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Genetic, Immunological, and Cytotoxic Comparisons of *Legionella* Proteolytic Activities

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Several strains of *Legionella pneumophila* and other species of *Legionella* with proteolytic activities were compared by assays, including Southern hybridizations and Western immunoblots, to determine their proteolytic, hemolytic, and cytotoxic activities. Only proteases from strains of *L. pneumophila* were both hemolytic and cytotoxic, and proteolytic activities extracted from other species of *Legionella* possessed only hemolytic activity. A 4.0-kilobase DNA sequence encoding the 38-kilodalton metalloprotease from *L. pneumophila* Philadelphia 1 that we showed previously was responsible for the observed hemolytic and cytotoxic phenotypes (F. D. Quinn and L. S. Tompkins, Mol. Microbiol., 3:797–805, 1989) was used in Southern hybridizations to probe chromosomal DNA from several strains of *L. pneumophila* and other *Legionella* species. The probe hybridized to the chromosomal DNA of all serogroups of *L. pneumophila* but not to any strains of *L. dumoffii*, *L. micdadei*, *L. feeleii*, or *L. jordanis* that we examined. Additionally, Western immunoblots done with rabbit antisera made to the cloned *L. pneumophila* protease demonstrated cross-reactions among 38-kilodalton proteins from strains of *L. pneumophila*, but no reactions were observed with proteins from other species of *Legionella*. Similarly, the cloned protease from *L. pneumophila* reacted with convalescent-phase sera from patients infected with *L. pneumophila*, but not with antisera isolated from patients infected with other *Legionella* species. Thus, despite some similarities among the proteolytic activities of members of the genus *Legionella*, including proteolytic and hemolytic phenotypes, metal requirements for zinc or iron, sensitivity to EDTA, and temperature and pH optima, we documented distinct genetic, immunological, and cytotoxicity differences among the proteolytic activities produced by *Legionella* species.

Members of the family *Legionellaceae*, which now comprise 28 known species, are the etiologic agents responsible for the pneumonic illness known as Legionnaires disease and the nonpneumonic syndrome Pontiac fever. Although not all species have been isolated from humans, each is considered a potential human pathogen. The majority (85 to 90%) of human pneumonic infections attributed to *Legionella* species have been caused by *Legionella pneumophila* serogroup 1 (34), with *Legionella micdadei* contributing another 6%. Pontiac fever has been shown to be caused by strains of *L. pneumophila* (30), *L. micdadei* (21), and *Legionella feeleii* (24). It is likely that a combination of risk factors resulting in decreased host defenses and exposure to an environmental source produces the highest probability of infection (1, 15–17). However, the genetic basis for virulence of these organisms remains unknown.

Several bacterial products may play a role in the virulence of *Legionella* species. Extracellular enzymes, such as proteases (13, 23), cytotoxins (6), and hemolysins (3), have been implicated as factors that are responsible for the pulmonary (9) and extrapulmonary (9, 46) manifestations of Legionnaires disease. Proteases have been shown to be major contributing factors in the development of pulmonary lesions in experimental pneumonias caused by *Pseudomonas aeruginosa* (25) and *Serratia marcescens* (28). Several groups of investigators (6, 7, 18, 26, 40) have demonstrated that purified protease or culture supernatants containing proteolytic activity from *L. pneumophila* have cytolytic effects on tissue culture cells, while Conlan et al. (13), Belyi et al. (6), and Rosenfeld et al. (35) have demonstrated that a partially purified *L. pneumophila* protease administered into the lungs of guinea pigs or administered to guinea pigs intradermally could elicit hemorrhagic and necrotic lesions. In addition, Blander and Horwitz (10) have recently observed that infected guinea pigs develop cell-mediated immunity to the protease, while Williams et al. (45) have shown, by using double-labeling techniques, that *L. pneumophila* cells and protease proteins are located in the same intracellular location and are intimately associated with pulmonary lesions.

Recent work in several laboratories (6, 26, 32a, 35) has demonstrated that the 38-kilodalton (kDa) protease from *L. pneumophila* produces the observed hemolytic and cytotoxic activities. It is not known whether these characteristics are possessed by proteases from other *Legionella* species or whether their proteases differ structurally and functionally from the 38-kDa zinc metalloprotease of *L. pneumophila*. Some evidence indicates that species other than *L. pneumophila* produce hemolytic activities (3, 11), but no reports of cytotoxic activities have been published.

We have recently cloned the genetic sequence encoding a 38-kDa protease from *L. pneumophila* serogroup 1 (32a) and have shown, by transposon inactivation analysis, that a single trifunctional polypeptide encoded on a 1.2-kilobase (kb) cloned DNA fragment, designated *pro*, from *L. pneumophila* serogroup 1 is responsible for proteolytic, hemolytic, and cytotoxic properties. In this study we compared the proteolytic, hemolytic, and cytotoxic activities from several species within the genus *Legionella* using molecular genetic and immunoblot analyses.

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MATERIALS AND METHODS

Bacterial strains and growth media. Most of the clinical isolates of *Legionella*, as well as the *P. aeruginosa* Hak-1, *Aeromonas hydrophila*, and *S. marcescens* strains used in this study, were obtained from the Clinical Microbiology Laboratory, Stanford University Medical Center. Other clinical isolates, as well as environmental isolates of *Legionella jordanis* and *L. feeleii*, were kindly supplied by J. Barbaree (Centers for Disease Control, Atlanta, Ga.). Isolates of *L. pneumophila* Philadelphia 1 and *L. jordanis* were independently acquired from the Centers for Disease Control; their strain affiliations are unknown.

L broth and L agar (42) were used to grow *Escherichia coli* K-12 derivatives carrying cloned genes. Buffered charcoal-yeast extract agar and buffered yeast extract broth used for the cultivation of *Legionella* species were prepared as described previously (33). Skim milk plates were made by adding 1% powdered skim milk to basal L-broth medium before it was autoclaved. Antibiotics were added after the medium was thoroughly stirred and cooled to 50°C. Canine blood medium was formulated by adding heparin-treated canine whole blood to a final concentration of 5% to autoclaved and cooled L agar. Media and skim milk were purchased from Difco Laboratories (Detroit, Mich.). Canine whole blood was furnished by the Department of Laboratory Animal Medicine, Stanford University. Antibiotics, salts, buffers, and charcoal were purchased from Sigma Chemical Co. (St. Louis, Mo.). Restriction endonucleases were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). Nitrocellulose was acquired from Schleicher & Schuell, Inc. (Keene, N.H.). Reagents for agarose and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, Calif.).

Isolation and analysis of proteases from *Legionella* species. *Legionella* isolates were inoculated into 15 ml of buffered yeast extract broth and grown in a gyratory shaker at 37°C and 200 rpm until the stationary phase was attained (12 to 18 h). After cellular material was removed by high-speed centrifugation and filtered through 0.45-μm-pore-size disposable syringe filter units (Gelman Sciences, Inc., Ann Arbor, Mich.), the supernatants were concentrated 10-fold with mini-concentrator units (Amicon Corp., Danvers, Mass.). The concentrated supernatants were assayed and standardized for proteolytic activity by using the dye release assay described by Dreyfus and Iglewski (14). Proteolytic activity from concentrated supernatants or periplasmic osmotic shockates was added to 1 ml of 0.025 M NaH_2PO_4 (pH 6.0) and incubated at room temperature for 15 min in polypropylene microcentrifuge tubes before the addition of 25 mg of hide powder azure (Sigma). Assay tubes were incubated at 37°C for 30 min while they were shaken vigorously. After incubation, the samples were chilled in an ice bath and centrifuged for 5 min at 1,000 × *g*, and the supernatants were measured spectrophotometrically at 595 nm for dye release. One unit of protease activity was arbitrarily defined as the amount of enzyme that produced a change in the A_{595} of 1.0 during 30 min of incubation. The temperature stability of the protease was determined as follows. After the enzyme was incubated at various temperatures for 1 h, samples were assayed for protease activity as described above. The pH optimum for protease activity was determined by assaying dilutions of protease in the following buffers: 0.025 M acetate (pH 4.0, 4.5, 5.0, and 5.5), 0.025 M NaH_2PO_4 (pH 5.5, 6.0, 6.5, and 7.0), and 0.025 M Tris hydrochloride (pH 7.5 and 8.0).

It has been demonstrated by Baine (2) that canine erythrocytes are more susceptible to lysis by *Legionella* species-produced factors than are erythrocytes from several other mammalian species. In the assays described here, hemolysis was determined after the addition of 100-μl dilutions of the protease containing 0.1 to 2.0 U of activity into wells bored in 5% canine blood agar plates. Plates were subsequently scored for beta-hemolysis after incubation for 48 h at 37°C.

Cytotoxic activity was demonstrated by tissue culture bioassay by a modification of the procedure of Gentry and Dalrymple (20). Fresh monolayers of CHO cells were grown to a density of 5×10^5 isolates per well in 24-well, flat-bottom tissue culture dishes. Tissue culture medium consisted of minimal essential medium (GIBCO Diagnostics, Madison, Wis.) supplemented with L-glutamine and 5% fetal bovine serum at 37°C in a humidified CO_2 incubator. Dilutions of the concentrated supernatants containing 0.1 to 2.0 U of proteolytic activity in 100-μl volumes were added to the tissue culture wells containing CHO cells in minimal essential medium. The dishes were incubated for 36 h at 37°C in a CO_2 incubator. The CHO cells were observed microscopically before they were rinsed in phosphate-buffered saline, fixed with Formalin, and stained with crystal violet. The amount of crystal violet extracted from the monolayers by the addition of 1 ml of 50% ethanol was determined spectrophotometrically at 595 nm. In the assay the concentration of extracted dye was directly proportional to the number of viable cells present.

Comparison of *Legionella* and other bacterial proteases by immunoblotting. Rabbit anti-*Legionella* protease antiserum was obtained as a generous gift from the laboratory of Paul Hoffman (University of Tennessee, Memphis) (26). Antiserum was also raised in rabbits to periplasmic osmotic shockates from *E. coli* clones containing a recombinant plasmid described previously (32a). Human convalescent-phase sera were obtained from B. Fields and H. Wilkinson (Centers for Disease Control) and from several patients in the Stanford University Hospital who were recovering from documented infections caused by *L. pneumophila*, *Legionella dumoffii*, and *L. micdadei* (41). Antibody titers of the convalescent-phase sera were determined by using indirect immunofluorescence by the methods of the Centers for Disease Control or the California State Laboratories, Berkeley.

Immunoblot analysis of the recombinant protease and wild-type proteases were performed by the electrophoretic transfer procedure described by Towbin et al. (43). Recombinant and wild-type proteases were treated with 5% β-mercaptoethanol and run on sodium dodecyl sulfate-polyacrylamide gels (27). Recombinant protease was obtained by periplasmic osmotic shock of *E. coli* strains containing the cloned protease gene as described previously (32a). Wild-type proteases were obtained from concentrated *Legionella* culture supernatants as described above. Protein bands were transferred to nitrocellulose paper with a transblot apparatus (Bio-Rad). Positive bands were detected by immunoperoxidase staining with a 1:200 dilution of rabbit antisera prepared against either the recombinant or wild-type proteases and a 1:3,000 dilution of horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (Bio-Rad).

Analysis of *Legionella* and other proteolytic bacteria by Southern hybridization. *Legionella* isolates were propagated directly from frozen stock cultures on buffered charcoal-yeast extract agar plates. Scrapings from three plates were combined in phosphate buffered saline (pH 7), and genomic

TABLE 1. Comparison of Southern hybridizations with supernatant activities from *Legionella* species, other proteolytic bacteria, and *E. coli* containing a recombinant plasmid[a]

Organism	Source	Proteolysis	Hemolysis	Cytotoxicity	Southern reaction with a 4-kb probe
E. coli LE392		−	−	−	−
P. aeruginosa Hak-1	SUMC	+	w	w	−
A. hydrophila	SUMC	+	ND	ND	−
S. marcescens	SUMC	+	ND	ND	−
E. coli LE392(pSP106)		+	+	+	+
L. pneumophila Philadelphia 1, CDC-1 (parent)	CDC	+	+	+	+
L. pneumophila Philadelphia 1, CDC-2	CDC	+	+	+	+
L. pneumophila Glouchester 1	CDC	+	+	+	+
L. pneumophila Indianapolis 1	CDC	+	+	+	+
L. pneumophila Naples 1	CDC	+	+	+	+
L. pneumophila Alameda 1	CDC	+	+	+	+
L. pneumophila serogroup 1, SUMC 1p	SUMC	+	+	+	+
L. pneumophila serogroup 1, SUMC 1ap	SUMC	+	+	+	+
L. pneumophila serogroup 3, CDC 3	SUMC	+	+	+	+
L. pneumophila serogroup 6, CDC 6	SUMC	+	+	+	+
L. pneumophila serogroup 8, CDC 8	SUMC	+	+	+	+
L. dumoffii Tex-KL	CDC	+	+	−	−
L. dumoffii SUMC 1d	SUMC	+	+	−	−
L. micdadei 31B	Pittsburgh[b]	+	+	−	−
L. micdadei SUMC 1m	SUMC	−	−	−	−
L. feeleii serogroup 1, CDC 1f	CDC	−	−	−	−
L. feeleii serogroup 2, CDC 2f	CDC	−	−	−	−
L. jordanis BL-540	CDC	+	+	−	−
L. jordanis CDC 1j	CDC	+	+	−	−

[a] Abbreviations: SUMC, Stanford University Medical Center; CDC, Centers for Disease Control; W, weak activity; ND, not determined.
[b] This strain was obtained from V. Yu, University of Pittsburgh Hospital.

DNA was isolated by the method of Roussel and Chabbert (36). Total genomic DNA was cleaved by *Sal*I-*Bgl*II endonuclease digestions and electrophoresed overnight at 30 V on 0.7% agarose. The fragments were transferred to nitrocellulose sheets by the method of Southern (38). A 4.0-kb *Sal*I-*Bgl*II fragment encoding the recombinant protease from *E. coli* LE392(pSP106) (32a) was isolated from low-melting-point agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) by the method of Struhl (39) and was radiolabeled with [α-^{32}P]dCTP (DuPont, NEN Research Products, Boston, Mass.) by nick translation with a kit (Bethesda Research Laboratories). Hybridization with the radiolabeled probe was carried out under high stringency in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate–0.2% EDTA at 68°C for 18 h, before blots were washed twice in 2× SSC at 68°C (31, 42). Filters were exposed to X-AR5 film (Eastman Kodak Co., Rochester, N.Y.) at −70°C in the presence of an intensifying screen for 12 to 48 h.

RESULTS

Detection of proteolytic, hemolytic, and cytotoxic phenotypes from *Legionella* species. Osmotic shockates from *E. coli* and *E. coli*(pSP106) expressing the *L. pneumophila* recombinant protease and concentrated supernatants from 28 strains of *Legionella*, *Pseudomonas*, *Aeromonas*, and *Serratia* were assayed for the presence of proteolytic, hemolytic, and cytotoxic activities. Within 24 h, zones of proteolysis were observed on casein agar plates inoculated with the osmotic shockate from the *E. coli* strain containing the *L. pneumophila* protease gene and concentrated supernatants from all strains of *L. pneumophila*, *L. dumoffii*, and *L. jordanis*; one strain of *L. micdadei*; and control strains of *Pseudomonas*, *Aeromonas*, and *Serratia* (Table 1). The osmotic shockates and the concentrated supernatants from all the proteolytic strains of *Legionella* showed hemolysis on canine blood agar plates within 24 to 48 h (Table 1).

Cytotoxic activity was observed within 24 to 48 h after the addition of 1.0 U of proteolytic activity from the shockates or supernatants from *L. pneumophila* strains. Cells of CHO cell monolayers became rounded and detached from the surface of the microdilution wells, and total cell lysis of the monolayer occurred by 48 h, as demonstrated by microscopy and the crystal violet retention assay (Table 1). Even after 128 h and proteolytic activity of up to 2 U, proteolytic activities from species other than *L. pneumophila* had no effect on CHO cell monolayers (Table 1).

Stability of activities of the *Legionella* proteases. Osmotic shockate from *E. coli* LE392(pSP106), which expressed the recombinant protease and concentrated supernatants from several strains of *L. pneumophila*, *L. dumoffii*, *L. micdadei*, and *L. jordanis*, which were determined to be proteolytic (Table 1), were assayed for pH and temperature optima and sensitivity to the metalloprotease inhibitor EDTA. With the addition of 1.0 mM EDTA, proteolytic, hemolytic, and cytotoxic activities from all examined proteolytic strains were reduced substantially. The optimum pH for the proteolytic activities was found to be approximately 6.0; activity decreased rapidly when the pH was raised ≥2 units above the optimum. Assays were conducted by increasing the pH from 4.0 to 8.0 in 0.5-unit increments. The optimum active temperature for the cloned and wild-type proteases was 35 to 42°C. Temperatures of ≥60°C caused substantial decreases in proteolytic activities for all examined species of *Legionella*. Analysis of the temperature and pH optima for hemolytic and cytotoxic activities of the various proteases could not be determined because of autolysis of the test cells in deleterious environments. Proteolytic, hemolytic, and cytotoxic activities of the cloned protease and *L. pneumophila*

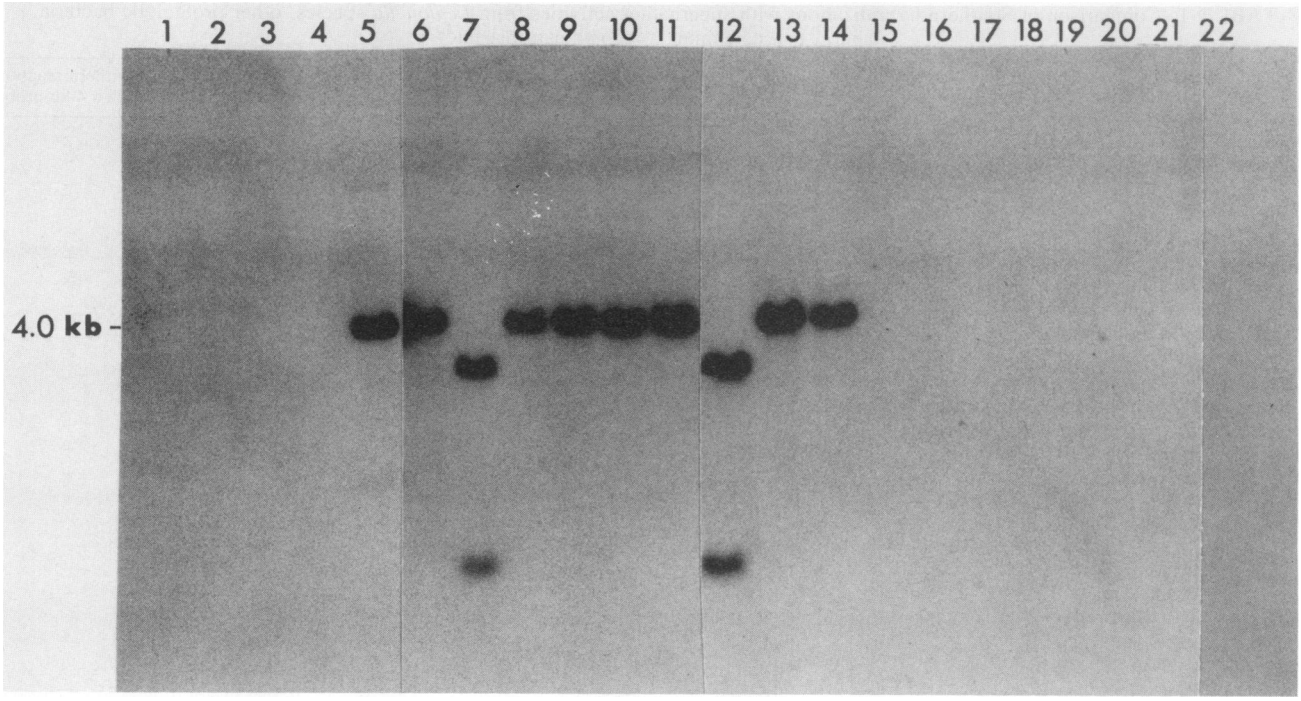

FIG. 1. Southern blot analysis of *Sal*I-*Bgl*II chromosomal digestions from several strains of *Legionella* and control bacteria. The protease gene containing the 4.0-kb *Sal*I-*Bgl*II fragment was used as the probe. Lanes: 1, *E. coli* LE392; 2, *P. aeruginosa* Hak-1; 3, *A. hydrophila*; 4, *S. marcescens*; 5, purified 4.0-kb *Sal*I-*Bgl*II fragment; 6, *L. pneumophila* Philadelphia 1, CDC 1 (parent strain); 7, *L. pneumophila* Philadelphia 1, CDC 2; 8, *L. pneumophila* serogroup 1 clinical isolate SUMC 1p; 9, *L. pneumophila* serogroup 1 clinical isolate SUMC 1ap; 10, *L. pneumophila* Indianapolis 1; 11, *L. pneumophila* Naples 1; 12, *L. pneumophila* serogroup 3, CDC 3; 13, *L. pneumophila* serogroup 6, CDC 6; 14, *L. pneumophila* serogroup 8, CDC 8; 15, *L. dumoffii* Tex KL; 16, *L. dumoffii* clinical isolate SUMC 1d; 17, *L. micdadei* clinical isolate SUMC 1m; 18, *L. micdadei* 31-B; 19, *L. feeleii* serogroup 1, CDC 1f; 20, *L. feeleii* serogroup 2, CDC 2f; 21, *L. jordanis* CDC 1j; 22, *L. jordanis* BL-540.

wild-type concentrated supernatant were blocked by the addition of a 1:2 dilution of polyclonal rabbit antibodies made against either the native or recombinant proteases. The same dilution of antibodies made against the recombinant protease had no effect on activities from the concentrated culture supernatants of *L. dumoffii*, *L. micdadei*, *L. jordanis*, or *P. aeruginosa*.

Southern hybridization of the protease gene from *L. pneumophila* with other *Legionella* species. The 4.0-kb *Sal*I-*Bgl*II fragment encoding the 38-kDa protease from *L. pneumophila* hybridized under high-stringency conditions with restriction fragments from all strains of *L. pneumophila* (Fig. 1). Chromosomal DNA restriction fragments of *Legionella* species other than *L. pneumophila* and control bacteria consisting of representatives from other genera known to produce extracellular proteases did not hybridize with the probe under similar stringency conditions. The 4.0-kb fragment hybridized exclusively to DNA fragments from cytotoxic strains of *L. pneumophila* (Table 1).

Immunoblot analysis of *Legionella* strains expressing proteolytic activity. Western immunoblots were used to determine antigenic similarities among proteolytic activities from several species of *Legionella* and the 38-kDa zinc metalloprotease of *L. pneumophila* cloned in *E. coli*. Rabbit antibodies raised against *E. coli* osmotic shockates containing the cloned protease were reacted with concentrated supernatants from 25 strains of *Legionella* (Table 1) and control bacteria. Only proteases from strains of *L. pneumophila* reacted with the antisera (Fig. 2). The *L. pneumophila* strains which reacted on Western immunoblots correlated with positive reactions from Southern hybridizations. The high-molecular-weight bands present in the control lanes (lanes 4 and 5) of Fig. 2 were the result of antibodies that reacted with the *E. coli* periplasmic proteins that were present in osmotic shockates.

The cloned protease was also used to detect the presence of anti-protease antibodies in convalescent-phase human sera. The cloned protease reacted with convalescent-phase antisera with high titers (≥1:2,048) from patients infected with *L. pneumophila* but not with sera from patients infected with strains of *L. micdadei* or *L. dumoffii* (Fig. 3). The high-molecular-weight bands present in lanes 3 to 7 of Fig. 3 were the result of overexposure and were also observed in control lanes of osmotic shockates from *E. coli* strains containing vector alone (data not shown).

DISCUSSION

We have recently cloned and characterized a DNA sequence encoding the 38-kDa zinc metalloprotease produced by a strain of *L. pneumophila* (32a) that was initially described by Dreyfus and Iglewski (14). In addition to the protease, the cloned sequence, designated *pro*, also encodes both a cytotoxin and a canine erythrocyte hemolysin. Our genetic analyses indicated that these three activities are likely produced by a single protein (32a). These same findings have been reported recently by several groups of investigators (6, 26, 35) with purified *L. pneumophila* prote-

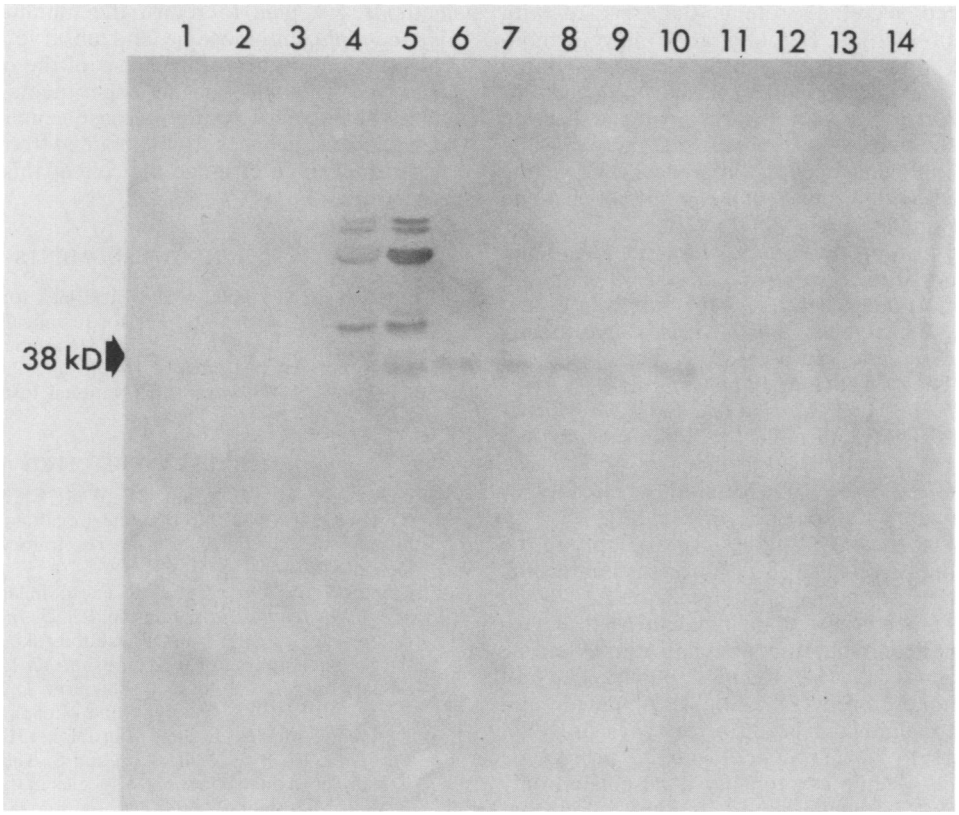

FIG. 2. Western immunoblot demonstrating cross-reactivity at 38 kDa between the cloned protease from osmotic shockate (*E. coli* containing pSP106) and concentrated supernatants from several species of *Legionella* and protease-producing non-*Legionella* strains of bacteria. Reacting antiserum was made against the osmotic shockate from a recombinant protease clone (*E. coli* LE392 containing plasmid pSP106). Lanes: 1, *P. aeruginosa* Hak-1; 2, *A. hydrophila*; 3, *S. marcescens*; 4, *E. coli* LE392 containing cloning vector pREG153 alone; 5, *E. coli* LE392 containing pSP106; 6, *L. pneumophila* Philadelphia 1, CDC 1; 7, *L. pneumophila* Philadelphia 1, CDC 2; 8, *L. pneumophila* serogroup 3, CDC 3; 9, *L. pneumophila* serogroup 6, CDC 6; 10, *L. pneumophila* serogroup 8, CDC 8; 11, *L. dumoffii* Tex KL; 12, *L. micdadei* 31B; 13, *L. feeleii* serogroup 1, CDC 1f; 14, *L. jordanis* BL-540. kD, Kilodaltons.

ase. In the present report we described the results of studies to determine whether other *L. pneumophila* strains and other *Legionella* species express similar proteases and contain genetically related DNA sequences. We examined strains of *L. pneumophila*, *L. dumoffii*, *L. micdadei*, *L. feeleii*, and *L. jordanis* for proteolysis, hemolysis, and cytotoxicity by bioassays to determine whether these strains contained genetically related sequences and produced antigenically similar proteases. All *L. pneumophila* strains that were examined secreted metalloproteases that reacted immunologically with specific polyclonal antibodies raised against the recombinant 38-kDa protease and contained either 3.7- and 0.3-kb fragments or a 4.0-kb *Sal*I-*Bgl*II restriction fragment that hybridized with the 4.0-kb probe under high-stringency conditions. These results show the conservation of the 4.0-kb DNA fragment containing the protease gene among all strains of *L. pneumophila*. In addition, we also observed that cytotoxicity for CHO cells was only observed in these strains, further supporting the conclusion that both activities are contained within a single protein.

In contrast, strains of other *Legionella* species, including *L. dumoffii*, *L. jordanis*, and *L. micdadei*, produced proteolytic and hemolytic activities that biochemically resembled those of *L. pneumophila* cloned protease and the protease secreted by wild-type strains of *L. pneumophila*. However, none of these strains reacted with the cloned protease probe,

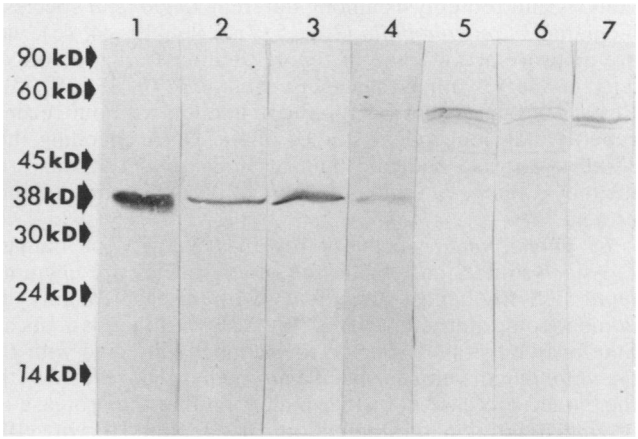

FIG. 3. Western immunoblot demonstrating cross-reactivity at 38 kDa between the cloned protease from osmotic shockates (*E. coli* containing pSP104) and human convalescent-phase sera from patients infected with several species of *Legionella*. The lane designations correspond to antisera listed as follows: rabbit antiserum made to purified native protease of *L. pneumophila* (lane 1); *L. pneumophila* serogroup 1; titer, >1:2,048 (lane 2); *L. pneumophila* serogroup 1; titer, >1:2,048 (lane 3); *L. pneumophila* serogroup 1; titer, 1:2,048 (lane 4); *L. dumoffii*; titer, >1:2,048 (lane 5); *L. dumoffii*; titer, 1:2,048 (lane 6); and *L. micdadei*; titer, 1:2,048 (lane 7). kD, Kilodaltons.

and none produced secreted proteins that reacted with specific antisera. In addition, Berdal et al. (8) have demonstrated that proteolytic activities from the concentrated supernatants of several *Legionella* species produced different proteolytic cleavage patterns on synthetic peptide substrates. From these data, we conclude that there must be structural, functional, and genetic differences among the proteases produced by different *Legionella* species, in spite of some observed similarities.

Although the 56-kDa protease of *S. marcescens* (29) and the hemolysins of *Actinobacillus pleuropneumoniae* (G. LaLonde and P. O'Hanley, submitted for publication) and *Vibrio vulnificus* (22) have been shown to act as cytotoxins, in general it has been found that proteolysis, hemolysis, and cytotoxicity from bacteria such as *P. aeruginosa* (25), *Aeromonas salmonsida* (4), *V. vulnificus* (32), and *Staphylococcus epidermidis* (37) occur through the actions of distinct proteins. Therefore, the observation that one polypeptide from *L. pneumophila* is responsible for all three phenotypes may be novel among bacterial proteases.

Hemolysis may be an inappropriate term to apply to the effect caused by the *Legionella* 38-kDa protease on canine erythrocytes. There is little or no activity of the *Legionella* proteases against other types of mammalian erythrocytes (2), and there is no detectable lysis of any erythrocyte type in tube suspension assays (M. Keen, unpublished data). Additionally, canine erythrocytes are more osmotically unstable than other mammalian erythrocyte types are (26). Hemolysis appears to be associated with all proteolytic strains of *Legionella*, while cytotoxicity is associated only with proteases from *L. pneumophila*. It may follow that hemolysis of canine erythrocytes, because of the inherent instability of this erythrocyte type, is caused by proteolysis, while cytotoxicity may occur by a more complicated mechanism. Thus, the concordance between proteolysis and hemolysis probably does not imply relatedness between protease proteins produced by *L. pneumophila* versus those produced by other *Legionella* species.

Although several investigators have previously noted hemolysis and proteolysis among different *Legionella* species, including *L. pneumophila* (2, 3, 5, 7, 12–14, 19, 44, 45), we are unaware at this time of reports demonstrating cytotoxicity in species other than *L. pneumophila* (6, 13, 18, 45). Results of the present study, in conjunction with our recent genetic and biochemical studies of the DNA encoding the 38-kDa protease, indicate that *L. pneumophila* strains are the only *Legionella* species capable of producing this unique protein.

L. pneumophila accounts for the majority of human *Legionella* infections, while the other species are encountered less frequently in patients with disease, mainly in immunocompromised patients. We observed that patients at Stanford University Hospital nosocomially infected with *L. pneumophila* developed specific antibodies that reacted with the cloned protease, a further indication that the protease is produced during natural infection. Blander and Horwitz (10) have noted that guinea pigs infected with *L. pneumophila* develop specific cell-mediated immunity to the protease.

The detection of protease during *L. pneumophila* infections, coupled with the results of our experimental studies, suggests that the virulence of *L. pneumophila* is somehow related to the elaboration of the protease-cytotoxin. In order to determine whether this protein is indeed an essential virulence determinant, we have begun genetic studies involving the construction of isogenic *Legionella* mutants deficient in protease production. Using genetic exchange methods, we plan to return the mutated gene to the *L. pneumophila* chromosome and observe the effect of this construction on the pathogenesis of the organism in guinea pigs. We have also begun experiments to introduce the wild-type *L. pneumophila* protease gene into species which lack related sequences. Data obtained from these studies will also permit us to examine the role of this protein in pathogenesis.

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