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Effects of Cultural Conditions on Protease Production by *Aeromonas hydrophila*

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Production of extracellular proteolytic activity by *Aeromonas hydrophila* was influenced by temperature, pH, and aeration. Conditions which produced maximal growth also resulted in maximal protease production. Enzyme production appeared to be modulated by an inducer catabolite repression system whereby NH_4^+ and glucose repressed enzyme production and complex nitrogen and nonglucose, carbon energy sources promoted it. Under nutritional stress, protease production was high, despite poor growth.

Aeromonas hydrophila is a common inhabitant of aquatic environments. It is both a primary pathogen and a secondary "cooperative" pathogen in fish (8, 9). *A. hydrophila* is a part of the normal microbial flora of fish and converts from a commensal state to a pathogenic state under stress conditions (7, 14, 20). *A. hydrophila* infections in humans have also been reported; however, only compromised hosts have been infected (4, 18).

A. hydrophila produces a number of potential virulence factors, including enterotoxins, cytotoxins, hemolysins, lipases, and proteases (19). The proteases from *A. proteolytica*, *A. salmonicida*, and *A. liquefaciens* have been studied in detail (3, 5, 16, 17, 23), and attention has now turned to the proteases of *A. hydrophila* (1, 5; J. B. Reeves, and E. L. Wright, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B156, p. 44).

Although the hemolytic activity of *A. hydrophila* (in the absence of protease) has been suggested to be a significant lethality factor in trout (1), Ellis et al. (6) implicated proteolytic and collagenolytic activities in the histopathology produced in experimental trout infections. Protease also has been demonstrated to be essential in the induction of *A. salmonicida* infection in fish (15).

Although purification and characterization of *Aeromonas* protease(s) and evidence suggesting protease involvement in pathogenicity have been reported, the regulation of protease production by *A. hydrophila* has not been thoroughly investigated. This paper reports on the effects of cultural conditions on the production of extracellular proteolytic activity by *A. hydrophila* UV 108.

The culture (obtained from the University of Victoria Collection of Isolates, Victoria, British Columbia, Canada) was maintained on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) slants at 25°C. This organism showed typical *A. hydrophila* reactions in standard biochemical tests and the API 20E rapid test system (Analytab Products, Plainview, N.Y.). Growth of the organism for enzyme production was carried out in 200-ml volumes (in 1-liter Erlenmeyer flasks) of a medium with the following composition: 0.06 M K_2HPO_4, 0.02 M KCl, and 0.5% (wt/vol) complex nitrogen. The solution was adjusted to pH 7.0 with HCl. After autoclaving the medium, 1.6 mM $MgSO_4$ and 0.5% (wt/vol) carbohydrate were added. Cells were normally grown at 25°C and 300 rpm on a gyratory shaker. Cells were harvested by centrifugation (10,000 × *g*, 15 min). Proteolytic activities of the cell-free supernatants were measured by the method of Charney and Tomarelli (2) on azocasein at a concentration of 25 mg/ml. The unit of activity (enzyme unit [EU]) was defined as the change in absorbance at 440 nm × 100 per minute per milligram of protein at 25°C.

Proteolytic activity was found in the culture supernatant but not in the cells throughout most of the growth period; however, an increase in activity did not directly parallel cell growth (Fig. 1). The proteolytic activity remained constant in the culture filtrate for at least 12 h after the onset of the stationary phase. In the late logarithmic phase, cell-associated proteolytic activity was detected. An increase in proteolytic activity was associated with a drop in medium pH during the active phase of growth. Both the initial and the final pH values of the medium affected the amount of protease detected (Table 1). The highest protease activity was detected when the final pH of the medium was >6.9. Maximal cell yields and protease production were achieved

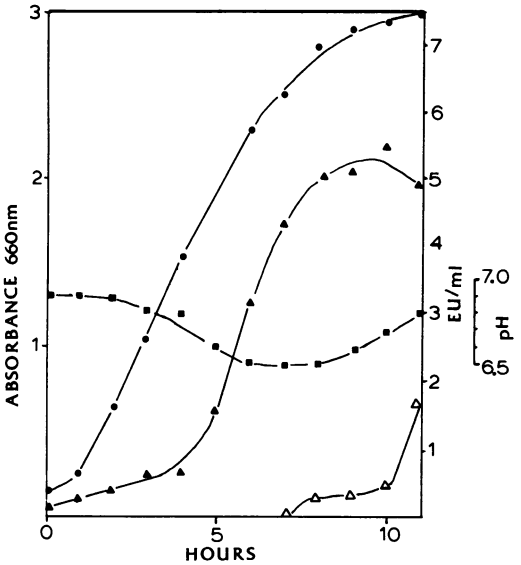

FIG. 1. Growth of and protease production by *A. hydrophila* UV 108. Absorbance at 660 nm (●) and pH (■) were monitored during growth. We measured protease activity in cell-free culture filtrates (▲) and washed (10 mM Tris-hydrochloride, pH 7.0), and sonicated cells (△).

under high aeration at the lowest temperature tested (20°C) (Table 2).

Available carbon and nitrogen sources also had profound effects on the production of protease (Table 3). On poorly utilized carbon sources, the cells produced larger amounts of protease, compared with amounts produced on more readily utilizable carbon sources. Organic nitrogen sources were found to have differential effects on growth stimulation, protease production, or both (Table 3). When supplemented with a complex nitrogen source, inorganic nitrogen (NH_4^+) stimulated growth but inhibited protease production. A 26% reduction in protease production occurred when NH_4Cl was added to a final concentration of 50 mM; a 66% reduction occurred at 100 mM. At these levels, NH_4Cl had no effect on the activity of preformed protease (data not shown). When NH_4^+ was the sole nitrogen source, protease production was completely inhibited.

Generally, conditions which produced maximal growth of *A. hydrophila* UV 108 resulted in maximal protease production. Protease production by *A. proteolytica* requires aerated medium (12). Contrary to our findings for *Aeromonas* spp., Wiersma et al. (21) found that low O_2 tension stimulates protease production by *Vibrio* spp. The proper culture pH has been shown to be crucial for protease production by *A. liquefaciens* (3): pH 7.0 allows protease production, whereas pH 6.2 restricts enzyme production. Protease production by UV 108 behaved similarly.

The effects of nitrogen sources on the production of protease enzymes by *Aeromonas* spp. have been examined in some detail. Although in our study Difco Proteose Peptone did not produce prolific growth, it provided the best nutrition for protease synthesis. This was in contrast to the results obtained by Dahle (3), who found that for strains of *A. liquefaciens* and *A. salmonicida*, Neopeptone (Difco Laboratories, Detroit, Mich.) is the best nitrogen source for both growth and protease production, whereas proteose peptone is the poorest. Compared with other *Aeromonas* spp., UV 108 produced large amounts of protease when grown on casein as the nitrogen source, whereas on Casamino Acids it produced poor enzyme yields. Dahle did

TABLE 1. Influence of initial pH on growth and total protease produced at late stationary phase (24 h)[a]

pH		Dry wt of cells (mg/ml)	EU/mg (dry wt)
Initial	Final		
5.5	4.9	4.32 ± 0.13	0.16 ± 0.004
6.0	5.4	5.74 ± 0.22	0.21 ± 0.001
6.5	6.4	6.02 ± 0.18	0.25 ± 0.001
7.0	6.9	7.24 ± 0.36	3.04 ± 0.040
7.5	6.9	7.21 ± 0.21	ND[b]
8.0	7.1	7.23 ± 0.18	2.70 ± 0.020
8.5	7.2	7.32 ± 0.19	2.77 ± 0.021

[a] Cells were grown in basal salts plus 0.5% (wt/vol) peptone and 0.5% (wt/vol) glucose. Values are means of replicate samples ± standard deviations.
[b] ND, Not determined.

TABLE 2. Influences of temperature and aeration on total protease produced at late stationary phase (24 h)[a]

Temp (°C)	Aeration (300 rpm)	Dry wt of cells (mg/ml)[b]	EU/mg (dry wt)
20	+	6.90 ± 0.17	3.91 ± 0.07
	−	3.60 ± 0.05	0.53 ± 0.01
25	+	6.85 ± 0.17	1.24 ± 0.02
	−	2.90 ± 0.07	0.72 ± 0.01
30	+	3.65 ± 0.09	0.66 ± 0.01
	−	2.92 ± 0.06	0.65 ± 0.01
35	+	4.00 ± 0.10	0.43 ± 0.008
	−	2.52 ± 0.06	0.52 ± 0.008
37	+	4.00 ± 0.10	0.50 ± 0.008
	−	3.62 ± 0.09	0.65 ± 0.01

[a] Cells were grown in basal salts plus 0.5% (wt/vol) peptone and 0.5% (wt/vol) glucose. Values are means of replicate samples ± standard deviations.

TABLE 3. Influences of carbon and nitrogen sources on *A. hydrophila* total protease production

Nutrient (0.5% [wt/vol])	Cell growth (mg [dry wt]/ml)[a]	Protease production (EU/mg of cell wt)
Carbon source		
None	0.60 ± 0.03	5.55 ± 0.33
Lactate	1.71 ± 0.04	4.74 ± 0.12
Sucrose	3.37 ± 0.08	10.01 ± 0.20
Glycerol	5.38 ± 0.13	3.49 ± 0.09
Mannitol	6.16 ± 0.12	3.14 ± 0.06
Glucose	6.39 ± 0.16	1.68 ± 0.04
Fructose	6.50 ± 0.16	6.10 ± 0.12
Glycogen	8.46 ± 0.17	5.36 ± 0.11
Maltose	8.69 ± 0.22	2.91 ± 0.07
Nitrogen source		
None	0.11 ± 0.006	1.82 ± 0.09
Casein	4.12 ± 0.10	1.13 ± 0.02
Casamino Acids	6.12 ± 0.15	0.26 ± 0.006
Proteose Peptone	7.92 ± 0.16	1.45 ± 0.03
Neopeptone	7.95 ± 0.20	0.68 ± 0.02
Tryptose	8.15 ± 0.16	1.01 ± 0.02

[a] Cells were grown in basal salts supplemented with 0.5% (wt/vol) peptone (carbon source studies) or 0.5% (wt/vol) glucose (nitrogen source studies). Values are means of replicate samples ± standard deviations determined at 24 h (late stationary phase).

not find protease during growth of *Aeromonas* spp. on intact casein, but the enzyme was shown to be produced with trypsinized casein (3). Merkel et al. (12) and Litchfield and Prescott (10) found casein digests to be potent inducers of protease production by *A. proteolytica*. Using an NH_4^+-containing chemically defined medium, Riddle et al. (13) showed that glutamic acid increases both growth of and protease production by *A. hydrophila*. The differences in protease production on different nitrogen sources may reflect differences in the amino acids that induce the protease, if indeed it is both partially constitutive and inducible (10). Complex organic nitrogen sources vary in concentration of free amino acids and amount of small peptides present. Thus, each nitrogen source may have the required amino acids present in (i) amounts insufficient to induce activity, (ii) optimal induction concentrations, or (iii) excessive induction amounts, which would repress protease synthesis; such effects were observed by Wiersma and Harder (22). The concentrations of trace elements in the complex nitrogen sources may also cause changes in protease production; for example, Riddle et al. (13) found Zn^{2+} to be stimulatory and Fe^{2+} to be inhibitory to protease synthesis by *A. hydrophila*.

Protease production by UV 108 was decreased in the presence of NH_4^+ although it was less sensitive to ammonium repression than were other microbial proteases. Liu and Hsieh (11) found that 0.5% (wt/vol) $(NH_4)_2SO_4$ (78 mM NH_4^+) inhibits *A. salmonicida* and *A. liquefaciens* protease production 50 and 75%, respectively. *Vibrio* sp. strain SAI endopeptidase production is inhibited 58% by 0.2% (wt/vol) NH_4Cl (37 mM) (22).

A. hydrophila grew slowly in chemically defined media and showed lower growth yields but higher levels of protease production, compared with growth and production on complex undefined media (13). It has been suggested that protease production by *Vibrio* sp. strain SAI is under an inducer catabolite repression that is actually reflected by the growth rate and energy status of the cell (22). When *Vibrio* sp. strain SAI was stressed to grow at a low rate, protease production was abundant. Similarly, when UV 108 was stressed by limiting the carbon or nitrogen source, large amounts of protease were produced, despite the very sparse growth. These kinds of stresses may induce the cell to produce a series of "scouting enzymes" (e.g., proteases, amylases, phosphatases, etc.), the purpose of which is to digest potential polymeric nutrient sources to provide readily metabolizable substrates for microbial growth (W. Harder, personal communication). *A. hydrophila* UV 108 required the proper pH, temperature, and aeration for optimal protease production. It also required a limited carbon or nitrogen source. It appears that protease production regulation in UV 108 may be similar to that in *Vibrio* spp. in that an inducer catabolite repression system that is relatively insensitive to free ammonium ions and that is reflected by a low cell energy status operates, but only under permissive physical conditions.

During stressful periods, the fish host harboring commensal *A. hydrophila* may be depleting tissues of a particular nutrient(s). This can invoke the production of a series of enzymes, including protease, by the commensal microbe. This inducer environment, along with an impaired immunological defense, may be the stress-related factor that allows initiation of the transition to the disease state. Continued research into the effects of stress on predisposition of fish to infection, enhancement of the *A. hydrophila* virulence, or both and the role of protease in disease induction and lesion formation may provide the basis for *Aeromonas* disease control.

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