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Pathogenicity Factors and Virulence for Rainbow Trout (*Salmo gairdneri*) of Motile *Aeromonas* spp. Isolated from a River

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Ninety-seven motile *Aeromonas* strains were isolated over a period of a year from samples of water and sediment collected at different sites along a river. Strains were regularly recovered from all samples, regardless of the source of isolation or seasonal conditions. Isolates were biochemically characterized by the API 20NE system (Analytab Products, Plainview, N.Y.) and classified as *Aeromonas hydrophila* (74 strains), *Aeromonas sobria* (11 strains), and *Aeromonas caviae* (12 strains). Despite the high level of homogeneity observed in their biochemical patterns, they displayed different degrees of virulence for fish; 72.02% of *A. hydrophila* isolates and 63% of *A. sobria* isolates were virulent for fish by intramuscular challenge, but lower frequencies of virulence were observed when intraperitoneal injections were used. All *A. caviae* strains proved to be avirulent. Caseinases, hemolysins, and Vero cytotoxins were produced by 100, 91, and 94.59%, respectively, of *A. hydrophila* strains and with lower frequencies and lower caseinase activities by *A. sobria* isolates. No correlation was found between these activities and the degree of virulence of the strains for fish. Most hydrophobic strains seem to be concentrated in *A. caviae*, *A. sobria*, and avirulent *A. hydrophila* groups. Known virulence markers commonly associated with virulent strains (acriflavine negative and self-pelleting negative and precipitation after boiling positive phenotypes) had a low representation in the total of strains studied and were not associated with virulence.

Among the etiological agents of bacterial fish diseases, the motile *Aeromonas* group, and especially *Aeromonas hydrophila*, is considered one of the most important. This microorganism is responsible for hemorrhagic septicemia, a disease affecting a wide variety of freshwater fish species and occasionally marine fish (18). It has also been reported as a cause of infections in humans (6).

Several extracellular toxins and enzymes have been described that may be associated with the virulence of *A. hydrophila*. These include hemolysins, cytotoxins, enterotoxins, and proteases (1, 20). Additional protein layers, O antigens, fimbriae, and other nonspecific adherence mechanisms (8, 17, 21) seemed to be related to the pathogenic capacity of the strains, contributing to the colonization of animal tissues. Nevertheless, the exact mechanism of virulence and the particular role of the factors involved have not yet been established.

Motile aeromonads have been isolated from their natural aquatic habitats, where they survive and multiply in favorable nutritional conditions (5, 9, 11). Pathogenicity attributes have proved to be present in a high percentage of waterborne strains (31), but their virulence for fish appears to be lower than that displayed by strains isolated from fish (7).

The purpose of this study was to examine the biochemical phenotypes of motile aeromonads isolated from different microhabitats in the River Porma (León province, Spain) together with their virulence factors, such as extracellular products and surface characteristics, and to determine the relationships between these factors and the virulence of these bacteria for fish.

MATERIALS AND METHODS

Sampling procedures. Four locations along the River Porma were sampled over a year. Sampling sites were situated at elevations of between 800 and 1,195 m above sea level. Water temperature fluctuated between 0.5 and 24°C. Samples of water and sediments were collected in sterile containers and placed on ice for transport to the laboratory. The time from collection to sample processing never exceeded 1 h.

Isolation and identification. Samples of 0.1 ml of undiluted and 10-fold-diluted water and sediment were spread on plates of starch-ampicillin medium (24) and incubated for 24 h at 28°C. Yellow and amylolytic colonies 2 to 5 mm in diameter were considered presumptive *Aeromonas* species and underwent the following protocol for biochemical characterization: Gram stain, motility, oxidase, DNase, resistance to O/129 agent, and growth on *A. hydrophila* confirmation medium (14). Identification was completed by using the API 20NE system as recommended by the manufacturer (Analytab Products, Plainview, N.Y.). Strips were incubated at 28°C. The production of SH_2 from cysteine was added to the complementary test of the API 20NE system as described by Popoff (26).

Strains were stored at −80°C in 15% (vol/vol) glycerol for further characterization.

Virulence for fish. The strains were grown in 10 ml of Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) for 18 h at 28°C without shaking. Cells were pelleted at 4,000 × *g* and 4°C and suspended in sterile saline (0.85%). Inocula were prepared by diluting the bacterial suspension to the adequate optical density (at 540 nm) for 3×10^7 CFU to be contained in a 0.1-ml volume. Ten rainbow trout (average length, 10 cm), previously anesthetized with MS-222 (Sigma Chemical Co., St. Louis, Mo.), were inoculated with 0.1 ml containing 3×10^7 cells. Both intramuscular and intraperitoneal injections were used. The trout were maintained in 70-liter tanks of water provided with individual filtration and forced aeration systems. Tanks were placed in a temperature-controlled room where the

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water temperature was stable at 15°C. Deaths were monitored up to 72 h after injection. When necessary, the 50% lethal dose was calculated by the method of Reed and Muench (28).

Hemolysin assays. Strains were evaluated for their ability to produce hemolysins by the plate method. Each individual isolate was streaked on tryptic soy agar (TSA) medium containing 5% (vol/vol) sheep erythrocytes. Hemolytic activity was measured basically as described by Asao et al. (3). Strains were grown in 50 ml of TSB in a rotatory shaker at 28°C and 250 rpm for 24 h. Cultures were centrifuged at 4°C, and supernatants were filtered through a 0.45-μm-pore-size filter. Then 50-μl samples of twofold serial dilutions of the filtrates in 0.01 M Tris hydrochloride buffer (pH 7.2) containing 0.9% NaCl were mixed with an equal volume of a 1% suspension of sheep erythrocytes in a microdilution plate and incubated at 28°C for 1 h. The hemolytic activity was expressed as the highest dilution of filtrate showing total hemolysis.

Cytotoxin assay. Supernatants obtained as described above were tested for the presence of cytotoxin on Vero cell monolayers as previously described by Notermans et al. (22), with slight modifications. Samples (25 μl) of twofold dilutions of the supernatants in TSB were added to Vero monolayers grown in 24-well tissue culture plates and incubated at 37°C in a 5% CO_2 atmosphere. Plates were read after 24 h under an inverted microscope. Wells showing totally or partially destroyed monolayers were considered positive.

Cytotoxic titers were expressed as the highest dilution giving a positive response.

Caseinase assays. Caseinase production was detected by streaking strains on TSA-skim milk (2% final concentration). The caseinase activity was semiquantified as follows. Two serial dilutions (100 μl) of the culture supernatants in 0.1 M Tris (pH 7.2) buffer were placed in 8-mm wells made on a petri dish filled with 30 ml of 2% skim milk and 2% agar in the same buffer. Plates were maintained at 4°C to permit diffusion of the enzyme and then incubated for 24 h at 28°C. Caseinase activity was expressed as the highest dilution showing a halo of digestion.

Autoagglutination test. The autoagglutination test for self-pelleting (SP^+) and precipitation after boiling (PAB^+) phenotype characterization was carried out as described by Janda et al. (13). Each strain was grown in 6 ml of brain heart infusion broth for 18 h at 28°C. At the end of the incubation period, cultures were observed for evidence of self-pelleting, which was manifested as a large aggregate of cells at the bottom of the tube and the absence of turbidity in the medium. A 3-ml fraction was heated for 1 h at 100°C in a water bath and then cooled for 10 min and compared with the samples kept at room temperature for a control. Reduction in turbidity was considered positive for precipitation after boiling.

Hemagglutination test. The method described by Atkinson and Trust (4) was followed for the evaluation of the hemagglutination ability of strains. Sheep blood was collected and placed in Alsever solution. The erythrocytes were washed three times in Dulbecco phosphate-buffered saline (pH 7.4). Then 20 μl of a 3% (vol/vol) blood cell suspension was mixed with an equal volume of bacterial suspension (1.5×10^9 cells per ml of the same buffer) on a glass slide. The test was performed at room temperature and read within 10 min.

Acriflavine agglutination. Agglutination in acriflavine (0.2%) was effected by the method of Mittal et al. (21) on a glass slide with colonies grown on TSA supplemented with 5% sheep blood.

Congo red uptake test. The Congo red uptake test was basically performed as described by Statner and George (32). Strains were streaked on plates of TSA containing Congo red (final concentration, 50 μg/ml) and incubated at 28°C. Colonies were examined under obliquely reflected light on a black background. Bright orange or red colonies were considered positive. Different intensities in the dye uptake were expressed as + and ++.

Crystal violet binding. The ability of *Aeromonas* species to bind crystal violet was determined as follows. Individual strains were incubated under standard conditions on TSA plates for 24 h and then flooded with a solution of 0.5 mg of crystal violet per ml. The dye was removed after 2 min of contact, and the crystal violet uptake was qualitatively determined.

Salt aggregation test. Salt aggregation was tested as reported by Qadri et al. (27). Fresh colonies of strains obtained after growth on TSA for 18 h at 28°C were mixed with solutions of ammonium sulfate in a 0.02 M sodium phosphate buffer (pH 6.8) in concentrations ranging from 0.5 to 3 M.

Statistical analysis. To establish the relationships between different characteristics of strains and their virulence, the chi-square test was applied. Relationships between the degree of virulence and caseinase, hemolytic, and cytotoxic activities were assessed by linear regression analysis.

TABLE 1. Isolation frequency of motile *Aeromonas* species

Motile *Aeromonas* species	No. of isolates (%)
Presumptive *Aeromonas*	239
Aeromonas hydrophila	74 (30.96)
Aeromonas sobria	11 (4.60)
Aeromonas caviae	12 (5.02)
A. hydrophila-Vibrio fluvialis[a]	16 (6.69)
A. sobria-Vibrio cholerae[a]	4 (1.67)
A. sobria-A. hydrophila[a]	7 (2.92)

[a]Strains with biochemical characters belonging to different species or genera.

RESULTS

From a total of 239 presumptive *Aeromonas* species isolated on starch-ampicillin medium, 124 isolates (51.88%) were biochemically characterized and classified as shown in Table 1.

Six different API 20NE profile numbers were observed among the 74 strains of *A. hydrophila* (Table 2), the most

TABLE 2. API 20NE profile numbers of *A. hydrophila*, *A. caviae*, and *A. sobria* isolated from water and river sediments

A. hydrophila (n = 74)		*A. caviae* (n = 12)		*A. sobria* (n = 11)	
Profile	No. of strains	Profile	No. of strains	Profile	No. of strains
7577754	66	7577754	9	7176755	8
5177754	1	7477754	1	7177755	2
7476754	1	3577754	2	7176754	1
7575754	1				
7576754	4				
5577754	1				

TABLE 3. Predominant biochemical patterns of *A. hydrophila*, *A. sobria*, and *A. caviae* strains isolated from river water and sediment

Test	Test result for:		
	A. hydrophila	*A. caviae*	*A. sobria*
Reduction of nitrates to nitrites	+	+	+
Indole formation	+[a]	+	+
Glucose fermentation	+	+	+
Arginine dehydrolase	+[a]	+	+
Urease	−	−	−
Esculin hydrolysis	+[a]	+	−
Gelatin hydrolysis	+	+	+
β-Galactosidase	+	+	+
Glucose assimilation	+	+	+
Arabinose assimilation	+[a]	+	−[a]
Mannose assimilation	+[a]	+	+
Manitol assimilation	+	+	+
N-Acetylglucosamine assimilation	+	+	+
Maltose assimilation	+	+	+
Gluconate assimilation	+[a]	+	+
Caprate assimilation	+	+	+
Adipate assimilation	−	−	−
Malate assimilation	+	+	+
Citrate assimilation	−	−	+[a]
Phenyl-acetate assimilation	−	−	−
Oxidase	+	+	+
Voges-Proskauer[b]	+	−	
Gas production from glucose[b]	+	−[a]	
Lecithinase[b]	+	+[a]	
Growth at 42°C[b]	+	−[a]	
SH_2 production from cysteine[b]	+	−[a]	

[a] Variable responses to the test were observed in some strains.
[b] Complementary tests.

frequent of which was represented by 66 strains (89.17%) and corresponded to the biochemical pattern shown in Table 3. Only eight strains differed from it in their responses to the following tests: indole, esculin hydrolysis, arginine dehydrolase, mannose assimilation, arabinose assimilation, and gluconate assimilation. The biochemical patterns of *Aeromonas sobria* and *A. caviae* strains are given in Table 3.

The group of *A. caviae* isolates included 12 strains, which, although Voges-Proskauer negative, gave variable results in the production of gas from glucose, growth at 42°C, and the production of SH_2 from cysteine. Nevertheless, they were all unable to produce hemolysin and cytotoxins and showed very little or no caseinolytic activity. SH_2 production, when detected, was slower than that for *A. hydrophila* and was manifested as the blackening of the medium but only at the top of the tube. However, the isolates were finally considered to be *A. caviae*, because their biochemical characters correspond to those given by Kuijper et al. (15) for hybridization groups 4 and 5A.

Motile *Aeromonas* species were regularly isolated from each water and sediment sample at the four sampling sites.

Virulence for rainbow trout (*Salmo gairdneri*). Virulence of 74 *A. hydrophila*, 12 *A. caviae*, and 11 *A. sobria* isolates was assayed for rainbow trout by using both intraperitoneal and intramuscular injections. Fifty-seven (77.02%) *A. hydrophila* strains were virulent for trout upon intramuscular challenge, with mortalities ranging from 20 to 100%. The 50% lethal dose of strains randomly chosen from the most virulent (100% mortality) was about 10^7 cells; 63.6% of *A. sobria* isolates tested were positive in the pathogenicity test, whereas all *A. caviae* strains were avirulent.

The frequency of virulent strains was lower when intraperitoneal injections were used, and mortality never exceeded 60% (Table 4).

TABLE 4. Virulence for rainbow trout of motile *Aeromonas* species by using intraperitoneal and intramuscular injections

Species	n[a]	No. of isolates (%)			
		Intramuscular route[b]		Intraperitoneal route[b]	
		Virulent	Avirulent	Virulent	Avirulent
A. hydrophila	74	57 (72.02)	17 (22.97)	29 (39.18)	45 (60.81)
A. caviae	12	0	12 (100.0)	0	12 (100.0)
A. sobria	11	7 (63.63)	4 (36.36)	5 (45.45)	6 (54.54)

[a] Number of strains tested.
[b] Cells (3×10^7) were inoculated by the intramuscular or intraperitoneal route.

Death occurred within 48 to 72 h postinjection. A postmortem examination revealed the following pathological signs: hemorrhages at the internal abdominal wall, pale-colored liver, pronounced and sometimes sanguinous ascites, and, when intramuscular injections were used, a necrotic lesion affecting the skeletal muscle at the site of injection at 24 h after challenge. The lesion usually became hemorrhagic at the end of the infectious process. Strains were always recovered from both external necrotic lesions and kidneys.

Hemolytic, cytotoxic, and caseinase activities. Caseinolytic capacity was a common feature among both virulent and avirulent *A. hydrophila* strains. No relationship was observed between the caseinase activity of the supernatants (titer, 1/64 to 1/1,024) and the virulence of strains (r = 0.0694). *A. sobria* isolates generally displayed little or no activity (titer, <1/64). Some of the noncaseinolytic strains caused the highest levels of mortality.

Sixty-eight (91.89%) *A. hydrophila* strains and 7 (68.6%) *A. sobria* strains were hemolytic, as shown on sheep blood agar. The hemolysis type was different for the two species: whereas *A. hydrophila* produced a halo of hemolysis formed by two zones, one of total hemolysis and another of incomplete lysis, *A. sobria* produced only one of partial hemolysis. Hemolytic activity ranged from titers of 1/4 to 1/128, 1/16 being the most common. No higher activity was found in the less virulent strains, as reported by Lallier et al. (16).

Cytotoxic activity on Vero cells was displayed by culture supernatants of 70 (94.59%) *A. hydrophila* strains and 8 (72.72%) *A. sobria* strains (titers, 1/4 to 1/128). No correlation was observed between individual activities and the virulence of the strains.

A. caviae isolates produced neither cytotoxins nor hemolysins and were not caseinolytic or were very weak producers (titers, <1/16).

Different combinations of the three extracellular products were observed among both virulent and avirulent strains of the three motile *Aeromonas* species (Table 5).

Surface properties of the strains. Relative frequencies of the different surface characteristics of *Aeromonas* spp. are given in Table 6. Hemagglutination ability tested on sheep blood cells was exhibited by 41 (42.2%) of the strains. This property was equally distributed among *A. caviae* and *A. hydrophila*. All but one of the *A. sobria* strains were negative.

Spontaneous agglutination represented by the SP^+ or PAB^+ phenotype was not a very common feature among the strains; PAB^+ occurred only in 14 strains, 8 of which were avirulent. However, 66 (68.04%) of the strains were SP^-

TABLE 5. Caseinolytic (CAS), hemolytic (HEM), and cytotoxic (CYT) activities of virulent and avirulent *A. hydrophila*, *A. sobria*, and *A. caviae* strains

Extracellular products			Virulent strains (*n* = 64)			Avirulent strains (*n* = 33)		
CAS	HEM[a]	CYT[a]	*A. hydrophila*	*A. sobria*	*A. caviae*	*A. hydrophila*	*A. sobria*	*A. caviae*
+	+	+	52[b]	2[c]	0	11[b]	0	0
+	+	−	2[b]	0	0	0	0	0
+	−	+	0	0	0	2[b]	1[c]	0
+	−	−	0	0	0	7[b]	1[c]	0
−	+	+	0	4	0	0	1	0
−	−	−	0	1	0	0	1	12[d]

[a] Hemolysin and cytotoxin titers ranged from 1/4 to 1/64.
[b] Caseinolytic activity titer, 1/28 to 1/1,024.
[c] Caseinolytic activity titer, <1/64.
[d] Caseinolytic activity titer, <1/8.

PAB$^-$. Hydrophobicity as measured by the salt aggregation test seemed to be more associated with *A. sobria* and *A. caviae* strains. The highest frequencies of salt aggregation test values of 0.5 were observed for both groups of strains, whereas values of 2 were only encountered among *A. hydrophila* strains. The acriflavine agglutination test was positive for all *A. sobria* strains and in 72.97 and 75% of *A. hydrophila* and *A. caviae* strains, respectively.

Congo red was bound by all strains to a variable extent; *A. sobria* was the most efficient in the uptake of the dye.

Surface characters and virulence. No significant differences were observed in the frequency of distribution of the following factors among virulent and avirulent strains within the *A. hydrophila* strains: hemagglutination in sheep erythrocytes, SP PAB phenotypes, crystal violet binding, and acriflavine agglutination. The more hydrophobic strains (salt aggregation test value, 0.5) and those that incorporated Congo red dye to the highest extent associated significantly with the avirulent group (Table 7).

All virulent *A. sobria* strains presented the phenotype SP$^-$ PAB$^-$, did not agglutinate sheep blood cells, and were able to bind crystal violet and to aggregate in acriflavine (0.2%), whereas all but one were scored as ++ on Congo red. The avirulent group contained the highest frequencies of the more hydrophobic strains.

DISCUSSION

A high ability of *A. hydrophila* to live under a wide variety of environmental conditions in natural waters has been observed by Hazen et al. (11). The microorganism has been isolated over wide ranges of salinity, conductivity, temperature, pH, and turbidity. We also found *Aeromonas* strains distributed throughout the river environment, and we observed a high degree of similarity in the biochemical traits of the 97 strains studied regardless of the source of isolation or seasonal conditions. A single biochemical profile was exhibited by 89.18% of *A. hydrophila*, 75% of *A. caviae*, and 72% of *A. sobria* isolates. Homogeneity of environmental strains with regard to biochemical characteristics has already been reported by Fliermans et al. (9). The finding of 27 strains with phenotypes sharing characters belonging to different species or genera (*A. hydrophila* and *Vibrio fluvialis*, *A. sobria* and *Vibrio cholerae*, *A. sobria* and *A. hydrophila*) and the difficulties encountered in trying to separate *A. caviae* from *A. hydrophila* by using biochemical tests appear to

TABLE 6. Surface characters of *Aeromonas* species

Surface markers	No. of isolates (%) with marker			
	A. hydrophila (*n* = 74)	*A. caviae* (*n* = 12)	*A. sobria* (*n* = 11)	Total
SP$^-$ PAB$^+$	9 (12.60)	4 (33.34)	1 (9.09)	14 (14.43)
SP$^-$ PAB$^-$	50 (67.56)	8 (66.66)	8 (72.72)	66 (68.04)
SP$^+$ PAB$^-$	11 (14.86)	0	1 (9.09)	12 (12.37)
SP$^+$ PAB$^+$	4 (5.40)	0	1 (9.09)	5 (5.15)
Salt aggregation test[a]				
2	6 (8.10)	0	0	6 (6.18)
1.5	29 (39.18)	1 (8.33)	1 (9.09)	31 (31.95)
1	31 (41.89)	6 (50.00)	4 (37.37)	41 (42.26)
0.5	8 (10.81)	5 (41.66)	6 (63.63)	19 (19.58)
Crystal violet binding				
+	44 (59.45)	8 (66.66)	9 (81.81)	61 (62.88)
−	30 (40.54)	4 (33.34)	2 (19.19)	36 (37.11)
Acriflavine agglutination				
+	54 (72.97)	9 (75.00)	11 (100.0)	74 (76.28)
−	20 (27.02)	3 (25.00)	0	23 (23.71)
Congo red uptake[a]				
++	27 (36.48)	7 (58.33)	8 (72.72)	42 (43.29)
+	47 (63.51)	5 (41.67)	3 (27.27)	55 (56.70)
Sheep erythrocyte agglutination				
+	34 (45.94)	6 (50.00)	1 (9.09)	41 (42.26)
−	40 (54.05)	6 (50.00)	10 (90.90)	56 (57.74)

[a] Significant differences ($P < 0.05$).

TABLE 7. Surface properties of virulent and avirulent *Aeromonas* species

Surface marker	No. of isolates (%) with marker				
	A. hydrophila (*n* = 74)		*A. sobria* (*n* = 11)		*A. caviae* (*n* = 12)
	Virulent (*n* = 57)	Avirulent (*b* = 17)	Virulent (*n* = 7)	Avirulent (*n* = 4)	
SP^- PAB^+	6 (10.52)	3 (17.64)	0	1 (25.00)	4 (33.34)
SP^- PAB^-	39 (68.42)	11 (64.70)	7 (100.00)	1 (25.00)	8 (66.66)
SP^+ PAB^-	9 (15.78)	2 (11.76)	0	1 (25.00)	
SP^+ PAB^+	3 (5.26)	1 (5.88)	0	1 (25.00)	
Salt aggregation test[a]					
2	5 (8.87)	1 (5.88)	0	0	0
1.5	23 (40.35)	6 (35.29)	0	0	1 (8.33)
1	25 (43.85)	6 (35.29)	3 (42.85)	1 (25.00)	6 (50.00)
0.5[a]	4 (7.01)	4 (23.52)	4 (57.14)	3 (75.00)	5 (41.66)
Crystal violet binding					
+	34 (59.64)	10 (58.82)	7 (100.00)	2 (50.00)	8 (66.66)
−	23 (40.35)	7 (41.17)	0	2 (50.00)	4 (33.34)
Acriflavine (0.2%) aglutination					
+	42 (73.68)	12 (70.58)	7 (100.00)	4 (100.00)	9 (75.00)
−	15 (26.31)	5 (29.40)	0	0	3 (25.00)
Congo red uptake[a]					
++	17 (29.82)	10 (58.82)	6 (85.71)	2 (50.00)	7 (63.63)
+	40 (70.17)	7 (41.17)	1 (14.28)	2 (50.00)	5 (41.66)
Sheep erythrocyte agglutination					
+	25 (43.85)	10 (58.82)	0	1 (25.00)	6 (50.00)
−	32 (56.14)	7 (41.17)	7 (100.00)	3 (75.00)	6 (50.00)

[a] Significant differences ($P < 0.05$)

indicate the absence of a sharp dividing line between species and that biochemical means for the identification of motile *Aeromonas* isolates at the species level are, at the moment, imprecise. Our findings are borne out by both the interspecific and intergeneric antigenic relationships reported by Leblanc et al. (19) and Sakazaki and Shimada (29) for members of the family *Vibrionaceae* and the 11 DNA hybridization groups recently established by Kuijper et al. (15) for the motile *Aeromonas* species.

Despite the relatively high similarity of the strains from the biochemical point of view, they displayed variable degrees of virulence (mortalities from 0 to 100%) by intramuscular challenge. The lower virulence of strains found when intraperitoneal injections were used strongly suggests that the environmental strains studied lacked the protective surface characteristics described by Dooley and Trust (8), Janda et al. (13), and Mittal et al. (21) for highly virulent strains, which contribute to overcoming the peritoneal phagocytic barrier and the natural defenses of the host, permitting an easier establishment in the tissues.

Great variations in the virulence test conditions (water temperature, fish size, and injection route) used by different authors make it difficult for us to compare quantitative virulence results. However, everything seemed to indicate that our strains should be considered weakly virulent (50% lethal dose, $>10^7$) and having a low invasive capacity.

It has been reported by Sakazaki et al. (29) that serogroup O:11 is highly represented among *A. hydrophila* strains from human sources and rare among environmental strains. On the other hand, it is commonly associated with S layer, PAB^+, and acriflavine-negative characters in highly virulent strains (25), which seem to associate more readily with invasive infections than with localized illnesses (13). It is not surprising, then, to observe a lack of association between the presence of established virulence markers and the virulence of our strains and thus to find that non-acriflavine-agglutinating and -hemagglutinating strains are distributed homogeneously among virulent and avirulent groups of *A. hydrophila* and that SP^- PAB^+ phenotypes were more frequent among the avirulent *A. hydrophila* and *A. caviae* isolates than in the most virulent group. It is possible that known virulence factors of *A. hydrophila* for warm-blooded animals may not be attributable to its fish pathogenicity; as indicated by Santos et al. (30), a different mechanism of virulence may be involved in the invasion of the bacteria in poikilothermic and homoiothermic hosts.

Congo red uptake, described as a good virulence marker in *Aeromonas salmonicida* (12) and other species (27), was incorporated by all strains at various levels, as already reported by Statner and George (32), but the avirulent *A. hydrophila* and *A. caviae* groups contained the highest frequencies of ++ strains. This character was associated with higher values of hydrophobicity as measured by the salt aggregation test in the groups of strains cited.

Factors of virulence represented by extracellular proteases, hemolysins, and Vero cytotoxins were produced by nearly all virulent and avirulent strains of *A. hydrophila* to the same extent. This fact and the absence of correlation between the corresponding activities and the degree of virulence (*r*, 0.069) may indicate that qualitative factors rather than quantitative ones are involved in the pathologic process.

A different situation was found in the *A. sobria* group, in which some strains, although lacking even the ability to

excrete all or some of the products cited above, were able to induce fish death. This again suggests that other extracellular enzymes or toxins different than those studied here may be involved in the virulence mechanism of the microorganism. Differences in toxigenic profiles of *A. hydrophila* and *A. sobria* have been reported by Olivier et al. (23).

Physical and nutritive water parameters such as conductivity, pH, temperature, redox potential, and phosphate, nitrate, or total organic carbon concentration seem to increase the survival period of *A. hydrophila* and to affect its distribution in natural aquatic environments (9, 10). Structural and morphological changes have been observed in marine bacteria during survival periods (2), which leads us to imagine that the prolonged presence of *Aeromonas* species in river water can cause changes in the virulence of the strains as a result of loss or alteration of pathogenicity attributes, the synthesis of which can constitute a waste of energy for the microorganism. The low level of virulence found among the strains we studied and the differences in surface characters with regard to those described by other authors for highly virulent microorganisms suggest the possibility of the changes mentioned above.

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