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In Vitro and In Vivo Antibacterial Activities of CS-807, a New Oral Cephalosporin

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CS-807 is a new oral prodrug of R-3746, a cephalosporin derivative, with potent in vitro and in vivo antibacterial activity against both gram-positive and gram-negative bacteria. The susceptibility of about 1,200 clinical isolates to R-3746 was determined by the agar dilution method. Ninety percent or more of pathogens such as *Staphylococcus aureus*, streptococci, *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, indole-positive and indole-negative *Proteus* spp., *Providencia rettgeri*, and *Haemophilus influenzae* were inhibited at concentrations ranging from ≤0.01 to 1.56 μg/ml. Furthermore, at a concentration of 3.13 μg/ml, 50% or more of *Staphylococcus epidermidis*, *Morganella morganii*, *Citrobacter freundii*, and *Serratia marcescens* strains were also inhibited. *Pseudomonas aeruginosa* and *Xanthomonas maltophilia* were resistant to R-3746. The activity of R-3746 was scarcely influenced by several growth conditions. R-3746 was highly resistant to hydrolysis by β-lactamases derived from various species of bacteria. Killing-curve studies demonstrated bactericidal activity of R-3746 at concentrations above the MIC. R-3746 showed high affinity for penicillin-binding proteins 1, 3, and 4 of *Staphylococcus aureus* and 1A, 1Bs, and 3 of *Escherichia coli*. Systemic infections in mice caused by various pathogens, including β-lactamase-producing strains, responded well to therapy with oral doses of CS-807.

Various oral cephem antibiotics having relatively broad antibacterial spectra have been developed and used worldwide (2, 3, 15, 18, 19, 34). The effectiveness, however, of the oral antibiotics in the treatment of bacterial infections is somewhat limited because of their poor activity against gram-negative bacilli, in particular against opportunistic pathogens belonging to the *Enterobacteriaceae* or *Pseudomonadaceae*.

Recently, new oral cephem antibiotics such as cefixime (7), T-2588 (T. Yasuda, A. Yotsuji, S. Okamoto, and S. Mitsuhashi, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 224, 1984), and ME-1207 (M. Inoue, A. Tamura, T. Yoshida, R. Okamoto, K. Atsumi, K. Nishihata, and S. Mitsuhashi, 25th ICAAC, abstr. no. 582, 1985) have been developed, and clinical efficacy studies of cefixime and T-2588 are in progress. Those compounds have the characteristics of much higher in vitro and in vivo antibacterial activity than any other oral antibiotics, such as cephalexin, cefadroxil, cefroxadine, cefradine, cefatrizine, and cefaclor, against gram-negative bacteria, including opportunistic pathogens, and of enhanced resistance to various types of β-lactamase.

CS-807 is a new oral prodrug of R-3746; its proper chemical name is 1-(isopropoxycarbonyloxy)ethyl(6*R*, 7*R*)-7-[2-(2-amino-4-thiazolyl)-(*Z*)-2-(methoxyimino)acetamido]-3-methoymethyl-8-oxo-5-thia-1-azabicyclo[4,2,0]-oct-2-ene-2-carboxylate. Chemical structures of CS-807 and R-3746 are shown in Fig. 1.

The present report deals with the in vitro antibacterial activity of R-3746 together with the therapeutic effect of CS-807 on systemic infections in mice compared with cefaclor, amoxicillin, cefixime, and T-2588 (T-2525).

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MATERIALS AND METHODS

Chemicals. CS-807 and R-3746 were prepared by Sankyo Co., Ltd., Tokyo, Japan. The other antibiotics were gifts: penicillin G, amoxicillin, and methicillin from Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; cephaloridine from Nihon Glaxo Co., Ltd., Tokyo, Japan; cefaclor from Shionogi & Co., Ltd., Osaka, Japan; cefixime from Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan; and T-2588 and T-2525 from Toyama Chemical Co., Ltd., Tokyo, Japan. All antibiotics were prepared daily with distilled water to the desired concentrations. ^{14}C-labeled penicillin G (potassium 6-phenyl [1-^{14}C]acetamidopenicillanate; 54 mCi/mmol) was purchased from Amersham International, Buckinghamshire, United Kingdom.

Bacterial strains. Standard strains stocked at our laboratories and clinical isolates of various species collected from several hospitals in a wide area of Japan were used.

Determination of MICs and MBCs. MICs were determined by a twofold serial agar dilution method. As media, unless otherwise specified, sensitivity test broth (STB) and sensitivity disk agar (SDA) (both from Nissui Seiyaku Co., Ltd., Tokyo, Japan) were used for culture and MIC determinations, respectively. For streptococci, defibrinated horse blood (Nippon Bio-Supp. Center, Tokyo Japan) was added to the agar medium to a final concentration of 5%. For *Haemophilus influenzae*, brain-heart infusion broth (BHIB) and agar (BHIA) (both from Difco Laboratories, Detroit, Mich.) supplemented with 10 μg of hemin and 5 μg of β-NAD per ml were used. For *Bacteroides fragilis*, Gifu anaerobic medium broth and agar (Nissui Seiyaku) were used. The other media were STB containing 0.4% potassium nitrate for *Pseudomonas aeruginosa* and BHIB for culture of streptococci. Overnight cultures of test strains were diluted with a buffered saline–0.01% gelatine solution to a final concentration of approximately 10^6 CFU/ml. One spot (10 μl) of the diluted cultures, corresponding to approximately 5

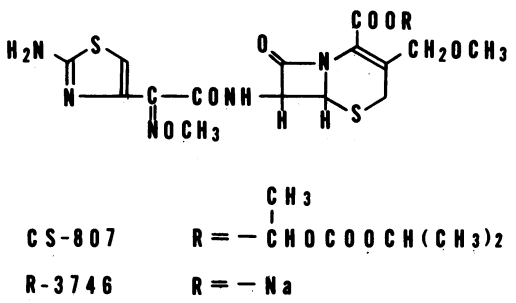

FIG. 1. Chemical structures of CS-807 and R-3746.

$\times 10^4$ CFU, was applied to 10-ml agar layers containing various concentrations of antibiotics with an inoculator (Microplanter; Sakuma Seisakusho, Tokyo, Japan). The plates were incubated at 37°C for 18 h, whereas those to which *Bacteroides fragilis* was spotted were incubated for 24 h at 37°C under anaerobic conditions. The MIC was defined as the lowest concentration of antibiotic which prevented visible growth of bacteria.

MBCs were determined by the twofold dilution method in heart infusion broth (HIB; Eiken Chemical Co., Ltd., Tokyo, Japan) with a final inoculum of 5×10^5 CFU/ml. After 18 h of incubation at 37°C, the lowest drug concentration which allowed no visible growth was defined as the MIC, and the MBC was defined as the lowest concentration which yielded no colony formation after incubation overnight at 37°C on drug-free SDA plates to which one spot (about 10 μl) from clear subcultures after MIC determinations was applied.

Killing-curve studies. Overnight cultures in HIB were diluted 100-fold with fresh HIB, and 9.9-ml portions of the diluted samples were incubated at 37°C for 2 h with shaking until they reached ca. 5×10^5 CFU/ml. Portions (0.1 ml) of antibiotics were added to the cultures at various concentrations around the MIC determined by the broth dilution method. Subcultures were further incubated at 37°C with shaking. Samples (0.1 ml) were removed at 0, 1, 2, 4, 6, and 24 h, diluted 10-fold serially with 0.9% physiological saline solution, and plated on SDA to determine the CFU remaining.

Stability to β-lactamase. Various types of β-lactamase (14) used in this study were purified by the methods reported previously (5, 6, 11–13, 16, 25–27, 31) and stored at −70°C until assay. The enzymatic reaction was carried out with 3 ml of the reaction mixture containing 0.1 mM substrate, 50 mM phosphate buffer at pH 7.0, and an appropriate amount of β-lactamase at 30°C in a water-jacketed spectrophotometer (model 24; Beckman Instruments, Inc., Fullerton, Calif.). β-Lactamase activity was determined by the spectrophotometric method (33). For determining the rate of hydrolysis, the molecular absorbancy difference ($\Delta\varepsilon$) and the specific wavelength for R-3746 were 6.75 M^{-1} cm^{-1} and 265 nm, respectively. For other substrates, the $\Delta\varepsilon$ and specific wavelength were also determined. Substrate specificity was expressed as the relative hydrolysis rate of each substrate, taking the hydrolysis rate of penicillin G for plasmid-mediated penicillinases and that of cephaloridine for chromosomally mediated β-lactamases as 100%.

Assay of PBPs. Preparation of membrane fractions was performed by the methods of Utsui and Yokota (32). The membrane suspension was adjusted to a concentration of 8 and 15 mg of protein per ml for *Staphylococcus aureus* and *Escherichia coli*, respectively. Penicillin-binding proteins (PBPs) were assayed by the method of Spratt (29) with some modifications. Experiments to determine the binding affinity of β-lactam antibiotics for PBPs were performed by preincubation of the membrane fractions with nonradioactive drugs at 30°C for 10 min before addition of ^{14}C-labeled penicillin G. Fluorographs of PBPs were analyzed quantitatively with a densitometer (model CS-910; Shimadzu Corp., Kyoto, Japan), and the I_{50} (concentration required to inhibit ^{14}C-labeled penicillin G binding by 50%) was calculated.

Protective effect. Test organisms for systemic infections in mice were cultured in HIB containing 5% horse serum (*Streptococcus pneumoniae* 2132 and *Streptococcus pyogenes* 1412) or in BHIB (other organisms) at 37°C for 18 h. Subcultures, after being suitably diluted with fresh HIB or BHIB, were suspended in the same amount of 8% gastric mucin (Difco Laboratories), except *E. coli* ML4707. Four-week-old ICR male mice weighing 19 to 21 g were infected intraperitoneally with 0.2-ml portions of bacterial suspension, corresponding to 2 to 10 times the minimal lethal dose. Under these conditions, untreated mice died within 48 h. Immediately after infection, 10 mice for each dose level were treated orally with 0.1 ml of a single-dose regimen (six dose levels or more) of test drugs which were diluted with 0.5% carboxymethylcellulose solution by intragastric tube. The 50% effective dose (ED_{50}) was calculated by the Litchfield-Wilcoxon probit method (22) according to the survival rates at 5 days after infection.

RESULTS

Susceptibility of clinical isolates. The susceptibility of clinical isolates to R-3746 and the reference drugs is listed in Table 1. The lowest concentration of R-3746 at which 90% of the clinical isolates were inhibited (MIC_{90}) ranged from ≤0.01 to 1.56 μg/ml for methicillin-susceptible *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and several members of the *Enterobacteriaceae* such as *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Proteus vulgaris*, *Proteus inconstans*, *Providencia rettgeri*, and *H. influenzae*. Approximately 50% of *Staphylococcus epidermidis*, *Morganella morganii*, *Citrobacter freundii*, and *Serratia marcescens* strains were inhibited at concentrations ranging from 0.39 to 3.13 μg/ml. The MIC_{90}s for these organisms were 50, 12.5, >100, and 25 μg/ml, respectively. R-3746 had rather weak antibacterial activity against *Enterococcus faecalis*, *Enterobacter cloacae*, and *Bacteroides fragilis*. *Pseudomonas aeruginosa* and *Xanthomonas maltophilia* were resistant to R-3746.

The activity of R-3746 was roughly comparable to that of T-2525 against staphylococci, *Streptococcus pyogenes*, and several members of the *Enterobacteriaceae* such as *E. coli*, *K. pneumoniae*, *K. oxytoca*, *Proteus* spp., *S. marcescens*, and *Bacteroides fragilis*. R-3746 exerted much greater activity than cefixime against staphylococci and streptococci, whereas the activity of R-3746 was somewhat less potent than that of cefixime against most of the gram-negative organisms except *Enterobacter cloacae* and *Bacteroides fragilis*. Cefaclor showed activity similar to that of R-3746 against staphylococci, but it had much weaker activity than R-3746 against all gram-negative bacteria tested, in particular indole-positive *Proteus* spp., *Providencia rettgeri*, *Morganella morganii*, *Enterobacter cloacae*, *Serratia marcescens*, and *Bacteroides fragilis*. The activity of amoxicillin against *Enterococcus faecalis* was much better than that of R-3746, whereas amoxicillin, in general, displayed weaker

TABLE 1. Susceptibility of clinical isolates to R-3746

Organism (no. of isolates)	Drug[a]	MIC (μg/ml)		
		Range	50%	90%
Methicillin-susceptible *Staphylococcus aureus* (100)	R-3746	0.78–12.5	1.56	1.56
	CCL	0.78–100	1.56	1.56
	AMPC	0.10–25	0.78	1.56
	CFIX	3.13–>100	12.5	12.5
	T-2525	0.78–25	1.56	3.13
Methicillin-resistant *Staphylococcus aureus* (45)	R-3746	25–>100	>100	>100
	CCL	25–>100	>100	>100
	AMPC	6.25–100	25	50
	CFIX	≥100	>100	>100
	T-2525	25–>100	100	>100
	Methicillin	3.13–>100	12.5	>100
Staphylococcus epidermidis (79)	R-3746	0.10–>100	3.13	50
	CCL	0.05–>100	3.13	25
	AMPC	0.025–100	1.56	12.5
	CFIX	1.56–>100	25	>100
	T-2525	0.10–>100	3.13	50
Streptococcus pyogenes (79)	R-3746	≤001	≤0.01	≤0.01
	CCL	0.025–0.20	0.10	0.10
	AMPC	≤0.01	≤0.01	≤0.01
	CFIX	0.025–0.20	0.10	0.10
	T-2525	≤0.01	≤0.01	≤0.01
Streptococcus pneumoniae (24)	R-3746	0.025–0.39	0.025	0.05
	CCL	0.39–0.78	0.39	0.78
	AMPC	≤0.01–0.78	0.025	0.025
	CFIX	0.20–0.78	0.20	0.20
	T-2525	≤0.01–0.025	≤0.01	≤0.01
Enterococcus faecalis (49)	R-3746	1.56–>100	6.25	>100
	CCL	3.13–100	50	100
	AMPC	0.39–1.56	0.39	1.56
	CFIX	3.13–>100	25	>100
	T-2525	6.25–>100	6.25	>100
Escherichia coli (91)	R-3746	0.05–1.56	0.39	0.78
	CCL	0.39–6.25	1.56	3.13
	AMPC	0.39–>100	3.13	>100
	CFIX	0.025–1.56	0.10	0.39
	T-2525	0.05–0.78	0.20	0.39
Klebsiella pneumoniae (49)	R-3746	0.05–1.56	0.10	0.20
	CCL	0.10–12.5	0.20	0.39
	AMPC	6.25–>100	25	100
	CFIX	≤0.01–0.78	0.025	0.05
	T-2525	0.05–1.56	0.10	0.20
Klebisella oxytoca (50)	R-3746	0.05–3.13	0.10	0.39
	CCL	0.39–>100	0.78	12.5
	AMPC	12.5–>100	50	>100
	CFIX	≤0.01–0.10	≤0.01	0.05
	T-2525	0.025–3.13	0.10	0.78
Proteus mirabilis (54)	R-3746	≤0.01–0.20	0.05	0.10
	CCL	0.39–12.5	1.56	3.13
	AMPC	0.10–>100	0.78	0.78
	CFIX	≤0.01	≤0.01	≤0.01
	T-2525	≤0.01–0.10	0.025	0.05
Proteus vulgaris (54)	R-3746	≤0.01–25	0.05	0.39
	CCL	3.13–>100	100	>100
	AMPC	6.25–>100	100	>100
	CFIX	≤0.01–0.39	≤0.01	0.025
	T-2525	≤0.01–12.5	0.05	0.39
Proteus inconstans (54)	R-3746	≤0.01–6.25	0.05	0.39
	CCL	0.20–>100	12.5	>100
	AMPC	0.39–>100	50	>100
	CFIX	≤0.01–0.78	≤0.01	0.20
	T-2525	≤0.01–12.5	0.10	0.78
Providencia rettgeri (53)	R-3746	≤0.01–1.56	≤0.01	0.39
	CCL	0.10–>100	100	>100
	AMPC	0.10–>100	100	>100
	CFIX	≤0.01–0.78	≤0.01	0.10

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TABLE 1—*Continued*

Organism (no. of isolates)	Drug[a]	MIC (μg/ml)		
		Range	50%	90%
	T-2525	≤0.01–3.13	0.05	1.56
Morganella morganii (81)	R-3746	0.05–100	0.39	12.5
	CCL	12.5–>100	>100	>100
	AMPC	12.5–>100	>100	>100
	CFIX	≤0.01–50	0.10	6.25
	T-2525	0.025–12.5	0.10	1.56
Enterobacter cloacae (77)	R-3746	0.20–>100	12.5	>100
	CCL	6.25–>100	>100	>100
	AMPC	6.25–>100	>100	>100
	CFIX	0.10–>100	12.5	>100
	T-2525	0.10–>100	3.13	>100
Citrobacter freundii (71)	R-3746	0.39–>100	1.56	>100
	CCL	1.56–>100	12.5	>100
	AMPC	1.56–>100	100	>100
	CFIX	0.10–>100	0.78	>100
	T-2525	0.10–>100	0.39	50
Serratia marcescens (81)	R-3746	0.20–>100	0.78	25
	CCL	12.5–>100	>100	>100
	AMPC	12.5–>100	>100	>100
	CFIX	0.05–>100	0.20	6.25
	T-2525	0.20–>100	1.56	12.5
Haemophilus influenzae (54)	R-3746	0.025–0.78	0.05	0.10
	CCL	0.78–50	1.56	6.25
	AMPC	0.20–>100	6.25	12.5
	CFIX	0.025–6.25	0.025	0.05
	T-2525	≤0.01–1.56	≤0.01	0.025
Bacteroides fragilis (27)	R-3746	0.78–>100	12.5	100
	CCL	12.5–>100	>100	>100
	AMPC	1.56–>100	12.5	100
	CFIX	0.39–>100	25	>100
	T-2525	1.56–>100	12.5	>100

[a] CCL, cefaclor; AMPC, amoxicillin; CFIX, cefixime.

activity than R-3746 and the other cephalosporins against almost all of the gram-negative bacteria.

Influence of growth conditions. The type of medium (sensitivity disk, Mueller-Hinton, heart infusion, and nutrient agar media) did not make more than a twofold difference in the activity of five strains of *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*, *Providencia rettgeri*, *Morganella morganii*, and *Serratia marcescens*, including β-lactamase-producing strains. Changes in the pH of SDA from 5.5 to 8.5 affected the activity of R-3746; the MICs of R-3746 for *Staphylococcus aureus* were 4- to 16-fold lower at acidic pH, and conversely the MICs for *Proteus mirabilis* and *Serratia marcescens* tended to be 4- to 8-fold lower at neutral and alkaline pHs. With the addition of horse serum to SDA to final concentrations of 10 and 25%, the MICs of R-3746 were little affected. The effect of inoculum size was determined with final inocula of 10^3, 10^4, 10^5, and 10^6 CFU on SDA. At 10^4 CFU, the MICs of R-3746 were identical to the MICs at 10^3 CFU, and at 10^5 CFU the MICs were at most twofold greater than the MICs at 10^4 CFU. An increase in the inoculum size from 10^5 to 10^6 CFU caused a fourfold or greater rise in the MICs for all the organisms tested, but the MICs were not changed more than twofold between β-lactamase-positive and β-lactamase-negative strains. The effects of several factors on MICs of reference drugs tended to be similar to those of R-3746, with a few exceptions for cefaclor and amoxicillin for β-lactamase-producing strains

TABLE 2. Bactericidal effect of R-3746[a]

Organism	MBC/MIC (μg/ml)				
	R-3746	CCL	AMPC	CFIX	T-2525
Staphylococcus aureus FDA 209P JC-1	6.25/6.25	1.56/1.56	0.10/0.10	100/100	6.25/6.25
Escherichia coli NIHJ JC-2[b]	1.56/0.78	6.25/6.25	6.25/6.25	0.78/0.78	0.39/0.39
Klebsiella pneumoniae PCI-602	0.05/0.025	0.78/0.78	>100/>100	≤0.01/≤0.01	0.05/0.05
Serratia marcescens IAM1184[b]	1.56/1.56	>100/>100	50/25	0.78/0.39	1.56/1.56
Proteus vulgaris OX-19	0.10/0.10	100/12.5	100/50	≤0.01/≤0.01	0.025/≤0.01
Proteus mirabilis IFO3849	0.20/0.20	12.5/3.13	1.56/1.56	≤0.01/≤0.01	0.20/0.10
Providencia rettgeri IFO3850[b]	1.56/0.78	100/12.5	12.5/3.13	3.13/0.78	6.25/1.56
Morganella morganii IFO3848[b]	0.05/0.05	50/50	25/12.5	≤0.01/≤0.01	0.025/0.025

[a] MICs were determined by the twofold serial dilution method with HIB, and then MBCs were determined by the loop transfer method onto drug-free agar plates. See Table 1, footnote *a*, for abbreviations.

[b] β-Lactamase-producing strain.

TABLE 3. Stability of R-3746 to β-lactamases

Enzyme source	Type of β-lactamase[a]	Relative hydrolysis rate[b] (% of control)						
		R-3746	CCL	AMPC	CFIX	T-2525	CER	PCG
Rms212[c]	III	<0.1	1.6	110	<0.1	<0.1	—[d]	100
Rms213[c]	V	2.3	10	730	0.4	22	—	100
Rte16[c]	V	0.2	24	97	<0.1	0.1	—	100
pCR1::Tn*2101*[c]	V	<0.1	0.2	130	0.1	0.1	—	100
Escherichia coli GN5482	Ib	1.2	180	7.1	0.4	0.7	100	—
Citrobacter freundii GN7391	Ia	0.4	78	3.4	0.1	0.4	100	—
Enterobacter cloacae GN7471	Ia	0.6	80	1.5	0.2	0.4	100	—
Serratia marcescens GN10857	Ia	5.2	210	1.9	4.4	6.2	100	—
Providencia rettgeri GN4430	Ia	6.0	150	3.9	3.3	18	100	—
Morganella morganii GN5407	IIa	0.8	130	6.9	0.9	2.3	100	—
Pseudomonas aeruginosa GN10362	Id	2.2	110	6.2	0.8	1.4	100	—
Klebsiella oxytoca GN10650	IVb	4.2	84	290	0.8	7.3	100	—
Proteus vulgaris GN7919	Ic	11	140	27	1.4	28	100	—
Pseudomonas cepacia GN11164	Ic	54	97	180	1.3	65	100	—

[a] Richmond and Sykes classification.
[b] Relative rates of hydrolysis of substrates are expressed as the percentage of penicillin G (PCG) hydrolysis for plasmid-mediated β-lactamases or cephaloridine (CER) hydrolysis for chromosomally mediated β-lactamases. Other abbreviations: see Table 1, footnote *a*.
[c] Host bacteria: *Escherichia coli* W3630 for Rms212 and Rms213, *Escherichia coli* ML1410 for Rte16, and *Escherichia coli* ML4905 for pCR1::Tn*2101*.
[d] —, Not tested.

with a higher inoculum size. MBCs of R-3746 with a final inoculum of 10^5 CFU in HIB were identicial to or at most twofold greater than the MICs for *Staphylococcus aureus* and the members of the *Enterobacteriaceae*, including β-lactamase-producing strains (Table 2). The strains are representative of the five strains tested for each species. Cefixime, T-2525, and amoxicillin showed bactericidal effects (MBCs/MICs) similar to those of R-3746, but the MBCs of cefaclor were eightfold or more higher than the MICs for *Proteus vulgaris* and *Providencia rettgeri*.

Stability to β-lactamases. The stability of R-3746 against hydrolysis by various types of β-lactamase was compared with that of the reference drugs. The relative rates of hydrolysis are shown in Table 3. R-3746, as well as cefixime, showed a high stability to several types of plasmid-mediated β-lactamases. T-2525 and cefaclor were rather unstable to some of these β-lactamases. Amoxicillin was extremely unstable to all the plasmid enzymes. R-3746 was almost as stable as cefixime and T-2525 to β-lactamases produced by several members of the *Enterobacteriaceae* and *Pseudomonas aeruginosa* GN10362, whereas these compounds were only slightly hydrolyzed by β-lactamases produced by *Serratia marcescens* GN10857 and *Providencia rettgeri* GN4430. Cefaclor was far more unstable than R-3746, but amoxicillin showed less stability than R-3746 and cefixime to all the chromosomal β-lactamases. R-3746 and T-2525 were

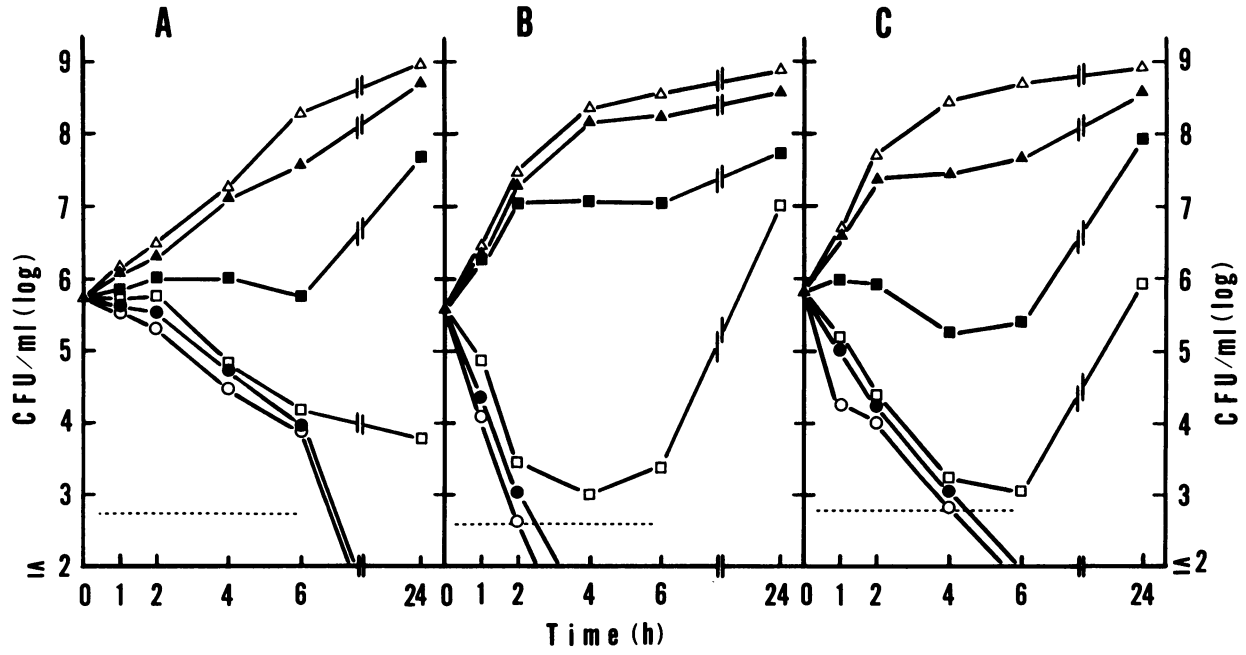

FIG. 2. Time-kill curves of R-3746 against *Staphylococcus aureus* FDA 209P JC-1 (A), *Escherichia coli* NIHJ JC-2 (B), and *K. pneumoniae* GN6445 (C). Symbols: △, no drug; ▲, 1/16 MIC; ■, 1/4 MIC; □, 1× MIC; ●, 4× MIC; ○, 16× MIC. The dotted line represents 99.9% killing.

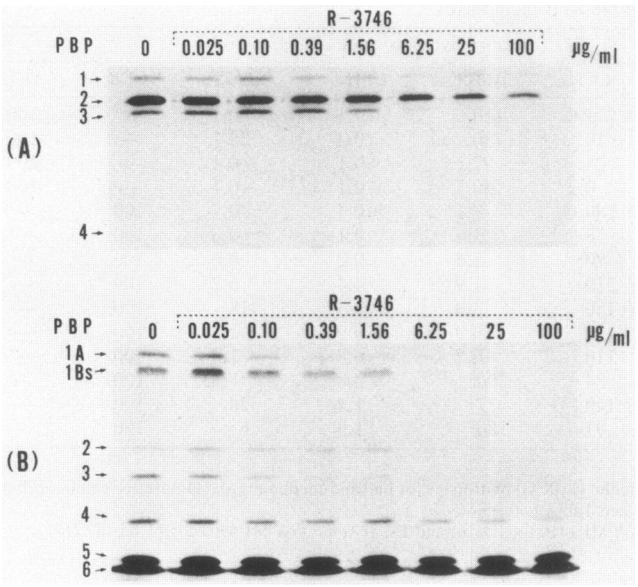

FIG. 3. Results of fluorography by competition of R-3746 at concentrations of 0.025, 0.10, 0.39, 1.56, 6.25, 25, and 100 μg/ml with ^{14}C-labeled penicillin G for binding to the PBPs of *Staphylococcus aureus* FDA 209P JC-1 (A) and *Escherichia coli* NIHJ JC-2 (B).

hydrolyzed by cefuroximases (14) produced by *K. oxytoca* GN10650, *Proteus vulgaris* GN7919, and *Pseudomonas cepacia* GN11164 to a greater extent than cefixime was. Cefaclor and amoxicillin were much more unstable to the cefuroximases than R-3746.

Time-kill studies. Time-kill curves of R-3746 are shown in Fig. 2. Bactericidal activity was compared with that of cefaclor and T-2525. R-3746 and the reference drugs exhibited bactericidal actions against *Staphylococcus aureus* FDA 209P JC-1 at concentrations above the MIC (MICs: R-3746, 6.25 μg/ml; cefaclor, 1.56 μg/ml; T-2525, 6.25 μg/ml). Moreover, no regrowth was observed after 24 h of incubation. Killing of more than 99.9% of the initial viable cells was achieved within 6 h of incubation only after exposure to cefaclor at concentrations higher than the MIC. Rapid reduction of CFU in *Escherichia coli* NIHJ JC-2 was found with the addition of drugs at concentrations above the MIC (MICs: R-3746, 1.56 μg/ml; cefaclor, 6.25 μg/ml; T-2525, 0.39 μg/ml). Reductions in viability greater than 99.9% were seen at concentrations of 4- and 16-fold the MIC of R-3746 and at concentrations above the MIC of cefaclor and T-2525. No regrowth after 24 h of incubation was observed at concentrations of 4- or 16-fold the MIC of these antibiotics. Against *K. pneumoniae* GN6445, an apparent decrease in CFU was seen after exposure to higher than the MIC of each antibiotic (MICs: R-3746, 0.10 μg/ml; cefaclor, 0.39 μg/ml; T-2525, 0.20 μg/ml). Decreases of 99.9% or more were seen with the addition of 4- and 16-fold the MIC of R-3746 and cefaclor and above the MIC of T-2525 within 3 to 4 h. No regrowth occurred after exposure to 4- and 16-fold the MIC of R-3746 and T-2525 and 16-fold the MIC of cefaclor up to 24 h.

Binding affinity for PBPs. The binding affinity of R-3746 for PBPs was compared with that of cefaclor. The results of fluorography by competition of R-3746 with ^{14}C-labeled penicillin G for binding to PBPs of *Staphylococcus aureus* FDA 209P JC-1 and *Escherichia coli* NIHJ JC-2 are shown in Fig. 3. R-3746 had higher affinities for PBP 1 (I_{50}, 0.3 μg/ml) and PBP 2 (I_{50}, 2.4 μg/ml) of *Staphylococcus aureus* than cefaclor did for PBP 1 (I_{50}, 0.6 μg/ml) and PBP 2 (I_{50}, 8.4 μg/ml). The affinities of R-3746, however, for PBP 3 (I_{50}, 0.9 μg/ml) and PBP 4 (I_{50}, 0.1 μg/ml) were comparatively lower than those of cefaclor for PBP 3 (I_{50}, 0.2 μg/ml) and PBP 4 (I_{50}, 0.1 μg/ml). R-3746 possessed high affinities for *Escherichia coli* PBP 3 (I_{50}, 0.4 μg/ml), PBP 1A (I_{50}, 0.6 μg/ml), and PBP 1Bs (I_{50}, 0.8 μg/ml), moderate affinity for PBP 2 (I_{50}, 2.2 μg/ml), and relatively low affinity for PBP 4 (I_{50}, 13.0 μg/ml). Cefaclor showed moderate affinities for PBP 1A (I_{50}, 2.3 μg/ml) and PBP 3 (I_{50}, 5.2 μg/ml) and rather low affinities for PBP 1Bs (I_{50}, 14.2 μg/ml), PBP 4 (I_{50}, 18.0 μg/ml), and PBP 2 (I_{50}, 29.7 μg/ml). R-3746 and cefaclor showed scarcely any affinity for PBP 5 (I_{50}, >100 μg/ml) and PBP 6 (I_{50}, >100 μg/ml) of *Escherichia coli*.

In vivo antibacterial activity. The protective effects of a single dose of CS-807 and the reference drugs on systemic infections in mice with a variety of pathogens are summarized in Table 4. CS-807 showed high protective activity against *Staphylococcus aureus* Smith infection, with an ED_{50} of 3.56 mg/kg; CS-807 was about 16 and 12 times more active than cefixime and T-2588, respectively, whereas it was less effective than cefaclor and amoxicillin. CS-807 demonstrated good in vivo activity against *Streptococcus pneumoniae* 2132 infection; it was 2 and >20 times more active than cefaclor and cefixime, respectively. Furthermore, against *Streptococcus pyogenes* 1412 infection, the protective activity of CS-807 was roughly comparable to that of cefaclor, amoxicillin, or T-2588 and about seven times greater than that of cefixime. Against the mice infected with *Escherichia coli* ML4707, CS-807 showed potent protective activity comparable to that of cefixime and T-2588 and about six times more potent than that of amoxicillin. The protective effect of CS-807 in an infection with *K. pneumoniae* GN6445 was as good as that of cefixime, and it was approximately 3 and 10 times better than T-2588 and cefaclor, respectively. CS-807 had an excellent protective effect, identical to cefixime, on mice infected with *Proteus mirabilis* GN4754, and it was about seven and three times more effective than cefaclor and amoxicillin, respectively. Against an infection with *Serratia marcescens* L-65, a β-lactamase-producing strain, CS-807 exhibited very potent in vivo activity, with an ED_{50} of 1.31 mg/kg; it was roughly similar to cefixime and T-2588.

DISCUSSION

Several new cephalosporins such as cefotaxime, ceftizoxime, cefmenoxime, and cefixime were reported to have significantly potent antibacterial activity against various species of gram-negative bacteria by the introduction of an α-methoxyimino aminothiazole side chain at the 7-position of the cephem nucleus (7, 8, 10, 17, 20, 24). The potent antibacterial activity has been shown to be due to their enhanced affinity for the target enzymes and their high stability to various types of β-lactamase (21). We have confirmed in the present study that R-3746, a methoxyimino aminothiazolyl cephalosporin, had characteristics similar to those of the other new cephalosporin derivatives; it was highly stable to both the plasmid-mediated β-lactamases and the chromosomally mediated β-lactamases, with a few exceptions. The cephalosporin derivatives with an imino-ether group in the 7-substituent in general were shown to be less stable to the cefuroximase classified by Mitsuhashi and Inoue (14), which includes the chromosomal β-lactamases of *Bacteroides fragilis*, *Proteus vulgaris*, and *Pseudomonas*

TABLE 4. Protective effect of CS-807 on systemic infections in mice

Organism	Challenge dose (CFU/mouse)	Drug[a]	ED_{50} (mg/kg)[b]	MIC (μg/ml)
Staphylococcus aureus Smith	1.1×10^7 (+ mucin)	CS-807	3.56 (2.26–5.33)	1.56[c]
		CCL	0.14 (0.09–0.23)	0.78
		AMPC	0.06 (0.04–0.09)	0.10
		CFIX	55.4 (38.3–80.9)	12.5
		T-2588	41.7 (29.8–58.9)	1.56[d]
Streptococcus pyogenes 1412	4.1×10^6 (+ mucin)	CS-807	0.16 (0.09–0.28)	≤0.01[c]
		CCL	0.13 (0.08–0.20)	0.05
		AMPC	0.08 (0.05–0.13)	≤0.01
		CFIX	1.15 (0.74–1.83)	0.10
		T-2588	0.15 (0.10–0.23)	0.025[d]
Streptococcus pneumoniae 2132	3.6×10^2 (+ mucin)	CS-807	0.57 (0.38–0.76)	0.025[c]
		CCL	1.31 (0.78–2.15)	0.10
		AMPC	0.09 (0.06–0.13)	≤0.01
		CFIX	13.7 (9.69–19.4)	0.20
		T-2588	0.65 (0.44–1.04)	0.01[d]
Escherichia coli ML4707	1.1×10^7	CS-807	1.38 (0.85–2.23)	0.20[c]
		CCL	3.68 (2.08–7.25)	0.78
		AMPC	8.65 (5.52–13.0)	6.25
		CFIX	1.71 (1.09–2.26)	0.20
		T-2588	2.29 (1.30–5.02)	0.10[d]
Klebsiella pneumoniae GN6445	2.0×10^7 (+ mucin)	CS-807	1.12 (0.71–1.83)	0.05[c]
		CCL	11.9 (7.69–18.2)	0.39
		AMPC	>100	25
		CFIX	0.75 (0.48–1.20)	0.025
		T-2588	3.61 (2.24–5.74)	0.10[d]
Proteus mirabilis GN4754	1.9×10^7 (+ mucin)	CS-807	0.67 (0.42–1.09)	0.025[c]
		CCL	4.68 (2.95–8.22)	0.39
		AMPC	1.98 (1.29–3.18)	0.39
		CFIX	0.54 (0.35–0.84)	≤0.01
		T-2588	1.90 (0.44–4.27)	0.025[d]
Serratia marcescens L-65[e]	1.1×10^7 (+ mucin)	CS-807	1.31 (0.29–2.60)	1.56[c]
		CCL	>100	>100
		AMPC	>100	>100
		CFIX	0.63 (0.35–1.05)	0.78
		T-2588	4.04 (1.89–7.01)	0.78[d]

[a] Abbreviations are defined in Table 1, footnote *a*.
[b] Numbers in parentheses are the 95% confidence limits.
[c] MIC of R-3746.
[d] MIC of T-2525.
[e] β-Lactamase-producing strain.

cepacia, though the level of stability is dependent on derivatives. R-3746, having the same imino-ether group, would have shown a similar tendency in stability to cefuroximases. The antibacterial activity of R-3746, being free from the effect of plasmids encoding penicillin resistance, is ensured by the high stability to each type of plasmid-mediated penicillinase. On the other hand, its antibacterial activity against the organisms producing species-specific β-lactamases is supposed to be affected by its stability to these β-lactamases. Relatively weak activity of R-3746 against *Bacteroides fragilis* may reflect the moderate instability of R-3746 to the β-lactamase produced by this species, as reported previously (28, 30). This would not always be a disadvantage for CS-807 (R-3746) as an oral antibiotic for the following reasons. One of the most serious adverse effects is pseudomembranous colitis. It is supposed that this colitis is caused by enterotoxin-producing *Clostridium difficile*, which increases under alterations of intestinal microflora (1). Thus, it may be useful for oral antibiotics to have less potent activity against the major species, such as *Bacteroides fragilis*, which constitute the intestinal microflora.

R-3746 showed higher affinity than cefaclor did for PBP 3, 1A, and 1Bs of *Escherichia coli*, in that order. Of the PBPs in *Escherichia coli*, PBP 1Bs and its compensating PBP 1A are thought to function in cell elongation, whereas PBP 3 is thought to function in septum formation (22, 29). The affinity of β-lactam antibiotics for either of these PBPs was well correlated with their MICs, suggesting that those PBPs are essential targets for β-lactam antibiotics (4, 23). Hence, a lower MIC of R-3746 than of cefaclor for *Escherichia coli* could be explained by its higher binding affinity for PBP 1A, 1Bs, and 3. Conversely, the affinity of R-3746 for PBP 3, one of the essential target enzymes of *Staphylococcus aureus*, was lower than that of cefaclor. It was reported that the rather low affinity of cefixime for PBP 3 of *Staphylococcus aureus* was responsible for its weaker antibacterial activity against this species (30). The fact that the activity of R-3746 against clinical isolates of *Staphylococcus aureus* was almost equal to or slightly less than those of cefaclor and amoxicillin may be due to the relatively lower affinity of R-3746 than these β-lactams for PBP 3 of *Staphylococcus aureus*.

New methoxyimino aminothiazolyl cephalosporins such as cefotaxime, ceftizoxime, and cefmenoxime have not been developed as oral drugs because of poor absorption of these agents from the gastrointestinal tract. Recently, extensive investigations of new oral cephalosporins have resulted in the development of cefixime, T-2588 (T-2525), ME-1207 (ME-1206), CS-807 (S. Sugawara, M. Iwata, M. Tajima, T. Magaribuchi, H. Yanagisawa, H. Nakao, J. Kumazawa, and

S. Kuwahara, 26th ICAAC, abstr. no. 592, 1986), and other derivatives. These compounds were reported to have in vitro and in vivo antibacterial activity against common clinical isolates such as *Escherichia coli*, *K. pneumoniae*, *Proteus mirabilis*, and the opportunistic pathogens which are resistant to β-lactam antibiotics used orally at present. Moreover, CS-807 (R-3746) and ME-1207 (ME-1206) were reported to be active against *Staphylococcus aureus* as well. The excellent in vivo efficacy of CS-807 seems to be based not only on high stability to β-lactamases and high affinity for PBPs of R-3746, but also on the high levels and long duration of this compound in serum after oral administration in mice (T. Komai, K. Fujimoto, M. Iwata, M. Sekine, and H. Masuda, 26th ICAAC, abstr. no. 593, 1986). Furthermore, the binding of the active form of CS-807 with mouse serum protein at relatively low rates (47%) may have brought about the good therapeutic efficacy of CS-807.

Based on these features, CS-807 can be considered a prominent candidate for an oral agent for further studies aiming at clinical trials.

LITERATURE CITED

1. **Bartlett, J. G., T. W. Chang, M. Gurwith, S. L. Gorbach, and A. B. Onderdonk.** 1978. Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. N. Engl. J. Med. **298**:531–534.
2. **Bill, N. J., and J. A. Washington II.** 1977. Comparison of in vitro activity of cephalexin, cephradine, and cefaclor. Antimicrob. Agents Chemother. **11**:470–474.
3. **Buck, R. E., and K. E. Price.** 1977. Cefadroxil, a new broad-spectrum cephalosporin. Antimicrob. Agents Chemother. **11**: 324–330.
4. **Curtis, N. A. C., D. Orr, G. W. Ross, and M. G. Boulton.** 1979. Affinities of penicillins and cephalosporins for the penicillin-binding proteins of *Escherichia coli* K-12 and their antibacterial activity. Antimicrob. Agents Chemother. **16**:533–539.
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. **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.
7. **Kamimura, T., H. Kojo, Y. Matsumoto, Y. Mine, S. Goto, and S. Kuwahara.** 1984. In vitro and in vivo antibacterial properties of FK 027, a new orally active cephem antibiotic. Antimicrob. Agents Chemother. **25**:98–104.
8. **Kamimura, T., Y. Matsumoto, N. Okada, Y. Mine, M. Nishida, S. Goto, and S. Kuwahara.** 1979. Ceftizoxime (FK 027), a new parenteral cephalosporin: in vitro and in vivo antibacterial activities. Antimicrob. Agents Chemother. **16**:540–548.
9. **Litchfield, J. T., and F. Wilcoxon.** 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. **96**:99–113.
10. **Masuyoshi, S., S. Arai, M. Miyamoto, and S. Mitsuhashi.** 1980. In vitro antimicrobial activity of cefotaxime, a new cephalosporin. Antimicrob. Agents Chemother. **18**:1–8.
11. **Matsubara, N., A. Yotsuji, K. Kumano, M. Inoue, and S. Mitsuhashi.** 1981. Purification and some properties of a cephalosporinase from *Proteus vulgaris*. Antimicrob. Agents Chemother. **19**:185–187.
12. **Matsuura, M., H. Nakazawa, M. Inoue, and S. Mitsuhashi.** 1980. Purification and biochemical properties of β-lactamase produced by *Proteus rettgeri*. Antimicrob. Agents Chemother. **18**: 687–690.
13. **Minami, S., M. Inoue, and S. Mitsuhashi.** 1980. Purification and properties of a cephalosporinase from *Enterobacter cloacae*. Antimicrob. Agents Chemother. **18**:853–857.
14. **Mitshuashi, S., and M. Inoue.** 1981. Mechanisms of resistance to β-lactam antibiotics, p. 41–56. *In* S. Mitsuhashi (ed.), Beta-lactam antibiotics. Japan Scientific Societies Press, Tokyo.
15. **Muggleton, P. W., C. H. O'Callaghan, R. D. Foords, S. M. Kirby, and D. M. Ryan.** 1969. Laboratory appraisal of cephalexin, p. 353–360. Antimicrob. Agents Chemother. 1968.
16. **Murata, T., S. Minami, K. Yasuda, S. Iyobe, M. Inoue, and S. Mitsuhashi.** 1981. Purification and properties of cephalosporinase from *Pseudomonas aeruginosa*. J. Antibiot. **34**:362–365.
17. **Nakano, H.** 1981. Structure-relationships related to ceftizoxime (FK 749). Med. Res. Rev. **1**:127–157.
18. **Neu, H. C., and K. P. Fu.** 1978. Cefaclor: in vitro spectrum of activity and beta-lactamase stability. Antimicrob. Agents Chemother. **13**:584–588.
19. **Neu, H. C., and K. P. Fu.** 1979. Cefatrizine activity compared with that of other cephalosporins. Antimicrob. Agents Chemother. **15**:209–212.
20. **Nishida, M., T. Kamimura, N. Okada, Y. Matsumoto, Y. Mine, and T. Murakawa.** 1979. Comparison of antibacterial activity of a new cephalosporin, ceftizoxime (FK 749) with other cephalosporin antibiotics. J. Antibiot. **32**:1319–1327.
21. **Nishida, M., and Y. Shigi.** 1982. The role of permeability and affinity for penicillin-binding proteins in the antibacterial activity of the third-generation cephalosporins, p. 65–81. *In* H. C. Neu (ed.), New β-lactam antibiotics: a review from chemistry to clinical efficacy of the new cephalosporins. College of Physicians of Philadelphia, Philadelphia.
22. **Noguchi, H., M. Matsuhashi, T. Nikaido, J. Itoh, N. Matsubara, M. Takaoka, and S. Mitsuhashi.** 1978. Affinities of beta-lactam antibiotics to penicillin-binding proteins in *Escherichia coli* and *Pseudomonas aeruginosa* in relation to their antibacterial potencies, p. 361–387. *In* S. Mitsuhashi (ed.), Microbial drug resistance, vol. 2: microbial drug resistance and related plasmids. University of Tokyo Press, Tokyo.
23. **Nozaki, Y., A. Imada, and M. Yoneda.** 1979. SCE-963, a new potent cephalosporin with high affinity for penicillin-binding proteins 1 and 3 of *Escherichia coli*. Antimicrob. Agents Chemother. **15**:20–27.
24. **Okazaki, 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.
25. **Saino, Y., M. Inoue, and S. Mitsuhashi.** 1982. Purification and properties of an inducible cephalosporinase from *Pseudomonas maltophilia* GN12873. Antimicrob. Agents Chemother. **25**:362–365.
26. **Saino, Y., F. Kobayashi, M. Inoue, and S. Mitsuhashi.** 1982. Purification and properties of inducible penicillin β-lactamase isolated from *Pseudomonas maltophilia*. Antimicrob. Agents Chemother. **22**:564–570.
27. **Sato, K., T. Fujii, R. Okamoto, M. Inoue, and S. Mitsuhashi.** 1984. Biochemical properties of β-lactamase produced by *Flavobacterium odoratum*. Antimicrob. Agents. Chemother. **27**:612–614.
28. **Shigi, Y., Y. Matsumoto, M. Kaizu, Y. Fujishita, and H. Kojo.** 1984. Mechanism of action of the new orally active cephalosporin FK 027. J. Antibiot. **37**:790–796.
29. **Spratt, B. G.** 1975. Distinct penicillin binding protein involved in the division, elongation, and shape of *Escherichia coli* K12. Proc. Natl. Acad. Sci. USA **72**:2999–3003.
30. **Tajima, M., K. Sawa, K. Watanabe, and K. Ueno.** 1983. The β-lactamases of genus *Bacteroides*. J. Antibiot. **36**:423–428.
31. **Toda, M., M. Inoue, and S. Mitsuhashi.** 1981. Properties of cephalosporinase from *Proteus morganii*. J. Antibiot. **34**:1469–1475.
32. **Utsui, Y., and T. Yokota.** 1985. Role of an altered penicillin-binding protein in methicillin- and cephem-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **28**:397–403.
33. **Waley, S. G.** 1974. A spectrophotometric assay of β-lactamase action on penicillins. Biochem. J. **139**:780–789.
34. **Zak, O., W. A. Vischer, C. Schenk, W. Tosch, W. Zimmermann, J. Regos, E. R. Suter, F. Kradorlfer, and J. Gelzer.** 1976. CGP-9000: a new orally active, broad-spectrum cephalosporin. J. Antibiot. **29**:653–655.