ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1990, p. 44–51
0066-4804/90/010044-08$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 34, No. 1

Purification and Characterization of Inducible β-Lactamases in *Aeromonas* spp.

JOSEPH P. IACONIS AND CHRISTINE C. SANDERS*

Department of Medical Microbiology, Creighton University School of Medicine, Omaha, Nebraska 68178

Received 26 June 1989/Accepted 19 October 1989

β-Lactamases from *Aeromonas hydrophila* and *A. sobria* were purified and characterized. Both species produced β-lactamases that were inducible by either cefoxitin or imipenem. These species were resistant to ampicillin and cephalothin but not imipenem. Isoelectric focusing of sonic extracts revealed one band at pI 8.0 and a second band at pI 7.0 for *A. hydrophila*. Likewise, *A. sobria* produced two bands, one at pI 8.4 and the other at pI 7.0. Two enzymes from each species were separated by flatbed electrofocusing gel and purified to homogeneity. The molecular weight of the pI 7.0 enzyme (A1) from both species was estimated to be 42,500, whereas the pI 8.0 (A2h) and 8.4 (A2s) enzymes of *A. hydrophila* and *A. sobria* had molecular weights of 31,500 and 35,000, respectively, on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The relative V_{max} values for cephalothin, penicillin, and imipenem for these enzymes indicated that A1 was primarily a cephalosporinase while A2h and A2s were penicillinases highly active against carbapenems. A1 was susceptible to inhibition by cloxacillin, while the A2 enzymes were inhibited by clavulanic acid and EDTA and required zinc for activity. Thus, there appear to be two distinct inducible β-lactamases in *A. hydrophila* and *A. sobria* that play an important role in the β-lactam resistance of these species.

Members of the genus *Aeromonas*, belonging to the family *Vibrionaceae*, are gram-negative facultative anaerobes that have long been known to cause disease in freshwater fish and cold-blooded animals (21, 25). On the basis of molecular genetic data, however, investigators now propose to place this genus in a new family, *Aeromonodaceae* (12). The role of *Aeromonas* spp. as enteric pathogens in humans has increased in recent years, in part because of better methods and isolation techniques. These bacteria are implicated in a wide spectrum of enteric diseases ranging in clinical severity from mild diarrhea to acute gastroenteritis in both pediatric and adult populations (9, 14, 17, 20, 30, 35). Furthermore, various *Aeromonas* species can be isolated from individuals with wound infections (17, 18), and recovery of these organisms from severe infections, like septicemia, often involves patients with other underlying illnesses (3, 18, 19). Antibiotic resistance in *Aeromonas* spp. poses a potential problem for the therapeutic management of infections caused by these organisms. Inducible β-lactamase activity has been associated with resistance to penicillin and many of the early cephalosporins in clinical isolates of *Aeromonas* spp. (10, 23, 30, 33). We recently reported the existence of two apparently distinct inducible β-lactamases in *Aeromonas sobria* and *A. hydrophila* (1). However, the specific catalytic activity of each enzyme remained uncertain, since only crude enzyme preparations were examined. Therefore, we sought to purify and characterize these two enzymes from *A. hydrophila* and *A. sobria* and assess their roles in resistance to β-lactam antibiotics.

MATERIALS AND METHODS

Bacteria. *A. hydrophila* AER 19 and *A. sobria* AER 14 were described in a previous study (1). *Xanthomonas maltophilia* (formerly *Pseudomonas maltophilia*) GM55 was obtained from St. Joseph Hospital, Omaha, Nebr. *Aeromonas* mutants (M) constitutive for β-lactamase expression were derived from their wild-type strains by serial twofold passages in Mueller-Hinton Broth (MHB) containing cefuroxime (32 to 128 μg/ml). Each strain was kept frozen in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.)-horse serum (1:1, vol/vol) at −70°C until used.

Antibiotics. Working solutions were prepared on the day of use from laboratory standard powders of all compounds as specified by the manufacturers. All isolates were tested for susceptibility to the following compounds: ampicillin trihydrate (Bristol Laboratories, Syracuse, N.Y.), aztreonam (E. R. Squibb & Sons, Princeton, N.J.), benzylpenicillin (Sigma Chemical Co., St. Louis, Mo.), cefotaxime (Hoechst-Roussel Pharmaceuticals, Somerville, N.J.), cefoxitin and imipenem (Merck Sharp & Dohme, West Point, Pa.), cefuroxime and ceftazidime (Glaxo Group Research, Ltd., Greenford, United Kingdom), cephalothin (Eli Lilly & Co., Indianapolis, Ind.), and potassium clavulanate (Beecham Laboratories, Bristol, Tenn.).

Susceptibility tests. Antibiotic susceptibility tests were performed by serial twofold dilution in Mueller-Hinton II agar (BBL). The inoculum consisted of 10^4 CFU per spot applied with a Steers replicator (34). The MIC was defined as the lowest concentration that prevented growth after incubation for 18 h at 35°C in air.

β-Lactamase induction. Each wild-type and mutant strain was grown overnight in MHB at 37°C. A 1:20 dilution of each culture was made into fresh MHB, and the diluted culture was shaken at 37°C in air for 90 min. For induction, cefoxitin or imipenem at 1/4 of the MIC was then added to the broth and incubated for an additional 120 min. After this induction period, 1 mM 8-hydroxyquinoline (Sigma) was added to each culture. The cells were harvested by centrifugation at 4°C, washed twice in 0.1 M phosphate buffer (pH 7.0), and disrupted with a Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Sussex, United Kingdom). The supernatant obtained after centrifugation at 6,000 rpm at 4°C was dialyzed overnight in 0.1 M phosphate buffer (pH 7.0) at 4°C. The β-lactamases obtained in sonic extracts were examined by isoelectric focusing (IEF) and spectrophotometry as described previously (1).

* Corresponding author.

Preparation of crude enzymes. Solutions of crude β-lactamases were purified from *A. hydrophila* AER 19M and *A. sobria* AER 14M, both of which were constitutive for enzyme expression. Each species was grown in 2 liters of MHB for 18 h at 37°C in air. The cells were harvested by centrifugation at 8,000 × *g* for 30 min, washed twice in 0.1 M phosphate buffer (pH 7.0), and resuspended in the same buffer at 1/20 of the original volume. An overnight MHB culture of *X. maltophilia* GM55 (10 ml) was inoculated into 2 liters of MHB and induced with imipenem (3.2 μg/ml) after 90 min of growth. Approximately 4 h after induction, the cells were harvested as described above. The cell suspension obtained from each preparation was disrupted with a Soniprep 150 ultrasonic disintegrator (MSE), followed by ultracentrifugation at 30,000 × *g* for 20 min. The supernatant was dialyzed against 1% glycine (adjusted to pH 7.0) and concentrated to 3 ml with a Micro-ProDiCon apparatus (Bio-Molecular Dynamics, Beaverton, Oreg.) on dialysis membranes with a nominal molecular weight (MW) limit of 10,000.

Purification of β-lactamase. Approximately 3 ml (≅120 mg of protein) of each of the concentrated enzyme preparations described above was loaded onto a Multiphor preparative flatbed electrofocusing (PFBE) unit (LKB, Bromma, Sweden) to separate the pI 7.0 from the pI 8.0 and pI 8.4 β-lactamases from *A. hydrophila* AER 19M and *A. sobria* AER 14M, respectively. The dextran granulated gel (LKB) was focused lengthwise at a constant power of 18 W (4°C) for a total of 4 h. The gel contained 5% pH 6.5 to 10.5 ampholytes (Sigma). At completion, the gel bed was fractionated into 30 equal compartments and β-lactamase activity was estimated by using nitrocefin, cephalothin, and benzylpenicillin. Enzyme-positive gel fractions were loaded onto fiberglass-plugged columns (1.0 by 15 cm) and eluted with 3 ml of 0.01 M phosphate buffer (pH 7.0) containing 1 mM $ZnCl_2$ at 4°C. The pH 8.0 and 8.4 eluates of *A. hydrophila* AER 19M and *A. sobria* AER 14M, respectively, were stored at −70°C until further use. These β-lactamases were designated as A2h and A2s, respectively. PFBE also served to separate the pI 6.5 β-lactamase from the pI 9.2 β-lactamase of *X. maltophilia* GM55. The 6.5 β-lactamase was referred to as L-1 as described by Saino et al. (29).

The pI 7.0 β-lactamase (designated A1) from each species was further purified to remove any contaminating A2 enzyme and nonspecific protein associated with this enzyme after electrofocusing. Approximately 3.0 ml of the eluate (≅15 mg of protein) recovered after electrofocusing was applied to a carboxymethyl (CM)-Sephadex C-50 column (1.5 by 10 cm) equilibrated with 0.01 M phosphate buffer (pH 7.0). The column was washed with 50 ml of the same buffer to immediately elute the unadsorbed A1 β-lactamase. Fractions that hydrolyzed nitrocefin were pooled and concentrated to 5 ml by ultrafiltration at 4°C with a PM 10 membrane (nominal MW limit, 10,000; Amicon Corp., Lexington, Mass.). The concentrate was applied to a Sephadex G-75 column (1.5 by 50 cm) preequilibrated with 0.01 M phosphate buffer (pH 7.0) and eluted at 5 ml/h with the same buffer. The eluates that hydrolyzed nitrocephin were pooled (30 ml), concentrated to 2 ml by ultrafiltration (PM 10), and stored at −70°C. The material retained was passed through a column (0.5 by 10 cm) containing AG 501-X8 ion-exchange bed resin (Bio-Rad Laboratories, Richmond, Calif.) preequilibrated with 0.01 M phosphate buffer (pH 7.0) to separate protein from ampholine before use.

The MW of each purified enzyme was estimated on a Sephadex G-75 Superfine column (2 by 70 cm) previously equilibrated with 0.05 M phosphate buffer containing 0.02% sodium azide. The enzymes were eluted under constant pressure at a descending flow rate of 15 ml/h. The A_{280} of the effluent was monitored, and 2.0-ml fractions were collected. Each fraction was assayed for β-lactamase activity with nitrocefin as the substrate. The column was standardized with RNase A (MW, 13,700), chymotrypsinogen (MW, 25,000), ovalbumin A (MW, 43,000), and bovine serum albumin (MW, 67,000). Blue dextran was used to determine the void volume.

β-Lactamase assays. Hydrolysis of β-lactam antibiotics was examined by UV spectrophotometric assays (Beckman DU-7) in 1-cm light path cuvettes with readings recorded at 10-s intervals for 5 min at the wavelength of maximal absorbance for the β-lactam ring of each drug, i.e., cephalothin, 265 nm; cefuroxime, 274 nm; cefotaxime, 254; ceftazidime, 254; imipenem, 299 nm; cloxacillin, 260 nm; aztreonam, 292 nm; and nitrocefin, 489 nm. The rate of benzylpenicillin hydrolysis was measured at 233 nm. Antibiotic solutions were prepared in 0.1 M phosphate buffer (pH 7.0). For substrate profiles, all antibiotics were examined at a concentration of 100 μM, except for benzylpenicillin, which was assayed at 500 μM. One unit of β-lactamase activity was defined as the amount of enzyme which hydrolyzed 1 nmol of substrate per min in the linear phase of the reaction at 37°C in 0.1 M phosphate buffer (pH 7.0). The Michaelis constant (K_m) and the maximum rate of hydrolysis (V_{max}) were determined by the Lineweaver-Burk plot of initial velocity (v) at six different substrate concentrations. The susceptibilities of β-lactamases to inhibition by potassium clavulanate and cloxacillin were determined quantitatively by preincubating the enzyme with various concentrations of inhibitors for 10 min. Benzylpenicillin (500 μM) or imipenem (100 μM) was then added as the substrate, and residual enzyme activity was measured. The concentration of inhibitor required for 50% inhibition of enzyme activity was determined by probit analysis.

Analytical gels. IEF was performed with 7% polyacrylamide gels (Sigma) containing pH 3 to 10 ampholytes (Sigma) by the method of Vecoli et al. (37). The gels were focused

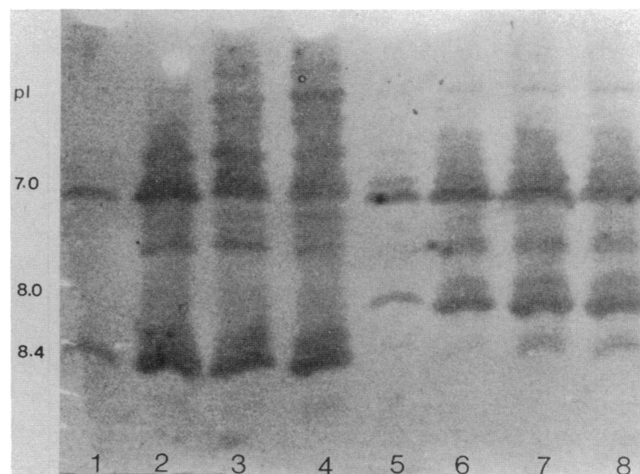

FIG. 1. IEF patterns of sonic extracts from *A. sobria* AER 14 and *A. hydrophila* AER 19. Lanes 1, 3, 5, and 7 contained uninduced samples; lanes 2, 4, 6, and 8 contained samples from strains induced with cefoxitin. Lanes: 1 and 2, *A. sobria* AER 14 wild type; 3 and 4, *A. sobria* AER 14M; 5 and 6, *A. hydrophila* AER 19 wild type; 7 and 8, *A. hydrophila* AER 19M.

TABLE 1. β-Lactam susceptibilities of *A. hydrophila* AER 19 and *A. sobria* AER 14

Isolate	MIC (μg/ml)						β-Lactamase activity[a]		
	Piperacillin	Cephalothin	Ceftazidime	Cefotaxime	Cefuroxime	Imipenem	Uninduced	Induced with: Cefoxitin	Induced with: Imipenem
A. hydrophila AER 19									
Wild type	16	>128	0.12	<0.03	0.5	2	11	740	865
Mutant	>128	>128	32	128	>128	128	850	852	855
A. sobria AER 14									
Wild type	>128	>128	0.25	0.5	0.12	2	15	900	926
Mutant	>128	>128	128	128	>128	16	625	627	630

[a] Activities are expressed as nanomoles of nitrocefin hydrolyzed per minute per milligram of protein per milliliter. Only wild-type strains were inducible with cefoxitin or imipenem.

widthwise at 4°C and 1,600 V for 9 min with a Multiphor 2197 power unit (LKB). The focused gels were overlaid with molten agar containing 50 μg of nitrocefin per ml. MWs were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (38). The purified enzymes and MW protein markers (Bio-Rad) were treated with 1% sodium dodecyl sulfate and 3% 2-mercaptoethanol at 100°C for 5 min before electrophoresis in an 11% polyacrylamide gel. All samples were electrophoresed at a current of 15 mA for 4 h at 10°C. The gels were then stained with silver nitrate by the method of Wray et al. (39).

Protein determination. Protein concentration was estimated by the BCA protein assay (Pierce Chemical Co., Rockford, Ill.) with bovine serum albumin as the standard.

RESULTS

Inducible β-lactamase. The wild-type and mutant *Aeromonas* strains displayed two distinct inducible β-lactamases on IEF gels (Fig. 1, lanes 1, 2, 5, and 6). *A. hydrophila* AER 19 and *A. sobria* AER 14 shared a common β-lactamase at pI 7.0 (A1), and a second β-lactamase was detected at pIs 8.0

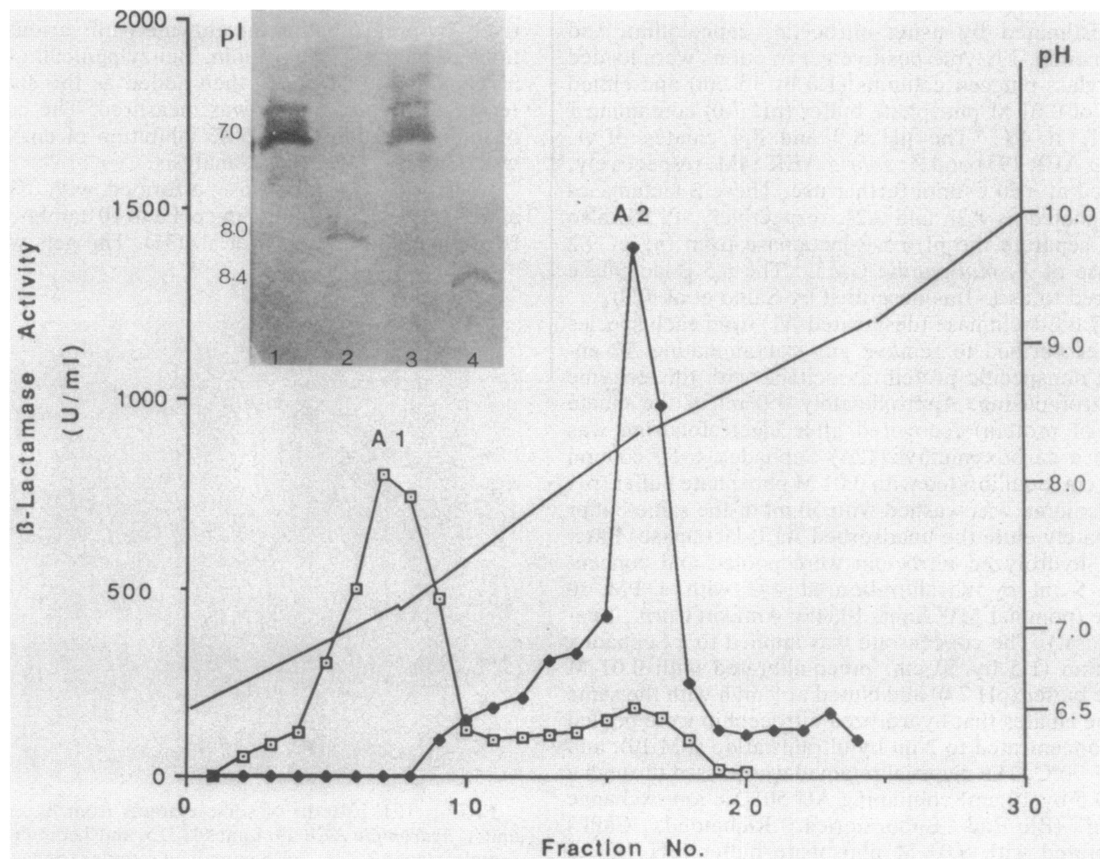

FIG. 2. Elution profile of *A. hydrophila* AER 19M A1 and A2 after PFBE. Symbols: □, A1 activity in the presence of nitrocefin; ◆, A2 activity in the presence of penicillin. The inset shows IEF of *A. hydrophila* AER 19M A1 and A2h and *A. sobria* AER 14 M A1 and A2s in lanes 1 to 4, respectively, after fractionation by PFBE.

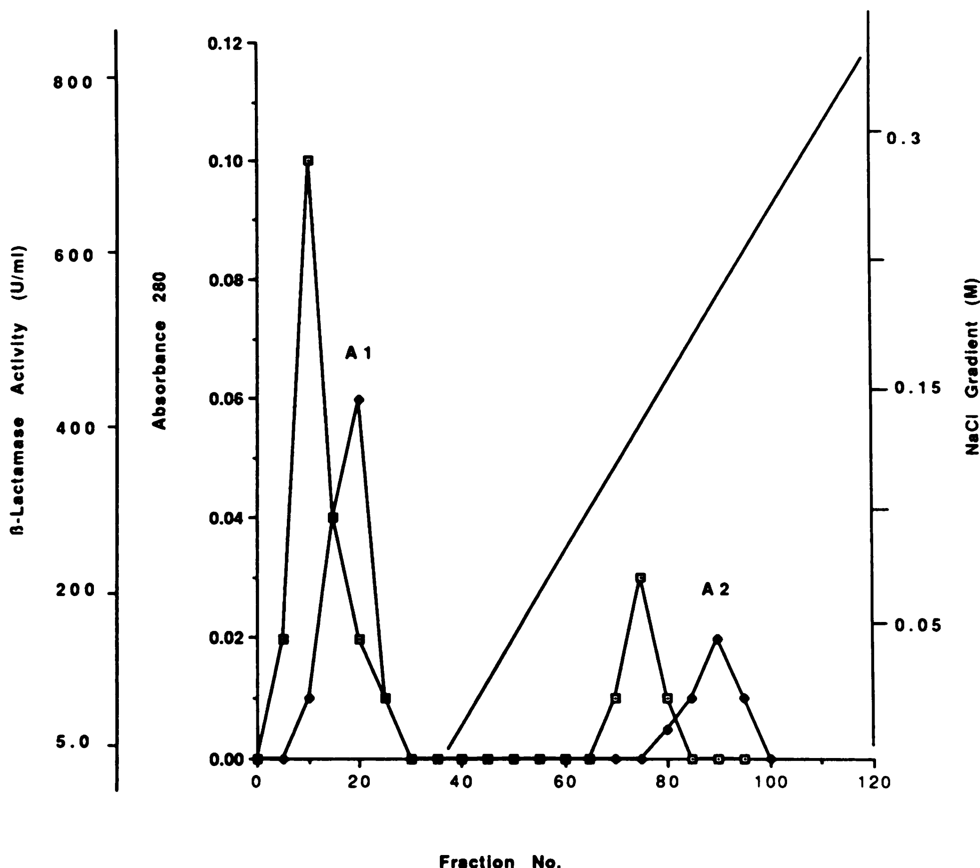

FIG. 3. CM–Sephadex C-50 column chromatography of *A. hydrophila* AER 19M A1 and A2 enzymes. Partially purified A1 enzyme obtained from PFBE was applied to a CM–Sephadex C-50 column (1.5 by 10 cm). The A2 enzyme was eluted with a linear NaCl gradient of 0.05 to 0.3 M. Symbols: ⊡, A_{280}; ——, NaCl gradient; ◆, activity of each fraction with nitrocefin as the substrate.

(Ah2) and 8.4 (A2s) for *A. hydrophila* AER 19 and *A. sobria* AER 14, respectively. Likewise, two major IEF bands were detected for *Aeromonas* mutants derived from their wild types, and these mutants were constitutive for β-lactamase production (Fig. 1, lanes 3, 4, 7, and 8). There was no selective induction of either β-lactamase band by cefoxitin or imipenem (Fig. 1). Additional minor bands at pI 7.4 observed in sonic extracts from induced wild types or mutants were subsequently found to represent satellite bands (see below).

The β-lactam susceptibility and enzyme expression of the inducible wild-type and mutant strains are listed in Table 1. Both wild-type isolates were highly susceptible to all of the β-lactams examined, except for piperacillin and cephalothin. The mutants were significantly more resistant to all of the β-lactams listed in Table 1. *A. hydrophila* AER 19M appeared to be eightfold more resistant than *A. sobria* AER 14M to imipenem.

Purification of *Aeromonas* A1 and A2 β-lactamases. Enzymes A1 and A2 were purified from *A. hydrophila* AER 19M and *A. sobria* AER 14M. PFBE was used as a major first step in the separation of A1 and A2 on the basis of pI. Two distinct peaks in enzyme activity were obtained by PFBE (Fig. 2). The first peak demonstrated optimal activity against nitrocefin, while the second peak displayed optimal activity against benzylpenicillin. Although PFBE resulted in good separation of the enzymes (Fig. 2), the A1 β-lactamases of both species required further purification for a homogeneous preparation. These additional steps included chromatography on CM-Sephadex to remove contaminating A2 enzyme (Fig. 3) and on Sephadex G-75 to remove contaminating protein (Fig. 4). The final purified A1 enzyme gave a single band at pI 7.0 on IEF (data not shown). The entire purification procedure for β-lactamases of *A. hydrophila* AER 19M is summarized in Table 2. The enzymes were purified to a high specific activity with a modest yield. The use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated major bands giving MWs of 42,500 for A1 from both species. The A2 enzymes of *A. hydrophila* AER 19M (A2h) and *A. sobria* AER 14M (A2s) had MWs of 31,500 and 35,000, respectively (Fig. 5). The MWs obtained by Sephadex G-75 chromatography for the β-lactamases of *A. hydrophila* AER 19M were 41,000 and 28,000 for the A1 and A2 enzymes, respectively, suggesting that the native enzymes were purified in their monomeric forms.

β-Lactam hydrolysis by purified enzymes. There were no significant differences in substrate profiles for the A1 β-lactamases purified from *A. hydrophila* AER 19M and *A. sobria* AER 14M. Comparable results were also obtained for the A2 β-lactamases purified from both species. Therefore, only A1 and A2 enzymes purified from *A. hydrophila* AER 19M were used for further analyses of enzyme kinetics. Differences in kinetic parameters were noted for both enzymes (Table 3). The A1 enzyme demonstrated activity primarily against cephalosporins. This enzyme possessed only minor activity against penicillin and no detectable activity against cloxacillin or imipenem. On the other hand, the A2h enzyme readily hydrolyzed penicillin and imipenem

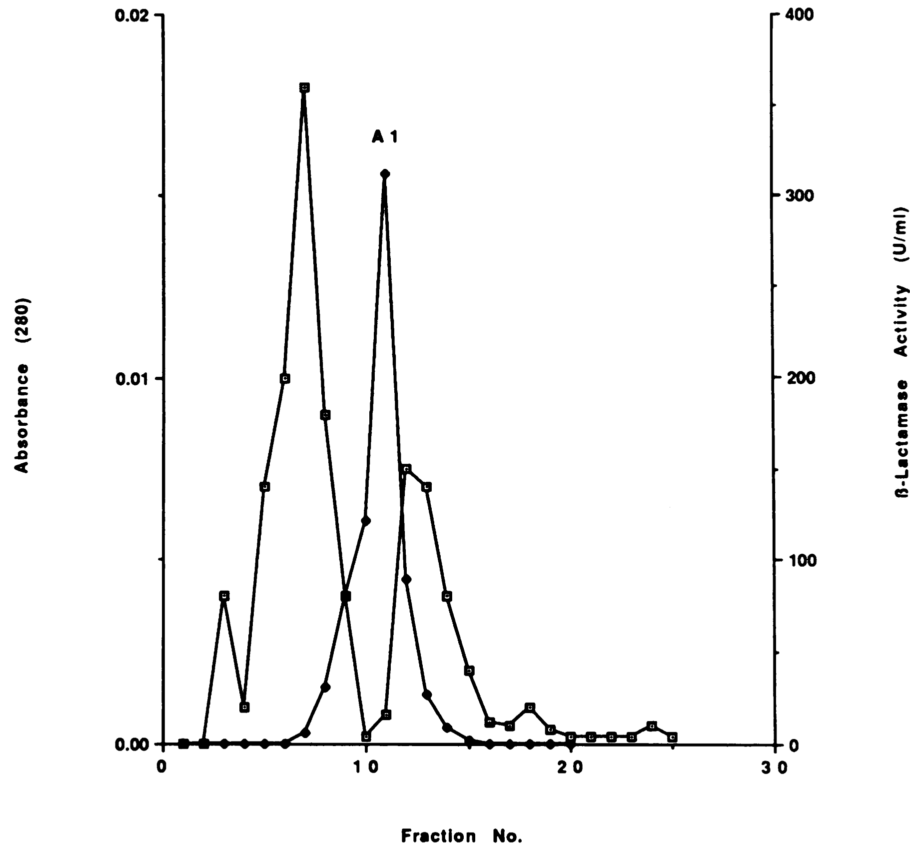

FIG. 4. Elution profile of *A. hydrophila* AER 19M A1 on Sephadex G-75. Approximately 15 mg of protein obtained from a CM–Sephadex C-50 column was loaded onto a Sephadex G-75 column (1.5 by 50 cm). □, A_{280}; ◆, β-lactamase activity measured with nitrocefin as the substrate.

but was much less active against cephalothin, cefotaxime, and ceftazidime. Both *Aeromonas* enzymes hydrolyzed cefuroxime slowly but obeyed Michaelis-Menten kinetics. The affinity (K_m) of the A2h enzyme for cefuroxime was approximately 10 times higher than that of the A1 enzyme. When assayed against nitrocefin, the A2h enzyme was poorly inhibited by cloxacillin but was moderately susceptible to clavulanic acid (Table 4). On the other hand, the A1 enzyme was susceptible to cloxacillin but not clavulanate.

There were similarities and differences between the ki-

TABLE 2. Purification of *A. hydrophila* AER 19M β-lactamases

Stage	Purification step[a] and product	Protein concn (mg/ml)	β-Lactamase activity[b] (U/min per ml)	Sp act (U/min per mg)	Fold purification[c]	% Yield
I	Sonication					
	A1	62	2,260	36	1.0	100
	A2	68	7,450	110	1.0	100
II	Ultracentrifugation at 30,000 × *g*					
	A1	47	3,170	670	1.8	88
	A2	38	9,280	240	2.2	72
III	PFBE					
	A1	1.7	1,420	840	23	4.7
	A2	0.085	1,670	19,640	178	1.8
IV	CM–Sephadex C-50, A1	0.1	390	3,900	108	0.86
V	Sephadex G-75, A1	0.056	380	6,780	188	0.63

[a] β-Lactamase activities from stages I to III were assayed spectrophotometrically with cephalothin (100 μM) and penicillin G (500 μM) to monitor A1 and A2 activities, respectively. The A2 enzyme was purified through stage III.

[b] One unit of activity was defined as the amount of enzyme which hydrolyzed 1 nmol of the substrate per min per ml at 37°C in 0.1 M phosphate buffer (pH 7.0).

[c] Purification was based on the specific activity at a purification step divided by the specific activity of the starting material.

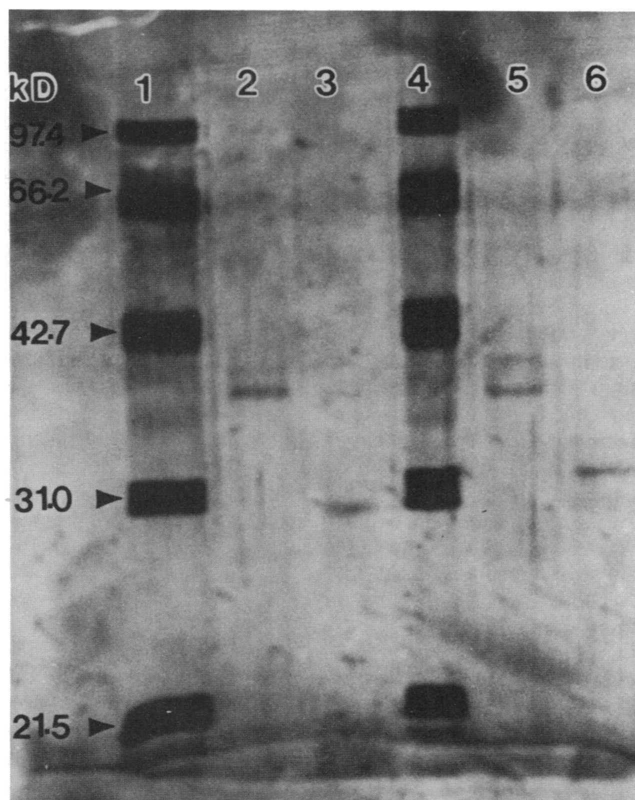

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analyses of stage V purified A1 and stage III A2 β-lactamases. Lanes: 1 and 4, standard MW markers; 2 and 5, A1 (0.5 μg); 3 and 6, A2 (0.3 μg). Enzymes from *A. hydrophila* AER 19M were in lanes 2 and 3, and those from *A. sobria* AER 14M were in lanes 5 and 6. kD, Kilodaltons.

netic properties of the *A. hydrophila* AER 19M A2h enzyme and the *X. maltophilia* L-1 enzyme. The major difference was inability of the L-1 enzyme to hydrolyze cephalosporins other than nitrocefin under the assay conditions used (Table 3). In contrast, the A2h enzyme demonstrated modest but detectable activity against these β-lactam antibiotics. The relative V_{max} of A2h for cloxacillin was 30 times lower than

TABLE 3. Hydrolysis of β-lactam antibiotics by A1 and A2 β-lactamases from *A. hydrophila* AER 19M and L-1 β-lactamase from *X. maltophilia* GM55

Substrate	A1		A2		L-1	
	Relative V_{max}[a]	K_m (μM)	Relative V_{max}	K_m (μM)	Relative V_{max}	K_m (μM)
Nitrocefin	100	433	100	30.6	100	1.9
Cephalothin	26.8	120	1.8	28	<0.01	—[b]
Cefuroxime	2.9	12.5	12.8	0.8	ND[c]	ND
Cefotaxime	0.3	5.0	0.5	15	<0.01	—
Ceftazidime	0.1	4.0	0.3	9	<0.01	—
Imipenem	<0.01	—	68.5	30	264	13
Penicillin G	0.8	0.4	170	124	824	145
Cloxacillin	<0.01	—	1.1	19	164	0.5
Aztreonam	0.4	0.3	3.2	53.3	34	66.5

[a] Relative rates of hydrolysis of substrates are expressed as percentages of nitrocefin hydrolysis.
[b] —, Not determined because of very low V_{max}.
[c] ND, Not determined.

TABLE 4. Inhibitory effects of cloxacillin and clavulanic acid on A1 and A2 β-lactamases from *A. hydrophila* AER 19M and L-1 β-lactamase from *X. maltophilia* GM55

Substrate	Inhibitor	Enzyme	I_{50} (μM)[a]
Nitrocefin	Cloxacillin	A1	0.26
		A2	>50
		L-1	>50
Nitrocefin	Clavulanate	A1	>40
		A2	0.4
		L-1	>40
Penicillin G	Clavulanate	A2	0.4
		L-1	>40
Imipenem	Clavulanate	A2	>40
		L-1	>40

[a] I_{50}, Inhibitor concentration required for 50% inhibition of enzyme activity.

that of the L-1 enzyme; however, both enzymes demonstrated high V_{max} values for penicillin and imipenem and similar K_m values for these antimicrobial agents. Interestingly, the A2h enzyme was inhibited by clavulanic acid when its activity against benzylpenicillin was assayed but not when its activity against imipenem was assayed. This differential inhibition of substrate hydrolysis was not observed for the L-1 enzyme (Table 4). To assess whether clavulanic acid acted as an inactivator, A2h was incubated with the inhibitor for up to 12 h and measured every hour (following dialysis) for residual activity against benzylpenicillin and imipenem as substrates. No reversal of enzyme inhibition was detected when activity against benzylpenicillin was assayed, but enzyme activity remained uninhibited in the presence of imipenem (data not shown).

Table 5 demonstrates the effects of various inhibitors on the activities of the purified enzymes. Treatment of the A2h and L-1 enzymes with EDTA resulted in almost complete inhibition of activity, but optimal activity was recovered after dialysis, followed by addition of zinc sulfate (final concentration, 1 mM) at 25°C for 30 min. The inhibition profiles were essentially the same for A2h and L-1, except that the L-1 enzyme was slightly less susceptible to $HgCl_2$ and the A2h β-lactamase was more susceptible to iodine than was L-1. In comparison with the metalloenzymes, addition of zinc or EDTA did not enhance or diminish the activity of the A1 enzyme. Furthermore, A1 was more sensitive to inhibition by copper sulfate and *p*-chloromercuribenzoate than was A2h or L1.

TABLE 5. Effects of various inhibitors and ions on the activities of the A1 and A2 β-lactamases of *A. hydrophila* AER 19M and the L-1 β-lactamase of *X. maltophilia* GM55[a]

Inhibitor	Concn (mM)	% Inhibition of:		
		A1	A2	L-1
EDTA	2	0	100	80
Iodine	1	82	71	23
Mercuric chloride	0.5	100	100	89
p-Chloromercuribenzoate	1	49	17	20
Zinc sulfate	1	0	0	0
Copper sulfate	1	54	0	0

[a] The enzyme was preincubated in 0.1 M phosphate buffer (pH 7.0) for 10 min at 37°C with each inhibitor at the indicated concentration, and the remaining activity was assayed with 100 μM nitrocefin as the substrate.

DISCUSSION

Although previous workers have studied the hydrolytic activities of β-lactamases from various *Aeromonas* species against β-lactam agents (16, 30, 32, 33), most investigators have not purified or fully characterized the enzymes. In the current study, *A. hydrophila* and *A. sobria* were found to produce two distinct types of inducible β-lactamases designated A1 (pI 7.0) and A2 (pIs 8.0 and 8.4) that were easily separated by PFBE. Only two other gram-negative organisms, *X. maltophilia* (L-1 and L-2) and *Yersinia enterocolitica* (A and B), have been reported to have two distinct inducible β-lactamases (13, 28, 29). Both genera produce one enzyme with strong cephalosporinase activity (L-2 and B) and a second enzyme (L-1 and A) that hydrolyzed a variety of penicillins. However, only the L-1 β-lactamase of *X. maltophilia* has been shown to be a metalloenzyme.

The substrate profiles revealed distinct enzymatic activity for each of the *Aeromonas* enzymes. The A1 β-lactamase typically behaved as a group 1 cephalosporinase (6). It hydrolyzed cephalothin and cefuroxime much faster than penicillin or the newer broad-spectrum cephalosporins and was inhibited by very low concentrations of cloxacillin. Although the MW of this β-lactamase falls within the range of reported cephalosporinases of other genera, the pI for this enzyme was neutral rather than basic (6).

The second type of inducible β-lactamase, A2, most rapidly hydrolyzed penicillin and imipenem. Minimal hydrolytic activity was observed for cephalosporins other than nitrocefin. Although the A2 enzymes showed some physical characteristics similar to those described by Sawai et al. for an inducible penicillinase that they purified from *A. hydrophila* (32), the A2 enzymes were much less active against oxacillin and had a much lower affinity for benzylpenicillin. Furthermore, no requirement for metal ions was reported by Sawai et al. (32). The hydrolytic activity of the A2 enzymes, in contrast, was inhibited by EDTA but restored upon addition of Zn^{2+}. Although restoration of activity by other divalent cations was not examined further in this study, the dependency of A2 enzymes on Zn^{2+} for optimal activity suggests that they are metalloenzymes. Interestingly, a metalloprotease with a pI of 7.0 was identified in *A. hydrophila*, underlining the importance of divalent cations in the metabolic evolution of this organism (24).

The A2h enzyme had several features in common with purified L-1 β-lactamase and other metalloenzymes described in the literature (5, 15, 27, 29, 31). These similarities include dependency on divalent cations for hydrolytic activity, especially for carbapenem hydrolysis; inhibition by EDTA; and relatively low native MWs. Although L-1 has been shown to exist in polymeric form in the active state (MW, 118,000), the subunit MW has been shown to be 26,000 (29). The A2h enzyme also possessed a basic pI, as observed for the metalloenzymes of *Bacillus cereus* and *Legionella gormanii* (4, 15). Unlike other metalloenzymes, however, A2h was moderately susceptible to clavulanic acid when benzylpenicillin, but not imipenem, was used as the substrate. A similar type of substrate-specific inhibition pattern by *p*-chloromercuribenzoate was described for SHV-1, SHV-2, and TLE plasmid-mediated β-lactamases (2, 22, 26). The activities of these enzymes were inhibited with nitrocefin or cephalothin as the substrate but not with benzylpenicillin as the substrate.

There are at least two explanations for selective inhibition by clavulanic acid. Clavulanic acid might bind close to but not at the active-site locus. Such a bond would create a steric hindrance for access of the larger penicillin molecule into the catalytic cavity but permit the smaller imipenem molecule into this site. A less probable explanation would require two active sites for substrate hydrolysis on A2h. One enzymatic site would contain an active serine residue that would eventually form an irreversible acylenzyme product with clavulanate (8, 11). This would prevent subsequent binding of a penicillin molecule with the serine-containing active site. The second enzymatic site would possess an active-site amino acid other than serine. Partial inhibition of A2h by *p*-chloromercuribenzoate may suggest the presence of catalytically important cysteine residues (7). X-ray crystallography on β-lactamase II of *B. cereus* has revealed the spatial orientation of the histidine residues and the cysteine residue that surround the zinc ion at the active site (5, 36). It is possible that the active site responsible for imipenem hydrolysis on A2h assumes a similar nucleophilic core. For either active site speculated above, zinc would function as an essential electrophilic catalyst in β-lactam hydrolysis, since A2 is nonfunctional in the presence of EDTA (5). Further work is required to determine the precise explanation for this substrate-specific inhibition.

There appear to be few molecular similarities among the known metalloenzymes (7, 15, 27, 29, 31). Thus, it is possible that the A2 enzymes represent a new structural class of β-lactamase. However, sequencing is necessary for a definitive determination.

The importance of β-lactamases in the resistance of *Aeromonas* spp. to various β-lactam antibiotics was also demonstrated in this study. Mutants of *A. hydrophila* AER 19 and *A. sobria* AER 14 that produced high levels of β-lactamases were significantly more resistant to the newer cephalosporins and imipenem than were the wild-type strains from which they were selected. Although the genetic locus of the enzymes was not investigated, their inducibility, coupled with the relatively rare occurrence of conjugative plasmids that confer antibiotic resistance in this genus (10), strongly suggests a chromosomal location. Since (i) a single inducer, cefoxitin or imipenem, caused increased production of both enzymes and (ii) mutations leading to elevated expression affected both enzymes, it is possible that the two structural genes are controlled by a common regulatory mechanism. However, further genetic studies are required to directly address these issues.

ACKNOWLEDGMENTS

This study was supported in part by Health Future Foundation, Omaha, Nebr.

We thank E. S. Moland for excellent technical assistance and Floyd C. Knoop for careful review of the manuscript.

LITERATURE CITED

1. **Bakken, J. S., C. C. Sanders, R. B. Clark, and M. Hori.** 1988. β-Lactam resistance in *Aeromonas* spp. caused by inducible β-lactamases active against penicillins, cephalosporins, and carbapenems. Antimicrob. Agents Chemother. **32:**1314–1319.
2. **Barthelemy, J., J. Peduzzi, H. B. Yaghlane, and R. Labia.** 1988. Single amino acid substitution between SHV-1 β-lactamase and a cefotaxime-hydrolyzing SHV-2 enzyme. FEBS Lett. **231:** 217–220.
3. **Bateman, J. L., R. P. Tu, M. J. Strampfer, and B. A. Cunha.** 1988. *Aeromonas hydrophila* cellulitis and wound infections caused by waterborne organisms. Heart Lung **17:**99–102.
4. **Bicknell, R., E. L. Manuel, J. Gagnon, and S. G. Waley.** 1985. The production and molecular properties of the zinc β-lactamase of *Pseudomonas maltophilia* II D 1275. Biochem. J. **229:**791–797.

5. **Bicknell, R., and S. G. Waley.** 1985. Cryoenzymology of *Bacillus cereus* β-lactamase II. Biochemistry **24**:6876–6887.
6. **Bush, K.** 1989. Classification of β-lactamases: groups 1, 2a, 2b, and 2b'. Antimicrob. Agents Chemother. **33**:264–269.
7. **Bush, K.** 1989. Classification of β-lactamases: groups 2c, 2d, 2e, 3, and 4. Antimicrob. Agents Chemother. **33**:271–276.
8. **Bush, K., and R. B. Sykes.** 1983. β-Lactamase inhibitors in perspective. J. Antimicrob. Chemother. **11**:97–107.
9. **Challapalli, M., B. R. Tess, D. E. Cunningham, A. K. Chapra, and C. W. Houston.** 1988. *Aeromonas* associated diarrhea in children. Pediatr. Infect. Dis. J. **7**:693–698.
10. **Chang, B. J., and S. J. Bottom.** 1987. Plasmids and resistance to antimicrobial agents in *Aeromonas sobria* and *Aeromonas hydrophila* isolates. Antimicrob. Agents Chemother. **31**:1281–1282.
11. **Charnas, R. L., J. Fisher, and J. R. Knowles.** 1978. Chemical studies on the inactivation of *Escherichia coli* R_{TEM} β-lactamase by clavulanic acid. Biochemistry **17**:2185–2189.
12. **Colwell, R. R., M. T. MacDonell, and J. deLey.** 1986. Proposal to recognize the family *Aeromonadaceae* fam. nov. Int. J. Syst. Bacteriol. **36**:473–477.
13. **Cornelis, G., and E. P. Abraham.** 1975. β-Lactamase from *Yersinia enterocolitica*. J. Gen. Microbiol. **87**:273–284.
14. **Diaz, A., A. C. Valasco, F. Hawkins, and F. Cabanas.** 1986. *Aeromonas hydrophila*-associated diarrhea in a neonate. Pediatr. Infect. Dis. **5**:704.
15. **Fuji, T., K. Sato, K. Meyata, M. Inoue, and S. Mitsuhashi.** 1986. Biochemical properties of β-lactamase produced by *Legionella gormanii*. Antimicrob. Agents Chemother. **29**:925–926.
16. **Hedges, R. W., A. A. Medeiros, M. Cohenford, and G. A. Jacoby.** 1985. Genetic and biochemical properties of AER-1, a novel carbenicillin-hydrolyzing β-lactamase from *Aeromonas hydrophila*. Antimicrob. Agents Chemother. **27**:479–484.
17. **Hickman-Brenner, F. W., K. L. MacDonald, A. G. Steigerwalt, G. R. Fanning, D. J. Brenner, and J. J. Farmer III.** 1987. *Aeromonas veronii*, a new ornithine decarboxylase-positive species that may cause diarrhea. J. Clin. Microbiol. **25**:900–906.
18. **Isaacs, R. D., S. D. Paviour, D. E. Bunker, and S. D. R. Lang.** 1988. Wound infection with aerogenic *Aeromonas* strains: a review of twenty-seven cases. Eur. J. Clin. Microbiol. Infect. Dis. **7**:355–360.
19. **Janda, M. J., and P. S. Duffey.** 1988. Mesophilic aeromonads in human disease: current taxonomy, laboratory identification, and infectious disease spectrum. Rev. Infect. Dis. **10**:980–997.
20. **Kuijper, E. J., A. G. Steigerwalt, B. S. C. I. M. Shoenmakers, M. F. Peeters, H. C. Zanen, and D. J. Brenner.** 1989. Phenotypic characterizaton and DNA relatedness in human fecal isolates of *Aeromonas* spp. J. Clin. Microbiol. **27**:132–138.
21. **Kulp, W. L., and D. G. Borden.** 1942. Further studies on *Proteus hydrophilus*, the etiological agent in "red-leg disease" of frogs. J. Bacteriol. **44**:673–685.
22. **Matthew, M.** 1979. Plasmid-mediated β-lactamase of gram-negative bacteria: properties and distribution. J. Antimicrob. Chemother. **5**:349–358.
23. **Motly, M. R., G. McKinley, and J. M. Janda.** 1985. In vitro susceptibilities of *Aeromonas hydrophila*, *Aeromonas sobria*, and *Aeromonas caviae*, to 22 antimicrobial agents. Antimicrob. Agents Chemother. **28**:151–153.
24. **Nieto, T. P., and A. E. Ellis.** 1986. Characterization of extracellular metallo- and serine-proteases of *Aeromonas hydrophila* strain B_{51}. J. Gen. Microbiol. **132**:1975–1979.
25. **Popoff, M.** 1984. Genus III: *Aeromonas*, p. 545–548. *In* N. R. Kreig and J. G. Holt (ed.), Bergey's manual of systemic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
26. **Reid, A. J., I. N. Simpson, P. B. Haiper, and S. G. B. Amyes.** 1987. Identification and characterization of a novel β-lactamase TLE-2, encoded by plasmid pUK702. FEMS Microbiol. Lett. **44**:125–128.
27. **Sabath, L. D., and E. P. Abraham.** 1966. Zinc as a cofactor for cephalosporinase from *Bacillus cereus* 569. Biochem. J. **98**: 11C–13C.
28. **Saino, Y., M. Inoue, and S. Mitsuhashi.** 1984. Purification and properties of an inducible cephalosporinase from *Pseudomonas maltophilia* GN12873. Antimicrob. Agents Chemother. **25**:362–365.
29. **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.
30. **San Joaquin, V. H., R. K. Scribner, D. A. Puckett, and D. F. Welch.** 1986. Antimicrobial susceptibility of *Aeromonas* species isolated from patients with diarrhea. Antimicrob. Agents Chemother. **30**:794–795.
31. **Sato, K., T. Fujii, R. Okamoto, M. Inoue, and S. Mitsuhashi.** 1985. Biochemical properties of β-lactamase produced by *Flavobacterium odoratum*. Antimicrob. Agents Chemother. **27**: 612–614.
32. **Sawai, T., K. Morioka, M. Ogawa, and S. Yamagishi.** 1976. Inducible oxacillin-hydrolyzing penicillinase in *Aeromonas hydrophila* isolated from fish. Antimicrob. Agents Chemother. **10**:191–195.
33. **Shannon, K., A. King, and I. Philips.** 1986. β-Lactamases with high activity against imipenem and Sch 34343 from *Aeromonas hydrophila*. J. Antimicrob. Chemother. **17**:45–50.
34. **Steers, E., E. L. Foltz, and B. S. Graves.** 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. **9**:307–311.
35. **Steffen, R., J. J. Mathewson, C. D. Ericsson, H. L. DuPont, A. Helminger, T. K. Balm, K. Wolff, and F. Witasser.** 1988. Travelers' diarrhea in West Africa and Mexico: fecal transport systems and liquid bismuth subsalicylate for self-therapy. J. Infect. Dis. **157**:1008–1013.
36. **Sutton, B. J., P. J. Artymicek, A. E. Cordero-Borloq, C. Little, D. C. Phillips, and S. G. Waley.** 1987. An X-ray crystallographic study of β-lactamase II from *Bacillus cereus* at 0.35 nm resolution. Biochem. J. **248**:181–188.
37. **Vecoli, C., F. E. Prerost, J. J. Ververis, A. A. Medeiros, and G. P. O'Leary, Jr.** 1983. Comparison of polyacrylamide and agarose gel thin-layer isoelectric focusing for the characterization of β-lactamases. Antimicrob. Agents Chemother. **30**:951–952.
38. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.
39. **Wray, W., T. Boulikas, V. P. Wray, and R. Hancock.** 1981. Silver staining of proteins on polyacrylamide gels. Anal. Biochem. **118**:197–203.