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Demonstration of Extracellular Chymotrypsin-Like Activity from Various *Legionella* Species

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Concentrated extracellular supernatants from *Legionella pneumophila, L. bozemanii, L. dumoffi, L. gormanii*, but not *L. micdadei*, exhibited a strong chymotrypsin-like activity upon synthetic chromogenic tri- and tetrapeptides. Bacterial cell sonic extracts showed low protease activities, different from those of the extracellular concentrates. Extracellular concentrates were also tested in the API ZYM system, where weak protease activity only was recorded. Protease inhibitors decreased the activity of the extracellular *Legionella* proteases upon the chromogenic peptides.

The metabolism of *Legionella* sp. strains depends in large part upon protein degradation, whereas carbohydrate degradation appears relatively unimportant.

Two possible pathogenic factors have been described, namely, a heat-stable cytotoxin (7) and a heat-stable hemolysin (1). Müller (8) has studied proteolysis of human serum proteins of *Legionella pneumophila* strains grown on agar plates. He found that *L. pneumophila* degraded several serum proteins, including macroglobulins such as alpha$_1$-antichymotrypsin.

In a later work, Müller (9) investigated aminopeptidases in bacterial suspensions of *L. pneumophila* strains, using several substrates including those present in the API ZYM system (API System S.A., La Balme les Grottes, Montalieu-Vercieu, France). Although he demonstrated several aminopeptidase activities, he did not detect trypsin- or chymotrypsin-like activity.

By measuring the extracellular protease activity in liquid broth of six *L. pneumophila* strains, Thompson et al. (12) found that a variety of substrates were degraded, including hide powder azure, but not elastin. Using the four then available synthetic chromogenic tri- and tetrapeptides, Berdal et al. (4) compared the protease activity patterns from different *Legionella* species and found similarities within strains of *L. pneumophila*.

In the present work, crude extracellular concentrates were used upon an enlarged selection of chromogenic peptides to investigate the presence of additional proteases.

MATERIALS AND METHODS

Bacterial strains. The strains *L. pneumophila* Philadelphia 1, Philadelphia 2, and Los Angeles 1, *L. bozemanii* Wiga and Mi 15, *L. dumoffi* NY 23, *L. gormanii* Ls 13, and *L. micdadei* Tatlock were all received from Robert Weaver, Special Bacteriology Laboratory, Centers for Disease Control, Atlanta, Ga. The strains were grown on charcoal-yeast extract agar plates (6) or kept frozen at −70°C in horse serum with 5% inositol added.

Production of bacterial extracellular concentrates and cellular sonic extracts. The *Legionella* strains were all grown in yeast extract broth (10). The production and concentration of supernatants were done by the procedures of Berdal and Fossum (3). The protein content in the concentrates was determined by optical density at 280 nm to be approximately 20 mg/ml.

The bacterial cell pellet (from *L. pneumophila* Philadelphia 1) left after centrifugation was diluted 1:2 in distilled water, sonicated for 15 s (Ultrasonic Disintegrator, model 100 Watt; Measuring & Scientific Equipment Ltd., London, England), and centrifuged at 5,000 × *g* for 10 min. The supernatant was assayed in the same way as the extracellular concentrates.

Chromogenic peptide assay. Synthetic tri- or tetrapeptides, with the chromophore *para*-nitroanilide (*p*NA) attached, were used. They were from KABI Peptide Research AB, Mölndal, Sweden, and are listed in Table 1.

To each well of a U-bottom, polyvinyl microtiter plate (Cooke, Alexandria, Va.) were added 25 μl of a Tris-hydrochloride buffer (0.2 M, pH 7.8) and 10 μl of crude extracellular concentrate or bacterial cell sonic extract. The buffer pH was chosen on the basis of earlier experiments (4). The optical density (OD) values at 410 nm, recorded at this stage (Minireader MR590; Dynatech, London, England), served for zero adjustment.

To the wells we then added 100 μl of a 1 mM solution of the chromogenic substrates in distilled water. For the substrates that did not readily dissolve, water containing 10% dimethyl sulfoxide (Mallinckrodt, St. Louis, Mo.) was used. This solvent did not significantly alter the protease activity when tried on other, readily soluble chromogenic peptides. When the *p*NA is liberated, a yellow color appears. The OD

TABLE 1. Chromogenic peptides used in the assays[a]

Code no.[b]	Molecular structure	Solvent
S-2160*	Bz-Phe-Val-Arg-*p*NA	Water + 10% DMSO[c]
S-2222*	Bz-Ile-Glu-Gly-Arg-*p*NA	Water
S-2238*	H-D-Phe-Pip-Arg-*p*NA	Water
S-2251*	H-D-Val-Leu-Lys-*p*NA	Water
S-2266*	H-D-Val-Leu-Arg-*p*NA	Water
S-2288*	H-D-Ile-Pro-Arg-*p*NA	Water
S-2294	Bz-Gly-Arg-Met-*p*NA	Water
S-2302*	H-D-Pro-Phe-Arg-*p*NA	Water
S-2327	H-D-Ser-Leu-Met-*p*NA	Water
S-2444*	pGlu-Gly-Arg-*p*NA	Water
S-2483	Ac-Ala-Pro-Ala-*p*NA	Water
S-2484*	pGlu-Pro-Val-*p*NA	Water + 10% DMSO
S-2494	O-Bzl-Ser-Thr-Pro-Pro-*p*NA	Water
S-2519	H-Ser-Pro-Pro-*p*NA	Water
S-2526	Bz-Ser-Pro-Pro-*p*NA	Water + 10% DMSO
S-2532	H-D-Arg-Val-Trp-*p*NA	Water
S-2545[d]	Suc-Ala-Pro-Phe-*p*NA	Water
S-2561	Suc-Ala-Pro-Tyr-*p*NA	Water
S-2586*	SucOMe-Arg-Pro-Tyr-*p*NA	Water
S-2591	H-D-Arg-Pro-Tyr-*p*NA	Water

[a] Abbreviations: Ac, acetyl; Ala, alanyl/alanine; Arg, arginyl/arginine; Bz, benzoyl; Bzl, benzyl; D, dextrostereoisomer; Glu, glutamyl; Gly, glycyl; H, hydrogen; Ile, isoleucyl; Leu, leucyl; Lys, lysine; Met, methionine; O, oxygen; pGlu, pyroGlutamyl; Phe, phenylalanyl/phenylalanine; Pip, pipecolyl; Pro, prolyl/proline; Ser, seryl; Suc, succinyl; SucOMe, methoxycarbonylpropionyl; Thr, threonyl; Trp, tryptophan; Tyr, tyrosine; and Val, valyl/valine.

[b] Commercially available products are marked with *.

[c] For the relatively water-insoluble peptides, 10% dimethyl sulfoxide (DMSO) served as cosolvent.

[d] Available in very limited quantity and demonstrated in Table 4 only.

values recorded were the differences between t_0 (OD before incubation of the plates at 37°C) and t_{10} (OD after 10 min of incubation). For the very fast reactions, which sometimes gave maximal OD value even before the 37°C incubation, the zero adjustment replaced t_0. Each test was done in duplicate. The time of 5 min required for one OD recording of one full microtiter plate could have some importance for the OD values of the fast reactions, but this was not taken into account. The recording was performed at room temperature. As a control, chymotrypsin was used (crystalline salt free) (National Biochemicals Corp., Cleveland, Ohio) at 0.1 mg/ml in distilled water.

API ZYM assay. The API ZYM test was performed according to the manufacturer's directions. Briefly, *L. pneumophila* Philadelphia 1 crude extracellular concentrate was diluted 1:10 in distilled water, and 70 μl of this solution was added to each well. The strip was then incubated for 4 h (the manufacturer's recommendation) before addition of the color-developing reagents. Positive results were estimated visually.

Antiprotease assays. The following protease inhibitors were used, dissolved or suspended in distilled water: chymostatin, 10 mg/ml (C-7268; Sigma, St. Louis, Mo.); purified leupeptin, 0.2 mg/ml (Sigma, L-3631); synthetic leupeptin, 10 mg/ml (Sigma, L-2884), aprotinin, 1 mg/ml (Sigma, A-1025), and phenylmethylsulfonyl fluoride (Sigma, P-7626). As the phenylmethylsulfonyl fluoride appeared rather water insoluble and also hydrophobic, 20 mg/ml was sonicated for 5 s and the resulting liquid phase was used.

In the assay, 10 μl of the antiprotease solutions was added to the microtiter plate wells immediately after 10 μl of *L. pneumophila* Philadelphia 1 concentrate. Ten microliters of distilled water served here as control. The mixture of crude concentrate and antiprotease was incubated for approximately 10 min at room temperature before the addition of substrates.

RESULTS

All *Legionella* strains, except *L. micdadei* Tatlock, showed a particularly rapid splitting of the substrates S-2586 and S-2561 (Table 2). These two substrates measure specifically chymotrypsin- and cathepsin G-like activities (manufacturer's information). Hydrolysis started immediately and was sometimes fast enough to reach the maximal OD value of 2.00 even before the incubation at 37°C had started. *L. pneumophila* concentrates also had a relatively fast activity upon S-2160 (known to be degraded by serine thrombin, trypsin, papain, and brinase), S-2222 (degraded by coagulation factor 10 A, duodenal trypsin, and acrosin), S-2302 (degraded by plasma kallikrein), and S-2327. *L. gormanii* LS 13 as well as *L. bozemanii* Mi 15 and Wiga very actively split S-2519 and S-2494, and these strains were also active upon S-2288 and S-2444 (used for the assay of plasmin activators like urokinase) (manufacturer's information).

When *L. pneumophila* Philadelphia 1 cellular sonic extracts were used, no notable substrate

TABLE 2. OD variation of 18 different *p*NA-derivatized peptides[a]

Species and strain	OD on peptide:																		
	S-2160 (Bz-Phe-Val-Arg)	S-2222 (Bz-Ile-Glu-Gly-Arg)	S-2238 (H-D-Phe-Pip-Arg)	S-2251 (H-D-Val-Leu-Lys)	S-2266 (H-D-Val-Leu-Arg)	S-2288 (H-D-Ile-Pro-Arg)	S-2294 (Bz-Gly-Arg-Met)	S-2302 (H-D-Pro-Phe-Arg)	S-2327 (H-D-Ser-Leu-Met)	S-2444 (pGly-Glu-Arg)	S-2483 (Ac-Ala-Pro-Ala)	S-2484 (pGlu-Pro-Val)	S-2494 (O-Bzl-Ser-Thr-Pro-Pro)	S-2519 (H-Ser-Pro-Pro)	S-2526 (Bz-Ser-Pro-Pro)	S-2532 (H-D-Arg-Val-Trp)	S-2561 (Suc-Ala-Pro-Tyr)	S-2586 (SucOMe-Arg-Pro-Tyr)	S-2591 (H-D-Arg-Pro-Tyr)
L. pneumophila Philadelphia 1	53	23	0	0	0	0	0	10	37	0	0	3	0	0	0	6	200	200	5
L. pneumophila Philadelphia 2	50	30	0	0	5	0	3	29	74	0	0	2	0	0	0	0	125	200	5
L. pneumophila Los Angeles 1	62	33	0	4	5	0	0	27	68	0	0	2	0	0	0	0	116	200	6
L. bozemanii Wiga	0	3	6	0	5	3	0	5	0	10	0	0	27	46	0	4	16	200	0
L. bozemanii Mi 15	0	12	16	10	31	30	6	21	5	71	0	0	134	200	4	3	28	200	0
L. dumoffi NY 23	5	0	0	0	0	0	5	0	2	0	0	0	13	22	2	0	7	139	2
L. gormanii Ls 13	31	20	0	3	3	0	3	5	3	2	0	2	200	200	15	0	21	200	5
L. micdadei Tatlock	0	0	7	0	0	0	0	4	0	0	0	0	0	2	0	6	0	7	2
L. pneumophila sonicated cell supernatant	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] The OD values are given after 10 min of incubation with different extracellular *Legionella* concentrates or an *L. pneumophila* Philadelphia 1 cell sonic extract supernatant at 37°C. The values are given as a mean of two assays. OD above the maximal level of 2.00 is indicated as 200. For peptide abbreviations, see Table 1, footnote *a*.

degradation after 10 min of incubation was recorded.

When the API ZYM was used for analysis of the crude extracellular concentrates, the enzymatic activities of *L. pneumophila* already described (9) were all confirmed. In addition to this, an uncertain trypsin- and chymotrypsin-like activity estimated to value 1 (according to the 0 to 5 value chart from the manufacturer) was registered.

In the assays with antiproteases, inhibition of the activity of *L. pneumophila* Philadelphia 1 concentrates was observed and varied with the different substrates. Chymostatin had a protease-inhibitory effect on the splitting of all substrates, especially of S-2327, S-2561, and S-2586. Both the leupeptins worked quite strongly against the splitting of S-2222 and S-2302. Aprotinin and phenylmethylsulfonyl fluoride had less pronounced inhibitory activity on the substrates assayed (Table 3). The protease inhibitor controls had no proteolytic activity on the peptides.

The substrates S-2586, S-2561, S-2545, S-2591, and S-2532 are similar in that they are all split by chymotrypsin and cathepsin G (manufacturer's information). Table 4 demonstrates how the substrate splitting by *L. pneumophila* Philadelphia 1 crude concentrate follows a chymotrypsin-like pattern.

DISCUSSION

The dominant substrate splitting pattern is the strong chymotrypsin-like activity of the majority of *Legionella* strains, expressed by the activity upon the substrates S-2586 and S-2561. The comparison in Table 4, intended to evaluate how closely to chymotrypsin the *L. pneumophila* Philadelphia 1 concentrate behaved, showed very similar but not identical activities. Considering the five peptides compared in Table 4, cathepsin G would be expected to have the strongest activity on S-2545 (manufacturer's information). As S-2545 remained unhydrolyzed, cathepsin G-like activity was not ascertained.

The very strong chymotrypsin-like activity demonstrated on some of these peptides, however, is in striking contrast to earlier results obtained by working with bacterial cells, which revealed no trace of chymotrypsin or chymotrypsin-like activities (9). We also were unable to detect chymotrypsin activity on the API ZYM substrate when we used the crude Philadelphia 1 concentrate. This may be explained by the specificity of the proteases upon the peptides. For instance, S-2586 and S-2591 have in common the –Arg–Pro–Tyr–*p*NA sequences. Where they differ, the methoxycarbonylpropionyl terminal of S-2586 apparently suits the *L. pneumophila* protease(s), whereas the H–D– terminal of S-2591 does not. Another parallel could be demonstrated by using *L. bozemanii* or *L. gormanii* concentrates on S-2519 and S-2526, which have a common -Ser-Pro-Pro-*p*NA peptide chain, differing only by an –H and a –Bz terminal.

The similarities between the bacterial proteases and the serine proteases (5, 11) were demonstrated by the effect of the protease inhibitors when *L. pneumophila* Philadelphia 1 extracellular concentrates were used on S-2327, S-2561,

TABLE 3. Effect of protease inhibitors upon a crude *L. pneumophila* Philadelphia 1 extracellular concentrate[a]

Protease inhibitor (concn)	Inhibition on peptide[b]:				
	S-2222 (Bz-Ile-Glu-Gly-Arg)	S-2302 (H-D-Pro-Phe-Arg)	S-2327 (H-D-Ser-Leu-Met)	S-2561 (Suc-Ala-Pro-Tyr)	S-2586 (SucOMe-Arg-Pro-Tyr)
Chymostatin (10 mg/ml)	+	++	+++	+++	+++
Synthetic leupeptin (10 mg/ml)	++	+++	+	+++	++
Purified leupeptin (0.2 mg/ml)	+++	+++	–	–	–
Supernatant of PMSF[c] sonic extract	–	++	–	–	+
Aprotinin (1 mg/ml)	–	–	–	+	–

[a] The recordings were made after 10 min of incubation with the *p*NA-derivatized peptides at 37°C.

[b] For the peptides S-2561 and S-2586, the extracellular concentrate was used diluted 1:10 in distilled water. Symbols: +++, inhibition of more than 75% of the control OD value; ++, between 75 and 50% inhibition; +, between 50 and 25% inhibition; –, less than 25% of the control OD value. For peptide abbreviations, see Table 1, footnote *a*.

[c] PMSF, Phenylmethylsulfonyl fluoride.

TABLE 4. Activity of crude *L. pneumophila* Philadelphia 1 extracellular concentrate on *p*NA-derivatized peptides split by chymotrypsin- or cathespin G-like activity[a]

Prepn	Temp (°C)	Time	Activity of peptide: S-2586 (SucOMe-Arg-Pro-Tyr)	S-2561 (Suc-Ala-Pro-Tyr)	S-2545 (Suc-Ala-Pro-Phe)	S-2591 (H-D-Arg-Pro-Tyr)	S-2532 (H-D-Arg-Val-Trp)
Concentrate	23	1 min	0.21	0.06	0.00	0.00	0.00
		3 min	0.61	0.18	0.00	0.00	0.00
		10 min	2.00	0.55	0.00	0.02	0.00
	37	10 min	2.00	1.50	0.00	0.06	0.00
		1 h	2.00	2.00	0.08	0.21	0.05
		2 h	2.00	2.00	0.14	0.38	0.10
Pure chymotrypsin	23	1 min	0.04	0.03	0.01	0.00	0.00
		3 min	0.13	0.09	0.01	0.02	0.02
		10 min	0.35	0.24	0.02	0.02	0.02
	37	10 min	0.77	0.53	0.05	0.07	0.05
		1 h	1.80	1.34	0.27	0.27	0.25
		2 h	2.00	1.74	0.50	0.51	0.48

[a] The Philadelphia 1 extracellular concentrate is compared with crystalline chymotrypsin at 0.1 mg/ml in distilled water. The assay was run for 10 min at room temperature before incubation at 37°C. For peptide abbreviations, see Table 1, footnote *a*.

and S-2586. The chymostatin inhibition of the hydrolytic activity of the *L. pneumophila* supernatants acting on S-2327, S-2561, and S-2586 reinforces the arguments for chymotrypsin or cathepsin G-like proteases (2). Similarly, the presence of trypsin- or kallikrein-like proteases (2) is suggested by the action of leupeptin in assays with S-2222, S-2302, or S-2561.

Proteases are thought to be pathogenic factors in *Legionella* infections, possibly acting through a mechanism of natural antiprotease deficiency in the lung (8). The proteases responsible for tissue destruction are commonly considered to be the elastase(s), cathepsin G, collagenase(s), and possibly plasmin and cathepsins B and D (2). None of these enzymes has been seen with certainty in this study in either *Legionella* culture supernatants or in cell extracts.

Sandvik (Thesis, Veterinary College of Norway, Oslo, 1962) found during his work with caseinate-precipitating proteases that for some bacteria, e.g., *Staphylococcus aureus*, the cultures should be arrested after 10 h of incubation to avoid inhibition of the proteases. Experiences with *Legionella* strains in yeast extract broth (3) gave an optimum of 14 h of incubation for the caseinate-precipitating proteinases, with strong or total enzyme inactivation after only a couple of hours more at 37°C.

Until now, major tissue-destructive proteases have not been detected from *Legionella* strains. We have not succeeded in ascertaining such enzymes. However, the methodology available with the chromogenic tri- and tetrapeptides looks promising in bacterial protease analysis. Apart from the diversity of the degradation pattern, which suggests its use for species identification (4), the enlarged selection of peptide molecular configurations presented here should be useful to specify bacterial protease activities.

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