JOURNAL OF BACTERIOLOGY, Oct. 1981, p. 333-340
0021-9193/81/100333-08$02.00/0

Vol. 148, No. 1

Loss of Virulence During Culture of *Aeromonas salmonicida* at High Temperature

E. E. ISHIGURO,* W. W. KAY, TERESA AINSWORTH, J. B. CHAMBERLAIN, R. A. AUSTEN, J. T. BUCKLEY, AND T. J. TRUST

Department of Biochemistry and Microbiology, University of Victoria, Victoria, B.C., Canada V8W 2Y2

Received 30 March 1981/Accepted 8 June 1981

The effect of growth temperature on the loss of virulence of the fish pathogen *Aeromonas salmonicida* was investigated. Three virulent strains were grown in Trypticase soy broth at temperatures ranging from 22 to 30°C. Growth at a higher-than-optimal temperature (26 to 27°C for the three strains studied) resulted in the selection of spontaneous attenuated derivatives in the initial bacterial population. For example, virulent bacteria represented less than 10% of the population of a culture grown at 30°C, and attenuated derivatives were easily isolated by streaking the culture on solid medium and picking single colonies. Virulent strains autoaggregated during growth and possessed a cell wall layer (A-layer) external to the outer membrane, as previously described. Attenuated strains did not autoaggregate and did not possess the A-layer. The A-layer apparently shielded bacteriophage receptors and a mannose-specific yeast agglutinin located in the outer membrane. Thus, virulent strains exhibited impaired adsorption of phages, whereas attenuated strains were phage sensitive. Furthermore, attenuated strains agglutinated yeast cells but virulent strains did not. The attenuated strains had higher maximum growth temperatures than their virulent parent strains, and this accounts for their selection at high temperatures. It is proposed that the A-layer contributes significantly to the physical properties of the *A. salmonicida* cell envelope and that these physical properties change upon loss of the A-layer to permit growth at a higher-than-usual temperature.

Aeromonas salmonicida is the etiological agent of the fish disease known as furunculosis and is probably the most important pathogen of salmonid species (7). The acute form of furunculosis is characterized by septicemia and high mortality. Subacute, chronic, and latent forms of the disease have also been recognized. Little is known about the virulence factors of *A. salmonicida* which contribute to the disease symptoms. Udey and Fryer (16) have shown that virulent strains possess an additional cell wall layer (A-layer) which is not present in attenuated strains. The A-layer appears to be external to the outer membrane of the cell envelope. It was also shown that cells with A-layers autoaggregate during growth, whereas cells without A-layers are nonaggregating. We have recently demonstrated that the A-layer is composed of a protein with a molecular weight of about 49,000 (49K protein), and the purification of this protein is reported elsewhere (8a).

This investigation was prompted by the observation that attenuated strains of *A. salmonicida* apparently have a higher maximum growth temperature than virulent strains. We show here that growth of virulent strains at higher-than-optimal temperature results in the eventual selection of attenuated derivatives within the starting population. In addition to confirming Udey and Fryer's (16) correlation between possession of A-layers, virulence, and autoaggregation, we report that virulent and attenuated strains can be differentiated by testing for the ability to agglutinate yeast cells and for bacteriophage sensitivity.

MATERIALS AND METHODS

Bacteria and growth conditions. The *A. salmonicida* strains used in this study are listed in Table 1. The attenuated strain A450-1 was derived from strain A450 after six serial subcultures in Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) at room temperature (ca. 22°C). The other attenuated strains, A449-3, A450-2, and A451-2, were isolated as described below from cultures of virulent strains grown once at 30°C in TSB. Bacterial stock cultures were prepared as 15% (vol/vol) glycerol suspensions which were stored at −70°C. Working stock cultures were maintained on plates on Trypticase soy agar (TSA; BBL Microbiology Systems) and stored at 10°C. Single colonies from these working stocks were transferred to fresh TSA plates every 2 to 4 weeks. No problems were encountered in maintaining the virulence of *A. salmonicida* by these methods.

TABLE 1. *Properties of A. salmonicida strains*

Strain	Source	Autoaggregation[a]	Yeast agglutination[a]	A-layer[b]	50% Lethal dose (bacteria per fish)
A440	American Type Culture Collection strain 14174	−	+	−	$>1 \times 10^8$
A449	C. Michel strain TG36/75[c]	+	−	+	8×10^3
A449-3	Attenuated derivative of A449	−	+	−	1×10^8
A450	C. Michel strain TG72/78	+	−	+	2×10^4
A450-1	Attenuated derivative of A450	−	+	−	5×10^7
A450-2	Attenuated derivative of A450	−	+	−	$>2 \times 10^8$
A451	C. Michel strain TG51/79	+	−	+	1×10^4
A451-2	Attenuated derivative of A451	−	+	−	1×10^8

[a] +, Positive; −, negative.
[b] Determined by electron microscopy. +, Present; −, absent.
[c] C. Michel, Institut National de la Recherche Agronomique, Thiverval-Grignon, France.

The inoculum for growth experiments was prepared by picking a single colony from a stock culture plate and inoculating it into 5 ml of TSB in a 50-ml Erlenmeyer flask. The culture was incubated without shaking at room temperature for about 18 h. The inoculum was diluted as described below in 10-ml quantities of TSB in 125-ml flasks. These cultures were incubated at the indicated temperatures in a Gyrotory water bath shaker (New Brunswick Scientific Co., model G76) at a shaking speed of 150 rpm. Growth was monitored with a Klett-Summerson colorimeter (green filter).

Bacteriophages. *A. salmonicida* bacteriophage strains 1-16AC, 43-10C, and 70-21 have been described previously (8). Bacteriophages 37, 40, 54, 55, and 59 were obtained from W. D. Paterson (Connaught Laboratories Ltd., Willowdale, Ontario). Phage stocks were prepared on *A. salmonicida* strain A440 by the confluent lysis technique (8). Phage susceptibilities and plaquing efficiencies were determined by methods described previously (8). Phage adsorption was determined by incubating designated concentrations of phages and bacteria in TSB at room temperature. At indicated times (see Fig. 5), 1-ml samples were taken, and the bacteria and adsorbed phages were removed by centrifugation at 12,000 × *g* for 5 min at 0°C. One drop of chloroform was added to the supernatant, and the unadsorbed phages were quantified by being plated on strain A440 (8). A control containing phage alone was routinely included in these experiments.

Estimation of attenuated cells. Attenuated derivatives were sensitive to bacteriophage 37, whereas their virulent parent strains were resistant. Thus, the degree of attenuation which occurred during growth could be estimated by determining the percentage of the population which was resistant to phage 37. Cultures were sampled at indicated times (see Table 2), and dilutions were plated on TSA for viable counts. Dilutions were also plated on TSA with phage 37 (4×10^9 plaque-forming units) to determine phage-resistant counts.

Autoaggregation. Autoaggregation was determined as described by Udey and Fryer (16).

Yeast agglutination. Suspensions of glutaraldehyde-fixed cells of *Saccharomyces cerevisiae* were prepared by the method of Eshdat et al. (4). Agglutination of yeast cells by *A. salmonicida* was determined by the method of Atkinson and Trust (1).

Electron microscopy. Bacteria from 24-h-old TSA cultures were negatively stained with 2% potassium phosphotungstate. Specimens for ultrathin sectioning were fixed for 1 h with 1.8% purified glutaraldehyde in 0.06 M cacodylate buffer (pH 7.4) containing 0.5 mg of ruthenium red per ml. After being washed three times in distilled water, specimens were treated for 3 h with 1.3% osmium tetroxide in cacodylate-ruthenium red. Specimens were washed three times, dehydrated through ethanol to propylene oxide, and embedded in Epon 812 resin. After being thin sectioned and stained with 0.2% lead citrate and 2% uranyl acetate, they were examined with a Philips EM300 electron microscope as described previously (15).

Analysis of outer membranes. The cell envelopes of *A. salmonicida* were differentially solubilized with sodium lauryl sarcosinate by the method of Filip et al. (5). Outer membrane protein concentrations were determined by the modified Lowry procedure developed by Markwell et al. (10), using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a standard. The protein composition of the outer membrane preparation was determined by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis as previously described (15).

Determination of virulence. The bacteria were grown at room temperature without shaking for 24 h. Cells were collected by centrifugation and suspended in sterile saline (0.85% NaCl). Dilutions of the bacterial suspension were prepared in saline, and the viable count was determined by plating on TSA. Virulence was tested in juvenile coho salmon (*Oncorhynchus kisutch*) weighing approximately 7 g. For each bacterial dose tested, five fish were anesthetized with tricaine methane sulfonate (1 mg/liter), and inoculated intraperitoneally with 0.1 ml of the bacterial suspension. The fish were maintained in fresh water at approximately 12°C for 10 days. Samples from the kidneys of dead fish were streaked on furunculosis agar (Difco Laboratories, Detroit, Mich.), and a positive result was recorded only in cases in which *A. salmonicida* could be isolated in pure culture. Virulence was expressed as the 50% lethal dose.

Determination of plasmid DNA content. Plasmid DNA was isolated by the method of Hansen and Olsen (6). The plasmid DNA samples were analyzed by the agarose gel electrophoretic method of Meyers et al. (11). Molecular weights were determined by use of a set of reference plasmids.

RESULTS

Effect of temperature on growth. Broth cultures of a virulent strain of *A. salmonicida*, A450, exhibited biphasic growth at 30°C when the starting cell density was about 5×10^6 to 8×10^7/ml. The amount of growth which occurred during the first phase was dependent on the inoculum size, as was the duration of the lag between the first and second growth phases (cultures 1 and 2, Fig. 1A). The first growth phase was not detectable by this method when the starting cell density was decreased to about 10^5/ml, and detectable growth in the second phase occurred only after a lag of over 30 h (culture 3, Fig. 1A). No growth was detected within 7 days when the inoculum was less than 10^5 cells per ml. In contrast, strain A450-1, a spontaneous attenuated derivative of A450, grew well at 30°C compared with its parent under all conditions tested (culture 4, Fig. 1A). When fully grown broth cultures of A450 were diluted into fresh medium and grown for a second time at 30°C, growth was monophasic and occurred after a brief lag of a few hours as shown in Fig. 1B. In this experiment, cultures 1, 2, and 3 in Fig. 1A were diluted into fresh TSB to a density of 2×10^7 cells per ml.

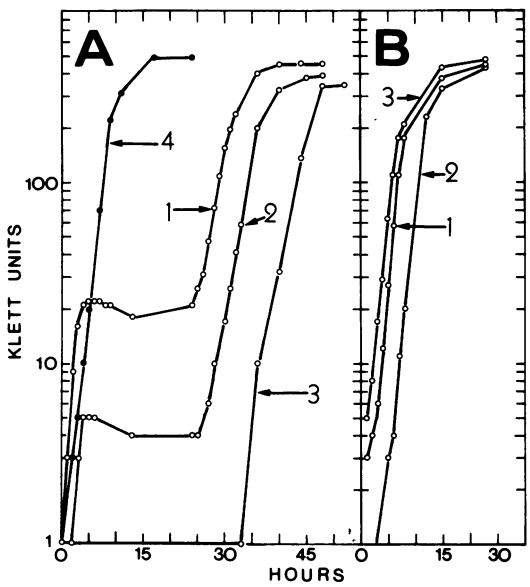

FIG. 1. *Growth of A. salmonicida at 30°C. (A) A culture of the virulent strain A450 grown at 22°C for 18 h was diluted into fresh TSB at 30°C. The initial cell densities were 6.5×10^7/ml for culture 1, 6.5×10^6/ml for culture 2, and 6.5×10^5/ml for culture 3. Growth of the attenuated stain, A450-1, at 30°C starting with a density of 7.5×10^6 cells per ml is shown for comparison (culture 4). (B) Cultures 1, 2, and 3 in (A) were diluted to 2.0×10^7 cells per ml in fresh medium and allowed to grow a second time at 30°C.*

As shown below, virulent *A. salmonicida* strains were completely resistant to bacteriophage strain 37, whereas attenuated strains were phage sensitive. Thus, it was possible to estimate the relative proportions of virulent and attenuated bacteria in the cultures described in the legend to Fig. 1 by using phage 37. As shown in Table 2, 93.8% of the cells in the inoculum used for the experiment in Fig. 1A were phage resistant (virulent). However, 90% or more of the cells in cultures grown once at 30°C (Fig. 1A) were phage sensitive (attenuated), and a second subculture at 30°C (Fig. 1B) resulted in further enrichment of phage-sensitive cells. These results suggested that the growth of virulent cells in the starting population was limited at 30°C and that attenuated derivatives were therefore selected for. In fact, pure cultures of attenuated derivatives were isolated from cultures grown once at 30°C by simply streaking on TSA and picking single colonies. Strains A449-3, A450-2, and A451-2 (Table 1) were obtained in this manner. Figure 2 shows the effect of growth temperature on the selection of attenuated derivatives. Significant enrichment for attenuated bacteria was seen at temperatures above 25°C for strains A449 and A450 and at 27°C or higher for strain A451.

Cell envelope structure. The ultrastructural properties of the cell envelopes of strains

TABLE 2. *Quantification of virulent (phage 37-resistant) and attenuated (phage 37-sensitive) cells in cultures of A. salmonicida strain A450 grown at 30°C*

Culture[a]	Viable cells per ml	Phage 37-resistant cells per ml	Phage resistant (%)
Fig. 1A: inoculum for cultures 1, 2, and 3	6.5×10^8	6.1×10^8	93.8
1	2.6×10^9	1.2×10^8	4.6
2	4.5×10^9	4.6×10^8	10.2
3	1.7×10^9	8.0×10^7	4.7
Fig 1B: inoculum for cultures 1, 2, and 3			
1	2.0×10^9	1.0×10^6	0.5
2	1.5×10^9	8.7×10^7	5.8
3	5.3×10^9	6.4×10^7	1.2

[a] Cultures 1, 2, and 3, whose growth is plotted in Fig. 1A, were tested at 50 h. Cultures 1, 2, and 3, whose growth is plotted in Fig. 1B, were tested at 24 h.

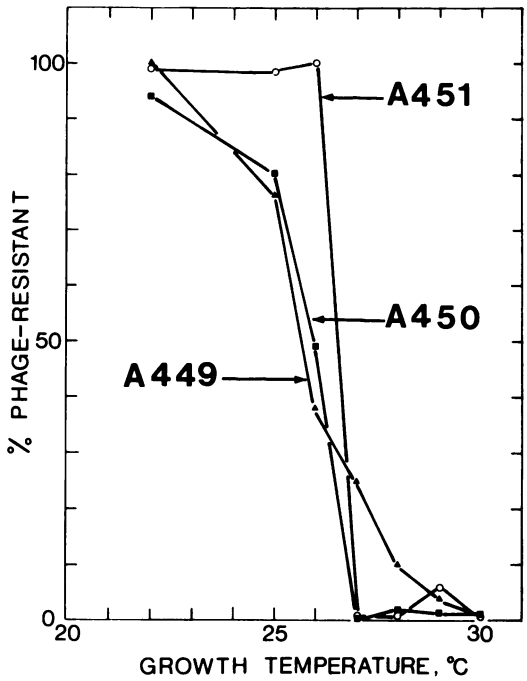

FIG. 2. *Effect of growth temperature on the selection of attenuated derivatives. A. salmonicida strains A449, A450, and A451 were grown to the stationary phase at various temperatures. Viable cell counts and total phage 37-resistant cell counts were determined on samples on these cultures. The percentages of phage 37-resistant cells, i.e., virulent cells, in the final populations are shown as functions of growth temperatures.*

A449, A450, and A451 and their attenuated derivatives were compared. The A-layer was present on the external surface of the outer membrane in sections of the virulent strains (Fig. 3A) but was not found in the attenuated strains (Fig. 3B). In negatively stained preparations, the A-layer appeared as a sheet composed of tetragonal subunits (5 by 5 nm) which appeared to completely envelope the bacteria. The ultrastructural details were best observed in cases in which the layer had accidentally sloughed off the cell surface during preparation of the sample for electron microscopy (Fig. 3C and D). No such structure was observed on the surfaces of negatively stained attenuated *A. salmonicida*.

The protein compositions of the cell envelopes were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The virulent strains A450 and A451 exhibited a major protein with a molecular weight of about 49,000 (Fig. 4) which was not present in the attenuated derivatives of these strains, A450-2 and A451-2. Identical results were obtained with the virulent strain A449 and its attenuated derivative, A449-3 (data not shown). As mentioned above, the 49K protein is the component of the A-layer (8a). There were differences in the cell envelope proteins of virulent and attenuated strains other than the 49K band. The significance of these additional differences is not known and is currently under investigation.

Other cell envelope-related properties. A direct correlation was made between the properties of autoaggregation and virulence (Table 1). Furthermore, the attenuated strains agglutinated yeast cells, whereas virulent strains did not (Table 1). Yeast cell agglutination was inhibited by mannose and appeared to be similar to the yeast agglutination system of *Aeromonas hydrophila* described by Atkinson and Trust (1).

Virulent and attenuated strains could also be differentiated on the basis of bacteriophage sensitivity. Virulent *A. salmonicida* strains A449, A450, and A451 exhibited either decreased plating efficiencies or complete resistance to eight phage strains (Table 3). In contrast, their attenuated derivatives were phage sensitive. These differences in phage sensitivity were all attributed to differences in phage adsorption. For example, Fig. 5 shows that phage 70-21 failed to adsorb to the virulent *A. salmonicida* strains A449, A450, and A451, but the phage readily absorbed to the attenuated derivatives of these strains and the attenuated strain, A440.

Plasmid composition. Plasmids were detected in all of the *A. salmonicida* strains examined (Fig. 6). The plasmid compositions of the attenuated strains were apparently identical to those of their parent strains. Two plasmids with molecular weights of 91×10^6 and 4.8×10^6 were common to all strains, and, in addition, strains A451 and A451-2 carried a plasmid with a molecular weight of 6.2×10^6.

DISCUSSION

The results of this study indicate that growth temperature is an important factor in maintaining the virulence of *A. salmonicida* in the laboratory, and the practical implications of this are manifest. Growth of *A. salmonicida* at a higher-than-optimal temperature resulted in the loss of virulence. We showed that this was caused by the selection of spontaneous attenuated derivatives in the starting population. These attenuated derivatives exhibited higher growth temperature maxima than did their virulent parents. The basis for this is not known, but we speculate that the attenuation of virulence may be accompanied by alterations in the physical properties of the cell envelope. There is ample evidence that the function of the outer membrane of

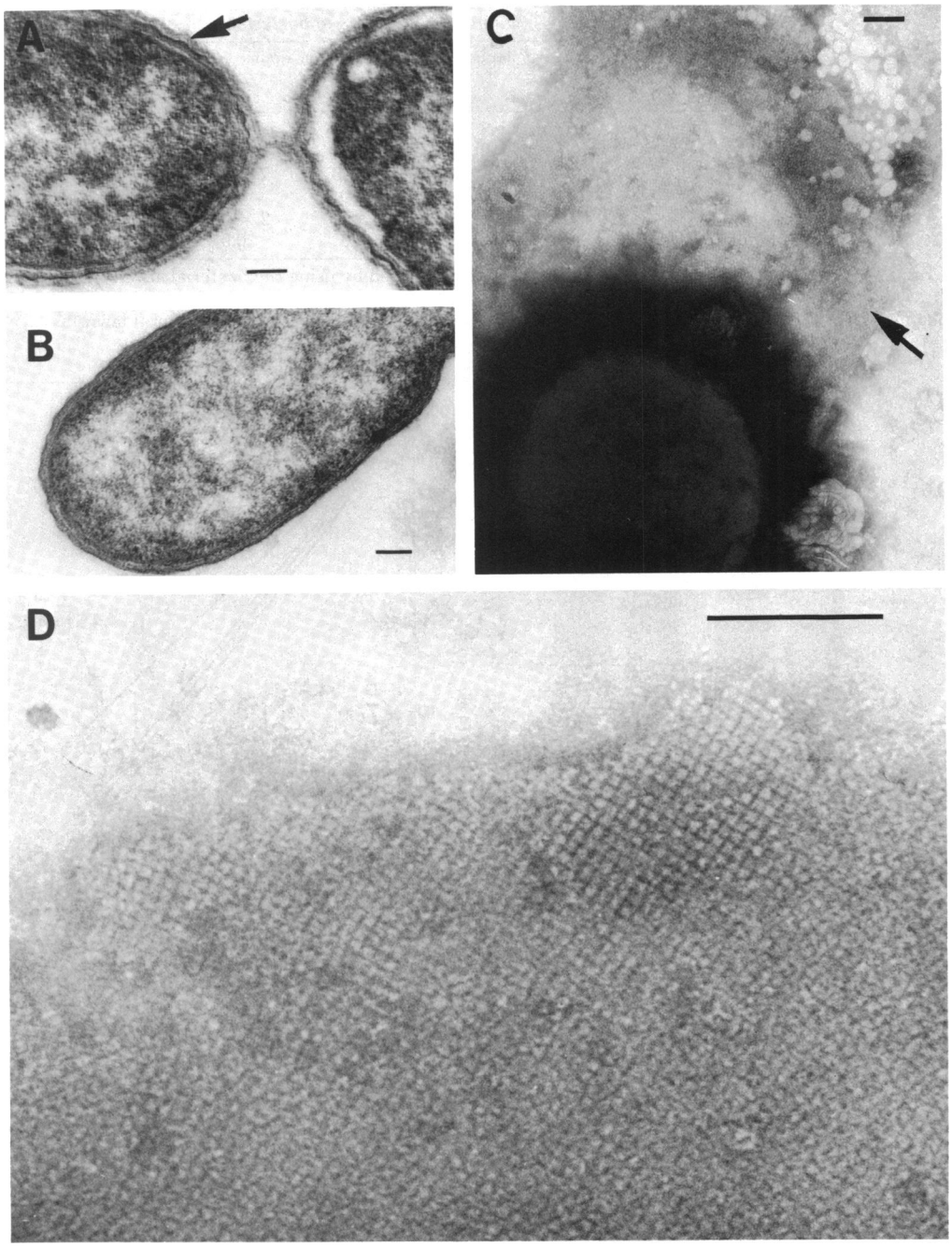

FIG. 3. *Thin sections of (A) virulent strain A450 showing the A-layer (arrow) and (B) attenuated strain A450-2 showing the absence of an A-layer. Negatively stained preparations of (C) virulent strain A450 showing the A-layer separated from a cell (arrow) and of (D) the A-layer of strain A450 showing tetragonal subunits. Bars represent 0.1 μm.*

gram-negative bacteria is influenced by the association of proteins with lipids and lipopolysaccharides (13, 14). Thus, it is quite possible that the A-layer contributes significantly to the physical state of the cell envelope of virulent *A. salmonicida*. These physical properties may change upon loss of the A-layer to permit growth at a higher-than-usual temperature.

TABLE 3. *Sensitivities of A. salmonicida strains to bacteriophages*

Bacterial strain	Relative plating efficiencies on phage strains[a]:							
	37	40	54	55	59	1-16AC	43-10C	70-21
A449	R	4×10^{-7}	2×10^{-3}	1×10^{-2}	2×10^{-5}	R	8×10^{-4}	R
A449-3	1.0	1.0	0.7	0.5	0.4	1.0	0.8	1.0
A450	R	6×10^{-7}	3×10^{-3}	4×10^{-2}	3×10^{-5}	7×10^{-3}	8×10^{-4}	R
A450-2	1.0	0.7	0.2	0.5	1.0	0.3	0.1	1.0
A451	R	1×10^{-3}	1×10^{-3}	2×10^{-2}	0.1	2×10^{-2}	8×10^{-4}	R
A451-2	1.0	0.7	1.0	1.0	1.0	0.9	1.0	1.0

[a] For each phage, the relative plating efficiencies were calculated by using the bacterial strain exhibiting the highest plating efficiency on that phage (assigned a value of 1.0) as a reference. R (resistant) indicates absence of plaques when a 10-μl drop containing 10^7 plaque-forming units was deposited on a bacterial lawn.

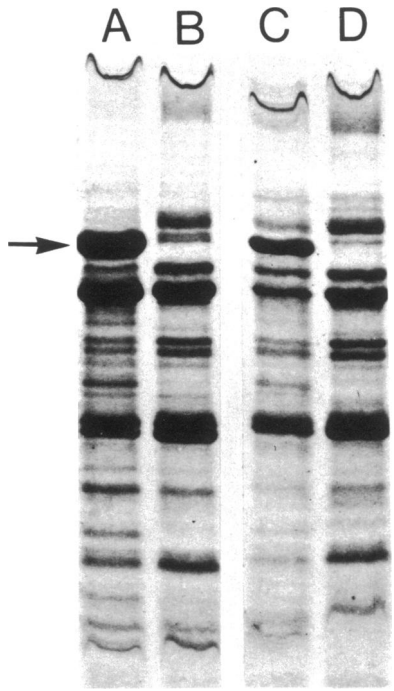

FIG. 4. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins of strain (A) A450, (B) A450-2, (C) A451, and (D) A451-2. The arrow indicates the position of the major protein (molecular weight, 49,000) present in virulent strains but absent in attenuated strains. This protein has recently been purified to homogeneity and shown to be the component of the A-layer.*

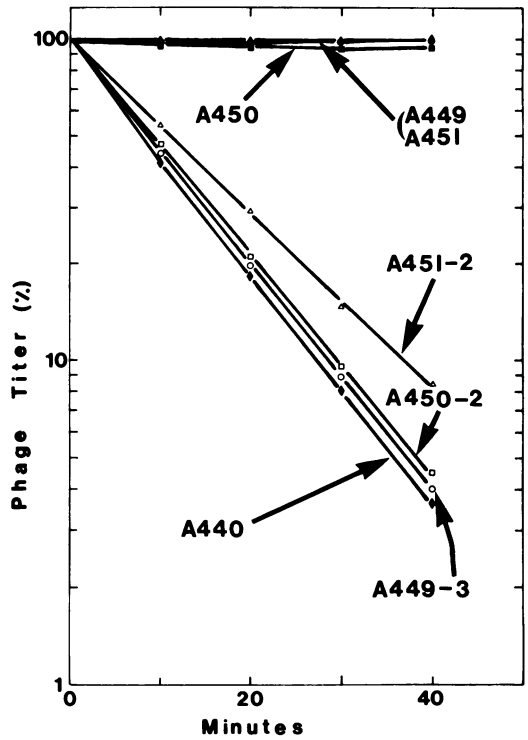

FIG. 5. *Adsorption of bacteriophage strain 70-21 to virulent strains A449, A450, and A451 and to attenuated strains A449-3, A450-2, A451-2, and A440.*

The molecular basis for the attenuation of virulence has not been determined. Growth at a high temperaure may result in the curing of plasmids in bacteria. Crosa et al. (2, 3) have implicated a plasmid in the virulence of *Vibrio anguillarum*, another fish pathogen. They have shown that growth of *V. anguillarum* at a high temperature results in the simultaneous curing of the plasmid and a loss of virulence (2). Our preliminary results suggested that a plasmid was not involved in the virulence of *A. salmonicida*, since the plasmid compositions of attenuated strains were identical to those of the virulent strains from which they were derived. The products of these plasmids are not known. On the other hand, the frequency of occurrence of the attenuated derivatives suggested that loss of virulence could well be attributed to a single mutational event. Irrespective of the mechanism, it is clear that attenuation of virulence was a consequence of the loss of the A-layer, since there

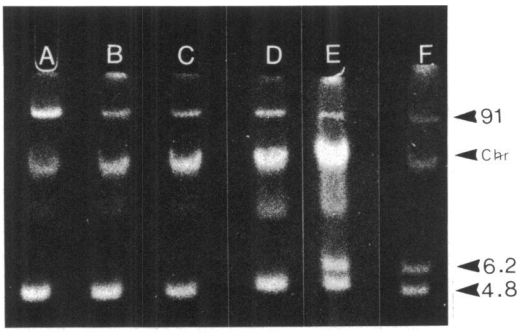

FIG. 6. *Agarose gel electrophoresis of plasmid DNA from strains A449 (A), A449-3 (B), A450 (C), A450-2 (D), A451 (E), and A451-2 (F). Chr indicates position of chromosomal DNA. Numerals refer to molecular weights (10^6).*

was a direct correlation between virulence, autoaggregation, and the presence of the A-layer, as was reported previously by Udey and Fryer (16).

The exact role of the A-layer in virulence is unclear at this time. It has been suggested that the A-layer may mediate the adherence of the bacteria to host tissues (16). On the other hand, the A-layer may also serve as a protective barrier against host defense systems. The A-layer clearly shielded at least most of the outer membrane of *A. salmonicida* and, therefore, was responsible for several cell surface-related properties unique to virulent strains. Virulent strains could be distinguished from attenuated strains on the basis of bacteriophage sensitivity. Furthermore, attenuated strains agglutinated yeast cells, whereas virulent strains did not. We have previously presented preliminary results indicating that the yeast agglutinin is an outer membrane protein (T. J. Trust, E. E. Ishiguro, and J. T. Buckley, Abstr. FEMS Symp. Bacterial Envelopes, abstr. no. 74, 1980). These results suggest that the A-layer masks the phage receptor sites and the yeast agglutinin in the virulent strains of *A. salmonicida*. These properties may be useful as rapid preliminary tests to determine virulence of *A. salmonicida* isolates (E. E. Ishiguro and T. J. Trust; Dev. Biol. Stan., in press). We show elsewhere that the A-layer is composed of a novel protein with a molecular weight of 49,000 which shields other cell surface proteins from chemical modification (8a).

The attenuation of virulence appeared to be a stable trait. We were unable to select for virulent revertants by serial passage of attenuated strains in fish, but this may have been due to the inadequacy of this selection technique. The problem is that attenuated cells in high doses (about 10^7 bacteria per fish or more) killed fish, and the *A. salmonicida* isolates obtained from these dead fish were always of the attenuated type. Thus, if virulent revertants existed in cultures of attenuated strains, they apparently occurred at frequencies below that which could be detected by passage in fish.

It should be noted that the three virulent strains used in this study exhibited similar growth-temperature relationships. Recent studies with additional strains indicate that the growth temperature range of virulent *A. salmonicida* is more variable than that reported here. For example, virulence of some strains could not be maintained at temperatures above 22°C. In our experience, the degree of virulence of any given strain does not change, provided that appropriate precautions are taken in the laboratory culturing of the organism (i.e., growth at low temperature and maintenance of stock cultures according to the procedures outlined above). In this respect, the practical value of serial passages in fish (9, 12) as a means of enhancing the virulence of *A. salmonicida* is at least questionable.

ACKNOWLEDGMENTS

This work was supported by a Strategic Grant from the Natural Sciences and Engineering Council of Canada.

We thank Barry Phipps for technical assistance.

LITERATURE CITED

1. **Atkinson, H. M., and T. J. Trust.** 1980. Hemagglutination properties and adherence ability of *Aeromonas hydrophila*. Infect. Immun. **27**:938-946.
2. **Crosa, J. H., L. L. Hodges, and M. H. Schiewe.** 1980. Curing of a plasmid is correlated with an attenuation of virulence in the marine fish pathogen *Vibrio anguillarum*. Infect. Immun. **27**:897-902.
3. **Crosa, J. H., M. H. Schiewe, and S. Falkow.** 1977. Evidence for plasmid contribution to the virulence of the fish pathogen *Vibrio anguillarum*. Infect. Immun. **18**:509-513.
4. **Eshdat, Y., I. Ofek, Y. Yashouv-Gan, N. Sharon, and D. Mirelman.** 1978. Isolation of a mannose-specific lectin from *Escherichia coli* and its role in the adherence of the bacteria to epithelial cells. Biochem. Biophys. Res. Commun. **85**:1551-1559.
5. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. J. Bacteriol. **115**:717-722.
6. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227-238.
7. **Herman, R. L.** 1968. Fish furunculosis, 1952-1966. Trans. Am. Fish. Soc. **97**:221-230.
8. **Ishiguro, E. E., W. W. Kay, and T. J. Trust.** 1980. Temperate bacteriophages for *Aeromonas salmonicida*. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. **8**:247-250.

8a. **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.

9. **Klontz, G. W., W. T. Yasutake, and A. J. Ross.** 1966. Bacterial diseases in the salmonidae in the Western United States: pathogenesis of furunculosis in rainbow trout. Am. J. Vet. Res. **27**:1455–1460.
10. **Markwell, M. A. K., S. M. Haas, L. L. Biaber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87**:206–210.
11. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.
12. **Michel, C.** 1980. A standardized model of experimental furunculosis in rainbow trout (*Salmo gairdneri*). Can. J. Fish. Aquat. Sci. **37**:746–750.
13. **Nikaido, H.** 1980. Nonspecific transport through the outer membrane, p. 400–407. *In* M. Inouye (ed.), Bacterial outer membranes. Biogenesis and functions. John Wiley and Sons, New York.
14. **Osborn, M. J., and H. C. P. Wu.** 1980. Proteins of the outer membrane of gram-negative bacteria. Annu. Rev. Microbiol. **34**:369–422.
15. **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 (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. **9**:35–38.
16. **Udey, L. R., and J. L. Fryer.** 1978. Immunization of fish with bacterins of *Aeromonas salmonicida*. Marine Fish. Rev. **40**:12–17.