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Aeromonas salmonicida Resistance to Complement-Mediated Killing

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The resistance of *Aeromonas salmonicida* to complement-mediated killing was investigated by using different strains and their isogenic mutants that had been previously characterized for their surface components. We found that the classical complement pathway is involved in serum killing of susceptible *A. salmonicida* strains, while the alternative complement pathway seems not to be involved. All of the *A. salmonicida* strains are able to activate complement, but the smooth strains (with or without the A-layer) are resistant to complement-mediated killing. The reasons for this resistance are that C3b may be bound far from the cell membrane and that it is rapidly degraded; therefore, the lytic final complex C5b-9 (membrane attack complex) is not formed. Isogenic rough mutants are serum sensitive because they bind more C3b than the smooth strains, and if C3b is not completely degraded, then the lytic complex (C5b-9) is formed.

The complement system plays a crucial role in humoral defense against microbial pathogens and has recently been reviewed elsewhere (34, 35). This series of serum proteins which are sequentially activated produces two major effects in terms of host defense, i.e., deposition of proteins (C3b or iC3b) onto the microbial surface which (i) serve as opsonins for C3b receptor-bearing phagocytes and (ii) act as a stable C5 convertase, resulting in the formation and assembly of a membrane attack complex (C5b-9) that is capable of lysing susceptible bacteria. The latter effect of direct bacterial killing is known as the serum bactericidal reaction.

Complement activation may take place by either of two pathways (classical or alternative [CPC or APC, respectively]), resulting in activation of the vital third component of complement, C3. Bacterial resistance to complement-mediated killing may result from the failure or limitation of complement activation by either of the two pathways or by the failure of activated complement to exert its effect. Various surface antigens which render bacterial cells resistant to complement-mediated killing, such as lipopolysaccharide (LPS), outer membrane proteins, and capsules (13, 22, 33), have been identified.

Aeromonas salmonicida is an important pathogen of salmonid fishes, producing the systemic disease furunculosis. The principal virulence factor of this pathogen appears to be an S-layer (A-layer), principally consisting of a two-dimensional crystalline tetragonal protein (A-protein with a molecular weight [MW] of 49,000) array (14), which is tethered to the cell by LPS (2). Impermeant labeling studies have shown that the A-layer appears to cover most of the surface of virulent *A. salmonicida* (5), although some LPS may also be exposed (26). This structure has been shown to protect this bacterium from serum killing in a manner that somehow requires both LPS and the A-layer (21); however, although A-layer is not completely necessary for the bacterium to be resistant to serum killing it is an important barrier for opsonophagocytosis.

In the present study, we have investigated the mechanism of complement activation by *A. salmonicida* strains and the role of LPS and the A-layer in the susceptibility of these strains to the bactericidal activity of nonimmune serum. Furthermore, we have investigated the role of high-MW LPS (HMW-LPS) (O-antigen-enriched fraction) and the low-MW LPS (LMW-LPS) (core and lipid A fraction) isolated by LPS fractionation in complement-mediated killing.

MATERIALS AND METHODS

Bacterial strains. The *A. salmonicida* strains used in this study have been previously described (21) and were a gift from W. W. Kay (University of Victoria, Victoria, British Columbia, Canada). Strains A450 and A449 showed an A-layer (A^+) and a complete LPS (with O antigen [O^+]). Strains A450-3 and A449-3 were isogenic mutants from A450 and A449, respectively, unable to assemble an A-layer (A^-) with a complete LPS (O^+), and at least strain A450-3 was also unable to synthesize A protein (11). Strains A450-1 and A449-3R were rough LPS mutants (O^-) from strains A450 and A449, respectively, and were unable to assemble an A-layer (A^-). The last strain was a deep rough mutant. Cultures were maintained and grown as previously described (21).

Bacterial survival in fresh NIS. The survival of exponential-phase bacteria in fresh nonimmune serum (NIS) was measured as previously described (36). Control measurements with bacteria in phosphate-buffered saline (PBS) (containing 0.15 M sodium chloride and 0.15 M sodium phosphate, pH 7.2) or heat-inactivated NIS (56°C for 30 min) were performed.

Treatment of serum. CPC activity in serum was selectively inhibited by chelation with 20 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) plus 2 mM $MgCl_2$ as previously described (8). Serum was treated with inulin (2 mg/ml) by the method described by Gotze and Muller-Eberhad (10) or was heated at 50°C for 20 min to inactivate factor B, which is required for APC activity in serum (7). After each serum treatment, we assayed the anticomplement activity of the treated serum by the method described by Morrison and Kline (20) as modified in a microtiter assay developed by Vukajlovich et al. (41) to be sure that the treatment was correct. Both pathways were inhibited either by the treatment of serum with 20 mM EDTA or by heating the

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serum at 56°C for 30 min. EGTA, EDTA, and inulin at the concentrations mentioned above had no effect on the survival of *A. salmonicida* strains in PBS during 3 h of incubation.

LPS and A-layer isolation. LPS from *A. salmonicida* strains was purified by the method described by Westphal and Jann (42) as modified by Osborn (24). Purified A-layers were isolated from different *A. salmonicida* wild-type strains (A^+) as previously described (6). By using this methodology, we isolated assembled A-layers that could be observed by negatively stained electron microscopy.

Subfractionation of LPS by column chromatography. Lyophilized LPS from *A. salmonicida* A450-3 (serum resistant; O^+:A^-) was solubilized at a final concentration of 7.5 mg/ml in buffer containing 3% (wt/vol) sodium deoxycholate, 0.2 M NaCl, 5 mM EDTA, 8 M urea, and 20 mM Tris hydrochloride (pH 8.3) and was applied at room temperature to a column of Sephacryl S-300 (Pharmacia Fine Chemicals) equilibrated in buffer containing 0.25% (wt/vol) sodium deoxycholate, 0.2 M NaCl, 5 mM EDTA, and 10 mM Tris hydrochloride (pH 8.0). Fractions (2.5 ml) were collected at a flow rate of 12 ml/h and were analyzed directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Before chemical analyses and serum inhibition studies, fractions were extensively dialyzed against distilled water, first at room temperature and then at 4°C.

Electrophoretic techniques. SDS-PAGE was performed by the procedure described by Laemmli (17). Samples were mixed 1:1 with sample buffer (containing 4% SDS) and boiled for 5 min, and 10-μl portions were applied to the gel. LPS bands were detected by the silver staining method described by Tsai and Frasch (40).

Analytical procedures. Total carbohydrates were measured by the phenol procedure (12) with glucose as the standard. 2-Keto-3-deoxyoctulosonic acid (KDO) was measured by the thiobarbituric acid method after hydrolysis of samples in 4 M HCl for 30 min (16). Monosaccharides were also analyzed to their alditol acetate derivatives by gas-liquid chromatography on a 3% SP-3840 column (Supelco) as previously described (38).

Inhibition of serum bactericidal activity. The effect of treating the serum with bacterial cells or purified cell components in the serum bactericidal reaction was studied as follows. (i) For whole cells, NIS (1.5 ml) was incubated at 37°C for 1 h with 10^8 cells and centrifuged for 5 min in an Eppendorf microcentrifuge. The supernatant was filtered through a 0.45-μm-pore-size filter to remove cells, and the treated serum (0.9 ml) was added to 0.1 ml of an exponential-phase culture (5×10^7 CFU) of *A. salmonicida* A450-1 (serum sensitive) or a similar strain, and the mixture was incubated at 37°C for 3 h. Samples were taken hourly, and bacterial concentrations were determined by dilution plating. (ii) Complete LPS or fractionated LPS was suspended in PBS to a final concentration of 1 to 5 mg/ml, and the suspension was briefly sonicated at 4°C until the solution cleared. LPS solutions in the concentrations range of 0.01 to 0.2 mg/ml were added to 1 ml of NIS, and the mixtures were incubated for 30 min at 37°C. After this treatment, the bactericidal activity of the serum was determined with strain A450-1 or a similar strain as described above. (iii) Purified A-layer was suspended in PBS at a final concentration of 5 mg/ml, and the suspension was briefly sonicated at 4°C until the solution cleared. The solution, in a final concentration range of 0.01 to 0.4 mg/ml, was added to 1 ml of NIS, and the mixture was incubated for 30 min at 37°C. After this treatment, the bactericidal activity of the serum was determined as described above.

Controls consisting of NIS incubated for 1 h at 37°C in PBS without cells, LPS, LPS fractions, or purified A-layer showed no inhibition of serum bactericidal activity.

Measurement of the anticomplement activity of whole cells or purified molecules. The anticomplement activity of whole cells, purified LPS (complete or fractionated), or purified A-layer was measured by the method described by Shafer et al. (30). Briefly, NIS was mixed either with whole cells or purified molecules in a final volume of 0.2 ml, and the mixture was incubated with shaking at 37°C for 30 min. Antibody-sensitized sheep erythrocytes were added to a fourfold dilution of treated NIS, and the mixture was incubated at the same temperature for an additional 30 min; then, the reaction was stopped by the addition of ice-cold saline, the cells were pelleted by centrifugation, and the A_{412} of the supernatant was measured. The positive control consisted of sensitized sheep erythrocytes plus NIS alone, and the negative control consisted of cells or purified molecules without added NIS.

Concentrations of C1q and C3 complement components were measured as previously described (18). Briefly, specific anti-C1q or anti-C3 antisera (Sigma) were coated onto a microtiter plate, and the plate was incubated overnight at 4°C, washed, and incubated for 1 h at 37°C with 1% bovine serum albumin. Meanwhile, NIS was treated with *A. salmonicida* whole cells, purified LPS (complete or fractionated), or purified A-layer for 30 min at 37°C. Untreated NIS was used as a standard with the same incubation period. After the plates had been washed, the treated or untreated NIS was added, and the plates were incubated for 90 min at 37°C. The plates were then washed again and incubated for 1 h at 37°C with protein A-alkaline phosphatase conjugate (Boehringer). After the plates had been washed, the color reaction was developed with 4-nitrophenyl phosphate (1 mg/ml) and the A_{405} was recorded.

Binding of C3b, C5b, and C5b-9 to whole cells. The interactions between whole *A. salmonicida* cells and complement components C3b, C5b, and C5b-9 were quantified by an enzyme immunoassay and were observed by immunogold electron microscopy. Bacteria that were preincubated for 5 to 20 min with 90% NIS at 37°C were washed twice with cold PBS by microcentrifugation; incubated for 45 min at 37°C in suspension with anti-C3b, anti-C5b, or anti-C5b-9 (Calbiochem) (1:100 dilution in PBS plus 1% bovine serum albumin); and washed again by microcentrifugation. Anti-C3b serum was able to react with C3b, iC3b, C3c, and C3d, as can be seen in lane 1 on Fig. 6A, B, and C. Then, in the case of the immunoassay, the bacteria were incubated with protein A-alkaline phosphatase (1:1,000 dilution in PBS) at 37°C for 45 min; in the case of immunogold electron microscopy, they were incubated with protein A conjugated to 14-nm gold particles (1:20 dilution in PBS). After the washing, the color reaction in the immunoassay was developed as described above and the A_{405} was recorded. In the case of immunogold electron microscopy, after the washing, the bacterial suspensions were placed on Formvar-coated copper grids, air dried, and examined in a Hitachi H600 transmission electron microscope.

Controls consisted of cells treated with protein A-alkaline phosphatase for the immunoassay or protein A conjugated to 14-nm gold particles for the immunogold electron microscopy in the absence of specific antibodies.

Analysis of bound C3 fragments. An *A. salmonicida* suspension (2×10^8 CFU/ml) was opsonized with NIS diluted in PBS (25% final concentration). Opsonization was carried out at 37°C for 0 to 90 min, and the reaction was stopped by the addition of ice-cold PBS. Serum-sensitive strains were opsonized only for 0 to 20 min. The tubes were centrifuged, and the pellets were washed three times with PBS. The pellets were resuspended in 1 M hydroxylamine in 50 mM carbonate-

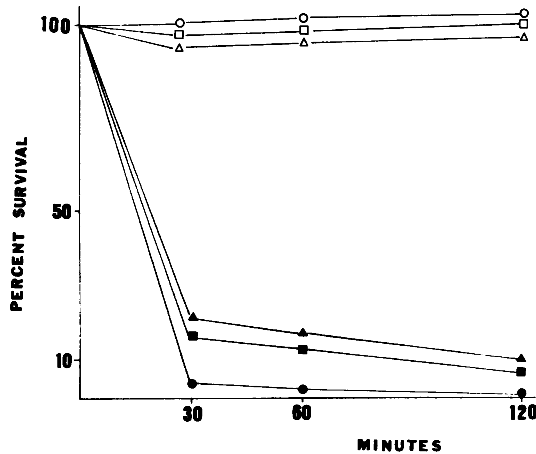

FIG. 1. The bactericidal effects of NIS on the serum-sensitive strain *A. salmonicida* A450-1. Control serum (●) was unaltered prior to the addition of cells. Otherwise, NIS was pretreated with heating at 56°C for 30 min (○), 20 mM EDTA (□), 20 mM EGTA plus 2 mM Mg_2Cl (△), 2 mg of inulin per ml (▲), or heating at 50°C for 20 min (■). The results showed are the averages of three independent experiments.

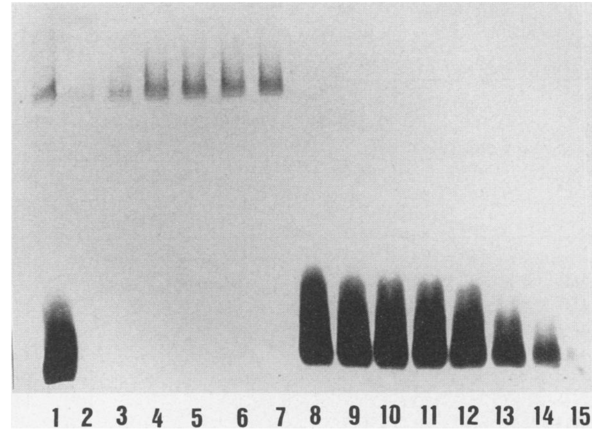

FIG. 2. Analysis of subfractionated LPS from *A. salmonicida* A450-3 (serum resistant) by SDS-PAGE. LPS was fractionated by gel filtration as described in Materials and Methods. Eluted fractions were analyzed with silver staining (40) for carbohydrates. Lanes: 1, purified whole LPS from *A. salmonicida* A450-3; 2 to 15, eluted fractions containing LPS. Fractions 2 to 7 showed HMW-LPS, while fractions 8 to 15 showed LMW-LPS.

bicarbonate buffer (pH 9.0) to disrupt ester bonds between the complement fragments and the bacterial surface (9). After 2 h at 37°C, the C3 fragment suspension was reduced with 10 mM dithiothreitol in 1% SDS at 37°C for 1 h and then alkylated with 22 mM iodoacetamide at 37°C for 1 h (9). Aliquots of the C3 fragment suspension were diluted 1:1 in sample buffer and subjected to SDS-PAGE. After electrophoresis, the gels were electroblotted to nitrocellulose membranes (39), and the membranes were blocked overnight with PBS plus 1% bovine serum albumin. After washing, the membranes were incubated with anti-C3 serum for 2 h, washed, and reincubated with protein A-alkaline phosphatase for 1 h. The C3 fragments were visualized on the membrane blots with BCIP-NBT (5-bromo-4-chloro-3-indolylphosphate disodium–nitroblue tetrazolium) (3). Two C3-purified preparations were used as controls (kindly provided by F. Vivanco, Fundación Jimenez Díaz, Madrid, Spain), one containing C3d (33-kDa band) and the other containing C3c (43- and 27-kDa bands) and the 75-kDa band common to C3b and iC3b (see lanes 1 in Fig. 6A, B, and C). The Western blots (immunoblots) were scanned using a Bio-Image densitometer (Millipore).

RESULTS

A. salmonicida strains were tested for their ability to resist the bactericidal activity of NIS from different sources (rabbit, human, and trout sera). The results obtained are similar to the ones reported before (21). Briefly, strains O^+:A^+ and O^+:A^- were resistant to human and rabbit sera and were partially resistant to trout serum, and strains O^-:A^- were sensitive to all sera (data not shown).

Mechanism of complement activation by *A. salmonicida* strains. The mechanism of complement activation by serum-sensitive *A. salmonicida* strains was examined. Trout or human NIS treated with Mg^{2+}-EGTA (which selectively inhibits the CPC) was nonbactericidal for *A. salmonicida* A450-1, while NIS pretreated with inulin or heated at 50°C for 20 min (which depletes the APC) was bactericidal for *A. salmonicida* A450-1 (Fig. 1). This strain was also rapidly killed in untreated NIS. However, NIS treated with EDTA or heated at 56°C for 30 min (which inhibits both complement pathways) was nonbactericidal for strain A450-1. Similar results were obtained with *A. salmonicida* A449-3R (data not shown). Furthermore, these strains were killed by complement factor B-deficient human serum (Sigma) but not by complement component C1q-deficient human serum (Sigma). These results suggested that serum killing of *A. salmonicida* strains is mediated by the CPC. Also, no specific anti-*A. salmonicida* antibodies were required for the CPC-dependent killing of the sensitive strains, because we were unable to detect specific antibodies against *A. salmonicida* in the NIS of trout or human origin.

Subfractionation of LPS. LPS from *A. salmonicida* A450-3 (serum resistant) was fractionated as described in Materials and Methods. The presence of LPS in column fractions was monitored by the determination of total carbohydrates and KDO (data not shown) and by SDS-PAGE. Fractions 2 to 7 showed homologous HMW-LPS, while fractions 8 to 14 showed LMW-LPS (Fig. 2). Fractions containing HMW-LPS (2 to 7) showed a high ratio of total carbohydrates to KDO (20:1) and also of rhamnose to KDO (13:1), since rhamnose is one of the sugars present on the O-antigen LPS of *A. salmonicida* (32). Fractions containing LMW-LPS (8 to 14) showed a low ratio of total carbohydrates to KDO (3:1) and also a glucose-to-KDO ratio of approximately 1:1 and no rhamnose, as can be expected from the fact that these fractions are free of O-antigen LPS whereas glucose is a sugar component of the core LPS of these strains (31).

Inhibition of serum bactericidal activity by whole cells or purified surface molecules. Preincubation of trout or human NIS with whole cells of *A. salmonicida* strains (O^+:A^+, O^+:A^-, or O^-:A^-) inhibited serum bactericidal activity when tested against serum-sensitive strains such as A450-1 or A449-3R, both of which lack O-antigen LPS.

Various concentrations of purified whole LPS (a mixture of O-antigen-containing and O-antigen-deficient LPS molecules) from *A. salmonicida* O^+:A^+ or O^+:A^- strains or various concentrations of purified LPS from *A. salmonicida* O^- strains (only with O-antigen-deficient LPS molecules) inhibited the bactericidal activity of trout or human NIS in a dose-dependent manner when tested against strain A450-1 (data not shown). For instance, these purified LPSs at 0.1 mg/ml were

TABLE 1. Inhibition of bactericidal activity of NIS against serum-sensitive strain A450-1 by different purified surface molecules of *A. salmonicida* strains

Surface molecule used for NIS treatment	% Survival of A450-1 after incubation for the indicated times with the following[a]:			
	Untreated NIS (3 h)	Treated NIS		
		1 h	2 h	3 h
LPS from strain[b]:				
A450 (O^+:A^+)	<0.1	96	102	109
A450-3 (O^+:A^-)	<0.1	98	104	112
A450-1 (O^-:A^-)	<0.1	102	110	120
A449 (O^+:A^+)	<0.1	95	99	105
A449-3 (O^+:A^-)	<0.1	99	108	118
A449-3R (O^-:A^-)	<0.1	100	109	117
HMW-LPS from strain A450-3[c]	<0.1	2	<0.1	<0.1
LMW-LPS from strain A450-3[c]	<0.1	102	116	127
Purified A-layer from strain[d]:				
A450 (O^+:A^+)	<0.1	<0.1	<0.1	<0.1
A449 (O^+:A^+)	<0.1	<0.1	<0.1	<0.1

[a] The bacteria were incubated at 20°C. The results are the averages of three independent experiments.
[b] Purified LPS at a concentration of 0.1 mg/ml.
[c] Purified HMW-LPS and LMW-LPS at a concentration of 0.05 mg/ml.
[d] Purified A-layer at a concentration of 0.2 mg/ml.

able to completely inhibit the bactericidal activity of human NIS against strain A450-1 (Table 1).

This fact prompted us to examine the interaction between HMW-LPS and LMW-LPS with NIS. As shown in Table 1, HMW-LPS-pooled fractions (2 to 7) from strain A450-3 were unable to inhibit the bactericidal activity of trout or human NIS, in addition to the fact that some residual activity was found, perhaps as a consequence of a minor amount of core LPS and lipid A still being present in this pooled fraction. By contrast, LMW-LPS-pooled fractions (8 to 14) from strain A450-3 were able to completely inhibit the bactericidal activity of trout or human NIS (Table 1).

Finally, purified A-layers (with LPS contamination of <1%, as determined with specific antibodies and gels) were obtained from strains A450 and A449. Even at high concentrations (0.2 or 0.4 mg/ml), none of these A-layers were able to inhibit the bactericidal activity of trout or human NIS when tested against the serum-sensitive strains of *A. salmonicida* (data shown in Table 1 for strain A450-1). Similar results were obtained with another serum-sensitive strain of *A. salmonicida* (A449-3R [data not shown]).

Anticomplement activities of whole cells and purified surface molecules. The complement-absorbing activities of whole cells and purified surface molecules were measured to determine whether inhibition of serum bactericidal activity was due to the depletion of serum complement. Whole cells of *A. salmonicida* strains inhibited complement-mediated hemolysis of sensitized sheep erythrocytes. Furthermore, complement components C1q and C3 were depleted when human NIS was treated with whole cells, either O^+:A^+, O^+:A^-, or O^-:A^- (Table 2).

The complement-absorbing activity of LPSs from all of the strains was dose dependent (Fig. 3). In addition, the C3 concentration was depleted when human NIS was treated with these purified LPSs (Table 3). Furthermore, HMW-LPS-pooled fractions (2 to 7) from A450-3 at the concentrations examined were unable to deplete C3 from human NIS, while LMW-LPS-pooled fractions (8 to 14) were able to do so (Table 3).

TABLE 2. Concentration of complement components C1q and C3 in untreated NIS and NIS treated with whole *A. salmonicida* cells

Strain used for treatment	Concn of component[a]	
	C1q	C3
None	1.31	1.95
A450	0.72	0.86
A450-3	0.68	0.74
A450-1	0.37	0.49
A449	0.71	0.81
A449-3	0.65	0.73
A449-3R	0.39	0.51

[a] Concentrations were determined by enzyme-linked immunosorbent assay (ELISA) and are given in arbitrary A_{405} units. Results are means from experiments done in triplicate at least twice. Standard deviations were all <0.08.

When we tested purified A-layers from different *A. salmonicida* strains (Table 3), we found that none of them were able to inhibit the complement-mediated hemolysis of sensitized sheep erythrocytes (Fig. 3 [data for the A-layer of strain A450]) or to deplete C3 from A-layer-treated human NIS (Table 3).

Binding of C3b, C5b, and C5b-9 to whole cells. As shown in Table 4, whole cells of *A. salmonicida* O^+:A^+ or O^+:A^- (serum-resistant) strains bound less C3b than those of serum-sensitive strains (O^-:A^- mutants). In addition, strains with A-layer (O^+:A^+) bound less C3b than isogenic strains lacking the A-layer (O^+:A^-). Identical results were obtained by immunogold electron microscopy (Fig. 4). It is important to note that O^+:A^+ or O^+:A^- strains seem to bind C3b further from the cell membrane than O^-:A^- strains (serum-sensitive strains) and also that there is less C3b bound on O^+:A^+ strains than on their isogenic A^- mutants (data shown for strains A450, A450-3, and A450-1 in Fig. 4).

In addition, serum-resistant strains did not bind C5b or C5b-9, while a high level of binding of complement components was observed for the serum-sensitive strains (Table 4). Identical results were obtained by immunogold microscopy (Fig. 5 [data shown for C5b-9 binding to strains A450, A450-3, and A450-1]). Similar immunogold electron microscopy results

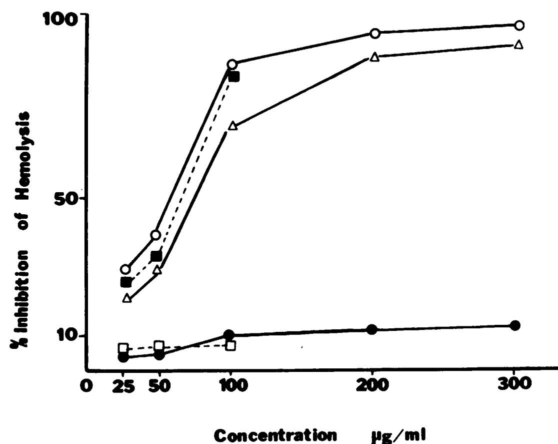

FIG. 3. Inhibition of complement-mediated hemolysis of sensitized sheep erythrocytes. Erythrocytes were incubated for 30 min in NIS as control or with NIS containing whole LPS from *A. salmonicida* A450 (△) or A450-1 (○), HMW-LPS from strain A450-3 (□), LMW-LPS from strain A450-3 (■), or purified A-layer from strain A450 (●). The results shown are the averages of three independent experiments.

TABLE 3. Concentrations of complement component C3 in untreated NIS and NIS treated with purified *A. salmonicida* surface molecules

Surface molecules used for NIS treatment	Concn of C3[a]
None	1.95
LPS from strain[b]:	
A450	0.62
A450-3	0.61
A450-1	0.48
A449	0.60
A449-3	0.59
A449-3R	0.49
HMW-LPS from strain A450-3[c]	1.89
LMW-LPS from strain A450-3[c]	0.47
Purified A-layer from strain[d]:	
A450	1.91
A449	1.92

[a] Concentrations were determined by ELISA and are given in arbitrary A_{405} units. Results are means from experiments done in triplicate at least twice. Standard deviations were all <0.07.
[b] Purified LPS at a concentration of 0.1 mg/ml.
[c] Purified HMW-LPS and LMW-LPS at a concentration of 0.05 mg/ml.
[d] Purified A layer at a concentration of 0.2 mg/ml.

were found with other serum-resistant and -sensitive *A. salmonicida* strains. No specific gold particles were found on control cells incubated in the absence of specific antibodies (data not shown).

Analysis of bound C3 fragments. The results of analysis of bound C3 fragments are shown in Fig. 6. As can be observed, the serum-resistant strains A450 (O^+:A^+) and A450-3 (O^+:A^-) (Fig. 6C and B, respectively) showed a large decrease and finally absence on the 105-kDa band (C3 fragment characteristic of C3b) over time, as well as a large amount of the 68-kDa band (C3 fragment characteristic of iC3b). Both of them also showed a band of 46 kDa, and strain A450-3 also showed a band of 43 kDa; all of these bands corresponded to different C3 degradation fragments. It seems clear, then, that both serum-resistant strains are able to degrade the deposited C3b to iC3b and to continue the C3b degradation. At 20 min, no practically C3b is observed.

However, the serum-sensitive strain A450-1 (Fig. 6A) did not show any decrease on the 105-kDa band over time; moreover, the relative amount of the 68-kDa band was rather inferior to those observed for the serum-resistant strains (Fig. 6B and C). These results suggested that in strain A450-1, some C3b degradation (to iC3b and more [see also the 46-kDa band for strain A450-1]) occurred, but to a lesser extent than in the serum-resistant strains, and also that bound C3b (105-kDa band) was still present at 20 min (without showing any reduction over time).

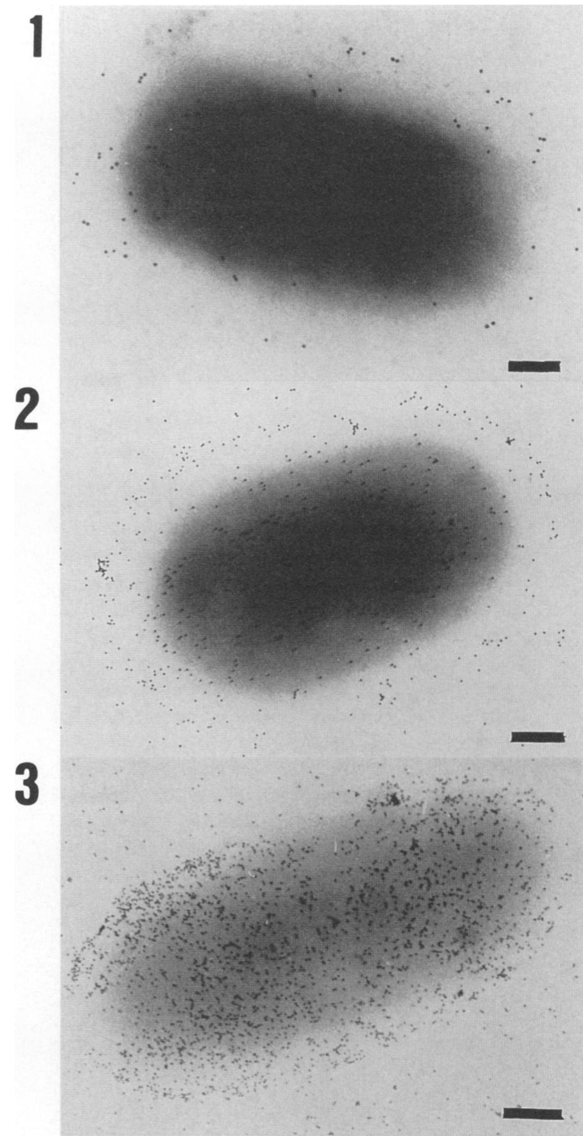

FIG. 4. Immunogold electron microscopy of *A. salmonicida* strains preopsonized with NIS, incubated with anti-C3b, and labeled with protein A–14-nm gold spheres. (1) Strain A450 (O^+:A^+) (serum resistant); (2) strain A450-3 (O^+:A^-) (serum resistant); (3) strain A450-1 (O^-:A^-) (serum sensitive). Bars, 200 nm.

TABLE 4. Interaction of complement components C3b, C5b, and C5b-9 with *A. salmonicida* whole cells

Strain	Relative concn (mean ± SD) of[a]:		
	C3b	C5b	C5b-9
A450 (O^+:A^+)	0.28 ± 0.06	0.08 ± 0.04	0.09 ± 0.03
A450-3 (O^+:A^-)	0.71 ± 0.09	0.13 ± 0.03	0.12 ± 0.04
A450-1 (O^-:A^-)	1.72 ± 0.18	1.51 ± 0.19	1.49 ± 0.15
A449 (O^+:A^+)	0.30 ± 0.03	0.09 ± 0.03	0.08 ± 0.04
A449-3 (O^+:A^-)	0.69 ± 0.07	0.11 ± 0.04	0.10 ± 0.05
A449-3R (O^-:A^-)	1.69 ± 0.16	1.56 ± 0.16	1.52 ± 0.13

[a] Results are given in arbitrary A_{405} units from ELISAs done in triplicate at least twice. When control cells were incubated in the absence of specific antibodies, the concentrations of C3b, C5b, and C5b-9 were always <0.1 A_{405} unit.

DISCUSSION

The bactericidal effects of immune sera and NIS are mediated by activated components of the CPC or APC (24, 31). Activation of either can lead to membrane damage culminating in cell death (35). Our study of the mechanism of complement activation by *A. salmonicida* strains indicates that the CPC is involved in serum killing of serum-sensitive strains. Selective inhibition of the CPC by treatment of serum with Mg^{2+}-EGTA abolished serum bactericidal activity. Sera treated with inulin or heated at 50°C for 20 min, which causes depletion of the APC, were bactericidal. Thus, the APC is

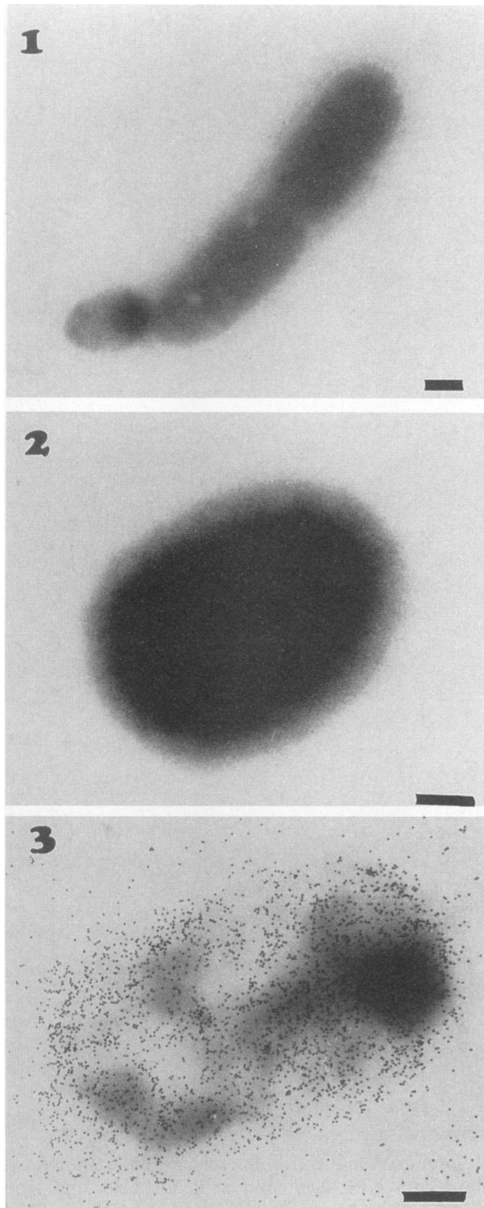

FIG. 5. Immunogold electron microscopy of *A. salmonicida* strains preopsonized with NIS, incubated with anti-C5b-9, and labeled with protein A–14-nm gold spheres. (1) Strain A450; (2) strain A450-3; (3) strain A450-1. Bars, 200 nm.

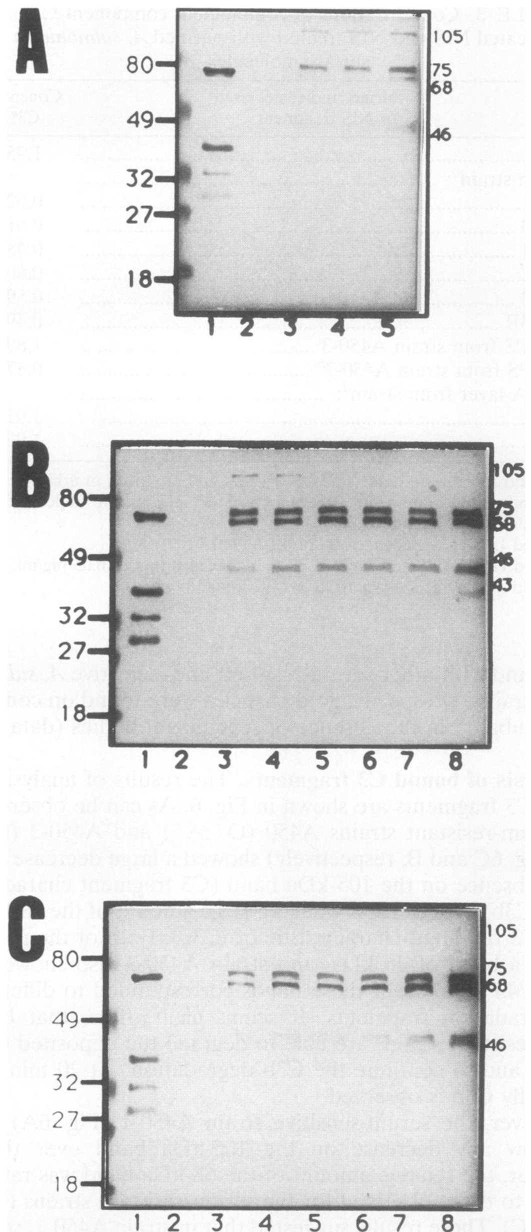

FIG. 6. Degradation pattern of C3 fragments released from serum-sensitive strain A450-1 (A) after NIS opsonization for 0, 5, 10, and 20 min (lanes 2 to 5, respectively); serum-resistant strains A450-3 (B) and A450 (C) after NIS opsonization for 0, 5, 10, 20, 30, 60, and 90 min (lanes 2 to 8, respectively). Lane 1 is the control of C3d (33-kDa band), C3c (43- and 27-kDa bands), and the band common to C3b and iC3b (75 kDa), as described in Materials and Methods. The presence of the 105-kDa band is characteristic of C3b, and the presence of the 68-kDa band is characteristic of iC3b.

probably not involved in the serum killing of serum-sensitive *A. salmonicida* strains. Other gram-negative bacteria, such as *Haemophilus influenzae* (27), *Salmonella* spp. (37), *Escherichia coli* (37), and *Klebsiella pneumoniae* (4), are known to activate both complement pathways; however, *Neisseria gonorrhoeae* (30), *Pseudomonas aeruginosa* (29), *Haemophilus ducreyi* (23), and *A. hydrophila* serotype O:34 (19) mainly activate the CPC. In addition, serum-sensitive *A. salmonicida* strains seem to activate the CPC in an antibody-independent manner, because whole cells or purified outer membranes are able to bind purified C1q (data not shown), as is the case for other bacteria (28) and, for instance, for *K. pneumoniae* as previously described by us (1).

Bacterial resistance to complement-mediated killing may be due to either of two main factors: (i) a complete or nearly complete inability to activate complement or (ii) a failure of activated complement to exert its effect (35). We clearly demonstrated that all of the *A. salmonicida* strains tested (including the A^+ strains) were able to activate complement, and the resistance to complement-mediated killing of the serum-resistant strains should be explained by the second factor. We clearly showed that these strains were able to

activate complement by measuring the inhibition of complement-mediated hemolysis of sensitized sheep erythrocytes or directly measuring C1q or C3 complement component depletion, which also occurs with their purified LPSs. It was also clear that purified A-layers from different wild-type strains were unable to activate complement. Therefore, it seems clear that although the A-layer covers most of the bacterial surface, some LPS molecules should be exposed at the bacterial surface. Furthermore, HMW-LPS (a fraction highly enriched in O antigen) is unable to activate complement, while LMW-LPS (a fraction containing lipid A and core LPS) is able to do so, as has been shown by different techniques (for instance, directly measuring C3 complement component depletion).

It has been postulated that some smooth enteric *Salmonella* and *Klebsiella* strains, which are serum-resistant organisms, fix C3b to the longest O-polysaccharide side chains of the LPS, preventing the formation of C5b-9 or the insertion of these side chains into the critical sites of the bacterial cell membranes, which causes membrane damage and cell death (15, 18).

Our study clearly shows that cells of *A. salmonicida* serum-resistant strains (O^+:A^+ or O^+:A^-) are able to bind C3b but are unable to form C5b or C5b-9. These cells, which have smooth LPS and are serum resistant, bind less C3b than cells of serum-sensitive strains (rough LPS devoid of O antigen), and the A^+ cells seem to bind less C3b than the A^- cells (always O^+). In addition, it seems by immunogold electron microscopy that C3b deposition on serum-resistant cells may be further from the cell membrane than it is in serum-sensitive cells and quantitatively different between A^+ and A^- cells that always have smooth LPS. Furthermore, these serum-resistant strains rapidly degraded C3b to iC3b and other C3 degradation fragments, and no bound C5b could be detected on these cells because no C3b was found. All of these findings suggested to us that C3b may be bound to the O-antigen polysaccharide units of LPS on smooth strains (the most external part of the LPS molecule, a nonactivating part of the LPS that rapidly degraded the bound C3b), and clearly no C5b was bound because no C3b was conserved. Our study clearly shows that no C5b or C5b-9 was formed when C3b was bound to smooth (serum-resistant) strains (Table 4 and Fig. 5 for C5b-9). Serum-sensitive strains bound more C3b than serum-resistant strains, and in addition this bound C3b was only partially degraded to iC3b and other C3 degradation fragments; however, some of the C3b was not degraded in the serum-sensitive strains (perhaps because it is bound more closely to the cell membrane). Because not all of the C3b was degraded, a large amount of C5b and C5b-9 could be easily observed on the cell membrane of the serum-sensitive strains (rough LPS).

We suggest that the difference in the amount of C3b bound among A^+ and A^- cells (always with smooth LPS) may be due to the fact that C3b binds to the LPS but not to the A-layer. Therefore, the A^+ strains would escape opsonization more effectively than the A^- strains, providing one of the possible pathogenic characteristics of this A-layer. We have thus clearly explained the defect of activated complement that renders *A. salmonicida* cells with smooth LPS resistant to complement-mediated killing.

This study has more closely defined the role of the different *A. salmonicida* surface molecules in the resistance of this bacterium to complement and provides additional evidence of the structural distribution of the LPS and the A-layer onto the bacterial surface.

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