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Surface Phenotypic Characteristics and Virulence of Spanish Isolates of *Aeromonas salmonicida* after Passage through Fish

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Eleven strains of *Aeromonas salmonicida* were passaged twice by intraperitoneal injection through rainbow trout and reisolated from the kidney of moribund fish. The surface characteristics and virulence of the strains changed following passage through fish. None of the in vitro tests used could effectively predict the in vivo virulence.

Aeromonas salmonicida causes the disease furunculosis in salmonids, a disease which can occur from the fry to adult stages.

The cell envelope of *A. salmonicida* is basically composed of an A-layer protein beyond the outer membrane and the repeating O-antigen subunit of bacterial lipopolysaccharide (LPS) (5). It has been shown (2) that the phenotype A^+ LPS^+ is an essential virulence factor, but avirulent A^+ strains have been reported. The A-layer is a 50-kDa protein (10, 23) and is apparently responsible for several cell surface properties, including autoaggregation (7, 18) and hydrophobicity (16, 25). However, some autoaggregating strains of *A. salmonicida* having no detectable A-layer have been reported (9).

Since long periods of maintenance in the laboratory can change surface properties and virulence of the strains, in the present study we have investigated the relationship between surface characteristics and virulence of the fish pathogen *A. salmonicida* after passage of the microorganism twice through fish. Eight *A. salmonicida* strains isolated in northwestern Spain were examined. Two collection strains (ATCC 14174 and ATCC 33658) and one strain from D. P. Anderson (3.93) were included in this study for comparative purposes. All strains were isolated from fish showing symptoms of furunculosis (Table 1). Before any experiment, the bacterial strains were passaged twice through rainbow trout by intraperitoneal injection and reisolated from the kidney of moribund fish (4). Fish with an average weight of 20 g and with no history of furunculosis were obtained from a hatchery and were maintained in aerated freshwater in 20-liter tanks at 18°C.

All bacterial strains were subjected to taxonomic analysis by a method previously described (23). The stock cultures were maintained in tryptic soy broth (Oxoid) at −20°C with 15% (vol/vol) glycerol. Since surface properties are affected by medium and growth phase (21), all assays were carried out on bacteria in logarithmic growth phase at 22°C in tryptic soy broth or tryptic soy agar (Oxoid).

Assays for pathogenicity were conducted by intraperitoneal inoculation of fingerling rainbow trout (*Oncorhynchus mykiss*) with an average weight of 8 g that were maintained in water at 18°C, as previously described (14). Tenfold dilutions (0.1 ml each) ranging from 10^3 to 10^8 CFU/ml were inoculated, using 10 fish per dose. After a 7-day period, the 50% lethal dose (LD_{50}) was calculated by the Reed and Muench method (17) as the number of bacteria needed to kill 50% of fingerling rainbow trout.

Total and outer membrane proteins were prepared (3). LPS was isolated (6) and treated with proteinase K (2 mg/ml). Protein and LPS samples were run in sodium dodecyl sulfate-polyacrylamide (12.5%) gels (SDS-PAGE) (11). The separated proteins and LPS were visualized by silver staining (13, 24).

The relative cell surface hydrophobicity of the strains was assayed by using the standard salt aggregation test (12). The salt aggregation test value was defined as the lowest molarity of $(NH_4)_2SO_4$ which caused a visible (white) clumping of bacteria.

Autoaggregation of strains was determined as described by Janda et al. (8). The adherence of the strains to host cells was tested by agglutination of brown trout (*Salmo trutta*) erythrocytes and yeast cells, according to a previous description (1).

TABLE 1. Origin and source of strains

Strain	Source of isolation	Place of origin	Donor[a]
Epizootics in freshwater			
NF-19.1	*Salmo trutta*	Spain[b]	A. E. Toranzo
NF-21.3	*Salmo salar*	Spain	A. E. Toranzo
AI_{127B}	*Salmo trutta*	Spain	Personal collection
AI_{130B}	*Salmo trutta*	Spain	Personal collection
Epizootics in seawater			
PP-42	*Oncorhynchus mykiss*	Spain	A. E. Toranzo
PP-91	*Oncorhynchus mykiss*	Spain	A. E. Toranzo
TO.11.1	*Oncorhynchus mykiss*	Spain	A. E. Toranzo
SCO 4.1	*Oncorhynchus kisutch*	Spain	A. E. Toranzo
Collection strains			
ATCC 33658	*Salmo salar*	United States	ATCC
ATCC 14174	*Salvenilus fontinalis*	United States	ATCC
3.93	*Salmo salar*	United States	D. P. Anderson

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[b] All strains were isolated in Galicia, northwest Spain.

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TABLE 2. Relation between cell surface properties of *A. salmonicida* strains and virulence for fish after two passages through fish

Strain	LD_{50}	Salt aggregation test (M)[a]	Minimum agglutinating dose[b]	Minimal sugar concn (wt/vol)[c]	
				L-Fucose	D-Mannose
NF-19.1	5×10^4	1	6.25×10^7	—	0.0625
SCO 4.1	10^5	0.4	1.25×10^8	0.125	1
AI_{130B}	2×10^5	1.5	1.6×10^6	—	0.125
ATCC 33658	$<3 \times 10^5$	2	1.25×10^8	0.25	0.0625
ATCC 14174	3.2×10^6	1	1.6×10^6	0.125	0.125
3.93	$<2 \times 10^7$	2	6.25×10^7	—	0.125
AI_{127B}	$>2 \times 10^7$	1	1.25×10^8	0.0312	0.0312
NF-21.3	3×10^7	1.5	3.12×10^7	1	1
PP-91	3.2×10^7	1	1.6×10^6	0.0625	0.0312
PP-42	6.3×10^7	1	6.25×10^7	0.25	0.25
TO.11.1	10^8	1	6.25×10^7	—	0.25

[a] Lowest molarity of $(NH_4)_2SO_4$ fielding a strong aggregation of bacteria.
[b] Lowest bacterial cell concentration giving visible agglutination of yeast cells within 10 min.
[c] Minimal concentration inhibiting yeast cell agglutination. —, no inhibition.

One strain of *Aeromonas hydrophila* (B_{51}) was used as a positive control (20). The minimum agglutinating dose was defined as the lowest level of bacterial cells giving visible agglutination of yeast cells within 10 min. The minimal sugar concentration inhibiting yeast cell agglutination was also assayed, using a dilution of 1% (wt/vol) D-mannose, L-fucose, D-galactose, or D-glucose (Sigma) in phosphate-buffered saline.

Regardless of the geographic origin and source, SDS-PAGE showed that all strains included in this study possessed the 50-kDa A protein and the repeating subunit of the high-molecular-weight O-antigen side chain of the bacterial LPS (data not shown). The phenotype A^+ LPS^+ has been described as virulent because it produced death of all fish (*Salvenilus fontinalis* with an average weight of 84 g) injected with 10^5 cells (2). Our results showed that not all *A. salmonicida* isolates of the A^+ LPS^+ phenotype are virulent in trout (Table 2). Only 4 of 11 isolates possessing the A^+ LPS^+ phenotype were virulent for trout at an LD_{50} of $<10^6$ cells per fish (*O. mykiss* with an average weight of 8 g).

Other results of this study are included in Table 2. Ten of 11 isolates agglutinated in 1 to 2 M $(NH_4)_2SO_4$, and only 1 A^+ LPS^+ virulent strain (SCO 4.1) was considered strongly hydrophobic because it agglutinated in 0.4 M $(NH_4)SO_4$ (20). We did not find a relationship between virulence and the existence of hydrophobic surfaces. These results are in accordance with those of Olivier (15), who found three A^+ avirulent strains that were not hydrophobic.

All of our isolates had the ability to autoaggregate and to agglutinate yeast cells. The latter capability has previously been associated with avirulent *A. salmonicida* strains that were not lethal for fish at doses of lower than 10^8 cells per fish (*O. mykiss* with an average weight of 35 g) (18). This correlation was not found in our study, and the ability of *A. salmonicida* to agglutinate yeast cells was present in A^+ avirulent and virulent strains.

Two different adhesive components for yeast are present among our isolates; 7 of the 11 isolates assayed were inhibited by L-fucose and D-mannose, and the other four isolates were inhibited only by D-mannose. In no case was yeast agglutination inhibited by D-galactose or D-glucose. It was thus confirmed that *Saccharomyces cerevisiae* agglutination is mediated by a D-mannose-recognizing lectin, results that had been described previously (7, 18).

The hemagglutination of human and fish erythrocytes by various bacterial species has been described before (7, 19). However, our results showed that all isolates of *A. salmonicida* tested were unable to agglutinate brown trout erythrocytes, in agreement with results obtained with rainbow trout erythrocytes (20).

Interestingly, the collection strains included in previous studies (7, 15, 19, 20) showed important changes in either surface properties or virulence after two passages through fish (Table 3). Strain ATCC 14174, unpassaged, showed yeast agglutination (7), was avirulent (15, 19) at an LD_{50} higher than 10^8 cells per fish (7-g fish) (7), was not autoaggregating (7, 15, 20), and did not have an A-layer (7, 15). With the same isolate, we have observed that, following passage, it maintains the yeast agglutination capacity, has an LD_{50} of 3.2×10^6 cells per fish for 8-g fish, shows an autoaggregating phenotype, and regains the A-layer.

From these results it can be concluded that the characteristics of autoaggregation, a yeast agglutination capacity, and the A^+ LPS^+ phenotype are common among the isolates we studied, whether they were virulent or avirulent for trout. Both surface characteristics and virulence of the strains changed following passage through fish. None of the in vitro tests used in this study could effectively predict the in vivo virulence of *A. salmonicida*.

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TABLE 3. Comparison of cell surface properties and virulence of *A. salmonicida* collection strains before and after passages through fish[a]

Strain	LD_{50}		Autoaggregation		Yeast agglutination		A-layer		Salt aggregation test (M)[b]	
	A	B	A	B	A	B	A	B	A	B
ATCC 33658	2.2×10^5 (19)	$<3 \times 10^5$	ND (20)	+	ND	+	ND	+	1.25–1.50 (19)	2
ATCC 14174	$>10^6$ (5) $>10^8$ (7) $>10^8$ (19)	3.2×10^6	− (7, 15, 20)	+	+ (7, 15)	+	− (7, 15)	+	1.75 (19)	2
3.93	1.4×10^3 (19)	$<2 \times 10^7$	− (20)	+	ND	+	ND	+	1.75 (19)	2

[a] A, results from earlier studies. Reference(s) is given in parentheses. B, results obtained in this study. +, positive; −, negative. ND, no data. Size of fish injected in each study: reference 19, rainbow trout, 4 to 6 g; reference 7, coho salmon, 7 g; reference 15, juvenile brook trout; our study, rainbow trout, 8 g.
[b] Lowest molarity of $(NH_4)_2SO_4$ fielding a strong aggregation of bacteria.

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REFERENCES

1. **Atkinson, H. M., and T. J. Trust.** 1980. Hemagglutination properties and adherence ability of *Aeromonas hydrophila*. Infect. Immun. **27:**938–946.
2. **Cipriano, R. C., and A. R. Blanch.** 1989. Different structural characteristics in the cell envelope of the fish pathogen *Aeromonas salmonicida*. Microbiol. Lett. **40:**87–95.
3. **Crosa, J. H., and L. L. Hodges.** 1981. Outer membrane proteins induced under conditions of iron limitation in the marine fish pathogen *Vibrio anguillarum* 775. Infect. Immun. **31:**223–227.
4. **Ellis, A. E., A. S. Burrows, and K. J. Stapleton.** 1988. Lack of relationship between virulence of *Aeromonas salmonicida* and the putative virulence factors: a layer, extracellular proteases, and extracellular haemolysins. J. Fish Dis. **11:**309–323.
5. **Evenberg, D., R. Versluis, and B. Lugtenberg.** 1985. Biochemical and immunological characterization of the cell surface of the fish pathogenic bacterium *Aeromonas salmonicida*. Biochim. Biophys. Acta **815:**233–244.
6. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
7. **Ishiguro, E. E., and T. J. Trust.** 1981. Differentiating characteristics of virulent and attenuated strains of *Aeromonas salmonicida*. Dev. Biol. Stand. **49:**163–168.
8. **Janda, J. M., L. S. Oshiro, S. L. Abbot, and P. S. Duffey.** 1987. Virulence markers of mesophilic aeromonads: association of the agglutination phenomenon with mouse pathogenicity and the presence of a peripheral cell-associated layer. Infect. Immun. **55:**3070–3077.
9. **Johnson, C. M., M. F. Tatner, and M. T. Horne.** 1985. Autoaggregation and extracellular A-layer protein in *Aeromonas salmonicida*. Aquaculture **46:**163–166.
10. **Kay, W. W., J. T. Buckley, E. E. Ishiguro, B. M. Phipps, J. P. L. Monette, and T. J. Trust.** 1981. Purification and disposition of a surface protein associated with virulence of *Aeromonas salmonicida*. J. Bacteriol. **147:**1077–1084.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
12. **Lindhal, M., A. Faris, T. Wadstrom, and S. Hjerten.** 1981. A new test based on "salting out" to measure relative surface hydrophobicity of bacterial cells. Biochim. Biophys. Acta **667:**471–476.
13. **Morrisey, J. M.** 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. **117:**307–310.
14. **Nieto, T. P., M. J. R. Corcobado, A. E. Toranzo, and J. L. Barja.** 1985. Relation of water temperature to infection of *Salmo gairdneri* with motile *Aeromonas*. Fish Pathol. **20:**99–105.
15. **Olivier, G.** 1990. Virulence of *Aeromonas salmonicida*: lack of relationship with phenotypic characteristics. J. Aquat. Anim. Health **2:**119–127.
16. **Parker, N. D., and C. B. Munn.** 1985. Cell surface properties of virulent and attenuated strains of *Aeromonas salmonicida*, p. 97–105. *In* A. E. Ellis (ed.), Fish and shellfish pathology. Academic Press, London.
17. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
18. **Sakai, D. K., and T. Kimura.** 1985. Relationship between agglutinative properties of *Aeromonas salmonicida* strains isolated from fish in Japan and their resistance to mechanisms of host defence. Fish Pathol. **20:**9–21.
19. **Santos, Y., I. Bandín, T. P. Nieto, J. L. Barja, and A. E. Toranzo.** 1991. Cell-surface-associated properties of fish pathogenic bacteria. J. Aquat. An. Health **3:**297–301.
20. **Santos, Y., I. Bandín, T. P. Nieto, D. W. Bruno, A. E. Ellis, and A. E. Toranzo.** 1990. Comparison of the cell surface hydrophobicity of bacterial fish pathogens by different procedures, p. 101–115. *In* F. O. Perkins and T. C. Cheng (ed.), Pathology in marine science. Academic Press, London.
21. **Statner, B., M. J. Jones, and W. L. George.** 1988. Effect of incubation temperature on growth and soluble protein profiles of motile *Aeromonas* strains. J. Clin. Microbiol. **26:**392–393.
22. **Toranzo, A. E., Y. Santos, S. Nuñez, and J. L. Barja.** 1991. Biochemical and serological characteristics, drug resistance and plasmid profiles of Spanish isolates of *A. salmonicida*. Gyobyo Kenkyu **26:**55–60.
23. **Trust, T. J., P. S. Howard, J. B. Chamberlain, E. E. Ishiguro, and J. T. Buckley.** 1980. Additional surface protein in autoaggregating strains of atypical *Aeromonas salmonicida*. FEMS Microbiol. Lett. **9:**35–38.
24. **Tsai, C. M., and C. E. Frasch.** 1982. Staining of lipopolysaccharide in SDS-polyacrylamide gels using silver staining method. Anal. Biochem. **119:**115–119.
25. **Van Alstine, J. M., T. J. Trust, and D. E. Brooks.** 1986. Differential partition of virulent *Aeromonas salmonicida* and attenuated derivatives possessing specific cell surface alterations in polymer aqueous-phase systems. Appl. Environ. Microbiol. **51:**1309–1313.

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