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Enzymatic Profile of *Legionella pneumophila*

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The enzyme activities of four strains of *Legionella pneumophila* were investigated by using the API ZYM system (API System S.A., F-38390 Montalieu Vercieu, France) and synthetic substrates. Aminopeptidases were detected specifically against L-alanine, L-arginine, L-aspartic acid, L-cystine, L-glutaminic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-tryptophan, L-tyrosine, and L-valine. Furthermore, the bacteria possessed esterase activity splitting propionate, butyrate, caproate, caprylate, and caprate, but not laurate, myristate, palmitate, and stearate, esters. The enzymes studied were inhibited partially by aprotinin. No inhibition of phosphatase (pH range, 5.4 to 8.5) or of phosphoamidase was observed. Activities of arylsulfatase, chymotrypsin, trypsin, and glycosidases could not be detected.

Legionella pneumophila shows only a few positive biochemical reactions that can be used for identification purposes. For example, with the exception of starch utilization, the organisms do not utilize carbohydrates (12). However, they use amino acids as their major source of energy and carbon. They appear, therefore, to possess a relatively rare type of metabolism among bacteria (8, 16, 21). This is confirmed by the finding of proteolytic action of *L. pneumophila* on human serum proteins (14, 15). In addition, exotoxin activity (1) and the ability of organisms to multiply intracellularly (2, 4, 5, 9, 13, 22) may be interpreted in this sense.

With the intention of increasing knowledge of the enzymatic profile of *L. pneumophila*, I investigated the reactions of nonproliferating cells with some synthetic substrates.

MATERIALS AND METHODS

Bacteria. Four strains of *L. pneumophila*, designated Philadelphia 2, Togus 1, Bloomington 2, and Los Angeles 1 (serovars 1, 2, 3, and 4, respectively, as described previously [14]) were used in this study.

Cultures. The strains were maintained at −70°C, and the cultures were obtained from stocks. Suspensions were plated on Mueller-Hinton medium supplemented with 5% chocolate-sheep blood and 1% (vol/vol) IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.). Incubation of agar plates was at 37°C in a candle extinction jar for 2 to 3 days.

Cell preparations. After the addition of about 3 ml of sterile distilled water, heavy cell growth on the plates was removed with glass rod spreaders. Smears were tested for purity by Gram staining and by growth on supplemented, but not on unsupplemented, Mueller-Hinton agar plates. All cell suspensions were adjusted to a turbidity approximating that of a McFarland no. 5 standard.

Enzyme assays. Enzyme assays were performed by using the API ZYM system (API System S.A., F-38390 Montalieu Vercieu, France). Each plastic strip contains 20 cupules; 19 of these have substrates and buffer impregnated into an inert supportive fabric, whereas one cupule contains no substrate and acts as a negative control. The substrates and enzymes are listed in Tables 1 and 2. Two drops of the prepared bacterial suspension was added with a Pasteur pipette to each of the 20 cupules of the API ZYM system strip. Each strip was placed in a moist chamber and incubated aerobically at 37°C for 4 h. After incubation, 1 drop from both API reagent A [tris(hydroxymethyl)aminomethane, 250 g; HCl (37%), 110 ml; lauryl sulfate, 100 g; distilled water to 1 liter] and API reagent B (Fast Blue BB, 3.5 g; 2-methoxyethanol to 1 liter) was added into each cupule. The resulting colors which developed within 4 min were read as positive reactions. In a similar manner, other synthetic substrates were placed into wells of U-shaped polystyrene microtiter plates (about 1 mg per well) in 0.1 ml of sterile 0.6 M phosphate buffer solution, pH 7.5 and, in some cases, pH 5.4 and pH 8.5. For testing enzymatic activity, 0.3 ml of the bacterial suspension was added and then incubated as described above. Hydrolysis of the different substrates by the corresponding enzymes was estimated by comparison of (i) yellow 4-nitrophenol and 4-nitroaniline, formed from colorless 4-nitrophenyl and 4-nitroaniline compounds, respectively, or (ii) differently colored diazo dyes, formed from naphthyl compounds after reaction with Fast Blue BB, as described previously for the API ZYM system (10, 11, 17, 20) and elsewhere (3).

Each strain was tested several times to ensure reproducibility of results.

Enzyme inhibition. Aprotinin is a known inhibitor of proteases, e.g., kallikrein, plasmin, and trypsin (7, 9), and was purchased from Bayer AG, Leverkusen, West Germany. It was used in a concentration of 20,000 U/ml.

Chemicals. Biochemicals and substrates were purchased from Serva GmbH, Heidelberg, West Germany, and Sigma Chemie GmbH, Munich, West Germany.

TABLE 1. *Enzymatic reactions of L. pneumophila serovars 1 to 4; amino acid metabolism*

Enzyme	Substrate	Result
L-Alanine AP[a]	L-Alanine-2-NA,[b,c] L-alanine-4-NI[d]	+
L-Arginine AP	L-Arginine-2-NA	+
L-Aspartic acid AP	L-Aspartic acid 2-NA	+
L-Cystine AP	L-Cystine-bis-2-NA,[b] L-cystine-bis-4-NI	+
L-α-Glutaminic acid AP	L-α-Glutaminic acid 2-NA, L-α-glutaminic acid 4-NI	+
L-γ-Glutaminic acid AP	L-γ-Glutaminic acid 2-NA, L-γ-glutaminic acid 4-NI	+
Glycine AP	Glycine-2-NA, glycine-4-NI	+
L-Histidine AP	L-Histidine-2-NA	+
L-Hydroxyproline AP	L-Hydroxyproline-2-NA	−
L-Isoleucine AP	L-Isoleucine-2-NA	+
L-Leucine AP	L-Leucine-2-NA,[b] L-leucine-4-NI	+
L-Lysine AP	L-Lysine-2-NA	+
L-Methionine AP	L-Methionine-2-NA	+
L-Phenylalanine AP	L-Phenylalanine-2-NA, L-phenylalanine-4-NI	+
L-Proline AP	L-Proline-2-NA	−
L-Tryptophan AP	L-Tryptophane-2-NA	+
L-Tyrosine AP	L-Tyrosine-2-NA	+
L-Valine AP	L-Valine-2-NA,[b] L-valine-4-NI	+
Chymotrypsin and chymotrypsin-like proteases	N-Benzoyl-DL-phenylalanine-2-NA,[b] naphthol AS hydrocinnamoate, naphthol AS phenylacetate, naphthol AS phenylbutyrate, naphthol AS phenylpropionate, naphthol AS propionate	−
Trypsin	N-Benzoyl-DL-arginine-2-NA[b]	−

[a] AP, Aminopeptidase.
[b] Tested by the API ZYM system.
[c] NA, Naphthylamide.
[d] NI, Nitroanilide.

All other chemicals were of analytical grade and were obtained from Merck AG, Darmstadt, West Germany.

RESULTS

Aminopeptidases and proteases. Table 1 shows the substrates used, the appropriate enzymes, and the results of the study with nonproliferating cells of *L. pneumophila*. The two types of substrates, i.e., 2-naphthylamide and 4-nitroanilide compounds, showed different levels of sensitivity. The color intensity of the liberated yellow 4-nitroaniline was less than that of the diazo dyes generated by reaction of free 2-naphthylamine and Fast Blue BB.

In regard to the enzymatic activities of the four strains investigated, Bloomington 2 seemed to be the strongest, but the differences were insignificant. All four strains possessed aminopeptidases with activities against L-alanine, L-arginine, L-aspartic acid, L-cystine, L-glutaminic acid, glycine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-phenylalanine, L-tryptophan, L-tyrosine, and L-valine. Activities against L-hydroxyproline and L-proline could not be detected. Neither trypsin nor chymotrypsin activities were detected by the substrates used.

Esterases, glycosidases, phosphatases and sulfatases. The results shown in Table 2 indicate that *L. pneumophila* splits esters of lower carboxylic acids, i.e., propionic acid, butyric acid, caproic acid, caprylic acid, and capric acid, but not those of higher fatty acids, i.e., lauric acid, myristic acid, palmitic acid, stearic acid, and oleic acid. Therefore, the organisms possess esterase activity, but no lipase. No enzymes which metabolize carbohydrates and no arylsulfatase could be detected. There was, however, strong phosphatase activity. The pH range of this enzyme is very broad, from pH 5.4 to 8.5. Activity of phosphoamidase was detected as well.

Assay for inhibition of enzyme. On the supposition that enzymes of *L. pneumophila* act as pathogenic factors, aprotinin (a potent inhibitor of proteases) was investigated (7, 19). Inhibition of aminopeptidases, i.e., cystine, leucine, and valine aminopeptidases, was shown by the API ZYM system. There was enzyme inhibition of two- to fivefold that was expressed as a diminution of the developed color intensity. The esterases were inhibited by a similar amount, but inhibition of phosphatase and phosphoamidase was not observed.

DISCUSSION

Knowledge of the enzymatic profile of *L. pneumophila* has several different aspects. One of these concerns is identification by means of

TABLE 2. *Enzymatic reactions of L. pneumophila serovars 1 to 4; esterases, glycosidases, phosphatases, and sulfatase*

Enzyme	Substrate	Result
Esterases		
Esterase (C_3)	2-Naphthylpropionate	+
Esterase (C_4)	1-Naphthylbutyrate, 2-naphthylbutyrate[a]	+
Esterase (C_6)	1-Naphthylcaproate, 2-naphthylcaproate	+
Esterase (C_8)	1-Naphthylcaprylate, 2-naphthylcaprylate[a]	+
Esterase (C_{10})	2-Naphthylcaprate	+
Esterase-lipase (C_{12})	1-Naphthyllaurate, 2-naphthyllaurate	−
Lipase (C_{14})	1-Naphthylmyristate, 2-naphthylmyristate[a]	−
Lipase (C_{16})	1-Naphthylpalmitate, 2-naphthylpalmitate	−
Lipase (C_{18})	1-Naphthylstearate, 2-naphthylstearate, 2-naphthyloleate	−
Glycosidases		
α-L-Fucosidase	2-Naphthyl-α-L-fucopyranoside[a]	−
α-D-Galactosidase	6-Bromo-2-naphthyl-α-D-galactopyranoside[a]	−
β-D-Galactosidase	6-Bromo-2-naphthyl-β-D-galactopyranoside, 2-naphthyl-β-D-galactopyranoside[a]	−
α-D-Glucosidase	2-Naphthyl-α-D-glucopyranoside[a]	−
β-D-Glucosidase	Arbutin, 6-bromo-2-naphthyl-β-D-glucopyranoside,[a] indoxyl-β-D-glucoside, salicin	−
β-D-Glucuronidase	Naphthol AS-BI-β-D-glucuronate[a]	−
α-D-Mannosidase	6-Bromo-2-naphthyl-α-D-mannopyranoside[a]	−
Phosphatases		
Phosphatase	1-Naphthylphosphate, 2-naphthylphosphate,[a] 3-indoxylphosphate, phenolphthaleindiphosphate	+
Phosphoamidase	Naphthol AS-BI-phosphodiamide[a]	+
Sulfatase		
Arylsulfatase	6-Bromo-2-naphthylsulfate potassium salt, 2-naphthylsulfate potassium salt, 4-nitrophenylsulfate potassium salt, phenolphthaleindisulfate potassium salt	−

[a] Tested by the API ZYM system.

enzymatic reactions, and another involves a better understanding of conditions for growth. This latter aspect includes occurrence in natural habitats, culture medium requirements, and, finally, the mechanism of virulence in the host.

Regarding identification, the API ZYM system seems to be useful as an additional resource, as described previously for other bacteria (10, 11, 17, 20). The specificities of the aminopeptidases were in some agreement with amino acid requirements that were recently described. According to these, *L. pneumophila* requires arginine, cysteine (or cystine), proline, serine, threonine, and valine (8). Examinations of serine and threonine could not be undertaken due to the unavailability of the corresponding substances as substrates. With the exception of proline, the other amino acids were split off from appropriate compounds by specific aminopeptidases. The role of other aminopeptidases liberating alanine, aspartic acid, glutaminic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, or tyrosine is still unclear. These amino acids support the growth of *L. pneumophila* as minor sources of carbon, energy, or both (8, 16, 21). Under natural conditions, all of the enzymes may be needed for the degradation of algal extracellular products in aquatic habitats (6, 18).

Finally, the virulence of *L. pneumophila* suggests some relation to its enzyme activities, i.e., the ability to degrade peptides and proteins of the infected host (14, 15). In addition, the activities of esterases and phosphatases may be involved in the virulence of the bacteria. Whether aprotinin can be used in the treatment of Legionnaires disease under the above-mentioned presuppositions should be investigated in more detail.

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