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Electrostatic Mechanism of Survival of Virulent *Aeromonas salmonicida* Strains in River Water

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The ecological mechanism of survival of *Aeromonas salmonicida*, the bacterial pathogen of fish furunculosis, in river water was investigated by laboratory-based experiments with two virulent strains (which were autoagglutinating) and two virulent strains (which were nonagglutinating). A difference in net electrical charge of *A. salmonicida* cells was detected by electrophoresis; cells of the virulent strains were negative, whereas cells of the avirulent strains were positive. Despite the loss of viable cells within a week in distilled water and physiological saline (0.85% sodium chloride), the cells of the virulent strains survived for more than 15 weeks in the presence of diluted humic acid (10 μg/ml), tryptone (10 μg/ml), and cleaned river sand (100 g/100 ml of medium), but loss of viable cells occurred within 5 weeks in the absence of sand. The cells of the avirulent strains lost viability within 2 weeks with no relation to the presence of sand. Using ion-exchange columns, humic acid and the amino acids of tryptone were found to be anionic and cationic in water (pH 7.0), respectively. Sand particles had a high capacity to adsorb humic acid alone and amino acid-humic acid complexes. Thirty to fifty times the environmental concentration of amino acids (10 μg/ml) were accumulated on the surface of sand particles, thereby permitting only bacterial cells carrying net negative electrical charges (virulent cells) to survive for a long period on the surface of the sand particles. These electrostatic interrelationships among river sand, humic acid, and bacterial cells are closely implicated in the mechanism of long-term survival of virulent *A. salmonicida* in river sediments.

Aeromonas salmonicida, a nonmotile aeromonad, causes fish furunculosis, which is an infectious disease in salmonid fishes (salmon and trout) and other fish species. In recent years, a few virulence factors of this pathogen, including extracellular products (protease, hemolysin, and leukocytolysin) and an additional protein layer in the cell envelopes, have been partly characterized (4, 5, 7–10, 12, 13, 16). In particular, it has been claimed that the extracellular protease and the additional protein layer are responsible for the formation of the skin lesions characteristic of the disease and the disposition to adhere to fish tissues, respectively (17–20). Fish affected with furunculosis shed virulent bacterial cells in large numbers, but the fate of the cells liberated from the infected hosts is poorly understood, although long-term persistence in small numbers in river sediments was reported (3, 6, 11). The mechanism of the survival of *A. salmonicida* cells in river water environments, however, is largely unknown. Understanding the mechanism for survival is of importance in naturally occurring outbreaks of fish furunculosis in wild and farmed fish as well as in research into routes of infection and source of infection in the epidemiology of *A. salmonicida* infections. Also, the survival of *A. salmonicida* is considered to be significant in the ecology of aquatic bacteria insofar as understanding how these heterotrophic bacteria live in nutrient-poor environments such as river water.

In this study, river water-related environmental factors associated with the survival of *A. salmonicida* and its possible mechanism for long-lasting persistence were investigated with cells of virulent and avirulent strains. In addition, the significance of the difference in the net electrical charge of the cells in relation to their survival was studied.

MATERIALS AND METHODS

Bacteria. Two virulent *A. salmonicida* strains, A-7301 and E-7609-3, and two avirulent strains, NCMB 1102 and ATCC 14174 (which are the reference strains of *A. salmonicida* [14]), were used. Their sources and characteristics are described in Table 1.

Media and culture. Bacteria were usually subcultured on nutrient agar slants at 20°C for 3 days and maintained at 15°C. Nutrient agar plates were used for viable counts. For the shake culture, Casamino Acids-tryptone (CT) broth contained the following (grams per liter): Casamino Acids (Difco Laboratories, Detroit, Mich.), 2; tryptone (Difco), 2; NaCl, 5; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.01. CT broth was buffered with 40 mM Tris hydrochloride (pH 7.4) before autoclaving at 121°C for 20 min. Fresh cultures on agar slants (at 20°C for 3 days) were used to inoculate 200 ml of CT broth in 500-ml flasks, which were then incubated by shake culture at 20°C for 48 h. Growth in CT broth was regularly measured by turbidimetry at an optical density of 610 nm with culture samples. To determine the capacity of *A. salmonicida* strains to survive in water, small amounts of humic acid, lignin, and tannic acid (Tokyo Chemical Industry Co., Tokyo, Japan) dissolved in distilled water were used with sand. Sand (measuring 1 to 3 mm in diameter) was collected from a river bed and then cleaned well with 0.01 N sodium hydroxide, 0.01 N hydrochloric acid, and distilled water. Cleaned sand was dried and sterilized at 160°C for 2 h in a dry oven. Sand (100 g) was added to 500-ml flasks containing tryptone at 10 μg to 1 mg/ml and humic acid at 1 μg to 50 μg/ml, lignin at 10 μg/ml, or tannic acid at 10 μg/ml dissolved in 100 ml of distilled water, which was adjusted to pH 7.0 with sodium hydroxide. The flasks were then autoclaved at 121°C for 20 min. After the addition of 10^9 viable cells (= 1 ml) in sterile physiological saline (0.85% sodium chloride), the flasks were incubated at 7 to 20°C for 5 to 15 weeks with occasional shaking (approximately 2-day intervals). Viable cells were enumerated on agar plates at the appropriate time intervals with small portions of cultures

TABLE 1. Source and characteristics of *A. salmonicida* strains used

Strain	Source	Autoagglutination	LD_{50}[a] (viable cells/fish)
A-7301	Sockeye salmon	+	3.5×10^5
E-7609-3	Pink salmon	+	9.0×10^4
NCMB 1102	NCMB[b]	−	$<10^8$
ATCC 14174	ATCC[c]	−	$<10^8$

[a] Rainbow trout weighing 30 to 50 g were used, and bacterial cells were injected intraperitoneally.
[b] NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland.
[c] ATCC, American Type Culture Collection, Rockville, Md.

after the flasks were vigorously shaken at each sampling time.

Humic substance infusion. Humic substance was collected from the surface of river gravel and infused from 50 g (wet weight) with 500 ml of 0.01 N sodium hydroxide at 60°C for 1 h. A supernatant as a humic substance infusion was obtained by centrifugation at 6,000 × *g* for 10 min at 4°C. The infusion, adjusted to pH 7.0 with hydrochloric acid and then autoclaved at 121°C for 20 min, was used as a medium.

Autoagglutination. Small portions of cell suspensions (dispersed cell turbidity, 0.9 at 610 nm in physiological saline [0.85% sodium chloride]) were used to determine the capacity for autoagglutination. A 2-ml sample of each suspension was set in a spectrophotometer equipped with a recorder. After the cell suspension was dispersed with a pipette, the change in turbidity due to agglutination and subsequent precipitation to the bottom of the cuvette was monitored at an optical density of 610 nm for 10 min at room temperature.

Net electrical charges of cells. A drop of bacterial cell suspension was placed in the center of filter strips (1 by 8 cm) dipped in physiological saline (0.85% sodium chloride) buffered with 40 mM Tris hydrochloride (pH 7.0). A direct current of 5 mA (per paper strip) was applied across the strips, and the movement of the cells was determined by culture of the strips on the surface of agar plates at 20°C for 3 days.

Ion-exchange resins. Amberlite IRA 410 and IR 120B (Sigma Chemical Co., St. Louis, Mo.) were used as anion-exchange and cation-exchange resins, respectively, to determine the electrical charges of dissolved humic acid, lignin, and tannic acid. Both resins were activated with 0.01 N sodium hydroxide and 0.01 N hydrochloric acid and finally washed with distilled water. Humic acid, lignin, and tannic acid solutions (20 ml at 10 μg/ml each) were added to ion-exchange resin columns (1.6 by 8 cm) at a flow rate of 2 ml/min. UV spectra of eluates were observed. Subsequently, the anion and cation exchangers were eluted with 0.01 N sodium hydroxide and 0.01 N hydrochloric acid, respectively. The adsorption of tryptone to both ion-exchange resins was investigated in the same way.

Sand column and gel filtration. Cleaned sand (600 g) was added to 300 ml of humic acid solution at 30 mg/ml (the pH was adjusted to 7.0 with sodium hydroxide) and allowed to stand for 1 h at room temperature. Sand columns (1.6 by 30 cm) were made with sand treated with humic acid. Untreated sand was also used as a control. Each column was well washed with distilled water until humic acid became undetectable at an optical density of 280 nm. Tryptone solution (100 ml at 50 μg/ml, pH 7.0) was added to the sand columns at a flow rate of 5 ml/min and then washed with distilled water until UV absorbance at 280 nm disappeared. These columns were eluted with 100 ml of 0.01 N sodium hydroxide–0.01 N hydrochloric acid at 5 ml/min. Eluates were lyophilized and redissolved in 2 ml of 0.01 N sodium hydroxide to overlay them on Sephadex G-100 (Pharmacia, Uppsala, Sweden) columns (1.2 by 35 cm). Elution was performed with 500 ml of 0.01 N sodium hydroxide at 20 ml/h.

LD_{50} determination. Rainbow trout weighing 30 to 50 g were used to determine 50% lethal doses (LD_{50}s). These fish were acclimatized for several weeks in water tanks supplied with well water at 10°C and fed daily with commercial dry pellet food. Groups comprising three fish each received intraperitoneal injections of 0.1 ml of viable bacterial cell suspensions, which were serially 10-fold diluted with sterile physiological saline (0.85% sodium chloride). Injected fish were held for 2 weeks, and mortality rates were recorded. LD_{50}s were calculated by the method of Reed and Muench (15).

RESULTS

Autoagglutination and virulence. Two strains, A-7301 and E-7609-3, were agglutinating, whereas the other strains, NCMB 1102 and ATCC 14174, were nonagglutinating. Virulence was determined by the LD_{50} in rainbow trout. The autoagglutinating strains had LD_{50}s of ca. 10^5 viable cells per fish and were therefore virulent, whereas the nonagglutinating strains were avirulent, since their LD_{50}s were more than 10^8 cells per fish.

Survival of bacteria in water. The survival of strain A-7301 cells in water was investigated by viable counts when 10^7 viable cells per ml were added and preserved at 20°C. Viable counts drastically decreased to 10^3 cells per ml after a day in distilled water, physiological saline (0.85% sodium chloride), or 3% sodium chloride solution and within 7 days in physiological saline.

Effects of humic acid on survival of cells. Effects of humic substance-related components, including humic acid, lignin, and tannic acid, on the survival of strain A-7301 cells in the presence of tryptone (10 μg/ml) and sand (100 g/100 ml in each flask) were investigated. When ca. 10^7 viable cells per ml were inoculated, the addition of humic acid (10 μg/ml) enabled the cells to maintain a level of 10^3 to 10^4 cells per ml after 10 weeks, although the addition of either lignin (10 μg/ml) or tannic acid (10 μg/ml) caused reduced cell numbers ($<10^2$/ml) after 5 weeks. Of the three components humic acid was the most effective for survival of A-7301 cells. Subsequently, a range of 1 to 50 μg of humic acid per ml was examined to determine the optimal concentration of humic acid for survival. When 1 μg/ml was added, the viable cells disappeared (fewer than 10 cells per ml) after 5 weeks. A level of 10^3 cells per ml after 5 weeks was held by the addition of 20 and 50 μg of humic acid per ml. The optimal concentration was determined to be 5 to 10 μg/ml in the presence of tryptone and sand. This optimal concentration was used thereafter.

In the presence of humic acid, tryptone, and sand, the viability of the autoagglutinating strains A-7301 and E-7609-3 persisted at 10^3 to 10^4 cells per ml after 10 and 15 weeks at 20°C, whereas the viability of the nonagglutinating strains NCMB 1102 and ATCC 14174 was reduced to less than 10 cells per ml within 2 weeks (Fig. 1). The autoagglutinating strains, however, lost viability in a short time (within 5 weeks) in the presence of tryptone alone and humic acid alone. In the absence of sand, both groups of strains lost their viability by 2 or 5 weeks, even though the cells were cultured with a combination of humic acid and tryptone (Fig. 1). Similar results were obtained when the cells were incu-

bated at a low temperature of 7°C for 15 weeks and when humic substance infusion prepared from river gravel was used in place of humic acid.

To maintain a level of 10^3 to 10^4 cells per ml 10 weeks after incubation with 10^7 cells per ml, 300 to 500 μg of tryptone per ml was required when A-7301 cells were incubated without humic acid and sand (Fig. 2).

Difference in net electrical charges of cells. Cell suspensions of the autoagglutinating strains A-7301 and E-7609-3 produced cell flocks (or clumps, which were also confirmed by microscopic observations) and subsequent precipitation, resulting in the loss of turbidity at pH 7, whereas cell suspensions of the nonagglutinating strains NCMB 1102 and ATCC 14174 produced no change in turbidity at pH 7 (Fig. 3). The degree of loss of turbidity in the agglutinating strains was greater at pH 4 than at pH 7 and was weaker at pH 10. The changes in turbidity in the nonagglutinating strains were the opposite of those in the agglutinating strains at pHs 4 and 10 (Fig. 3). The difference in the net electrical charges of the cells was investigated since the difference in the turbidity changes was suggested to be due to the property of net electrical charges of the cells. During electrophoresis, the cells of the agglutinating strains were attracted to the cathode at pH 7.0, whereas the cells of the nonagglutinating strains moved to the anode at pH 7.0. The difference in the movement of the cells directly demonstrated the difference in the net electrical charges of the cells of these strains.

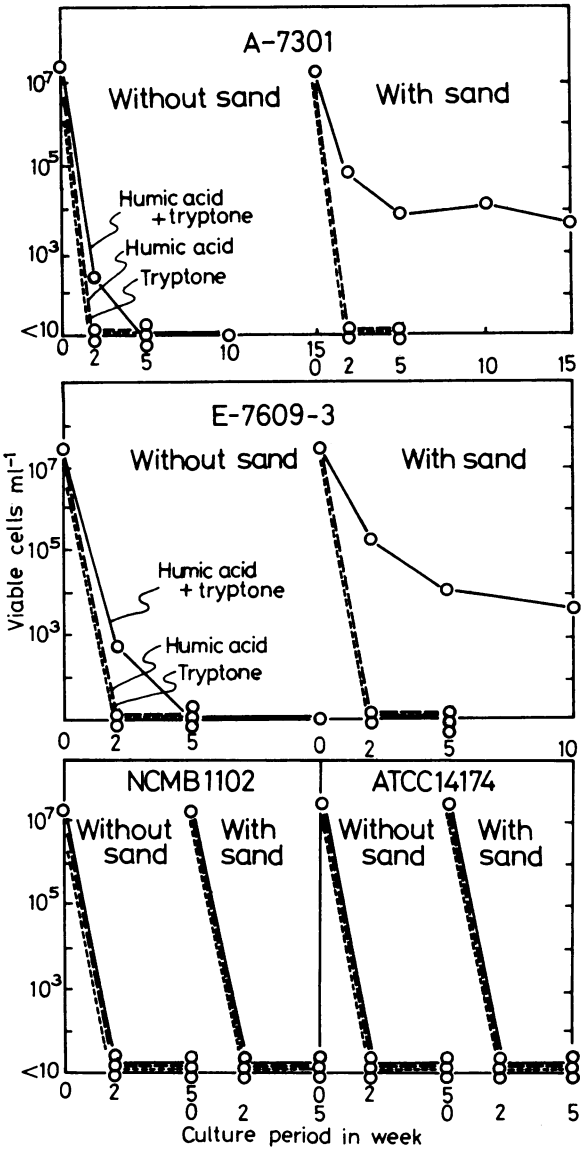

FIG. 1. Effects of sand, humic acid, and tryptone on the survival of *A. salmonicida* strains. Cells were incubated with humic acid (10 μg/ml) alone or tryptone (10 μg/ml) alone or with humic acid (10 μg/ml) plus tryptone (10 μg/ml) at 20°C for 15 weeks in the presence or absence of sand. Viable counts were determined on agar plates at the appropriate time intervals.

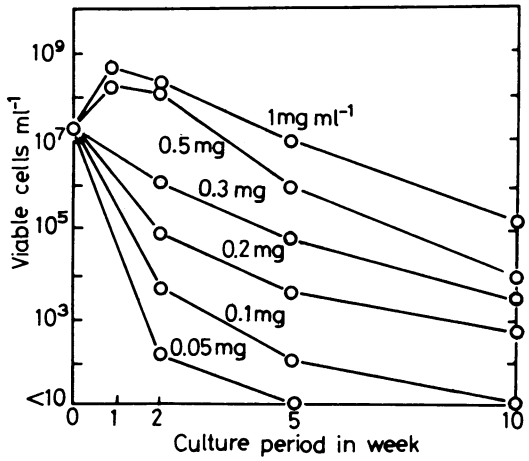

FIG. 2. Effects of tryptone on survival of *A. salmonicida* A-7301 in the absence of sand. Cells were incubated at 20°C for 10 weeks.

Adsorption of humic acid and tryptone to ion-exchange resins. The electrical charges of dissolved humic acid and tryptone at pH 7.0 were investigated by adsorption to columns of Amberlite IRA 410 (an anion-exchange resin) and Amberlite IR 120B (a cation-exchange resin). Humic acid,

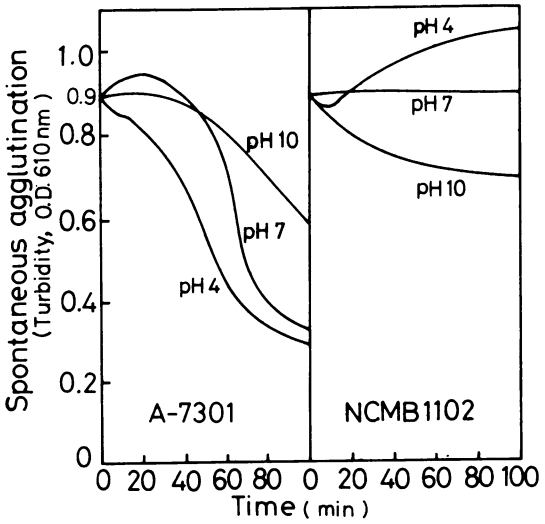

FIG. 3. Effects of pH on autoagglutination of *A. salmonicida* strains. Cell suspensions in physiological saline (0.85% NaCl) were adjusted to pHs 4, 7, and 10 with hydrochloric acid and sodium hydroxide. The changes in turbidity resulting from agglutination were recorded with a spectrophotometer. OD, optical density.

TABLE 2. Adsorption of humic acid and tryptone to sand columns

Sand column treatment	Eluate[a] (mg [dry wt]/100 g of sand)	
	Expt 1	Expt 2
Humic acid and tryptone	7.1	5.8
Humic acid	6.3	4.8
Tryptone	0.5	0.3

[a] The dry weight of eluates (100 ml of 0.01 N NaOH and 100 ml of 0.01 N HCl) was subtracted from the total dry weight of the eluates. The eluates contained humic acid, tryptone, or humic acid-tryptone conjugates (see Fig. 6).

lignin, and tannic acid dissolved in water at pH 7.0 were strongly adsorbed by the anion exchanger, whereas they completely passed through the cation exchanger. Adsorbed humic acid, lignin, and tannic acid were eluted with sodium hydroxide. On the contrary, tryptone was captured exclusively by the cation exchanger at pH 7.0 and was eluted from the column by hydrochloric acid.

Adsorption of humic acid and tryptone to sand columns. Sand columns treated with humic acid alone, tryptone alone, and humic acid-tryptone were used to measure the dry weight of adsorbed materials after elution. Eluates from sand columns treated with either humic acid or humic acid-tryptone were greater in dry weight (Table 2). The eluates from tryptone-treated columns were very small when the columns were eluted with hydrochloric acid. When the eluted materials from the sand columns treated with humic acid-tryptone were gel filtered, UV-absorbing materials were fractionated as a single large peak in the proximity of the void volume of a Sephadex G-100 column (Fig. 4). The UV spectrum of this peak was consistent with that of a solution comprising both humic acid and tryptone (Fig. 4).

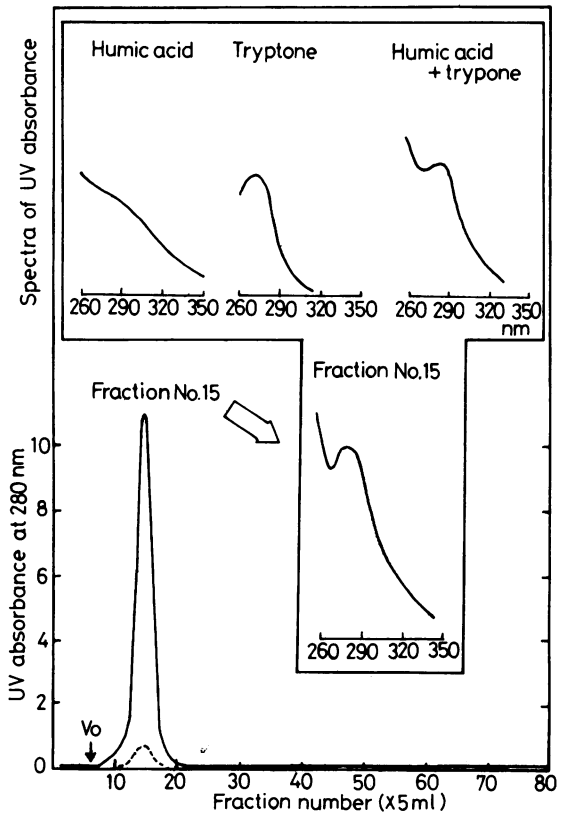

FIG. 4. Gel filtration of materials obtained from sand columns treated with humic acid and tryptone. Eluates from sand columns treated with both humic acid and tryptone (——) and nontreated columns (----) were gel filtered with Sephadex G-100 columns with 0.01 N sodium hydroxide.

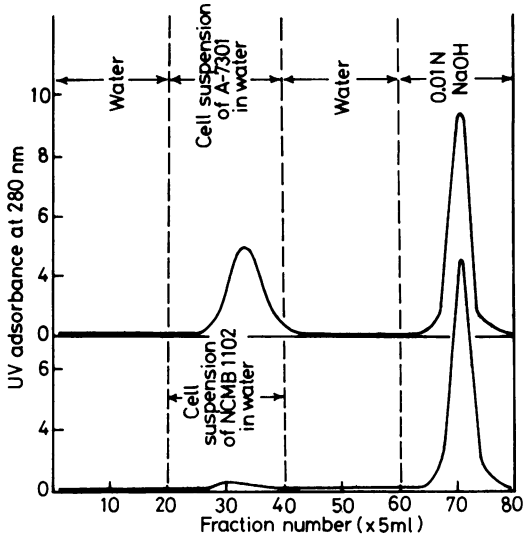

FIG. 5. Elution of humic acid from sand columns by dense cell suspensions of strains A-7301 and NCMB 1102. Cell suspensions were added to sand columns. Eluates were centrifuged at 10,000 × *g* for 10 min at 4°C, and humic acid contents of the supernatants were followed by UV absorption at 280 nm.

Elution of humic acid from sand columns by cell suspension. The capacity of the cell suspensions to elute humic acid from sand columns pretreated with humic acid was investigated. Humic acid adsorbed to sand columns was partly eluted by the dense cell suspension (ca. 10^9 viable cells per ml) of strain A-7301 in water (pH 7.0) but not by the cell suspension of strain NCMB 1102 (Fig. 5).

Characteristics of surviving cells in prolonged cultures. Characteristics of isolates from cultures incubated at 20°C with humic acid and tryptone in the presence or absence of sand were compared. Isolates after 5 to 15 weeks from the cultures of strains A-7301 and E-7609-3 in the presence of sand were autoagglutinating and pathogenic (or attenuated) to rainbow trout in LD_{50} determinations (Table 3). The cells

TABLE 3. Comparison of cell characeristics during survival experiments

Strain and culture condition for colony isolation[a]	Autoagglutination[b]	LD_{50}[c] (viable cells/fish)
A-7301-SHT5w	+	1.2×10^6
A-7301-SHT15w	+	8.6×10^6
A-7301-HT2w	−	$>10^8$
E-7609-3-SHT10w	+	4.7×10^6
E-7609-3-HT2w	−	$>10^8$

[a] Cell suspensions were prepared from 50 colonies on agar plates isolated from the cultures. Abbreviations indicate the following: e.g., A-7301 (strain number used), S (with sand), H (with humic acid at 10 μg/ml), T (tryptone at 10 μg/ml), 5w (5-week incubation at 20°C).

[b] Autoagglutination was determined by the method described in the legend to Fig. 3 (in physiological saline at pH 7.0).

[c] Rainbow trout received viable cell suspensions intraperitoneally.

incubated for 2 weeks in the absence of sand were nonagglutinating and nonpathogenic (Table 3). Quantitative changes in the characteristics of surviving cells were investigated with strain A-7301. Although 20 isolates from the culture before incubation were autoagglutinating and net negatively charged, the number of nonagglutinating, net positively charged cells increased as the incubation progressed from 5 to 15 weeks in the presence of humic acid, tryptone, and sand (Table 4). However, pathogenic (autoagglutinating and net negatively charged) cells were maintained in small numbers even after 15 weeks. In the absence of sand, nonagglutinating and net positively charged cells were exclusively detected after 2 weeks (Table 4), and consequently, their viability disappeared after 5 weeks.

TABLE 4. Changes in properties of cells of strain A-7301 during survival experiments

Culture condition for colony isolation[a]	No. of colonies isolated[b]			
	Agglutination[c]		Net electrical charges of cells[d]	
	Autoagglutinating	Nonagglutinating	Negative	Positive
A-7301 (before culture)	20	0	20	0
A-7301-HT2w	0	20	0	20
A-7301-SHT5w	8	12	9	11
A-7301-SHT15w	4	16	4	16

[a] See Table 3, footnote *a*.
[b] Twenty colonies isolated from each culture in the presence or absence of sand were subcultured on agar slants at 20°C for 3 days and used to determine agglutination and net electrical charges.
[c] See Fig. 3 (determined at pH 7.0).
[d] Net electrical charges of cells were determined by electrophoresis at pH 7.0. Negative, Cells were attracted to the cathode; positive, cells were attracted to the anode.

DISCUSSION

In this study, the autoagglutinating strains were pathogenic to fish (rainbow trout), whereas the nonagglutinating strains were nonpathogenic (Table 1). These results are consistent with the past literature (8, 9, 12). Also, there was a large difference between the bacterial strains with respect to net electrical charges; the living cells of the agglutinating strains possessed net negative charges, and the cells of the nonagglutinating strains showed net positive charges. The difference in the net electrical charges was clearly demonstrated by electrophoresis, since *A. salmonicida* is nonmotile (14). This difference was implicated by the finding that agglutination of the pathogenic strains was more rapid at acidic pH and weaker at alkaline pH than at neutral pH and that agglutination of the nonpathogenic strains was the converse (Fig. 3). These findings are interpreted as showing that at a neutral pH the difference in the agglutinating property is due to the difference in the net electrical charges of *A. salmonicida* cells.

The cells of the agglutinating strains remained viable for longer than 10 or 15 weeks at 7 and 20°C in water and sand, a trace of humic acid, and tryptone (the concentrations used are considered to be similar to those found in rivers). However, the viability was completely lost within 5 weeks in the absence of sand. The cells of the nonagglutinating strains lost viability within 2 weeks even though the cells were incubated with sand, humic acid, and tryptone (Fig. 1). The same results were obtained when humic substance infusion prepared from river gravel was used in place of humic acid. The possibility that humic acid as a nutrient supports the survival of agglutinating cells can be excluded because the presence of humic acid alone or humic acid plus tryptone had no effect on survival in the absence of sand (Fig. 1). It is probable that the long-lasting survival of the cells of the autoagglutinating strains is closely associated with the coexistence of humic acid, tryptone, and sand, thereby suggesting complicated interrelationships among these three components in the survival of autoagglutinating (pathogenic) *A. salmonicida*.

River sand had the capacity to adsorb humic acid and humic acid-tryptone on its surface (Table 2). Humic acid and tryptone dissolved in water (pH 7.0) were anionic and cationic, respectively. This was shown by their adsorption to anionic- and cationic-exchange resins. Humic acid and amino acids (of tryptone) will therefore produce humic acid-amino acid conjugates by ionic bonding when they are mixed. This interpretation was supported by the result that the material eluted from a sand column treated with both humic acid and tryptone, upon subsequent gel filtration, gave a single peak showing a UV absorption spectrum identical to that of a solution composed of humic acid and tryptone (Fig. 4). The surface of cleaned sand particles appeared to carry net positive electrical charges, as suggested by the adsorption of humic acid and the elution of adsorbed humic acid from the particles by sodium hydroxide (Table 2). Thus, an amino acid layer on the surface of sand particles would be formed with the aid of humic acid (which is thought to be polyanionic) (Fig. 6). Such adsorbed amino acids appear to be readily utilized by autoagglutinating bacterial strains, as indicated by the greater viability of these strains in the presence (Fig. 1) than in the absence (Fig. 2) of sand. When bacterial cells approach the sand particles of an amino acid-rich humic acid layer, cells carrying net negative charges appear to be adsorbed to the surface of the particles by ion exchange with humic acid. This was indicated by the fact that cells carrying net negative electrical charges could elute humic acid from sand columns while net positively charged cells could not; the cells (negatively charged cells) of strain A-7301 showed a high activity to elute humic acid from sand columns, but the cells (positively charged cells) of strain NCMB 1102 did not (Fig. 5). The amino acid-rich humic acid layer would provide a continuously replenished source of nutrient amino acids, allowing the adsorbed cells to survive on the surface of the sand particles for long periods (Fig. 6). This is supported by the fact that *A. salmonicida* needs amino acids and requires no vitamin or growth factor for growth and persistence (18). On the other hand, nonagglutinating cells will be expelled from the surface of sand particles by virtue of their net positive electrical charges, depriving them of the adsorbed nutrients (Fig. 6).

The adsorption of virulent *A. salmonicida* cells to sand particles in association with humic acid and amino acids could wholly account for the long-term survival of the cells in river sediments. It is suggested that, even though cell division was not shown in this study, the virulent cells are in a normal living state on the surface of river sand (or sediments). Allen-Austin et al. (3) reported that *A. salmonicida* survives in river water by entering a dormant phase, since viable counts could be recovered by the addition of nutrients (tryptone soy broth) after plate counts had reached zero. Based on the present study, it is more likely that *A. salmonicida* (which is virulent) persists as normally living cells in river sediments but that the cells are considerably starved. Despite no recovery from surface water, algae, and zooplankton (1, 2), a few laboratory-based experiments have demonstrated that the persistence of *A.*

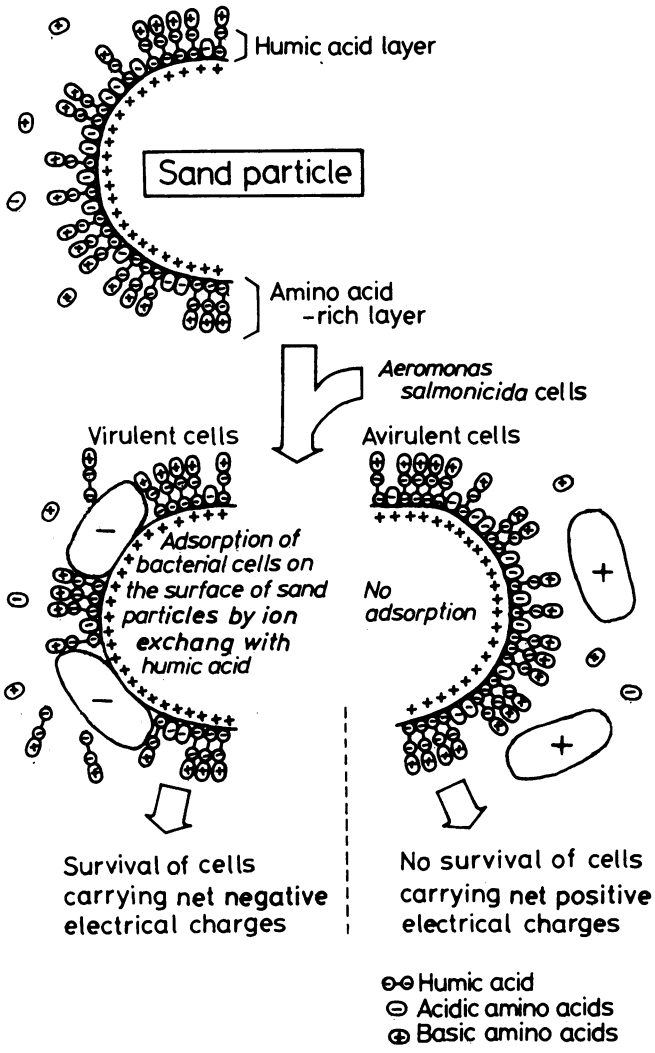

FIG. 6. A postulated mechanism for the long-term survival of virulent *A. salmonicida* in river sediments. Virulent cells which carry net negative charges and avirulent cells which carry net positive charges are separately depicted. The explanation is detailed in the text.

salmonicida and its virulence in river water depend on the presence of river sediments (6, 11). This is sufficiently explained by the foregoing electrostatic interrelationships among river sand, humic acid, amino acids, and bacterial cells. Michel and Dubois-Darnaudpeys (11) reported a decrease in the pathogenicity of *A. salmonicida* after prolonged incubation (9 months) with river sediments. A similar reduction of pathogenicity in strain A-7301 was observed in the present study (Table 3). This phenomenon was interpreted by the decrease of negatively charged (virulent) cells in long-term survival studies with the pathogenic strain even in the presence of sand (Table 4). This is considered to be due to the spontaneous occurrence of a free-living form (which has positively charged, avirulent cells) which is detached and derived from virulent cells attached to the surface of sand particles. This stage with the influence of sand particles implies agreement with the dormant stage reported for *A. salmonicida* survival by Allen-Austin et al. (3), since the viability of the free-living cells is decreasingly diminished by the lack of a nutrient supply, and since, despite no viable counts, the cells could be enumerated by direct counts (3). These cells would represent a transitional form and will ultimately be killed. However, a small number of cells attached to the surface of sand particles may still persist as live cells and be regarded as pathogenic cells.

The mechanism of the survival of virulent *A. salmonicida* in river sediments was experimentally characterized. A similar mechanism might be involved in the ecology of other aquatic microorganisms, including nonpolluted river water-, lake water-, and seawater-related heterotrophic bacteria, in association with suspended particles, detritus, and sediments.

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LITERATURE CITED

1. **Allen, D. A., B. Austin, and R. R. Colwell.** 1983. Numerical taxonomy of bacterial isolates associated with a freshwater fishery. J. Gen. Microbiol. **129**:2043–2062.
2. **Allen-Austin, D., B. Austin, and R. R. Colwell.** 1984. *Aeromonas media*, a new species isolated from river water. Int. J. Syst. Bacteriol. **33**:599–604.
3. **Allen-Austin, D., B. Austin, and R. R. Colwell.** 1984. Survival of *Aeromonas salmonicida* in river water. FEMS Microbiol. Lett. **21**:143–146.
4. **Brooks, D. E., and T. J. Trust.** 1983. Enhancement of bacterial adhesion by shear forces; characterization of the haemagglutination induced by *Aeromonas salmonicida* strain 438. J. Gen. Microbiol. **129**:3661–3669.
5. **Cipriano, R. C., B. B. Griffin, and B. L. Lidgerding.** 1981. *Aeromonas salmonicida*: relationships between extracellular growth products and isolate virulence. Can. J. Fish. Aquat. Sci. **38**:1322–1326.
6. **Dubois-Darnaudpeys, A.** 1977. Épidémiologie de furunculose des salmonidés. I. Etude expérimentale des conditions de survivie de *Aeromonas salmonicida* dans un environment abiotique. Bull. Fr. Piscic. **49**:121–127.
7. **Ellis, A. E., T. S. Hastings, and A. L. S. Munro.** 1980. The role of *Aeromonas salmonicida* extracellular products in the pathology of furunculosis. J. Fish Dis. **4**:41–51.
8. **Evenberg, D., R. Van Boxtel, B. Lugtenberg, F. Schurer, J. Blommaert, and R. Bootsma.** 1982. Cell surface of the fish pathogenic bacterium *Aeromonas salmonicida*. I. Relationship between auto-agglutination and presence of a major cell envelope protein. Biochim. Biophys. Acta **684**:241–248.
9. **Fuller, D. W., K. S. Pilcher, and J. L. Fryer.** 1977. A leukocytolytic factor isolated from cultures of *Aeromonas salmonicida*. J. Fish. Res. Board Can. **34**:1118–1125.
10. **Ishiguro, E. E., W. W. Kay, T. Ainsworth, J. B. Chamberlain, R. A. Buckley, and T. J. Trust.** 1981. Loss of virulence during culture of *Aeromonas salmonicida* at high temperature. J. Bacteriol. **148**:333–340.
11. **Michel, C., and A. Dubois-Darnaudpeys.** 1980. Persistence of the virulence of *Aeromonas salmonicida* strains kept in river sediments. Ann. Rech. Vet. **11**:375–380.
12. **Munn, C. B., E. E. Ishiguro, W. W. Kay, and T. J. Trust.** 1982. Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. Infect. Immun. **36**:1069–1075.
13. **Nomura, S., and H. Saito.** 1982. Production of the extracellular hemolytic toxin by an isolated strain of *Aeromonas salmonicida*. Bull. Jpn. Soc. Sci. Fish. **48**:1589–1597.
14. **Popoff, M.** 1984. Genus III. *Aeromonas*, p. 546–548. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
15. **Reed, L., and H. Muench.** 1938. A simple method of estimating fifty percent end points. Am. J. Hyg. **27**:493–497.

16. **Sakai, D. K.** 1984. The non-specific activation of rainbow trout, *Salmo gairdneri* Richardson, complement by *Aeromonas salmonicida* extracellular products and the correlation of complement activity with the inactivation of lethal toxicity products. J. Fish Dis. **7**:329–338.
17. **Sakai, D. K.** 1985. Loss of virulence in a protease-deficient mutant of *Aeromonas salmonicida*. Infect. Immun. **48**:146–152.
18. **Sakai, D. K.** 1985. Significance of extracellular protease for growth of a heterotrophic bacterium, *Aeromonas salmonicida*. Appl. Environ. Microbiol. **50**:1031–1037.
19. **Shieh, H. S.** 1982. Lethal toxicity of *Aeromonas salmonicida* protease to Atlantic salmon. Microbios Lett. **20**:137–139.
20. **Udey, L. R., and J. L. Fryer.** 1978. Immunization of fish bacterins of *Aeromonas salmonicida*. Mar. Fish. Rev. **40**:12–17.