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Patterns of Extracellular Proline-Specific Endopeptidases in *Legionella* and *Flavobacterium* spp. Demonstrated by Use of Chromogenic Peptides

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Some *Legionella* strains possess a strong extracellular proline-specific endopeptidase (PSE) activity. Using an enlarged selection of chromogenic peptides representing a variety of N-terminal amino acids binding to a -prolyl-proline.*para*-nitroanilide chain, PSE activity of *Legionella* and *Flavobacterium* strains was examined. Differences in PSE activity emphasized the importance of the chemical structure at the nonchromogenic end of the peptide substrates. There seem to be distinct patterns of N-terminal specificity of PSE in the two bacterial groups.

Synthetic, *para*-nitroanilide (*p*NA)-derivatized (chromogenic) tri- and tetrapeptides (13) are a sensitive and specific tool for demonstration of bacterial protease activity (5). The *p*NA is attached to the carboxy-terminal (C-terminal) end of a peptide chain by an amide bond. Some proteases, primarily endopeptidases (1, 2), recognize the peptide molecule from the amino-terminal (*N*-terminal) end (9, 12). At the C-terminal end, the amide bond is broken, liberating free *p*NA. Whereas the chromogenic peptide is colorless, the free *p*NA gives a yellow color, easily quantitated by optical density (OD) readings. The *p*NA itself is not recognized by the proteases, but the rest of the peptide molecule is active in the enzyme-protein interaction.

It was shown with chymotrypsin-like proteases from *Legionella* strains (5) that less elaborate configurations than tri- and tetrapeptides did not fulfill the requirements for detection of enzyme activity (14). As reported, analysis for chymotrypsin-like activity by the use of a chromogenic amino acid thus repeatedly failed to detect what appears to be a major extracellular protease in strains from several *Legionella* species (5).

Chromogenic peptide hydrolysis distinguishes between different *Legionella* species (4, 5). Some of these species were noted to have proline-specific endopeptidases (PSE), a group of proteases uncommon in mammals but present in some *Flavobacterium* strains (14). In the present investigation, the extracellular protease pattern comparison is extended to a larger material of proteolytic bacteria and a selected group of similar chromogenic peptides constructed around a -prolyl-proline.*p*NA (-Pro-Pro.*p*NA) chain. The differences in the N-terminal construction of the -Pro-Pro.*p*NA peptides were in some instances very slight. The intention with this group of peptides was to study the interference on protease activity of the N-terminal end of the chromogenic peptides.

MATERIALS AND METHODS

Bacterial strains. Twenty-six strains of *Legionella*, *Acinetobacter*, *Flavobacterium*, and *Pseudomonas* were included in the study. The *Legionella* strains were received from Robert E. Weaver, Special Bacteriology Laboratory, Centers for Disease Control, Atlanta, Ga. They were *Legionella bozemanii* strains Wiga and Mi 15, *L. dumoffii* NY 23, *L. gormanii* Ls 13, *L. longbeachae* LB 4 and Tucker 1, and *L. jordanis* BL 540.

The *Acinetobacter* strains were from the American Type Culture Collection (ATCC), Rockville, Md. They were *Acinetobacter calcoaceticus* ATCC 17906, ATCC 17986, ATCC 17988, and ATCC 23055.

The *Flavobacterium* and *Pseudomonas* strains were from the National Collection of Type Cultures (NCTC), London, England, or from personal collections with old as well as recent isolates. They were *Flavobacterium meningosepticum* NCTC 10016, NCTC 10585, and NCTC 10586, *F. saccharolyticum* NCTC 10753, *Flavobacterium* sp. IIb NCTC 10795 and NCTC 10796, *Flavobacterium* sp. IIf NCTC 10798 and NCTC 10799, and *F. anatipestifer* NCTC 11014. From Magne Bisgaard, Langaa, Denmark, were received *Flavobacterium* sp. from duck salpinx 4225/1, from duck nose 4238/2n, and from duck salpinx 4347/2. *F. odoratum* B 9856 was received from Robert E. Weaver. *Pseudomonas aeruginosa* B 10815 and B 10861 were from a personal collection. The strains named *F. saccharolyticum* (previously *Moraxella saccharolytica*) and *F. anatipestifer* (previously *Moraxel-*

la anatipestifer), as well as strains 4225/1, 4238/2n, and 4347/2, are assigned to *Flavobacterium* spp. in this publication on the basis of gas chromatographic detection of large cellular contents of branched fatty acids and other criteria (K. Bøvre and co-workers, personal communication; see also reference 6).

Although the natural distribution is not completely established for some species, these bacterial groups appear to share their soil and water natural habitat, while participating in disease production under certain circumstances.

The *Legionella* strains were grown on charcoal-yeast extract agar plates (7) or kept frozen at −70°C in horse serum with 5% inositol added. The strains originating from culture collections were kept lyophilized until plating on horse blood agar for 2 to 10 days before use.

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

Code No.	Molecular structure	Solvent
S-2160*[b]	Bz-Phe-Val-Arg · pNA	Water + 10% DMSO[c]
S-2222*	Bz-Ile-Glu-Gly-Arg · pNA	Water
S-2238*	H-D-Phe-Pip-Arg · pNA	Water
S-2251*	H-D-Val-Leu-Lys · pNA	Water
S-2266*	H-D-Val-Leu-Arg · pNA	Water
S-2288*	H-D-Ile-Pro-Arg · pNA	Water
S-2294	Bz-Gly-Arg-Met · pNA	Water
S-2302*	H-D-Pro-Phe-Arg · pNA	Water
S-2327	H-D-Ser-Leu-Met · pNA	Water
S-2444*	pGlu-Gly-Arg · pNA	Water
S-2483	Ac-Ala-Pro-Ala · pNA	Water
S-2484*	pGlu-Pro-Val · pNA	Water + 10% DMSO
S-2493[d]	H-Pro-Ser-Thr-Pro-Pro · pNA	Water
S-2494	O-Bzl-Ser-Thr-Pro-Pro · pNA	Water
S-2498[d]	Bz-Pro-Ser-Thr-Pro-Pro · pNA	Water
S-2507[d]	O-Bzl-Thr-Pro-Pro · pNA	Water
S-2518[d]	O-Bzl-Ser-Pro-Pro · pNA	Water
S-2519	H-Ser-Pro-Pro · pNA	Water
S-2524[d]	N-Bz-Thr-Pro-Pro · pNA	Water
S-2526	Bz-Ser-Pro-Pro · pNA	Water + 10% DMSO
S-2532	H-D-Arg-Val-Trp · pNA	Water
S-2533[d]	H-Ser-Thr-Pro-Pro · pNA	Water
S-2561	Suc-Ala-Pro-Tyr · pNA	Water
S-2586*	SucOMe-Arg-Pro-Tyr · pNA	Water
S-2591	H-D-Arg-Pro-Tyr · pNA	Water

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

[b] Commercially available products are marked with an asterisk.

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

[d] Demonstrated in Fig. 1 only.

Production of bacterial extracellular concentrates. Heavy inocula from overnight bacterial growth on agar plates were cultured in broth tubes rotating in a roller drum at 37°C according to the procedure of Berdal and Fossum (3). The *Legionella* strains, *F. meningosepticum* ATCC 10016 and 10586, and *Flavobacterium* spp. 4225/1 and 4347/2, were cultured in yeast extract broth (11) for an optimum of 14 h (3). All other bacteria, as well as the four *Flavobacterium* strains mentioned, were cultured in tryptic soy broth (Difco Laboratories, Detroit, Mich.) for 10 h, which permitted a satisfactory production of protease for these strains (Berdal, unpublished data). The viable count after incubation was more than 5×10^9 CFU/ml.

After centrifugation at 5,000 × *g* for 30 min, the supernatants were sterilized by filtration (pore size, 0.45 μm; Millipore Corp., Moselheim, France) before being concentrated 100 times on a Diaflo PM 10 filter (Amicon Corp., Lexington, Mass.) in a stirred cell (Amicon). The protein content of the concentrates was determined by OD at 280 nm to be between 15 and 30 mg/ml. The concentrates were stored at −70°C until use.

Chromogenic peptide assay. The chromogenic peptides (Table 1) were from KabiVitrum Peptide Research, Mölndal, Sweden. The assay followed closely the procedure previously described (4). Briefly, to each well of a U-bottom, polyvinyl microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.) was added 25 μl of 0.2 M Tris buffer (pH 7.8) and 10 μl of crude extracellular concentrate. The OD values at 410 nm recorded at this stage (Minireader MR 590; Dynatech, London, England) served for zero adjustment.

To the wells was then added 100 μl of a 1 mM solution of the chromogenic substrates in distilled water, and the OD at 410 nm was recorded. For the substrates which did not readily dissolve, 10% dimethyl sulfoxide (Mallinckrodt, St. Louis, Mo.) served as cosolvent. The mixture was incubated for 10 min, after which the amount of free *p*NA liberated was measured by again determining the OD at 410 nm. The values recorded are the differences between this reading and the reading taken at time zero. For the very fast reactions which sometimes gave a maximal OD value even before the 37°C incubation, the zero adjustment was considered as the start of reaction. Each test was done in duplicate. The readings were performed at room temperature.

RESULTS

The hydrolysis profiles of *L. jordanis* BL 540, the *Flavobacterium* strains, and the *P. aeruginosa* strains are given in Table 2. The complete profiles of the other protease-producing *Legionella* strains on the same selection of substrates have been reported previously (5).

L. longbeachae strains LB 4 and Tucker 1 were essentially negative on all substrates, resembling results with *L. micdadei* strain Tatlock (5). *L. jordanis* is *L. pneumophila*-like, with strong splitting of the substrate S-2586 (5).

The -Pro-Pro.*p*NA substrates S-2494, S-2519,

TABLE 2. Hydrolytic activity of extracellular proteases from *Legionella*, *Flavobacterium*, and *Pseudomonas* species[a]

Species and Strain	OD_{410} × 100 on given 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
L. jordanis BL 540	2	7	0	0	2	0	4	0	0
F. meningosepticum NCTC 10016	0	7	0	0	0	0	2	7	4
F. meningosepticum NCTC 10585	0	48	0	3	22	0	12	0	0
F. meningosepticum NCTC 10586	0	3	0	0	0	0	0	3	0
F. saccharolyticum NCTC 10753	0	2	0	0	0	0	0	0	23
Flavobacterium sp. IIb, NCTC 10795	0	48	0	2	3	0	10	6	7
Flavobacterium sp. IIb, NCTC 10796	5	0	0	3	5	2	4	37	21
Flavobacterium sp. IIf, NCTC 10798	0	0	11	122	129	131	2	0	4
Flavobacterium sp. IIf, NCTC 10799	0	0	61	200	200	200	3	10	19
F. anatipestifer NCTC 11014	10	200	4	6	7	12	16	28	16
Flavobacterium sp. from duck salpinx, 4225/1	0	49	2	3	2	0	0	11	44
Flavobacterium sp. from duck nose, 4238/2n	8	122	0	8	4	2	10	47	59
Flavobacterium sp. from duck salpinx, 4347/2	6	14	0	5	4	2	3	25	61
F. odoratum B 9856	0	7	0	0	5	0	0	0	7
P. aeruginosa B 10815	16	24	4	200	3	0	93	71	86
P. aeruginosa B 10861	0	0	0	200	0	0	6	20	8

[a] The *L. jordanis* strain was cultured on yeast extract broth; all other strains were cultured on tryptic soy broth (see text). Values represent OD variation at 410 nm on different *p*NA-derivatized peptides. Reactions which gave an OD_{410} of >2.0 (200) are listed as 200. See Table 1 for peptide abbreviations.

and S-2526 which are split by strains from the three *Legionella* species *L. bozemanii*, *L. dumoffii*, and *L. gormanii* (5) are also split by a number of *Flavobacterium* strains.

P. aeruginosa, represented by two strains, strongly split the substrate S-2251 which is used to determine streptokinase-activated plasminogen (manufacturer's information). The *Acinetobacter* strains were essentially negative, only *A. calcoaceticus* NCTC 17906 displaying a slight activity on substrate S-2586.

The activity of *Legionella* and *Flavobacterium* spp. on the enlarged selection of substrates constructed around the -Pro-Pro.*p*NA terminal chain is demonstrated in Fig. 1. There is a marked similarity of the hydrolysis profiles within each group and a marked difference between the groups.

Whereas two of the four *Flavobacterium* strains cultured for 14 h in yeast extract broth produced PSE at the same level as the 10-h cultures in tryptic soy broth, the two other strains produced somewhat less (Fig. 1). The hydrolysis profiles of the same strains on the -Pro-Pro.*p*NA substrates were generally similar (Fig. 1).

DISCUSSION

The PSE, reportedly quite unusual in mammals and bacteria (14), hitherto have eluded

TABLE 2—*Continued*

OD_{410} × 100 on given peptide									
S-2444 pGlu-Gly-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
0	3	0	0	0	0	3	5	200	4
6	32	2	46	80	47	0	71	69	0
0	0	0	7	11	6	0	14	10	0
0	0	0	10	18	13	0	12	2	0
2	0	0	12	36	13	0	2	2	0
4	2	0	10	21	12	3	67	66	4
6	6	0	53	91	85	0	84	64	10
0	0	0	0	3	0	0	0	0	0
2	0	0	0	5	0	0	0	0	0
7	20	2	15	87	13	7	23	91	104
6	4	0	8	84	16	4	5	26	2
12	44	5	37	112	32	2	28	79	22
11	34	2	19	85	20	0	16	21	7
0	0	0	0	3	0	0	0	0	0
3	24	0	0	0	0	0	72	81	70
6	12	0	0	0	0	0	18	23	8

detection due to lack of a convenient substrate. Our use of chromogenic peptides provides a suitable detection system for PSE activity. Within the limits of the present rather crude preparations of bacterial extracellular proteases assayed on chromogenic substrates, patterns of N-terminal specificity (8) become apparent independently of the culture medium chosen. These patterns are clearly different for *Legionella* and *Flavobacterium* spp. with the species and strains selected in the present study (Fig. 1). Thus, despite some overall similarities of protease patterns observed between the two groups, they appear to differ with respect to N-terminal specificity of their PSE.

Although previous studies (5) have shown great protease pattern homogeneity of strains belonging to the species *L. pneumophila*, the dissimilarities between some *Legionella* species are pronounced. Dissimilarities were also noted within the *L. bozemanii* species (5).

Analysis of peptide hydrolysis patterns of *Flavobacterium* strains may also distinguish subgroups corresponding to species as shown in this study by the clustering of the two *Flavobacterium* sp. IIf strains (Table 2). There are also relative similarities between the *F. anatipestifer* laboratory strain and recent isolates from ducks. The latter finding suggests that these organisms may belong to the same species, as expected from their origin and previous studies (see Materials and Methods).

That species of *Flavobacterium* may, on the other hand, be heterogeneous with this assay is exemplified by the patterns observed with the *F. meningosepticum* and *Flavobacterium* sp. IIb

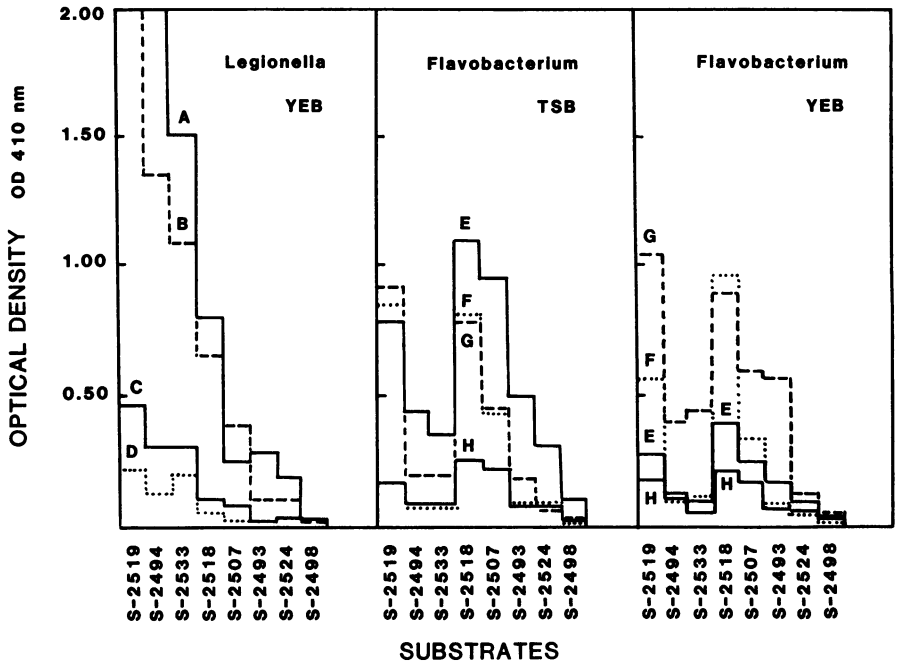

FIG. 1. Substrate hydrolysis patterns of PSE from strains (A) *L. gormanii* Ls 13, (B) *L. bozemanii* Mi 15, (C) *L. bozemanii* Wiga, (D) *L. dumoffii* NY 23, (E) *F. meningosepticum* 10016, (F) *Flavobacterium* sp. 4225/1, (G) *Flavobacterium* sp. 4347/2, and (H) *F. meningosepticum* 10586. The substrates represent different N-terminals binding to a -Pro-Pro.*p*NA chain. For the chemical configuration of the (chromogenic peptide) substrates, see Table 1. The *Flavobacterium* sp. PSE were analyzed from yeast extract broth (YEB) and tryptic soy broth (TSB) cultures. The *Legionella* sp. PSE were analyzed from YEB cultures only.

strains (Table 2). However, it is essential to note that the *Flavobacterium* sp. IIb strains used deviate genetically from each other as shown by DNA reassociation (10). Also, strain NCTC 10016 (type strain) of *F. meningosepticum* appears to be genetically distinct from the other two strains of this species examined (10).

We conclude that the use of chromogenic peptide substrate groups with very similar peptide configurations could be of value for characterization of bacterial genera and species. The substrates can be synthesized reproducibly and apparently have a great potential for use in the determination of various protease activities.

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