ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1977, p. 142-146

Vol. 11, No. 1
Printed in U.S.A.

Beta-Lactamase Activity in Strains of *Bacteroides melaninogenicus* and *Bacteroides oralis*

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Received for publication 10 August 1976

β-Lactamase from strains of *Bacteroides melaninogenicus* and *Bacteroides oralis* hydrolyzed penicillin more rapidly than ampicillin or carbenicillin. Cephalothin and a chromogenic cephalosporin (87/312) were also hydrolyzed by the enzyme. Activity was found only in β-lactam-resistant strains, but there was considerable variation in activity among strains having the same minimal inhibitory concentrations of antibiotic. β-Lactamase activity was cell bound and appeared to be tightly associated with the cell envelope since detergents were required to elute this activity.

Bacteroides melaninogenicus and *B. oralis* are components of the normal flora of the human upper respiratory tract and are frequently isolated from infections of that area. In the past, these species have been considered to be susceptible to β-lactam antibiotics, with resistance occuring in about 10% of the strains tested (5, 10). However, in a current survey, 40% of all clinical isolates of these species were resistant to penicillin (P. Murray and J. Rosenblatt, private communication).

Hydrolysis of penicillin and ampicillin by a strain of *B. oralis* was first demonstrated by Pinkus et al. (8). Recently, Hackman and Wilkins reported penicillinase activity by penicillin-resistant strains of both *B. melaninogenicus* and *B. oralis* (3). Since these strains are also resistant to other β-lactam antibiotics, we determined the ability of β-lactamases from these organisms to hydrolyze ampicillin, carbenicillin, and cephalothin. We also investigated the cellular location of the enzyme activity.

MATERIALS AND METHODS

Source of bacterial strains. Two strains of *B. melaninogenicus* were obtained from V. L. Sutter, Wadsworth Hospital, Los Angeles, Calif.: WAL 2721 (VPI 9465B) and WAL 2784 (VPI 9466). All other strains were obtained from the culture collection of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University (VPI), Blacksburg, Va. Identifications were made according to previously published procedures (4).

MIC determinations. Except for strains of *B. melaninogenicus* subsp. *asaccharolyticus*, minimal inhibitory concentrations (MICs) were determined by using an agar dilution technique (12) with brucella blood agar plates. All manipulations were carried out in an anaerobic chamber (Coy Manufacturing Co., St. Louis, Mo.), with an atmosphere of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. Since strains of *B. melaninogenicus* subsp. *asaccharolyticus* did not grow well on the brucella blood agar plates, MICs for these strains were determined by using a broth dilution method (13) with chopped-meat broth (4) substituted for brain heart infusion broth.

Source of antibiotics. Benzylpenicillin, potassium salt, was obtained from Sigma Chemical Co. (St. Louis, Mo.). Carbenicillin and ampicillin were provided by Bristol Laboratories (Syracuse, N.Y.). Cephalothin was obtained from the Eli Lilly & Co. (Indianapolis, Ind.). The chromogenic cephalosporin 87/312 was a gift from C. O'Callaghan (Glaxo Research Inc., Middlesex, England).

Preparation of cells. For determinations of β-lactamase in whole cells, 16-h cultures in 100 ml of Trypticase-yeast extract-glucose (TYG) medium (4) were harvested by centrifugation at 10,800 × *g* for 15 min at 4°C and then washed twice in 0.1 M potassium phosphate buffer (pH 5.9). Cells were resuspended in 10 ml of buffer, and the concentration of cells was determined by measuring optical density and comparing with a standard curve. The standard curve (optical density versus concentration of cells) was obtained using a *B. melaninogenicus* strain. Since standard curves for most *Bacteroides* species have the same slope, this standard curve was used for all strains tested. The final cell suspension had an optical density of approximately 10. Portions (0.1 ml) were assayed for enzyme activity.

Whole cells that had been washed and resuspended in buffer were mechanically disrupted by shaking aerobically with glass beads in a Bronwill cell disintegrator (Bronwill Scientific Inc., Rochester, N.Y.) for 3 min. Glass beads were allowed to settle and the lysed cell suspension was decanted. The lysed cell suspension was then centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatant was saved and the pellet was resuspended in 10 ml of buffer. Both the supernatant and pellet were assayed for enzyme activity.

Adsorption of enzyme to the glass beads used for breaking cells was estimated by adding 2-g glass beads to 5 ml of lysed cell suspension and mixing for 1 to 2 min. Enzyme activity after mixing was compared with enzyme activity before the addition of the glass beads. Approximately 10% of the activity was removed by adsorption to glass beads.

To determine the effect of growth phase on activity, cells growing in TYG medium were harvested at 4-h intervals after inoculation and prepared for assay as described above. Four strains (VPI 7795, VPI 9465B, VPI 9466, and VPI 9611) were tested.

β-Lactamase assays. β-Lactamase activity was determined using the microiodometric method of Sykes and Nordstrom (9) with penicillin, ampicillin, carbenicillin or cephalothin as the substrate. Cephalosporinase activity was also determined by using the chromogenic cephalosporin method of O'Callaghan et al. (7), except that assays were run at 30°C. Enzyme activities were determined by averaging triplicate assays. Activity obtained with washed whole cells was expressed as units per 10^{11} cells. One unit is defined as hydrolysis of 1 μmol of substrate/min.

To determine whether the different rates of hydrolysis obtained using the microiodometric method might be due, in part, to conditions of the iodometric assay (e.g., the presence of iodine), the hydrolysis of penicillin, ampicillin, and carbenicillin was also followed by using the thin-layer chromatographic method of Pinkus et al. (8). Washed whole cells were added to phosphate buffer (pH 7.0) containing 1 mg of antibiotic per ml. At 30-min intervals, 10-μl portions were spotted on silica gel plates and developed in ethanol-water (79:21, vol/vol). Spots were visualized using starch-iodine reagent (8). R_f values were as follows: penicillin, 0.8; penicilloic acid, 0.4; carbenicillin, 0.6; carbenicilloic acid, 0.3; ampicillin, 0.7; ampicilloic acid, 0.3. The amount of time required to hydrolyze 100% of the antibiotic was estimated and compared with that predicted by the rate obtained using the iodometric method.

Spheroplast formation. To determine whether cell-bound activity was in the periplasmic space, spheroplasts were made from washed whole cells using the lysozyme-ethylenediaminetetraacetate technique of Birdsell and Cota-Robles (1), except that cells were incubated 4 h. Spheroplast formation was checked microscopically. Spheroplasts were centrifuged and resuspended in 20% sucrose. β-Lactamase activities in the spheroplast supernatant and in the resuspended spheroplasts were determined by using the chromogenic cephalosporin method. Four strains (VPI 7795, VPI 9465B, VPI 9466, and VPI 9611) were used in these experiments.

Solubilization of β-lactamase activity. To elute β-lactamase activity from the lysed cell pellet, the pellet was resuspended in each of the following: 1 M NaCl in phosphate buffer (pH 7.0), 2% Triton X-100 (vol/vol) in phosphate buffer (pH 7.0), 2% sodium dodecyl sulfate (vol/vol) in tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.0), 10% sodium taurocholate (vol/vol) in phosphate buffer. After 1 h at room temperature, the mixture was centrifuged at 20,000 × *g* for 30 min (4°C). The supernatant and resuspended pellet were assayed for β-lactamase activity by using the chromogenic cephalosporin method.

Inducibility of β-lactamase activity. To determine whether exposure of strains to antibiotics prior to assay affected activity, four strains (VPI 9331, VPI 7795, VPI 9611, and VPI 9465B) were grown in the presence of 2 μg of penicillin or cephalothin per ml. β-Lactamase activities in these strains were compared with activities in the same strains grown in the absence of antibiotics.

RESULTS

MICs for the strains used in these experiments are given in Table 1. Penicillin-resistant strains were equally resistant to the other three antibiotics. Penicillin-susceptible strains were susceptible to the other antibiotics.

There was no detectable β-lactamase activity in any of the strains that were susceptible to β-lactam antibiotics. β-Lactamase activity was found only in the resistant strains. Activity appeared to be cell bound since no activity was detected in the culture supernatant.

TABLE 1. *MICs of penicillin, carbenicillin, ampicillin, and cephalothin for the Bacterioides strains used in this study*

Bacteroides strain (VPI no.)	MIC value (μg/ml)			
	Penicillin	Carbenicillin	Ampicillin	Cephalothin
B. melaninogenicus subsp. *intermedius*				
8944, 9145	<0.125	<0.125	<0.125	<0.25
9931, 9466	16-32	16-32	8-16	32
B. melaninogenicus subsp. *melaninogenicus*				
4196	<0.125	<0.125	<0.125	<0.125
9465B	32	16	16	64
B. melaninogenicus subsp. *asaccharolyticus*				
8107DP, 8544A	<0.125	<0.125	<0.125	<0.125
7795, 9848	16	16	8-16	32-64
B. oralis				
5933, 6733	<0.125	<0.25	<0.125	<0.25
4533C, 9611	16-32	16-32	8-16	16-32

Although the resistant strains were equally resistant to all four antibiotics, β-lactamase activity varied considerably, depending on the strain and the antibiotic used as substrate (Table 2). Strains of *B. oralis* and the two saccharolytic subspecies of *B. melaninogenicus* (*B. melaninogenicus* subsp. *intermedius* and *B. melaninogenicus* subsp. *melaninogenicus*) hydrolyzed penicillin much more rapidly than carbenicillin or ampicillin. Hydrolysis of cephalothin was less rapid than hydrolysis of penicillin but more rapid than hydrolysis of ampicillin or carbenicillin (Table 2). Strains of *B. melaninogenicus* subsp. *asaccharolyticus* had much lower activity on both penicillin and cephalothin than did strains of *B. oralis* or the other two *B. melaninogenicus* subspecies, but hydrolyzed ampicillin and carbenicillin at the same rate.

The different rates of hydrolysis of penicillin, ampicillin, and carbenicillin obtained by using the iodometric test were confirmed qualitatively by the thin-layer chromatographic method. When hydrolysis of penicillin, carbenicillin, and ampicillin was followed by using chromatography of the hydrolysis products, the conversion of penicillin to penicilloic acid by strains of *B. melaninogenicus* (*B. melaninogenicus* subsp. *intermedius* and *B. melaninogenicus* subsp. *melaninogenicus*) and *B. oralis* was completed in less than one-fourth the time required for the conversion of carbenicillin and ampicillin. Under these conditions, hydrolysis of all three antibiotics by strains of *B. melaninogenicus* subsp. *asaccharolyticus* occurred within the same time period. Since the qualitative results of the thin-layer chromatographic method agreed with the relative rates of hydrolysis obtained by the iodometric method, it appears that differences in activities on different substrates were not due to the special conditions of the iodometric assay, such as the presence of iodine.

Cephalosporinase activity, determined by using the chromogenic cephalosporin method, was at least 10-fold higher than activity obtained using the iodometric method with cephalothin as a substrate (Table 2). However, relative rates of cephalosporin hydrolysis by the different strains were the same with both methods.

Using logarithmically growing cells instead of stationary-phase cells did not affect the level of activity detected. β-Lactamase activity (per 10^{11} cells) of cultures sampled at 4-h intervals remained constant. β-Lactamase activity did not appear to be inducible by penicillin or cephalothin since the same activities were obtained with cells grown in penicillin or cephalothin as with cells grown in the absence of antibiotics.

In some cases, mechanical disruption of the cells increased activity. In Table 3, the percent increase in activity due to lysis is given for those strains in which such a difference occurred. No decrease in activity with lysis was observed in any of the strains tested. When lysed cell suspensions were centrifuged for 30 min at 20,000 × *g*, activity was found only in the pellet (cell envelope fraction). Except in the case of VPI 9465B, in which 3 to 10% of the activity appeared in the supernatant, no activity was detected in the supernatant. Enzyme activity could be extracted from the pellet with 2% Triton X-100 and 10% taurocholate but not with 1 M NaCl. Enzyme activity was lost when sodium dodecyl sulfate was added to the lysed cell suspension.

Treatment of cells with ethylenediaminetetraacetate-lysozyme resulted in the conversion of nearly all of the cells to spheroplasts. Spheroplast formation released 7 to 12% of the enzyme activity into the medium.

TABLE 2. *β-Lactamase activity in resistant strains of B. melaninogenicus and B. oralis*

Bacteroides (VPI no.)	β-Lactamase activity (μmol/min per 10^{11} cells)				
	Iodometric assay				Chromogenic cephalosporin 87/312
	Penicillin	Carbenicillin	Ampicillin	Cephalothin	
B. melaninogenicus subsp. *intermedius*					
9331	0.5	0.1	0.1	0.2	2.4
9466	1.1	0.4	0.2	0.6	10.0
B. melaninogenicus subsp. *melaninogenicus*					
9465B	1.4	0.2	0.1	1.3	12.8
B. melaninogenicus subsp. *asaccharolyticus*					
7795	0.2	0.1	0.1	<0.1	1.0
9848	<0.1	0.1	0.1	<0.1	0.8
B. oralis					
4533C	1.7	0.2	0.1	0.4	8.6
9611	1.8	0.3	0.1	0.6	8.0

TABLE 3. *Percent increase in β-lactamase activity in lysed cells of B. melaninogenicus*

Bacteroides (VPI no.)	β-Lactamase activity[a]			
	Penicillin	Carbenicillin	Ampicillin	Chromogenic cephalosporin 87/312
B. melaninogenicus subsp. *intermedius*				
9331	240	150	100	150
9466	210	110	120	140
B. melaninogenicus subsp. *melaninogenicus*				
9465B	210	110	100	110

[a] Expressed as a percentage of the activity in whole cells of the same strain on the same antibiotic.

DISCUSSION

Strains of *B. melaninogenicus* and *B. oralis* that are resistant to β-lactam antibiotics have enzymes that can hydrolyze both penicillins and cephalosporins. Since β-lactamase activity was found only in resistant strains of these species, it is probable that the production of β-lactamase is involved in the resistance of these organisms to β-lactam antibiotics. However, there was considerable variation in levels of activity found in strains with the same MIC values. The low β-lactamase activity on carbenicillin and ampicillin, as compared with penicillin, was not accompanied by a higher susceptibility to these antibiotics. Thus, production of β-lactamase may not be the only mechanism of resistance in these strains.

Levels of activity and hydrolysis patterns of *B. oralis* were almost identical with those of *B. melaninogenicus* subsp. *intermedius* and *B. melaninogenicus* subsp. *melaninogenicus*. *B. melaninogenicus* subsp. *asaccharolyticus*, which differs from the other *B. melaninogenicus* subspecies in a number of taxonomic characteristics, including deoxyribonucleic acid base composition (14), also differed from these subspecies and *B. oralis* in the level of enzyme activity on penicillin and cephalothin. These results indicate that *B. oralis* and the two saccharolytic *B. melaninogenicus* subspecies may have similar enzymes, whereas the enzyme produced by *B. melaninogenicus* subsp. *asaccharolyticus* may be distinct from these.

In all cases, β-lactamase activity appeared to be associated mainly with the cell envelope and could only be solubilized by detergents. This may be due in part to the method of mechanical disruption. The use of sonification rather than shaking with glass beads to disrupt cells may increase the percentage of soluble enzyme (R. G. Wilkinson, private communication). The fact that lysis of the cells increased activity indicates that there may be a permeability barrier separating the enzyme from the antibiotic. Also, spheroplast formation did not release significant amounts of activity into the medium. Thus, the enzyme may be associated with the inner membrane of the cell.

β-Lactamase activity in *B. melaninogenicus* and *B. oralis* differs from β-lactamase activities reported in other *Bacteroides* species. Whereas the enzymes from *B. melaninogenicus* and *B. oralis* were generally more active against penicillin than ampicillin, carbenicillin, or cephalothin, β-lactamase from *B. fragilis* has been reported to be more active on cephalosporins than on penicillins (2, 11). A β-lactamase from *B. clostridiiformis* was found to be active primarily on penicillin and ampicillin but not on carbenicillin and cephalosporins (11). Moreover, previous exposure to penicillin increased the activity of *B. clostridiiformis*, whereas previous exposure of *B. fragilis*, *B. melaninogenicus*, and *B. oralis* strains to penicillin did not increase β-lactamase activity.

β-Lactamases have now been found in resistant strains from a number of species of gram-negative bacteria. However, resistance to β-lactam antibiotics is not always correlated with high β-lactamase activities, and there is still some question about the true role of these enzymes (6). In the case of *B. melaninogenicus*, *B. oralis*, and *B. fragilis*, strains with similar patterns of resistance to β-lactam antibiotics produce β-lactamases that differ considerably in levels of activity and substrate specificity. Further work is needed to determine the significance of β-lactamase and the possible contribution of other mechanisms to penicillin and cephalosporin resistance in these *Bacteroides* species.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant GM14604 from the National Institute of General Medical Sciences and by a grant from the Upjohn Co.

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