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Adhesion of *Aeromonas salmonicida* Strains Associated with Net Electrostatic Charges of Host Tissue Cells

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The adhesion of *Aeromonas salmonicida*, the pathogenic bacterium of fish furunculosis in salmon and trout, to the surface of host tissue cells was investigated with two fish tissue culture cell lines (RTG-2 cells from rainbow trout, *Salmo gairdneri*, and CHSE-214 cells from chinook salmon, *Oncorhynchus tshawytscha*) and four *A. salmonicida* strains. Bacterial cells of pathogenic strains were highly adhesive to RTG-2 and CHSE-214 cells and were negatively charged in the net electrostatic charges, as determined by electrophoresis on filter paper strips at pH 7, whereas bacterial cells of nonpathogenic strains were nonadhesive and positively charged. The elctrophoresis of RTG-2 and CHSE-214 cells with balanced salt solution (BSS), phosphate-buffered saline, or fish serum diluted with BSS (pH 7) was carried out with an appropriate electrophoretic apparatus that was devised for this study. After electrophoresis with 20 mA of direct current for 15 min at pH 7, the elctrophoretic dispositions of these tissue culture cells were determined by the mode of frequency of occurrence of these cells in the partitioned chambers of the device. RTG-2 and CHSE-214 cells with BSS and fish serum were attracted from the central chamber (to which each cell sample was added) to the cathode chambers, but no attraction was detected when these cells were used with phosphate-buffered saline. Noradrenaline- and phosphoenolpyruvate-pretreated RTG-2 cells migrated more to the cathode chambers, whereas succinate- and valine-pretreated RTG-2 cells moved to the anode chambers. These movements to the cathode and anode were alleviated by the use of RTG-2 cells preincubated with pathogenic and nonpathogenic bacterial cells, respectively. The adhesion of the pathogenic bacteria to RTG-2 cells was enhanced by the use of RTG-2 cells pretreated with noradrenaline and phosphoenolpyruvate, whereas the nonpathogenic bacteria were adherent to RTG-2 cells pretreated with succinate and valine. These findings indicate that the adhesion of *A. salmonicida* strains to host tissue cells is closely associated with mutually converse net electrostatic charges.

The significance of adhesion to host tissues by pathogenic and nonpathogenic bacteria is well recognized (22). *Aeromonas salmonicida* adhesion is considered to be associated with its pathogenicity (18, 23), and the mechanism of this adhesion is still largely unclear.

The extracellular growth products of *A. salmonicida* have been shown to cause skin lesions (furuncles), lethality, and other signs of fish furunculosis that are characteristic of naturally occurring furunculosis in salmonid fish (1, 2, 10, 11). A few extracellular factors, including protease, hemolysin, and leukocytolysin, that are responsible for specific clinical signs of furunculosis have been reported (5, 13, 27, 28). In particular, the significant role of the extracellular protease in the capacity to produce furuncles, proliferation in hosts, and lethality was characterized by laboratory-based experiments (16, 19, 24, 25). Evidence that the protease is an effective protective antigen in furunculosis vaccination has also been found (17, 26). The protease is considered to be a major extracellular virulence factor because pathogenicity was lost by a protease-deficient mutant (which was positive for hemolysin and leukocytolysin production) that was induced from a highly virulent *A. salmonicida* strain by mutagenesis, although the mutant still preserved the properties of adhesion and infectivity (survival within the host). Avirulent strains were killed by the host in a short period of time (18). These results imply that certain cellular or intracellular factors, as well as the extracellular protease, are closely involved in the pathogenesis of furunculosis (7, 12).

It has been reported that cells of virulent *A. salmonicida* strains adhere to host tissue cells, while cells of avirulent strains do not (3, 7, 18, 29). Because virulent autoagglutinating strains possess an additional protein layer (A layer) that is external to the outer membrane of the cell envelope and avirulent strains lack it, the A layer has been considered to be responsible for adhesion of *A. salmonicida* to host tissue culture cells (3, 4, 7, 9, 29). The mechanism of adhesion, however, remains controversial and largely unsolved.

This study was performed in an attempt to investigate a possible mechanism for adhesion to the surface of host tissue cells with virulent and avirulent *A. salmonicida* strains and fish tissue culture cells.

MATERIALS AND METHODS

Bacteria. *A. salmonicida* strains were isolated from naturally occurring fish furunculosis in a variety of salmonid fishes: strain A-7301 was from sockeye salmon (*Oncorhynchus nerka*), E-7609-3 was from pink salmon (*Oncorhynchus gorbuscha*), and GH-7501 was from amago salmon (*Oncorhynchus rhodurus*). Strain NCMB 1102 (National Collection of Marine Bacteria, Aberdeen, Scotland) was also used as a reference strain (14).

Animals. Healthy rainbow trout (*Salmo gairdneri*; weight 30 to 50g) were used. These fish were acclimatized for several weeks in laboratory water tanks supplied with well water at 10°C and fed commercial dry pellets daily. These fish were used to determine the pathogenicity of bacteria to fish.

Media and cultures. Bacterial strains were usually subcultured on nutrient agar slants at 20°C for 3 days and main-

tained at 15°C. Nutrient agar plates were used for viable counts. For shake culture, Casamino Acids-tryptone (both from Difco Laboratories, Detroit, Mich.) broth was prepared and contained the following, in grams per liter: Casamino Acids, 2; tryptone, 2; NaCl, 5; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.01. Casamino Acids-tryptone (CT) broth was buffered at pH 7.4 with 40 mM Tris hydrochloric acid before it was autoclaved at 121°C for 20 min. Samples of cultures on agar slants were used to inoculate 250 ml of CT broth in 500-ml flasks, which were then incubated by shake culture at 20°C for 45 h.

Congo red test. To investigate the presence of the A layer in the cell envelopes of *A. salmonicida* strains, the capacity of bacterial colonies to bind Congo red was determined by the method described by Ishiguro et al. (6).

LD_{50} determination. Rainbow trout were used to determine the 50% lethal doses (LD_{50}s). Groups comprising five fish each received intraperitoneally 0.1 ml of viable bacterial cell suspensions which were serially diluted 10-fold with sterile physiological saline (0.85% sodium chloride). Injected fish were maintained for 2 weeks at 10°C in water tanks (50 liter), and mortalities were recorded. LD_{50}s were determined by the estimating method described by Reed and Muench (15).

Response to normal serum. The bactericidal action of normal serum prepared from pooled blood of rainbow trout was determined. The method has been described in a previous report (18).

Fish tissue culture cells. Two fish tissue culture cell lines, RTG-2 cells established from rainbow trout and CHSE-214 cells established from chinook salmon, were used as host tissue culture cells. The culture method of these cells has been previously described (18).

Adhesion of bacteria to tissue culture cells. The capacity of cells of *A. salmonicida* strains to adhere to the surface of monolayer cultures of RTG-2 and CHSE-214 cells was determined by a previously reported method (18). The number of adherent bacterial cells per flask (25 cm^2) was calculated. Monolayer cultures of RTG-2 cells in flasks were pretreated as follows, before use for the determination of the capacity of *A. salmonicida* strains for adhesion. Cultures were pretreated with Earle balanced salt solution (EBSS; GIBCO Laboratories, Grand Island, N.Y.) supplemented with mitomycin C (Boehringer GmbH, Mannheim, Federal Republic of Germany) at 25 μg/ml for 2 h at 15°C; EBSS supplemented with 25 μM noradrenaline (Sigma Chemical Co., St. Louis, Mo.) for 30 min at 15°C; Dulbecco phosphate-buffered saline (PBS) supplemented with 5 mM phosphoenolpyruvate (Sigma) for 30 min at 15°C; PBS supplemented with 5 mM sodium succinate for 30 min at 15°C; and PBS supplemented with 5 mM valine for 30 min at 15°C.

Net electrical charges of bacterial cells. Net electrical charges of live cells of the *A. salmonicida* strains used in this study were investigated by electrophoresis on filter paper strips (1 by 8 cm), which were then cultured on agar plates after electrophoresis. The method of electrophoresis and the determination of net electrical charges have been described previously (20).

Electrophoresis of tissue culture cells. Monolayer cultures of RTG-2 cells and CHSE-214 were dispersed with the dispersant EDTA-trypsin and collected. Cells were washed once with EBSS (pH 7.0) or PBS by centrifugation at 1,000 × *g* for 5 min and suspended in EBSS or PBS at 10^7 cells per ml. Washed RTG-2 and CHSE-214 cell suspensions (1 ml each) were added to the center of a hand-made electrophoretic device that was filled with 100 ml of EBSS or PBS (Fig. 1). Electrophoresis was performed in a thermo-bath at 15°C. An applied voltage was charged with direct current (DC) of 20 mA for 15 min. After electrophoresis the device was immediately partitioned by plastic plates fitted with magnetic ferrite-rubber tape at the edges to prevent diffusion of cells. The plates were held by strips of magnetic tape fitted with the wells of the electrophoretic tank (Fig. 1). This magnetic tape had no influence on electrophoresis. To avoid eddy currents, the partitioning plates were put into the apparatus as gently as possible. Each partitioned chamber (from the chambers of the anode side to cathode side, designated A4, A3, A2, A1, [where A is anode chamber], S[chamber for adding each cell sample], C1, C2, C3, C4 [where C is cathode chamber] was gently mixed by pipetting. The number of cells per milliliter enumerated with a hemacytometer (Fuchs-Rosenthal) with small portions of samples obtained from each chamber. The movement of cells was determined by calculating the relative frequency of occurence of the cells in these chambers. The modulation of electrical movement of RTG-2 and CHSE-214 cells was determined by using EBSS supplemented with 20 mM magnesium chloride and calcium chloride. In place of EBSS or PBS, 10% fetal bovine serum and rainbow trout serum diluted with EBSS were also used for electrophoresis.

Suspensions of RTG-2 and CHSE-214 cells (10^7 per ml) were incubated at a 1:1 volume at 20°C for 1 h with live *A.*

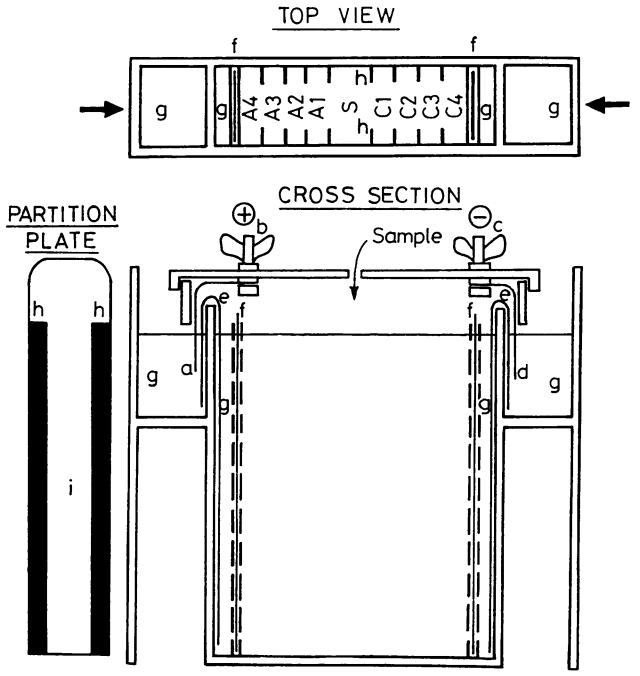

FIG. 1. Illustration of a hand-made device used for electrophoresis of tissue culture cells. Abbreviations: a, Anode (platinum); b, connection for the anode terminal; c, connection for the cathode terminal; d, cathode (platinum); e, filter paper strip of salt bridge; f, filter paper strip to prevent diffusion of cells; g, buffered saline; h, magnetic ferrite-rubber strip to attach plates that partition the electrophoretic tank (50 [width] by 20 [depth] by 80 [height] mm) of the apparatus into nine small chambers (A4, A3, A2, A1, S, C1, C2, C3, C4) after electrophoresis; i, plastic plates used for partitioning the device after electrophoresis. During the experiments the apparatus was cooled in a thermo-bath at 15°C. Arrows represent the position of the cross-section.

TABLE 1. Characteristics of the *A. salmonicida* strains used in this study

Strain	Auto-agglutination[a]	Response to normal serum[b]	Congo red test[c]	Net electrical charges of cells[d]	Pathogenicity to rainbow trout[e]
A-7301	+	R	+	−	+
E-7609-3	+	R	+	−	+
NCMB 1102	−	S	−	+	−
GH-7501	−	S	−	+	−

[a] Spontaneous agglutination in cell suspension.

[b] Fresh normal serum of rainbow trout was reacted with 10^8 viable bacterial cell suspensions at a 1:1 volume for 2 h at 20°C. R, Resistant (less than 80% reduction in viable counts due to serum); S, sensitive (more than 3 log units of reduction in viable counts due to serum).

[c] The capacity of bacteria to bind Congo red was determined by the method of Ishiguro et al. (7).

[d] Net electrical charges of bacterial cells were determined with an appropriate device for electrophoresis (20) at 7.0.

[e] Pathogenicity was determined by calculating the LD_{50}. +, Pathogenic (LD_{50} was 10^4 to 10^6 viable cells per fish); −, nonpathogenic (LD_{50} was more than 10^8 viable cells per fish).

salmonicida cells suspended in EBSS or PBS at 10^8 per ml, and electrophoresis was then conducted as described above to detect the electrophoretic modulation of these tissue culture cells by bacteria. RTG-2 cells pretreated with mitomycin C, noradrenaline, phosphoenolpyruvate, succinate, and valine also were used.

RESULTS

Characteristics of bacterial strains. The *A. salmonicida* strains used in this study were categorized as pathogenic or nonpathogenic by LD_{50} determination in rainbow trout, which is one of the host salmonid fishes in *A. salmonicida* infections. Cells of pathogenic strains A-7301 and E-7609-3 were highly adhesive to the surface of monolayer RTG-2 cell cultures, whereas cells of nonpathogenic strains NCMB 1102 and GH-7501 were nonadhesive (Fig. 2). With respect to net electrical charges of bacterial cells determined by electrophoresis, cells of the adhesive (pathogenic) strains were negatively charged at pH 7, whereas cells of the nonadhesive (nonpathogenic) strains were positively charged (Table 1). Based on Congo red tests, the pathogenic strains were A-layer positive, whereas the nonpathogenic strains were A-layer negative (Table 1).

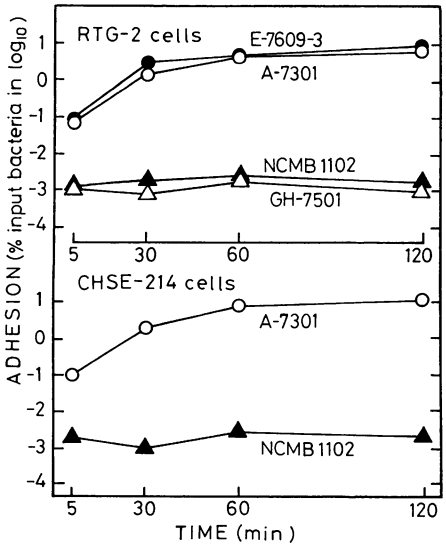

FIG. 2. Kinetics of adhesion of *A. salmonicida* cells to the surface of host tissue culture cells. Bacteria (approximately 10^8 viable cells) were inoculated into monolayers of the tissue cultures (RTG-2 cells and CHSE-214 cells) in 25-cm^2 flasks and then incubated at 20°C.

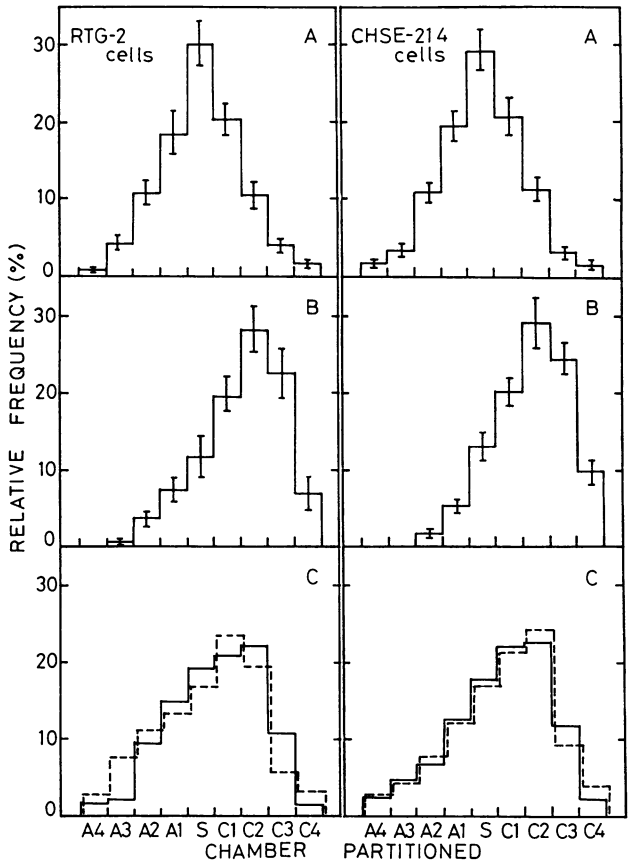

FIG. 3. Electrophoresis of live RTG-2 cells and CHSE-214 cells. A total of 10^7 RTG-2 or CHSE-214 cells (1 ml) were added to the center of an electrophoretic device (chamber S, see Fig. 1). (A) Cells were allowed to stand (in the absence of an applied voltage) with EBSS at pH 7.0 for 15 min (ranges represent the standard deviation of the relative frequency of occurrence of the cells determined in triplicate experiments). (B) Electrophoresis was performed with EBSS at pH 7.0 (an applied voltage with 20mA of DC for 15 min). (C) electrophoresis was performed with EBSS supplemented with fetal bovine serum (—) and rainbow trout serum (---) at 10% at pH 7.0.

Kinetics of adhesion. The kinetics of adhesion of *A. salmonicida* cells to the surface of RTG-2 and CHSE-214 cells was investigated in the absence of serum. Pathogenic strains A-7301 and E-7609-3 showed a higher capacity to adhere to the tissue culture cells than did nonpathogenic strains NCMB 1102 and GH-7501. The onset of adhesion appeared earlier when cells of the pathogenic strains were exposed to the monolayer cultures (viz. 5 min after exposure) (Fig. 2). The pathogenic strains attached in increasing numbers with exposure time and reached a plateau at 10^7 viable cells of monolayer culture per flask (25 cm^2) after 60 to 120 min, when 10^8 viable cells per flask were added (Fig. 2). The maximal levels in adhering cell populations were equiv-

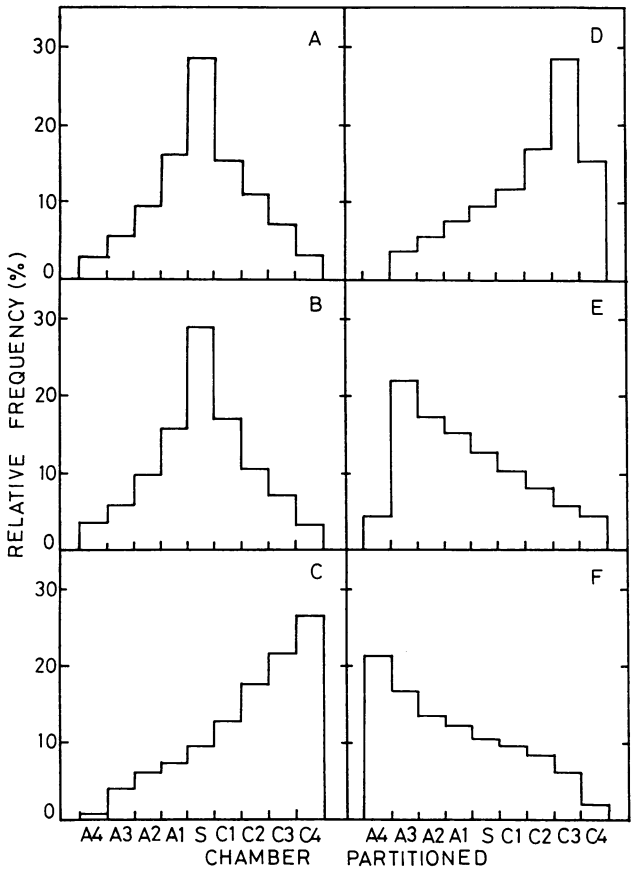

FIG. 4. Electrophoresis of pretreated RTG-2 cells. Electrophoresis was performed at pH 7.0 (an applied voltage with 20 mA of DC for 15 min). (A) Nontreated cells were used for electrophoresis with PBS; (B) mitomycin C-pretreated cells were used for electrophoresis with EBSS; (C) noradrenaline-pretreated cells were used for electrophoresis with EBSS; (D) phosphoenolpyruvate-pretreated cells were used for electrophoresis with PBS; (E) succinate-pretreated cells were used for electrophoresis with PBS; (F) valine-pretreated cells were used for electrophoresis with PBS.

alent to 4 to 11% of the initially exposed cells. The nonpathogenic strains maintained almost constant and lower levels of 10^3 to 10^4 cells per flask, equal to 0.001 to 0.01% of initial cell numbers (Fig. 2). The adhesion rates in the pathogenic strains were estimated to be 10^3 to 10^4 times as high as those in the nonpathogenic strains.

Electrophoresis of tissue culture cells. RTG-2 cells and CHSE-214 cells were normally distributed in the electrophoresis chambers when the device (Fig. 1) was allowed to stand (no applied voltage) with EBSS for 15 min at pH 7.0 (Fig. 3A). Distortion of distribution, however, was found when an applied voltage was charged with 20 mA of DC for 15 min (Fig. 3B). The peak of tissue culture cell distribution was shifted to the cathode and appeared in chamber C2. A similar cathodal migration under the influence of an applied voltage was seen for cells that were recovered from flasks, either EDTA or trypsin alone for cell dispersion. The results of electrophoresis were highly reproducible, as shown by each triplicate examination (Fig. 3A and B). The migration to the cathode was scarcely modulated by the addition of Mg^2 and Ca^2 to EBSS or by 10% fetal bovine serum or rainbow trout serum diluted with EBSS (Fig. 3C).

Alternate migration of tissue culture cells in electrophoresis. Although RTG-2 cells with EBSS showed cathodal migration (Fig. 3B), a normal distribution appeared when PBS was used for electrophoresis (Fig. 4A). Mitomycin C-pretreated RTG-2 cells resulted in a normal distribution, even though EBSS was used (Fig. 4B). Noradrenaline-pretreated RTG-2 cells with EBSS showed greater cathodal migration than nontreated RTG-2 cells, and the peak of cell distribution appeared in chamber C4 (Fig. 4C). When PBS was used for electrophoresis to prevent the influence of EBSS on cathodal migration (containing glucose), the peak of distribution of phosphoenolpyruvate-pretreated RTG-2 cells appeared in chamber C3 (Fig. 4D). In contrast, succinate-pretreated RTG-2 cells and valine-pretreated RTG-2 cells with PBS migrated to the anode, and the peaks of both cells appeared in chamber A3 and A4, respectively (Fig. 4E and F).

Electrophoretic modulation by bacterial cells. When RTG-2 cells were preincubated with bacterial cells, the peak of

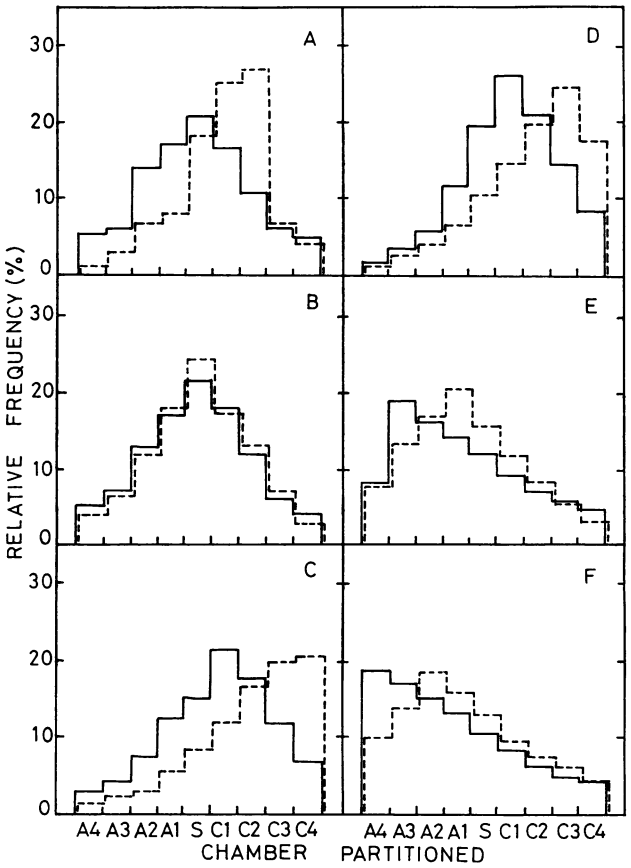

FIG. 5. Electrophoresis of live RTG-2 cells treated with *A. salmonicida* A-7301 and NCMB 1102. (A) RTG-2 cells preincubated with A-7301 (—) and NCMB 1102 (-----) cells in flasks were used for electrophoresis with EBSS; (B) mitomycin C-pretreated RTG-2 cells were preincubated with A-7301 and NCMB 1102 cells and then used for electrophoresis with EBSS; (C) noradrenaline-pretreated RTG-2 cells were preincubated with A-7301 and NCMB 1102 cells and then used for electrophoresis with EBSS; (D) phosphoenolpyruvate-pretreated RTG-2 cells were preincubated with A-7301 and NCMB 1102 and then used for electrophoresis with PBS; (E) succinate-pretreated RTG-2 cells were preincubated with A-7301 and NCMB 1102 cells and then used for electrophoresis with PBS; (F) valine-pretreated RTG-2 cells were preincubated with A-7301 and NCMB 1102 cells and then used for electrophoresis with PBS.

tissue culture cell distribution during electrophoresis with EBSS was shifted by pathogenic strain A-7301 from chamber C2 (Fig. 3B) to chamber S (Fig. 5A), and no shift was shown by nonpathogenic strain NCMB 1102 (Fig. 5A). Such a different electrophoretic modulation by preincubation with these bacterial cells was not shown when mitomycin C-pretreated RTG-2 cells were used in place of non-treated RTG-2 cells (Fig. 5B). During electrophoresis of noradrenaline-pretreated RTG-2 cells, the peak was shifted by preincubation with A-7301 cells from chamber C4 (Fig. 4C) to chamber C1 (Fig. 5C), but not by preincubation with NCMB 1102 (Fig. 5C). Similar electrophoretic modulation by bacterial cells was obtained during electrophoresis of phosphoenolpyruvate-pretreated RTG-2 cells (Fig. 5D). In contrast, the peaks of succinate-and valine-pretreated RTG-2 cells were shifted by NCMB 1102 cells from chambers A3 and A4 (Fig. 4E and F) to chambers A1 and A2 (Fig. 5E and F), but not by A-7301 cells (Fig. 4E and F and 5E and F).

Enhancement of adhesion. Bacterial strains A-7301 and NCMB 1102 did not adhere to monolayers of mitomycin C-pretreated RTG-2 cells, compared with their capacity to adhere to nontreated RTG-2 cells (Table 2). The capacity of strain A-7301 to adhere was enhanced two to three times by pretreatment with noradrenaline and phosphoenolpyruvate, but the capacity of NCMB 1102 was not enhanced (Table 2). In contrast, the enhancement of adhesion of strain NCMB 1102 was detected by pretreatment with succinate and with valine, although this strain was nonadhesive to nontreated RTG-2, whereas the capacity of strain A-7301 to adhere was lost by these pretreatments (Table 2).

DISCUSSION

In this study of a fish pathogenic bacterium *A. salmonicida*, there was a large difference in the net electrical charges of *A. salmonicida* strains. Pathogenic strains were negatively charged and nonpathogenic strains were positively charged (Table 1). Furthermore, the pathogenic strains were highly adhesive, whereas the nonpathogenic strains were nonadhesive to the surface of tissue culture cells (RTG-2 and CHSE-214 cells) of host fish (3, 7, 17, 21, 29). These data suggest that certain interrelationships exist between the charges on bacterial cells and host tissue cells and adhesion. Other workers have suggested that the A layer, which is characteristic of the cell envelopes of pathogenic strains, may be involved in adhesive mechanisms of *A. salmonicida* (3, 4, 7, 9, 28). Previously published data provide descriptions that the A layer has hydrophobicity because the amino acid composition of the A layer resembles that of fimbriae of other adhesive pathogenic bacteria (4, 9). On the basis of this concept, this study was carried out to investigate the implications of net electrostatic charges in *A. salmonicida* adhesion by an approach different from those used previously.

TABLE 2. Adhesion of *A. salmonicida* A-7301 and NCMB 1102 to RTG-2 cells

Pretreatment of RTG-2	Strain	Adhesion rate (%)[a]
None	A-7301	5.7
	NCMB 1102	0.008
Mitomycin C[b]	A-7301	0.05
	NCMB 1102	0.001
Noradrenaline[c]	A-7301	19.1
	NCMB 1102	0.002
Phosphoenolpyruvate[d]	A-7301	11.6
	NCMB 1102	0.001
Succinate[e]	A-7301	0.09
	NCMB 1102	1.7
Valine[f]	A-7301	0.03
	NCMB 1102	3.5

[a] Bacteria (10^8 viable cells) were added to 25-cm^2 flasks containing an RTG-2 monolayer with PBS and then incubated for 2 h at 20°C.

[b] RTG-2 monolayer cell cultures in flasks were pretreated with EBSS containing mitomycin C at 25 μg/ml for 2 h at 15°C.

[c] RTG-2 monolayer cell cultures in flasks were pretreated with EBSS containing 25 μM noradrenaline for 30 min at 15°C.

[d] RTG-2 monolayer cell cultures in flasks were pretreated with PBS containing 5mM phosphoenolpyruvate for 30 min at 15°C.

[e] RTG-2 monolayer cell cultures in flasks were pretreated with PBS containing 5 mM sodium succinate for 30 min at 15°C.

[f] RTG-2 monolayer cell cultures in flasks were pretreated with PBS containing 5 mM valine for 30 min at 15°C.

With regard to net electrostatic charges determined by electrophoresis, RTG-2 and CHSE-214 cells were attracted to the cathode in the live state at neutral pH in EBSS (Fig. 3B and C). No attraction was produced by electrophoresis of mitomycin C-treated cells (Fig. 4B). Further attraction to the cathode was evidenced by pretreating live cells with noradrenaline and phosphoenolpyruvate (Fig. 4C and D). Attraction to the anode was produced by pretreatment with succinate and valine (Fig. 4E and F). These results indicate that the fish tissue culture cells easily alter their electrostatic net charges, depending on the environmental situations, and that they are positively charged under usual environmental conditions such as in EBSS, fish serum, and fetal bovine serum (Fig. 3B and C). The net charge of the live cells was closely associated with the metabolic pathway of glycolysis (EBSS contains 1 g of glucose per liter) and probably the tricarboxylic acid cycle, because there was no attraction of the cells during electrophoresis with PBS (containing Ca^{2+} and Mg^{2+}) alone (Fig. 4A) and because there was severe anionic migration with succinate and valine (Fig. 4E and F). In particular, the implications of glycolysis were evidenced by further attraction to the cathode when noradrenaline-(which is an activator of glycolysis) and phosphoenolpyruvate-pretreated cells were used for electrophoresis (Fig. 4C and D). It is interpreted that negative charges (or the anionic migration) due to mucopolysaccharide (or sialic acid) are overwhelmed and cancelled by the positive charges (or the cationic migration of cells) in an excess of live tissue culture cells in the normal state, thereby allowing the tissue culture cells to be attracted to the cathode because of their predominant positive net electrostatic charges. Thus, RTG-2 and CHSE-214 cells are able to take different net charges in response to environmental nutritional conditions, and therefore, the movement of the live cells during electrophoresis is dynamic and alternative.

With respect to the mechanism of *A. salmonicida* adhesion, adhesion of bacterial cells of the pathogenic strains A-7301 and E-7609-3 used in this study to the surface of the monolayers of host tissue culture cells (RTG-2 and CHSE-214 cells) was considered to be due to the mutually opposite net electrostatic charges between the pathogenic bacterial cells and the tissue culture cells. The bacterial cells carry a negative charge and the tissue culture cells carry a positive charge (Table 1 and Fig. 3B and C). This interpretation was directly supported by the results that the usual attraction to the cathode (in the absence of bacterial cells) was lost following pretreatment with the pathogenic bacterial cells (but not by the nonpathogenic bacterial cells) or mitomycin C (Fig. 5A and B). The pathogenic bacterial cells strongly

inhibited the enhanced attraction of noradrenaline and phosphoenolpyruvate-pretreated tissue culture cells (Fig. 5C and D). Also, this interpretation was indirectly supported by the results that the nonpathogenic bacteria (positively charged) cells, strains NCMB 1102 and GH-7501, had no effect on cationic migration of live tissue culture cells or the nonmigration of mitomycin C-pretreated tissue culture cells. These effects of bacterial cells on host tissue culture cells during electrophoresis must be caused by the capacity of bacterial cells to adhere. In fact, the pathogenic bacterial cells were highly adhesive to normally cultured RTG-2 cells, and adherence was enhanced to noradrenaline- and phosphoenolpyruvate-pretreated RTG-2 cells (which carry stronger positive net charges than those of nontreated cells) (Table 2). Thus, the adhesion of pathogenic *A. salmonicida* strains (the net charges of which were negative) depends on and correlates with the degree of mutually converse net electrostatic charges on live host tissue culture cells (the net charges of which were positive).

There has been much interest in the interrelationship between *A. salmonicida* adhesion and the A layer, which is thought to be specific to virulent strains (3, 4, 7, 9, 29). It was recently reported (8), however, that the presence of the A layer incompletely paralleled adhesion and virulence in a comparison of seven strains of *A. salmonicida*, including strains lacking the A layer. A layer-positive cells can be identified by their capacity to stain with Congo red (6), and this method was used for a routine determination of the presence of the A layer. In this study, pathogenic and nonpathogenic strains were positive and negative for Congo red, respectively (Table 1). Although the A layer is negative (due to the Congo red test), the nonpathogenic strain (NCMB 1102) adhered to RTG-2 cells that were pretreated with succinate and valine (Table 2), which carried negative net electrostatic charges (Fig. 4E and F). Also, this strain inhibited the anionic migration of succinate- and valine-pretreated RTG-2 cells (Fig. 5E and F). These results strongly suggest that adhesion occurs because of mutually converse net electrostatic charges between bacterial cells and host tissue cells but not because of the A layer.

Thus, a new, unique modality of adhesion of pathogenic bacteria by mutually converse net electrostatic charges is strongly suggested by the fish pathogenic bacterium *A. salmonicida*. The findings do not necessarily rule out the specificity that is relevant to bacteria-to-host tissue cell relationships. The characterization of an interrelation between this electrostatic mechanism and the specificity (bacterial adhesins versus receptors of host cells) may be needed.

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