ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1987, p. 865–869
0066-4804/87/060865-05$02.00/0

Vol. 31, No. 6

Paradoxical Antibacterial Activity of Cefmenoxime against *Proteus vulgaris*

YASUSHI IKEDA,†* TAKESHI NISHINO, AND TERUO TANINO

Department of Microbiology, Kyoto Pharmaceutical University, Yamashina, Kyoto, 607, Japan

Received 24 October 1986/Accepted 5 March 1987

The growth-inhibitory effect of cefmenoxime against *Proteus vulgaris* was studied by using the broth dilution and paper disk diffusion methods. Cefmenoxime showed growth-inhibitory activity against *Proteus vulgaris* at low concentrations but not at high concentrations up to a certain limit. This paradoxical antibacterial activity was not observed with cefoperazone and cefbuperazone. The induction of β-lactamase by cefmenoxime and the rate of hydrolysis of cefmenoxime in the culture broth were proportional to the initial concentration of this antibiotic. At high initial concentrations, cefmenoxime was rapidly inactivated. On the other hand, neither cefoperazone nor cefbuperazone was inactivated irrespective of concentration. We conclude that cefmenoxime induces β-lactamase in *P. vulgaris*, perhaps accounting for its paradoxical antibacterial effect.

Cefmenoxime (CMX) is one of the new type of cephalosporins which have excellent antibacterial activity against gram-positive and gram-negative bacteria including *Proteus vulgaris* (10). However, like cefotaxime and cefotiam, CMX is comparatively unstable to β-lactamase produced by *P. vulgaris* (10).

In 1948, Eagle and Musselman (2) reported that the antibacterial activity of penicillin G against gram-positive cocci was paradoxically reduced as its concentration was increased. Similar phenomena against gram-positive cocci have been reported by others (9, 16). This effect is generally known as the "Eagle effect." Regarding gram-negative bacteria, a paradoxical effect of mecillinam against *Providencia stuartii* was reported in 1976 (4). However, the mechanism of this paradoxical effect has not been explained sufficiently.

In studying the antibacterial activity of β-lactam antibiotics, we observed an interesting phenomenon in *P. vulgaris*: whereas growth was found in the presence of CMX at high concentrations, no growth occurred at low concentrations.

This paper deals with the paradoxical effect of CMX on *P. vulgaris*.

MATERIALS AND METHODS

Antibiotics. CMX, prepared by Takeda Pharmaceutical Co., Ltd., Osaka, Japan, and cefoperazone (CPZ) and cefbuperazone (CBPZ), both prepared by Toyama Chemical Co., Ltd., Tokyo, Japan, were used as test antibiotics.

Bacterial strains. The strains of *P. vulgaris* used in this study were all derived from clinical materials obtained in Japan.

Paradoxical effect of drugs. (i) Broth dilution method. A series of tubes containing doubling dilutions of antibiotics in sensitivity broth (Eiken Chemical Co., Ltd., Tokyo, Japan) were inoculated with *P. vulgaris* at an inoculum size of 10^6 cells per ml. The tubes were incubated at 37°C for 18 h, and visible growth was observed.

(ii) Paper disk method. Sensitivity disk agar (Eiken Chemical Co., Ltd.) molten at 45°C was inoculated with organisms at 10^6 cells per ml. The medium was poured into 90-mm petri dishes. The paper disks, containing 60 μg of antibiotics, were placed centrally on the test plates. After 1 h of diffusion at 4°C, the plates were incubated at 37°C for 18 h, and the inhibition zones were observed.

Residual activity of antibiotics in cultures of *P. vulgaris* 11. A series of tubes containing doubling dilutions of each antibiotic in sensitivity broth were inoculated with *P. vulgaris* 11 at 10^6 cells per ml and incubated at 37°C. After 1, 3, and 5 h of incubation, cell suspensions (0.5 ml) were obtained. Then the β-lactamase was inactivated by the addition of 0.5 ml of methanol. The samples were stored at −20°C until bioassay. The assay of the antibiotic was performed in triplicate by the paper disk diffusion method. The standard curves were constructed with 0.05 M phosphate buffer (pH 7.0) containing methanol (1:1) as the diluent. The test organisms were *Proteus mirabilis* ATCC 21100 for CMX, *Micrococcus luteus* ATCC 9341 for CPZ, and *Klebsiella pneumoniae* ATCC 10031 for CBPZ.

Preparation of β-lactamase. An overnight culture in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) was diluted 10-fold with the same medium and incubated with shaking at 37°C for 2 h. Then 10 μg of cefmetazol per ml was added as a β-lactamase inducer. The incubation was continued with shaking for 2 h, and the bacterial cells were harvested by centrifugation at 4°C, washed twice with 0.05 M phosphate buffer (pH 7.0), and suspended in the same buffer. The cell suspension was treated with an ultrasonic disrupter (Tomy Seiko, model UR-200P) for 5 min in an ice bath. After centrifugation at 100,000 × *g* for 60 min at 4°C, the β-lactamase was purified on a CM-Sephadex C-50 (Pharmacia, Uppsala, Sweden) column as previously described (3).

Assay of β-lactamase. β-Lactamase activity was determined by the spectrophotometric method (11). The amount of enzyme that hydrolyzed 1 μmol of the substrate in 1 min at 30°C in 0.05 M phosphate buffer (pH 7.0) was defined as 1 unit of enzyme activity. The maximum rate of hydrolysis (relative V_{max}) and the affinity for the enzyme (K_m or K_i) were determined by a Lineweaver-Burk plot (5). For determination of K_i, cephalothin was used as the substrate.

Induction of β-lactamase. The β-lactamase-inducing activity was determined by the following method (8). An over-

* Corresponding author.
† Present address: Research Laboratory, Toyama Chemical Co., Ltd., 2-4-1, Shimookui, Toyama, 930, Japan.

TABLE 1. Growth-inhibitory effects of CMX, CPZ, and CBPZ against various strains of *P. vulgaris*

P. vulgaris strain	Antibiotic	Effect[a] at given concn (μg/ml) of antibiotic															
		100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	0.05	0.025	0.013	0.006	0
11	CMX	−	++	++	++	+	+	−	−	−	+	++	++	++	++	++	++
	CPZ	−	−	−	−	−	−	−	−	++	++	++	++	++	++	++	
	CBPZ	−	−	−	−	−	−	−	−	−	+	++	++	++	++	++	
25	CMX	−	−	−	−	−	−	−	−	−	−	−	++	−	++	++	++
	CPZ	−	−	−	−	−	−	−	−	−	+	++	++	++	++	++	
	CBPZ	−	−	−	−	−	−	−	−	−	−	+	++	++	++	++	
26	CMX	−	−	−	−	+	+	+	+	+	+	+	+	++	++	++	++
	CPZ	−	−	−	−	−	−	−	+	+	+	++	++	++	++	++	
	CBPZ	−	−	+	+	+	+	+	+	+	+	+	+	++	++	++	
113	CMX	−	++	++	−	−	−	−	−	++	++	++	++	++	++	++	++
	CPZ	−	−	−	−	−	−	−	+	+	++	++	++	++	++	++	
	CBPZ	−	−	−	−	−	−	−	−	+	+	++	++	++	++	++	
115	CMX	−	−	−	−	++	++	++	++	−	−	−	++	++	++	++	++
	CPZ	−	−	−	−	−	−	++	++	++	++	++	++	++	++	++	
	CBPZ	−	−	−	−	−	−	−	−	−	−	++	++	++	++	++	
120	CMX	−	−	−	−	−	++	−	++	−	+	++	++	++	++	++	++
	CPZ	−	−	−	−	−	−	+	+	+	+	+	+	++	++	++	
	CBPZ	−	−	−	−	−	−	−	−	−	+	+	+	++	++	++	

[a] ++, Good growth; +, growth; −, no growth.

night culture of *P. vulgaris* in sensitivity broth was diluted 20-fold with the same medium and incubated with shaking at 37°C for 2 h. Then various concentrations of CMX, CPZ, and CBPZ were added as the inducers. The incubation was continued. After 1 h of incubation, the cells were harvested and washed twice with 0.05 M phosphate buffer (pH 7.0), using a centrifuge. The cells were treated with an ultrasonic disrupter for 5 min in an ice bath and centrifuged at 15,000 × *g* for 30 min at 4°C. β-Lactamase activity was determined with cephaloridine as the substrate. The protein concentration of β-lactamase was determined by the method of Lowry et al. (6), using bovine serum albumin as the standard.

RESULTS

Effects of CMX, CPZ, and CBPZ on growth of *P. vulgaris*. Effects of antibiotics on the growth of six strains of *P. vulgaris* are shown in Table 1. CMX inhibited the growth of *P. vulgaris* 11 at comparatively low concentrations from 0.39 to 1.56 μg/ml. However, at higher concentrations, 3.13 to 50 μg/ml, CMX was not inhibitory. CMX did inhibit growth at 100 μg/ml, however. This paradoxical antibacterial effect of CMX was observed in other strains of *P. vulgaris* as well. On the other hand, neither CPZ nor CBPZ showed such a paradoxical effect in any of the *P. vulgaris* strains tested.

The above paradoxical effect of CMX was confirmed by the paper disk assay. The inhibition zone of CMX against *P. vulgaris* 11 is shown in Fig. 1. CMX produced a clear double inhibition zone against *P. vulgaris* 11. At lower concentrations, the zone was far from the disk; CMX inhibited the growth. However, at higher concentrations, the zone was near the disk; CMX did not inhibit the growth.

Residual activity in the culture of *P. vulgaris* 11. The residual activities of CMX, CPZ, and CBPZ in the culture broths of *P. vulgaris* 11 are shown in Fig. 2, 3, and 4, respectively. CMX was rapidly inactivated at high initial doses of 25 to 100 μg/ml. However, when the drug concentration was decreased, the inactivation slowed down. When 0.39 and 0.2 μg of CMX per ml were added to cultures of *P. vulgaris* 11, the initial concentration levels were maintained well for 5 h after initiation of incubation. On the other hand, CPZ and CBPZ were not inactivated at any concentration tested, and the residual activities of these agents did not decrease for 5 h following the start of incubation.

Stability and affinity for the β-lactamase produced by *P. vulgaris* 11. The relative rate of hydrolysis and the affinity for β-lactamase were determined. Table 2 shows the relative V_{max} and K_m or K_i of these three drugs for the purified β-lactamase preparation of *P. vulgaris* 11. Of the three substrates, CMX was the least stable to this β-lactamase, showing a relative V_{max} of 125, with that of cephaloridine

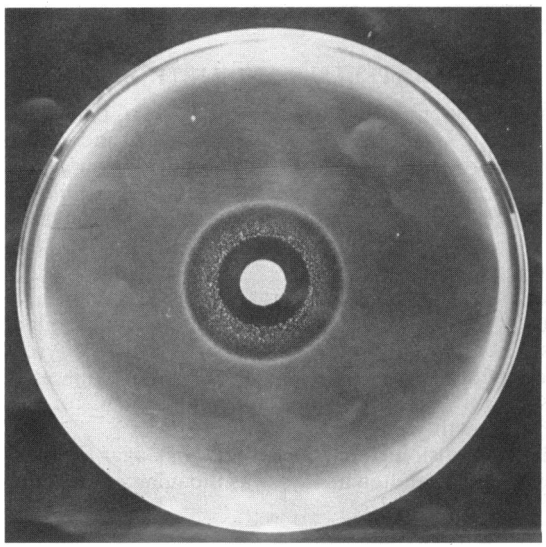

FIG. 1. Inhibition zone of CMX against *P. vulgaris* 11. The paper disk contained 60 μg of CMX.

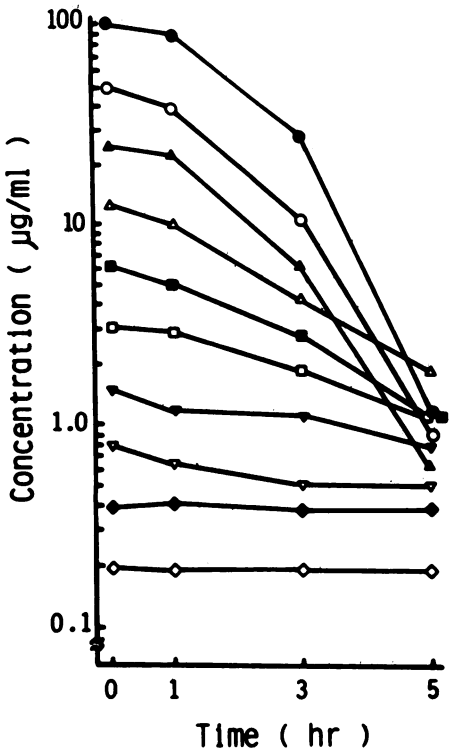

FIG. 2. Residual activity of CMX in *P. vulgaris* 11 culture. Initial amounts of CMX were 100 μg/ml (●); 50 μg/ml (○); 25 μg/ml (▲); 12.5 μg/ml (△); 6.25 μg/ml (■); 3.13 μg/ml (□); 1.56 μg/ml (▼); 0.78 μg/ml (▽); 0.39 μg/ml (◆); and 0.20 μg/ml (◇).

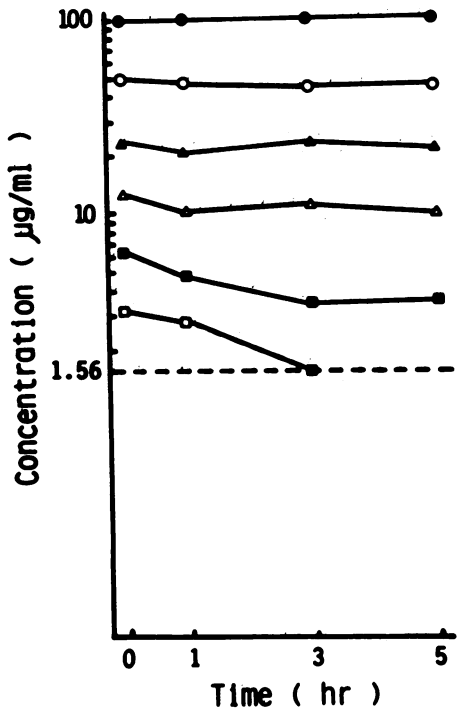

FIG. 3. Residual activity of CPZ in *P. vulgaris* 11 culture. Initial amounts of CPZ were 100 μg/ml (●); 50 μg/ml (○); 25 μg/ml (▲); 12.5 μg/ml (△); 6.25 μg/ml (■); 3.13 μg/ml (□); 1.56 μg/ml (▼); 0.78 μg/ml (▽); 0.39 μg/ml (◆); and 0.20 μg/ml (◇).

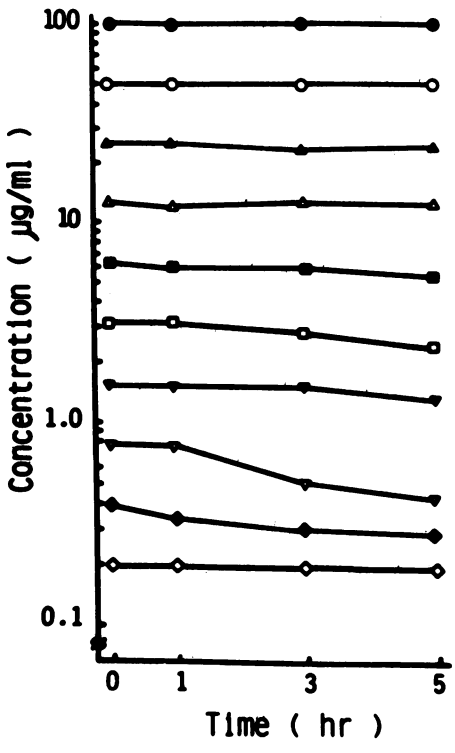

FIG. 4. Residual activity of CBPZ in *P. vulgaris* 11 culture. Initial amounts of CBPZ were 100 μg/ml (●); 50 μg/ml (○); 25 μg/ml (▲); 12.5 μg/ml (△); 6.25 μg/ml (■); 3.13 μg/ml (□); 1.56 μg/ml (▼); 0.78 μg/ml (▽); 0.39 μg/ml (◆); and 0.20 μg/ml (◇).

being 100. However, CMX showed the lowest affinity for the β-lactamase, with a K_m of 400 μmol. On the other hand, CPZ and CBPZ showed higher affinities for the β-lactamase, but CPZ showed moderate stability and CBPZ showed high stability.

Induction of β-lactamase. The β-lactamase-inducing activities of the drugs in culture broths were examined (Table 3). CMX had the highest inducer activity for β-lactamase formation, with CBPZ second. The maximum enzyme activity with CMX was 2.4 U/mg of protein at 100 μg/ml. In contrast, CPZ showed the lowest β-lactamase-inducing action, with a maximum of 0.14 U/mg of protein. CMX and CBPZ caused dose-dependent β-lactamase induction, but the inducer activity of CBPZ was suppressed at higher concentrations.

DISCUSSION

The antibacterial activity of a drug is generally proportional to its concentration. However, Eagle and Musselman

TABLE 2. Stability and affinity of CMX, CPZ, and CBPZ for β-lactamase of *P. vulgaris* 11

Antibiotic	Relative rate of hydrolysis (%)[a]	Affinity for enzyme (μmol)[b]
CMX	125	400 (K_m)
CPZ	4	8 (K_m)
CBPZ	<0.01	40 (K_i)

[a] The value for cephaloridine is taken as 100%. The specific activity of cephaloridine was 524 μmol/min per mg of protein.

[b] Represented by K_m or K_i value. For the determination of K_i, cephalothin was used as the substrate.

TABLE 3. Inducer activity of CMX, CPZ, and CBPZ for β-lactamase formation in *P. vulgaris* 11

Antibiotic	β-Lactamase activity (U/mg of protein)[a] at given antibiotic concn (μg/ml)						
	100	25	6.25	1.56	0.39	0.10	0
CMX	2.43	1.86	1.68	0.35	0.11	0.02	<0.01
CPZ	0.14	0.11	0.05	<0.01	<0.01	<0.01	
CBPZ	0.46	0.92	1.56	0.82	0.34	<0.01	

[a] Enzyme activity was determined with cephaloridine as the substrate.

(2) observed that the activity of penicillin G against gram-positive cocci such as *Staphylococcus* and *Streptococcus* spp. fell off as its concentration was increased. A similar phenomenon was noted with ampicillin against *Enterococcus faecalis* (16). However, these authors fail to offer a cogent account of the mechanism involved and, for that matter, do not suggest the role of β-lactamase in the phenomenon (2, 9, 16). On the other hand, few studies have explored the paradoxical antibacterial effect in gram-negative bacilli (4).

In this study, we observed a visually conspicuous paradoxical antibacterial effect in *P. vulgaris*. CMX showed growth-inhibitory activity at lower concentrations but not at high concentrations up to a certain limit. However, this paradoxical effect was not observed within 3 or 5 h of incubation, like the results of Shah et al. (12) (data not shown). In the culture broth, the amount of inactivated CMX and the induced β-lactamase activity were proportional to the concentration of CMX initially added. When a large amount of CMX was added to the culture broth, the β-lactamase activity was increased and the residual activity of CMX was rapidly decreased. In contrast, at lower concentrations, CMX was not inactivated in 5 h of incubation in the culture broth. Therefore, the inactivation of CMX in the culture broth led to regrowth, and CMX showed paradoxical antibacterial activity as a result. On the other hand, neither CBPZ nor CPZ showed this paradoxical effect or was inactivated in the culture broth. This is because CBPZ is highly stable to β-lactamase while CPZ is significantly stable to β-lactamase and, in addition, has a lower β-lactamase-inducing action.

Minami et al. (7) previously reported that, while carbenicillin and sulbenicillin were stable to purified β-lactamase of *P. vulgaris* in a cell-free experiment, they were inactivated in the culture broth because of their high β-lactamase-inducing activities. In our experiments, CMX caused dose-dependent and higher induction of β-lactamase. Moreover, CMX was not as stable to β-lactamase in cell-free experiments. These results appear to account for the observed paradoxical antibacterial effect.

New parenteral cephems such as CMX, cefotaxime, and ceftizoxime, which contain an aminothiazolyl-oxyimino group in their 7-acyl side chains, have expanded antibacterial spectra against gram-positive and gram-negative bacteria including *P. vulgaris*. However, they have relatively lower stability against β-lactamase in *P. vulgaris*. In this study, we used three types of new cephems. All of them have excellent activities against gram-positive and gram-negative organisms but are varied in stability and β-lactamase-inducing action. CPZ has low inducibility for β-lactamase in various organisms (7, 15). CBPZ is one of the cephamycin β-lactams and is highly stable to various types of β-lactamase (14). CMX, a typical aminothiazolyl cephalosporin, is stable to various types of β-lactamase, but unstable to the β-lactamase produced by *P. vulgaris*. According to our results, CMX induced elevated levels of β-lactamase. This increased induction of β-lactamase and the low stability are associated with the paradoxical antibacterial activity. These results suggest that β-lactams which show paradoxical antibacterial activity are those having high β-lactamase-inducing activity and low stability to this enzyme.

The β-lactamase produced in *P. vulgaris* 11 was classified as a typical Richmond-Sykes class Ic β-lactamase from the results of substrate specificity (data not shown) and was not a peculiar β-lactamase.

Eagle and Musselman had noticed in an animal infection model that the paradoxical antibacterial effect was also observed in vivo (1). It would be interesting to explore whether the paradoxical effect on *P. vulgaris* is also observed in vivo.

LITERATURE CITED

1. **Eagle, H., R. Fleischman, and M. Levy.** 1953. "Continuous" vs. "discontinuous" therapy with penicillin. The effect of interval between injections on therapeutic efficacy. N. Engl. J. Med. **248**:481–488.
2. **Eagle, H., and A. D. Musselman.** The rate of bactericidal action of penicillin in vitro as a function of its concentration, and its paradoxically reduced activity at high concentration against certain organisms. J. Exp. Med. **88**:99–131.
3. **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.
4. **Kerry, D. W., J. Hamilton-Miller, and W. Brunfitt.** 1976. Paradoxical effect of mecillinam on *Providencia stuartii*. J. Antimicrob. Chemother. **2**:386–388.
5. **Lineweaver, H., and D. Burk.** 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56**:658–666.
6. **Lowry, O. H., J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
7. **Minami, S., N. Matsubara, A. Yotsuji, H. Araki, Y. Watanabe, T. Yasuda, I. Saikawa, and S. Mitsuhashi.** 1983. Induction of cephalosporinase production by various penicillins in Enterobacteriaceae. J. Antibiot. **36**:1387–1395.
8. **Minami, S., A. Yotsuji, M. Inoue, and S. Mitsuhashi.** 1980. Induction of β-lactamase by various β-lactam antibiotics in *Enterobacter cloacae*. Antimicrob. Agents Chemother. **18**:382–385.
9. **Nishino, T., and S. Nakazawa.** 1976. Bacteriological study on effects of beta-lactam group antibiotics in high concentration. Antimicrob. Agents Chemother. **9**:1033–1042.
10. **Okonogi, K., M. Kuno, M. Kida, and S. Mitsuhashi.** 1981. β-Lactamase stability and antibacterial activity of cefmenoxime (SCE-1365), a novel cephalosporin. Antimicrob. Agents Chemother. **20**:171–175.
11. **Samuni, A.** 1975. A direct spectrophotometric assay and determination of Michaelis constants for the β-lactamase reaction. Anal. Biochem. **63**:17–26.
12. **Shah, P. M., G. Troche, and W. Stille.** 1979. Effect of concentration on bactericidal activity of cefotaxime. J. Antimicrob. Chemother. **5**:419–422.
13. **Shimpson, I. N., S. J. Plested, and P. B. Harper.** 1982. Investigation of the β-lactamase stability of ceftazidime and eight other new cephalosporin antibiotics. J. Antimicrob. Chemother. **9**:357–368.
14. **Tai, M., Y. Fukuoka, A. Yotsuji, K. Kumano, M. Takahata, H.**

Mikami, T. Yasuda, I. Saikawa, and S. Mitsuhashi. 1982. In vitro and in vivo antibacterial activity of T-1982, a new semisynthetic cephamycin antibiotic. Antimicrob. Agents Chemother. **22:** 728–734.

15. **Yotsuji, A., S. Minami, Y. Araki, M. Inoue, and S. Mitsuhashi.** 1982. Inducer activity of β-lactam antibiotics for the β-lactamase of *Proteus rettgeri* and *Proteus vulgaris*. J. Antibiot. **35:**1590–1593.
16. **Yourassowsky, E., M. P. Vanderlinder, and E. Schoutens.** 1976. A rapid simple method for demonstrating synergy of amikacin and penicillin against various microorganisms. J. Infect. Dis. **134:**275–277.