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Characterization of the Beta-Lactamases of Six Species of *Legionella*

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The beta-lactamases of six *Legionella* species were characterized by isoelectric focusing, gel filtration, and substrate profiles. Fifteen strains of *L. bozemanii*, *L. dumoffii*, *L. gormanii*, *L. longbeachae*, and *L. pneumophila* produced beta-lactamases active against nitrocefin. *L. micdadei* enzymes previously reported to be beta-lactamase negative caused a very slow pH-dependent breakdown of nitrocefin and degraded penicillin G at high substrate concentrations. The bioassay revealed predominantly penicillinase activity for all species except *L. micdadei*, which had no activity in this assay. The apparent molecular weights of enzymes of *L. bozemanii*, *L. gormanii*, and *L. pneumophila* were in the range of 15,000 to 32,000, and those of *L. micdadei* and *L. longbeachae* were greater than 250,000. The isoelectric focusing of extracts of *Legionella* strains in polyacrylamide gels showed beta-lactamase types specific for species (*L. bozemanii*, *L. gormanii*, and *L. pneumophila*) and serotype (*L. pneumophila*). It demonstrated four different beta-lactamase types in *L. pneumophila* and revealed close relationships among *L. pneumophila* serotypes 1, 3, and 6. *L. pneumophila* enzymes formed band patterns only in polyacrylamide gels containing 6 M urea, whereas *L. dumoffii* and *L. longbeachae* enzymes did not form bands in any of the gels. None of the band patterns resembled those of known plasmid-mediated beta-lactamases. These experiments suggest that isoelectric focusing of chromosomal beta-lactamases may be a valuable tool for taxonomic studies.

Beta-lactamases (penicillinase, EC 3.5.2.6) not only affect bacterial resistance to beta-lactam antibiotics, but are also useful as species or subspecies markers (10). Most of the strains of *Legionella* have been tested for beta-lactamase activity only by the qualitative nitrocefin test (1, 3, 7, 12, 15, 19). We studied the beta-lactamases of 17 strains (six species) of *Legionella* by enzyme activity assays, gel filtration, and isoelectric focusing (IEF) to determine whether they correlate with species and type designation as determined by DNA hybridization and immunological studies.

MATERIALS AND METHODS

Strains. The *Legionella* strains were obtained from R. M. McKinney, Centers for Disease Control, Atlanta, Ga. (*L. pneumophila* serogroup 2 [Togus 1], *L. pneumophila* serogroup 5 [Dallas 1E and Dallas 2E], and *L. longbeachae* serogroup 2 [Tucker 1]), P. H. Edelstein, Wadsworth Veteran's Administration Medical Center, Los Angeles, Calif. (*L. longbeachae* Los Angeles 24), and W. C. Winn, Medical Center Hospital of Vermont, Burlington, Vt. (*L. pneumophila* serogroup 3 [Burlington 4] and two strains of *L. pneumophila* serogroup 4 [Burlington 5 and Marsh]). The following strains were from the collection of one of us (A.W.P.): *L. bozemanii* MI-15 and WIGA, *L. dumoffii* NY-23 and Tex-KL, *L. gormanii* LS-13, *L. micdadei* EK and JC, and *L. pneumophila* Philadelphia 1 and JT.

Enzyme preparations. The *Legionella* strains were grown on charcoal-yeast-agar (4) incubated at 35°C in 3% CO_2 for 3 days. The bacteria were removed from the plates, suspended in distilled water, centrifuged at 13,800 × *g* for 20 min, resuspended in distilled water, and sonicated for 3 min (20-s bursts and 40-s intervals) with a Branson Sonifier cell disruptor 200. The crude enzyme preparations were cooled with ice water during sonication. The cell debris was removed by centrifugation (20 min at 24,000 × *g*), and the crude enzyme preparation was dialyzed and concentrated with a Micro-ProDiCon (Bio-Molecular Dynamics, Beaverton, Oreg.); membranes with a molecular weight cutoff of 10,000 were used. Protein concentrations were determined by the Lowry method (8). The enzymes were stored at −20°C before assay.

Analytical IEF. Polyacrylamide gels for IEF were prepared as described previously (11), with the exception of acrylamide, which was used at a concentration of 75 mg/ml instead of 70 mg/ml. Some gels contained 3 or 6 M urea, 10% glycerol, or 0.5% Triton X-100. The enzymes were usually placed 4 to 5 cm from the

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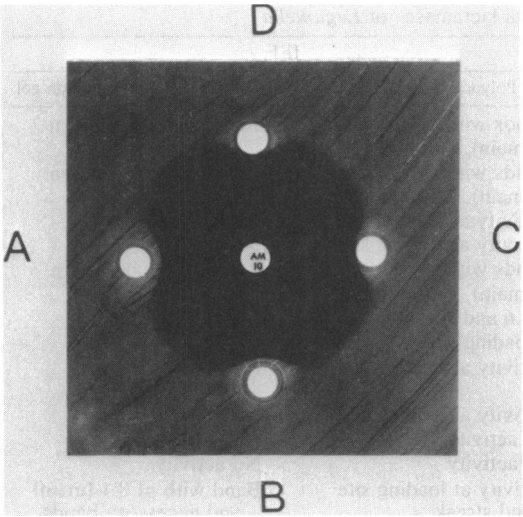

FIG. 1. Bioassay of the hydrolysis of ampicillin by *L. bozemanii* MI-15 beta-lactamase. Amount of protein per enzyme disk: (A) 150 μg, (B) 100 μg, (C) 50 μg, (D) 25 μg.

anode. IEF was carried out at a constant power of 1 W, maximal 320 V, for 15 h and 1 W, maximal 400 V, for 1 h with an LKB 2103 power supply (LKB Instruments Inc., Rockville, Md.). The pH values were measured with a surface electrode connected to a Corning pH meter. The gels were developed by using an enzyme assay. Filter paper soaked in nitrocefin, a chromogenic cephalosporin (0.5 mg/ml; Glaxo Research Ltd., Greenford, Great Britain), was laid onto the gel. The beta-lactamases that cause a breakdown of nitrocefin turned the yellow nitrocefin red.

Beta-lactamase activity. The activity of the crude enzyme preparations was tested against nitrocefin as substrate. Nitrocefin (75 μl; 50 μg/ml) and concentrated bacterial extracts (25 μl) were mixed and checked for color change. A positive reaction was recorded when the color change occurred within 1 min. The activity of some enzymes (see Table 2) was quantitated on a Hitachi 100-60 double-beam spectrophotometer. Enzyme (0.2 ml) was added to 3 ml of nitrocefin (0.1 mM; dissolved in phosphate buffer [pH 7.0; 0.1 M]), and the increase of absorption at a wavelength of 482 nm was measured at 37°C against a blank containing phosphate buffer. In one experiment, the enzyme activity was measured at pH 5.8 and 7.8. The enzyme activities were corrected for the spontaneous hydrolysis of nitrocefin and calculated as micromoles of nitrocefin hydrolyzed per minute per milligram of protein.

The activity of *L. micdadei* enzymes against penicillin was determined with Andrade indicator (14). The enzyme (25 μl) was added to Andrade indicator (75 μl), which contained penicillin G (32 mg/ml). A positive reaction was recorded when the color change, indicating the formation of acidic penicillin metabolites, occurred within 3 min.

The activities of the enzymes against seven substrates were determined by a bioassay performed by the method of Masuda et al. (9) and R. Labia (personal communication). The enzyme preparations were diluted in distilled water to give protein concentrations of 7.5, 5, 2.5, and 1.25 mg/ml. *Sarcina lutea* ATCC 6687 was streaked onto Mueller-Hinton agar plates. An antibiotic-containing disc was placed on the agar; the disks loaded with 20 μl of the enzyme preparation (giving 25 to 150 μg of protein per disk) were placed at the edge of the expected inhibition zone. The plates were incubated at 35°C for 18 h. A positive reaction was recorded when bacterial growth was noticed within the inhibition zone at the site of the enzyme disk (Fig. 1). The reactions were graded from 1 (low enzyme activity) to 4. A TEM-1 enzyme was included for comparison. The following antibiotics were tested: penicillin G (10 U per disk), ampicillin (10 μg per disk), carbenicillin (100 μg per disk), cephaloridin (30 μg per disk), cephalothin (30 μg per disk), cefamandole (30 μg per disk), and nitrocefin (100 μg per disk).

Gel filtration. Sephacryl S200 Superfine (Pharmacia, Uppsala, Sweden) and a G12 column (1,000 mm long) were used for the estimation of the apparent molecular weights. The columns were equilibrated with sodium phosphate buffer (0.1 M; pH 7.0; 0.1% sodium azide) and run at 5°C. Bovine serum albumin, ovalbumin, chymotrypsinogen, and RNase A were used for standardization of the column. The eluant was tested for

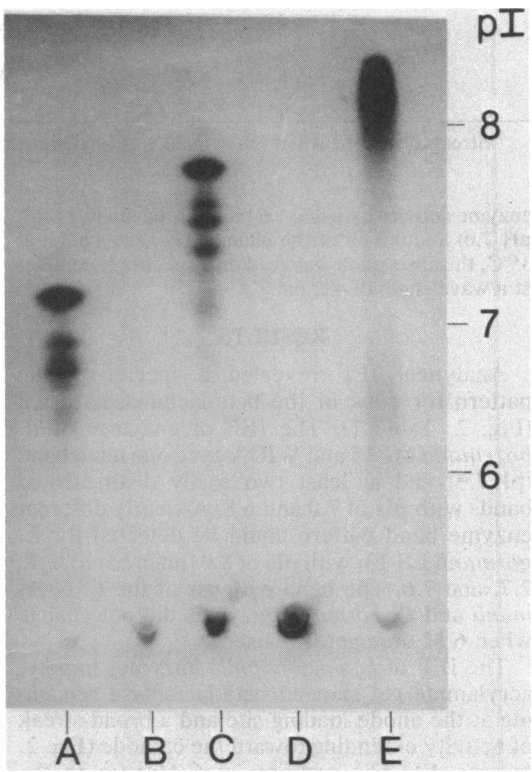

FIG. 2. Flat-bed IEF of beta-lactamases of *Legionella*. (A) *L. bozemanii* WIGA, (B) *L. dumoffii* Tex-KL, (C) *L. gormanii* LS-13, (D) *L. longbeachae* Los Angeles 24, (E) *L. pneumophila* Philadelphia 1.

TABLE 1. Flat-bed IEF of beta-lactamases of *Legionella*

Species	Serotype	Strain	IEF	
			Polyacrylamide gel	6 M urea polyacrylamide gel
L. bozemanii		MI-15	Bands with pI 7.3 (main), 7.0, and 6.8	Identical band pattern
		WIGA	Bands with pI 7.3 (main), 7.0, and 6.8	Identical band pattern
L. dumoffii		NY-23	Activity at loading site[a]	No activity
		Tex-KL	Activity at loading site[a]	No activity
L. gormanii		LS-13	Bands with pI 8.0 (main), 7.8, 7.7, and 7.6 and activity at loading site	Identical band pattern
L. longbeachae	1	Los Angeles 24	Activity at loading site	No activity
	2	Tucker 1	Activity at loading site	No activity
L. micdadei		EK	No activity	No activity
		JC	No activity	No activity
L. pneumophilia	1	Philadelphia 1	Activity at loading site and streak	Band with pI 8.1 (main) and accessory bands
	2	Togus 1	Activity at loading site and streak	Band with pI 8.24 (main) and accessory bands
	3	Burlington 4	Activity at loading site and streak	Band with pI 8.16 (main) and accessory bands
	4	Burlington 5	Activity at loading site and streak	Band with pI 8.24 (main) and accessory bands
	4	Marsh	Activity at loading site	Band with pI 8.24 (main)
	5	Dallas 1E	Activity at loading site and streak	Band with pI 8.36 (main) and accessory bands
	5	Dallas 2E	Activity at loading site and streak	Band with pI 8.36 (main) and accessory bands
	6	JT	Activity at loading site and streak	Band with pI 8.16 (main) and accessory bands

[a] Streak observed when the sample was loaded near the cathode.

enzyme activity by using 1.5 ml of nitrocefin (0.1 mM; pH 7.0) and 0.2 ml of the eluant. After incubation at 35°C, the absorption was read on a spectrophotometer at a wavelength of 482 nm.

RESULTS

Analytical IEF revealed a species-specific pattern for most of the beta-lactamases tested (Fig. 2; Table 1). The IEF of enzymes of *L. bozemanii* MI-15 and WIGA gave one main band (pI 7.3) and at least two easily distinguished bands with pIs of 7.0 and 6.8. A clearly different enzyme band pattern could be detected for *L. gormanii* LS-13, with pIs of 8.0 (main band), 7.8, 7.7, and 7.6. The band patterns of the *L. bozemanii* and *L. gormanii* enzymes did not change when 6 M urea gel was used.

The IEF of *L. pneumophila* enzymes in polyacrylamide gel showed beta-lactamase remaining at the anode loading site and a broad streak of activity extending toward the cathode (Fig. 2, column E). The addition of 6 M urea to the polyacrylamide gel resulted in the formation of discrete bands in four different patterns, with main bands of pIs 8.10, 8.16, 8.24, and 8.36 (Fig. 3). Enzymes of Philadelphia 1, Burlington 4, and

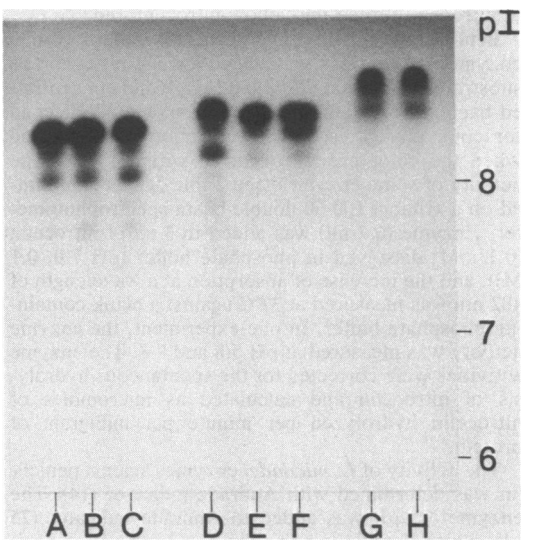

FIG. 3. Flat-bed IEF of beta-lactamases of *L. pneumophila* in a 6 M urea polyacrylamide gel. Serotypes and strains: (A) 1 (Philadelphia 1), (B) 3 (Burlington 4), (C) 6 (JT), (D) 2 (Togus 1), (E) 4 (Burlington 5), (F) 4 (Marsh), (G) 5 (Dallas 1E), (H) 5 (Dallas 2E).

JT of serotypes 1, 3, and 6 had identical band patterns, which differed from those of Togus 1, Burlington 5, Marsh, Dallas 1E, and Dallas 2E of serotypes 2, 4, and 5. Different strains of the same serotype had identical IEF patterns (Fig. 3, columns E and F and columns G and H).

Most of the enzymes of *L. dumoffii* stayed at the loading site. The beta-lactamase activity at the loading site increased, and a streak of low activity was detected when the loading site was nearer the cathode. The addition of glycerol or Triton X-100 to the gel did not result in the formation of enzyme bands. *L. longbeachae* enzymes also stayed at the loading site but, in contrast to those of *L. dumoffii*, did not produce a streak of activity. Both *L. longbeachae* and *L. dumoffii* enzymes showed no activity in a 6 M urea gel. Concentrates of enzymes prepared by the freeze-thaw method (2) (*L. dumoffii*) or by osmotic shock (16) (*L. longbeachae* Tucker 1) had insufficient activity for IEF.

The enzyme preparations of all strains except *L. micdadei* gave a positive nitrocefin reaction within 1 min. The activities, determined as micromoles of nitrocefin hydrolyzed per minute per milligram of protein (Table 2), ranged from very active (*L. gormanii* and *L. bozemanii*) to intermediately active (*L. dumoffii*, *L. longbeachae*, and *L. pneumophila*). *L. micdadei* enzymes showed a very slow increase in absorption at pH 7.0; the hydrolysis rate was three times greater at pH 7.8. A wavelength scan after the completed enzyme reaction showed a peak of absorption in the 480- to 490-nm range, typical of the hydrolysis of the beta-lactam bond of nitrocefin (17). The TEM-1 beta-lactamase had a considerably higher activity than did the *Legionella* enzymes. With penicillin G as the substrate, the *L. micdadei* enzymes caused a color change of Andrade indicator within 3 min.

The bioassay (Table 3) showed that only *L. micdadei* enzymes inactivated none of the substrates tested. All the other *Legionella* enzymes inactivated the penicillins (penicillin G, ampicillin, and carbenicillin) and to a lesser degree (*L. bozemanii*, *L. gormanii*, and *L. pneumophila*) the cephalosporins. The enzymes of *L. dumoffii* and *L. longbeachae* and the TEM-1 beta-lactamase included for comparison did not hydrolyze the cephalosporins.

The apparent molecular weights of the *L. bozemanii*, *L. gormanii*, and *L. pneumophila* enzymes were in the range of 15,000 to 32,000 (Table 2). In contrast, the enzymes of *L. dumoffii* and *L. longbeachae* were eluted with the void volume (molecular weight, ≥250,000). In experiments with *L. dumoffii* NY-23, a small enzyme peak was detected in the molecular weight range of 34,000 to 37,000; the amount of enzyme, however, was insufficient for IEF.

DISCUSSION

Our data confirm the findings of others (1, 3, 7, 12, 15, 19) that *L. bozemanii*, *L. dumoffii*, *L. gormanii*, *L. longbeachae*, and *L. pneumophila* produce beta-lactamases, as shown by the qualitative nitrocefin test. In contrast to previous studies (6), however, *L. micdadei* enzymes were able to break down nitrocefin, but at a very slow

TABLE 2. Hydrolysis rate and apparent molecular weights of *Legionella* beta-lactamases

Species	Strain	Nitrocefin hydrolysis rate (nmol/min per mg of protein; pH 7.0)	Apparent mol wt (10^3)
L. bozemanii	MI-15	73	15–17
	WIGA	66	15–16
L. dumoffii	NY-23	27	≥250
	Tex-KL	24	≥250
L. gormanii	LS-13	109	18–22
L. longbeachae	Los Angeles 24	20	≥250
L. micdadei	EK	0.12	ND[a]
	EK	0.07[b]	
	EK	0.36[c]	
	JC	0.14	ND
L. pneumophila	Philadelphia 1	36	28–31
	JT	41	28–32
Escherichia coli TEM-1	K-12	177	ND

[a] ND, Not determined.
[b] pH 5.8.
[c] pH 7.8.

TABLE 3. Activity of *Legionella* enzymes determined by a bioassay

Antibiotic	Enzyme activity[a]										
	L. bozemanii		*L. dumoffii*		*L. gormanii* LS-13	*L. longbeachae* Los Angeles 24	*L. pneumophila*		*L. micdadei*		*Escherichia coli* K-12 TEM-1
	MI-15	WIGA	NY-23	Tex-KL			Philadelphia 1	JT	EK	JC	
Penicillin G	4	4	2	2	4	2	2	3	NR[b]	NR	4
Ampicillin	3	3	1	1	3	1	2	3	NR	NR	3
Carbenicillin	4	4	1	1	3	1	1	2	NR	NR	3
Cephalothin	2	1	NR	NR	1	NR	NR	1	NR	NR	NR
Cephaloridine	3	2	NR	NR	2	NR	NR	NR	NR	NR	NR
Cefamandole	1	1	NR	NR	1	NR	1	2	NR	NR	NR
Nitrocefin	4	4	1	1	4	2	2	3	NR	NR	1

[a] Scale ranging from 4 (high activity) to 1 (low activity).
[b] NR, No reaction.

pH-dependent rate. A wavelength scan of the degraded nitrocefin revealed an absorption peak at 480 to 490 nm, typical of the hydrolysis of its beta-lactam bond (17). Penicillin G was hydrolyzed, as shown by the color change of the Andrade indicator. The very high substrate concentrations (32 mg/ml) and the basic pH of Andrade indicator might have accelerated the hydrolysis of penicillin. This reaction, however, could also be due to enzymes other than beta-lactamases, since only the production of acid is measured. Although the bioassay showed no activity for *L. micdadei* enzymes, we conclude that *L. micdadei* is probably a low-level producer of beta-lactamases active against nitrocefin and perhaps also against penicillin.

The activity of the *Legionella* beta-lactamases against different beta-lactam antibiotics was determined by a microbiological method, since the iodometric and spectrophotometric methods proved to be unsatisfactory (5, 19). Fu and Neu (5) and Thornsberry and Kirven (19) found that *L. pneumophila* enzymes were primarily cephalosporinases. Our assays, however, showed high penicillinase activity for the beta-lactamases of all *Legionella* strains, except *L. micdadei*, which was negative. These contradictory results might be due to the fact that in our assay the enzyme activities were determined at very low substrate concentrations, i.e., at the edge of the inhibition zone. Therefore, detectable substrate inactivation could only occur when there was sufficient affinity between substrate and enzyme. However, since this microbiological assay depends on several variables, such as the diffusion of the enzyme into the agar and the susceptibility of the indicator organism to the test antibiotic, these results cannot be compared with direct enzyme measurements of substrate profile.

None of the IEF patterns of the beta-lactamases of *Legionella* resembled those of known plasmid-mediated beta-lactamases. Therefore, either these enzymes represent a completely new group of plasmid-mediated beta-lactamases, which does not seem to be highly probable, or they are chromosomally mediated. Our analysis of these beta-lactamases supports the idea of Matthew et al. (10) that beta-lactamases can be used for taxonomic purposes. The IEF of extracts of *Legionella* strains showed beta-lactamase types specific for species (*L. bozemanii*, *L. gormanii*, and *L. pneumophila*) and partially specific for serotypes (*L. pneumophila*). It demonstrated four different beta-lactamase types in *L. pneumophila* and revealed a close relationship among *L. pneumophila* serotypes 1, 3, and 6. This supports the results of direct fluorescent-antibody tests, which showed cross-reactivity between serogroups 3 and 6 and irregular staining patterns of strains of serogroup 6 when stained with a serogroup 1 conjugate (13).

L. dumoffii and *L. longbeachae* beta-lactamases differed from each other only in their ability to produce a streak of enzyme activity when the enzymes were loaded near the cathode. However, other differences might be disguised by their inability to form bands on any of the gels. Because of their molecular weights of greater than 250,000, it appears likely that they are bound to larger molecules (e.g., membranes). Timewell et al. (20) made a similar observation regarding beta-lactamases of the *Bacteroides melaninogenicus* group which had molecular weights greater than 40×10^6 and did not form bands on IEF, possibly due to the attachment to lipopolysaccharides. They showed that these beta-lactamases could be focused if the enzymes were prepared by osmotic shock instead of sonication. However, concentrates of *L. dumoffii* and *L. longbeachae* did not yield detectable beta-lactamase activity after

osmotic shock or freeze-thaw treatment, indicating that their beta-lactamases are probably tightly attached to membranes.

The role of these beta-lactamases in determining antibiotic resistance is unclear. Although *L. bozemanii*, *L. dumoffii*, and *L. gormanii* appear to degrade penicillins more efficiently than cephalosporins, the minimal inhibitory concentrations of the penicillins are generally lower than those of the cephalosporins for these organisms (6, 18). Similarly, there is little correlation between levels of enzyme activity as measured by nitrocefin hydrolysis rates and susceptibility to beta-lactam antibiotics. An exception is *L. micdadei*, which produces very little beta-lactamase and is highly susceptible to both penicillins and cephalosporins in vitro.

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