awaits further purification of representative enzymes.

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