ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1980, p. 853-857
0066-4804/80/12-0853/05$02.00/0

Vol. 18, No. 6

# Purification and Properties of a Cephalosporinase from *Enterobacter cloacae*

SHINZABURO MINAMI,[1]* MATSUHISA INOUE,[2] AND SUSUMU MITSUHASHI[1,2]

*Department of Microbiology[1] and Laboratory of Bacterial Drug Resistance,[2] School of Medicine, Gunma University, Maebashi, Japan*

A cephalosporin β-lactamase (cephalosporinase) was extracted from *Enterobacter cloacae* GN7471 and purified by means of column chromatography. The resulting preparation gave a single protein band upon polyacrylamide gel electrophoresis. The enzyme's isoelectric point was 8.4, and its molecular weight was 44,000. The optimal pH was 8.5, and the optimal temperature was 40°C. The enzyme hydrolyzed cephalosporins much more readily than penicillins. The enzyme activity was inhibited by iodine, semisynthetic penicillins, cefuroxime-type cephalosporins, and cephamycin derivatives. The enzymological properties of the purified enzyme were compared with those of β-lactamases derived from other gram-negative enteric bacteria.

β-Lactamases have been considered to play a significant role in the high resistance of certain organisms to penicillins and cephalosporins (22, 26). It has already been shown that many *Enterobacter cloacae* strains produce a cephalosporin β-lactamase (cephalosporinase) which is considered to be a species-specific β-lactamase (12, 13). Some properties of these *E. cloacae* cephalosporinases have been investigated and compared with those of β-lactamases from other gram-negative bacteria by using crude or partially purified enzyme from bacterial cells (9, 11, 13, 15, 22, 23, 26).

This paper deals with the enzymological and physicochemical properties of a purified enzyme from *E. cloacae* GN7471 as compared with β-lactamases from other gram-negative bacteria.

## MATERIALS AND METHODS

**Bacterial strains.** *E. cloacae* GN7471 and other strains were isolated from a patient with urinary tract infection and stock cultures in this laboratory.

**Culture and harvesting of organisms.** *E. cloacae* was grown overnight in 200 ml of brain heart infusion broth at 37°C. The culture was diluted 10-fold with medium B and then grown at the same temperature under aeration for 5 h. When an inducer was required for the production of β-lactamase, benzylpenicillin was added 2 h after the commencement of culture, and the culture was allowed to continue growing for an additional 3 h. The cells were then harvested by centrifugation and washed once with 0.1 M sodium phosphate buffer (pH 7.0).

**Media.** Brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and heart infusion agar (Eiken, Tokyo) were used. Medium B was used for large-scale cultures and consisted of 2 g of yeast extract, 10 g of peptone, 8 g of $Na_2HPO_4 \cdot 12H_2O$, 2 g of $KH_2PO_4$, 1.2 g of $(NH_4)_2SO_4$, 2 g of glucose, 0.4 g of $MgSO_4$, and 1,000 ml of distilled water.

**Drugs.** The following cephalosporins and penicillins were used. Cephaloridine, cefazolin, cephalothin, cephalexin, benzylpenicillin, ampicillin, carbenicillin, cloxacillin, and methicillin were commercially available materials, and the following compounds were gifts from manufacturers: cefsulodin and cefotiam from Takeda Pharmaceutical Co., cefamandole and moxalactam (6059S) from Shionogi Pharmaceutical Co., cefuroxime from Shinnihon Jitsugyo Co., cefotaxime from Hoechst Japan Co., ceftizoxime (FK-749) from Fujisawa Pharmaceutical Co., cefoperazone from Toyama Chemical Co., cefoxitin from Daiichi Seiyaku Co., cefmetazole from Sankyo Co., clavulanic acid from Beecham Pharmaceutical, U.K., and CP-45899 from Pfizer Inc., New York, N.Y.

**Determination of minimum inhibitory concentration.** Minimum inhibitory drug concentrations were determined by the serial dilution technique (14, 27).

**Conjugation experiments.** The conjugation method for R plasmids has been previously reported (5). *Escherichia coli* K-12 ML1410 was used as the recipient.

**β-Lactamase induction.** To induce β-lactamase production, an overnight culture was diluted 10-fold with fresh medium and grown with shaking at 37°C. After 2 h, an appropriate concentration of inducer was added, and growth with shaking was continued for a further 3 h before the culture was harvested.

**Column preparation.** CM-Sephadex C-50 (Pharmacia, Uppsala, Sweden) was washed before use with sodium hydroxide (0.5 N) and hydrochloric acid (0.5 N), equilibrated with 0.005 M sodium phosphate buffer (pH 7.0), and packed onto a column. A Sephadex G-100 (Pharmacia, Uppsala, Sweden) column was prepared as has been described previously (25). The columns were equilibrated with 0.05 M sodium phosphate buffer (pH 7.0).

**Enzyme assay.** β-Lactamase activity was determined by either a spectrophotometric method of measuring the decrease in absorbance at an appropriate wavelength of the substrate (100 μM) in a temperature-controlled spectrophotometer (Beckman model

24) at 30°C as described by Hirai et al. (14) or by a modification of the Novick microiodometric method (19), using penicillin as a substrate.

One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 μmol of the substrate in 1 min at 30°C in 0.05 M sodium phosphate buffer (pH 7.0) (14).

**Protein determination.** The protein concentration in column eluates was estimated by measuring the absorbance at 280 nm. A more accurate estimation was arrived at by using the method of Lowry et al. with bovine serum albumin as the standard (17).

**Determination of molecular weight.** The molecular weight of the purified enzyme was determined by sodium dodecyl sulfate-discontinuous gel electrophoresis according to the method of Weber and Osborn (28), using albumin (molecular weight, 68,000), ovalbumin (45,000), chymotrypsinogen A (25,000), and cytochrome *c* (12,500) as the molecular weight standards. The gel concentration employed was 10%.

**Analytical polyacrylamide gel electrophoresis.** The purity of the enzyme preparation was determined by discontinuous gel electrophoresis as described by Davis (4), using a 7.5% acrylamide gel (pH 4.5) as the separation gel. Electrophoresis was carried out at 4°C.

**Determination of isoelectric point.** The isoelectric point of the enzyme was determined by electrofocusing (14).

## RESULTS

**Bacterial strains capable of producing cephalosporinase.** We randomly selected 28 *E. cloacae* strains that are highly resistant to various β-lactam antibiotics, especially to ampicillin. From these resistant strains we chose three, *E. cloacae* GN5797, GN7467, and GN7471, which did not have plasmids carrying resistance to ampicillin and whose β-lactamase could not significantly hydrolyze either ampicillin or carbenicillin. The 25 other bacterial strains had plasmids carrying resistance to ampicillin. For the purification experiments, *E. cloacae* GN7471 was chosen, because this strain produced slightly larger amounts of the enzyme than did the other strains whereas its substrate profile was the same as those of the two other strains. The substrate profiles as relative rates of hydrolysis against cephaloridine, cefazolin, cephalothin, cephalexin, cefotiam, and cefamandole were 100, 56, 440, 44, 63, and 2, respectively.

**Inducibility of β-lactamase production.** *E. cloacae* strains GN5797, GN7467, and GN7471 were grown in the presence of penicillin G. The activity of the enzyme was measured against cephalothin and compared with the activity of bacterial cells cultured without penicillin G.

Induction was observed with penicillin G only in strain GN5797, in which the hydrolysis of cephalothin was increased 570 times at a penicillin G concentration of 2,000 μg/ml (Table 1). In the other two strains, the enzyme activity was not enhanced in the presence of penicillin G.

**Purification procedure.** An outline of the purification procedure and the recovery after each step of the procedure is shown in Table 2. All operations were carried out at 4°C, and the pH of all buffers used was 7.0.

**Physicochemical properties of the purified enzyme.** The molecular weight of the pu-

TABLE 1. *Induction of β-lactamase formation in three Enterobacter strains*

| Strain | β-Lactamase activity[a] (uninduced) | β-Lactamase activity after induction with PCG at[b]: | | |
|---|---|---|---|---|
| | | 0.5 mg/ml | 1 mg/ml | 2 mg/ml |
| GN7471 | 14.9 | 16.8(1.1) | 19.7(1.3) | — |
| GN7467 | 15.5 | 16.4(1.1) | 16.3(1.1) | — |
| GN5797 | 0.02 | 5.4(270) | 6.8(340) | 11.4(570) |

[a] Hydrolytic activity, units per milligram of protein. Cephalothin was used as a substrate.

[b] Parentheses indicate ratio of the β-lactamase activity of cells with inducer to that without inducer. PCG, Penicillin G.

TABLE 2. *Summary of the purification of cephalosporinase from E. cloacae GN7471*

| | Procedure | Activity recovered[a] (U) | Sp act (U/mg of protein) | Purification factor | Recovery (%) | |
|---|---|---|---|---|---|---|
| | | | | | Per step | Overall |
| 1. | Ultrasonic disintegration | 2,940 | 4.27 | 1.0 | — | 100 |
| 2. | Streptomycin treatment | 2,479 | 6.40 | 1.5 | 84.0 | 84.0 |
| 3. | Chromatography on CM-Sephadex C-50 | 1,305 | 320 | 74.9 | 52.6 | 44.2 |
| 4. | Chromatography on CM-Sephadex C-50 | 959 | 578 | 135 | 73.5 | 32.5 |
| 5. | Gel filtration on Sephadex G-100 | 863 | 595 | 139 | 90.0 | 29.3 |

[a] β-Lactamase activity was determined photometrically using cephalothin (100 μM) as a substrate.

rified enzyme was estimated by disc electrophoresis to be 44,000. The isoelectric point of the enzyme was determined by electrofocusing, which indicated that *E. cloacae* cephalosporinase is a basic protein whose isoelectric point is 8.4.

**Properties of the purified enzyme.** The pH activity curve for the enzyme with cephalothin as a substrate was determined for a range of pH values from 6.0 to 9.0 in phosphate buffer (pH 6 to 8) and tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 8 to 9). The optimal pH was about 8.5. A temperature of 40°C was optimal for the enzyme activity when cephalothin was used as a substrate.

The Michaelis constant ($K_m$), the maximum rate of hydrolysis (relative $V_{max}$), and the dissociation constant of the enzyme-inhibitor complex ($K_i$) are shown in Table 3. The $K_m$ values and relative $V_{max}$ values were obtained from Lineweaver-Burk plots. The $K_i$ values for various compounds were determined with cephalothin as a substrate.

Higher relative $V_{max}$ values were obtained with cephalothin, cefazolin, and cephaloridine. Cephalexin, cefamandole, cefoperazone, and penicillin G showed lower $V_{max}$ values than the above cephalosporins. Cephamycin derivatives, cefuroxime-type cephalosporins, and penicillins except for penicillin G showed very low $V_{max}$ values and a high affinity to the enzyme, indicating their low $K_i$ values, and therefore they were competitive inhibitors of the enzyme action. Cefsulodin was highly resistant to hydrolysis by the enzyme, but it did not inhibit the enzyme activity.

A number of enzyme inhibitors were tested for their inhibitory effect on the activity of the purified enzyme (Table 4). The enzyme was preincubated in distilled water with each of the inhibitors and ions at the indicated concentrations for 10 min at 30°C, and the remaining enzyme activity was assayed using 100 μM cephalothin as a substrate. The enzyme activity was almost completely inhibited by 0.05 mM iodine, 1 mM mercury ion, 0.1 mM semisynthetic penicillins, 0.1 mM cephamycin derivatives, and 0.1 mM cefuroxime-type cephalosporins. Clavulanic acid (18, 21), CP-45899 (7), and *p*-chloromercuribenzoate had no inhibitory effect on the action of the enzyme even in presence of a 1 mM concentration of drug.

## DISCUSSION

All the β-lactam-resistant *E. cloacae* isolates tested produced detectable amounts of β-lactamase. Three of these resistant strains, GN5797, GN7467, and GN7471, produced only cephalosporinase, and the latter two strains produced a large amount of this enzyme without an inducer. *E. cloacae* GN5797 produced inducible cephalosporinase and required a higher concentration of inducer (2 mg of penicillin G per ml) for the optimal production of the enzyme than previ-

TABLE 3. *Kinetics of hydrolysis of various cephalosporins and penicillins by purified cephalosporinase from E. cloacae GN7471 and resistance levels of E. cloacae GN7471*

| Substrate | $K_m$ (μM) | $K_i$ (μM)[a] | Relative rate of hydrolysis[b] ($V_{max}$) | Drug resistance[c] (μg/ml) |
|---|---|---|---|---|
| Cephaloridine | 570 | — | 100 | 400 |
| Cefazolin | 800 | — | 100 | ≥3,200 |
| Cephalothin | 105 | — | 189 | 800 |
| Cephalexin | 61 | — | 14.3 | ≥3,200 |
| Cefotiam | 100 | — | 29.0 | 200 |
| Cefamandole | 6 | 2.00 | 0.37 | 200 |
| Cefoperazone | 250 | — | 1.25 | 6.3 |
| Cefuroxime | — | 0.18 | <0.01 | 200 |
| Cefotaxime | — | 0.05 | <0.01 | 25 |
| Ceftizoxime | — | 1.15 | <0.01 | 50 |
| Cefsulodin | — | — | <0.01 | 100 |
| Cefoxitin | — | 0.50 | 0.30 | 400 |
| Cefmetazole | — | 0.64 | 0.20 | 200 |
| Moxalactam | — | 0.81 | <0.10 | 25 |
| Penicillin G | 10 | — | 12.4 | ≥3,200 |
| Ampicillin | — | 15.00 | <0.10 | 800 |
| Carbenicillin | — | 0.48 | <0.10 | 100 |
| Methicillin | — | 0.02 | <0.10 | 1600 |
| Cloxacillin | — | 1.65 | <0.10 | 200 |

[a] $K_i$ values were determined using cephalothin as a substrate.
[b] Rates of hydrolysis of 19 substrates are expressed as the percentage of cephaloridine hydrolysis.
[c] Resistance to drugs is expressed as the minimum inhibitory concentration of each drug.

TABLE 4. *Effect of inhibitors and ions on the activity of cephalosporinase from E. cloacae GN7471*

| Innibitor (concn) | Inhibition (%)[a] |
|---|---|
| Iodine (0.05 mM) | 100 |
| $Mg^{2+}$ (1 mM) | 23 |
| $Zn^{2+}$ (1 mM) | 79 |
| $Co^{2+}$ (1 mM) | 50 |
| $Hg^{2+}$ (1 mM) | 100 |
| Cloxacillin (0.1 mM) | 100 |
| Methicillin (0.1 mM) | 100 |
| Carbenicillin (0.1 mM) | 100 |
| Ampicillin (0.1 mM) | 87 |
| Cefoxitin (0.1 mM) | 96 |
| Cefmetazole (0.1 mM) | 99 |
| Moxalactam (0.1 mM) | 99 |
| Cefuroxime (0.1 mM) | 100 |
| Cefotaxime (0.1 mM) | 100 |
| Ceftizoxime (0.1 mM) | 96 |
| Cefamandole (0.1 mM) | 91 |
| Cefoperazone (0.1 mM) | 50 |
| Cefsulodin (0.1 mM) | 8 |

[a] Cephalothin (100 μM) was used as a substrate. The relative rate of β-lactamase activity with inhibitor as compared to that without inhibitor is shown; 100 means the activity of the enzyme was not detected under the conditions of these experiments (see the results in the text).

ously reported by other workers (12, 13). From their substrate profiles, all the cephalosporinases from these strains can be considered to be the same as the typical enzyme according to the classification of Sawai et al. (24).

The enzyme was purified about 140-fold on the basis of the specific activity from *E. cloacae* GN7471 to apparent homogeneity (acrylamide gel electrophoresis) and had a specific activity of 595 U/mg of protein with cephalothin and a molecular weight of approximately 44,000. The molecular weight of the enzyme from *E. cloacae* GN7471 is close to those of β-lactamases from *E. cloacae* strains P99 and 214 and larger than those of other β-lactamases, including penicillinases from other gram-negative bacteria (26). The values of the molecular weights of purified penicillinases mediated by R plasmids, except for R-1818 (molecular weight, 44,600), are between 20,000 and 26,000. The species-specific cephalosporinases purified from *Pseudomonas aeruginosa* GN918 (29) and *Proteus morganii* 1510 (8) had molecular weights of 34,000 and 38,000, respectively (8, 24).

Sykes and Matthew (26) and Letarte et al. (16) reported that the isoelectric points of the cephalosporinases from *E. cloacae* strains P99, 214, and MULB250 were 7.5, 7.6 and 9.4, respectively, whereas the isoelectric point of the cephalosporinase from *E. cloacae* GN7471 was 8.4. This difference in values is due to the use of different methods for measuring the isoelectric point, as has been pointed out by Barthelemy et al. (1) and Couillard et al. (3).

Other properties (optimal pH, optimal temperature, inhibition by *p*-chloromercuribenzoate, and substrate profile) were quite similar to those of cephalosporinases from *E. cloacae* P99 and 214 (12, 13).

It has been reported by numerous investigators (2, 6, 10, 20, 24, 29, 30) that semisynthetic penicillins, particularly methicillin and cloxacillin, not only are resistant to hydrolysis by cephalosporinase but are strong inhibitors of the enzyme activity. These semisynthetic penicillins also inhibited the cephalosporinase of *E. cloacae* GN7471 in a competitive manner, with very low $K_i$ values. The same situation was observed with cephamycin derivatives such as cefoxitin, cefmetazole, and moxalactam and with cefuroxime-type cephalosporins such as cefuroxime, cefotaxime, and ceftizoxime. The $K_i$ values of these cephamycin derivatives and cefuroxime-type cephalosporins were two to three orders of magnitude lower than the $K_m$ values of cephalosporins such as cephaloridine, cephalothin, and others. These results suggest that cephamycin derivatives, cefuroxime-type cephalosporins, and some semisynthetic penicillins exert a synergistic effect when they are present along with cephalosporins which were hydrolyzed by cephalosporinase from *E. cloacae*. However, a preliminary experiment showed the unexpected result that cefoxitin exerted an antagonistic effect on the growth of *E. cloacae* GN5797 when present with cefoperazone, whereas methicillin had a synergistic effect. From these observations and from the low antibacterial activity of cephamycin derivatives (27), the resistance of *E. cloacae* to β-lactam antibiotics cannot be attributed solely to β-lactamase, but it seems likely that the contribution of β-lactamase to the resistance of *Enterobacter* strains to β-lactam antibiotics is one of the factors included in intrinsic resistance.

## LITERATURE CITED

1. **Barthelemy, M., M. Guionie, and R. Labia.** 1978. Beta-lactamases: determination of their isoelectric points. Antimicrob. Agents Chemother. **13**:695–698.
2. **Cole, M., S. Elson, and P. D. Fulbrook.** 1972. Inhibition of the β-lactamases of *Escherichia coli* and *Klebsiella aerogenes* by semi-synthetic penicillins. Biochem. J. **127**:295–308.
3. **Couillard, M., R. Letarte, J.-C. Pechere, and C. Morin.** 1980. Need of a combination of methods for identifying beta-lactamases: the case of *Enterobacter*, p. 764–766. *In* J. D. Nelson and C. Grassi (ed.), Current chemotherapy and infectious disease, vol. 1. American Society for Microbiology, Washington, D.C.
4. **Davis, B. T.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404–427.

5. **Egawa, R., T. Sawai, and S. Mitsuhashi.** 1967. Drug resistance of enteric bacteria. XII. Unique substrate specificity of penicillinase produced by R factor. Jpn. J. Microbiol. **11**:173–178.
6. **Fleming, P. C., M. Goldner, and D. G. Glass.** 1969. Inhibition of *Aerobacter* cephalosporin β-lactamase by penicillins. J. Bacteriol. **98**:394–397.
7. **Fu, K. P., and H. Neu.** 1979. Comparative inhibition of β-lactamases by novel β-lactam compounds. Antimicrob. Agents Chemother. **15**:171–176.
8. **Fujii-Kuriyama, Y., M. Yamamoto, and S. Sugasawa.** 1977. Purification and properties of beta-lactamase from *Proteus morganii*. J. Bacteriol. **131**:726–734.
9. **Goldner, M., D. G. Glass, and P. C. Fleming.** 1968. Characteristics of *Aerobacter* beta-lactamase. Can. J. Microbiol. **14**:139–145.
10. **Hamilton-Miller, J. M. T.** 1963. Inducible penicillinase in *Proteus morganii*. Biochem. Biophys. Res. Commun. **13**:43–48.
11. **Hamilton-Miller, J. M. T.** 1967. Hydrolysis of cephalosporins by β-lactamases from gram-negative bacteria. Nature (London) **214**:1333–1334.
12. **Hennessey, T. D.** 1967. Inducible β-lactamase in *Enterobacter*. J. Gen. Microbiol. **49**:277–285.
13. **Hennessey, T. D., and M. H. Richmond.** 1968. The purification and some properties of a β-lactamase (cephalosporinase) synthesized by *Enterobacter cloacae*. Biochem. J. **109**:469–473.
14. **Hirai, K., S. Iyobe, M. Inoue, and S. Mitsuhashi.** 1980. Purification and properties of a new β-lactamase from *Pseudomonas cepacia*. Antimicrob. Agents Chemother. **17**:355–358.
15. **Jack, G. W.** 1971. The purification and some properties of a β-lactamase sensitive to inhibition by *p*-chloromercuribenzoate. Biochim. Biophys. Acta **250**:428–436.
16. **Letarte, R., M. Devaud-Felix, J. C. Pechere, and D. A. Leprohon.** 1977. Enzymatic and immunological characterization of a new cephalosporinase from *Enterobacter aerogenes*. Antimicrob. Agents Chemother. **12**: 201–205.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
18. **Neu, H. G., and K. P. Fu.** 1978. Clavulanic acid, a novel inhibitor of β-lactamases. Antimicrob. Agents Chemother. **14**:375–377.
19. **Novick, R. P.** 1962. Micro-iodometric assay for penicillinase. Biochem. J. **83**:236–240.
20. **O'Callaghan, C. H., and P. W. Muggleton.** 1967. The action of cephaloridine with cloxacillin or methicillin against β-lactamase-producing gram-negative bacteria. J. Gen. Microbiol. **48**:449–460.
21. **Reading, C., and M. Cole.** 1977. Clavulanic acid, a beta-lactamase-inhibiting beta-lactam from *Streptomyces clavuligerus*. Antimicrob. Agents Chemother. **11**:852–857.
22. **Richmond, M. H., and R. B. Sykes.** 1973. The β-lactamases of gram-negative bacteria and their possible physiological role. Adv. Microb. Physiol. **9**:31–88.
23. **Ross, G. W., and M. G. Boulton.** 1973. Purification of β-lactamase on DEAE-Sephadex. Biochim. Biophys. Acta **309**:430–439.
24. **Sawai, T., S. Mitsuhashi, and S. Yamagishi.** 1968. Drug resistance of enteric bacteria. XIV. Comparison of β-lactamases in gram-negative rod bacteria resistant to α-aminobenzyl-penicillin. Jpn. J. Microbiol. **12**:423–434.
25. **Sawai, T., K. Takahashi, S. Yamagishi, and S. Mitsuhashi.** 1970. Variant of penicillinase mediated by an R factor in *Escherichia coli*. J. Bacteriol. **104**:620–629.
26. **Sykes, R. B., and Matthew.** 1976. The β-lactamases of gram-negative bacteria and their role in resistance to β-lactam antibiotics. J. Antimicrob. Chemother. **2**:115–157.
27. **Une, T., and S. Mitsuhashi.** 1977. Antimicrobial evaluation of cefoxitin: a new semisynthetic cephamycin. Comparative study with cefazolin and cephalothin. Arzneim. Forsch. **27**:89–93.
28. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determination by dodecyl sulfate polyacrylamide gel electrophoresis. J. Biol. Chem. **244**: 4406–4412.
29. **Yaginuma, S., T. Sawai, H. Ono, S. Yamagishi, and S. Mitsuhashi.** 1973. Biochemical properties of a cephalosporin β-lactamase from *Pseudomonas aeruginosa*. Jpn. J. Microbiol. **17**:141–149.
30. **Yaginuma, S., T. Sawai, S. Yamagishi, and S. Mitsuhashi.** 1974. β-Lactamase formation and resistance of *Proteus morganii* to various penicillins. Jpn. J. Microbiol. **18**:113–118.