

## Mesophilic *Aeromonas* sp. Serogroup O:11 Resistance to Complement-Mediated Killing

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**The complement activation by and resistance to complement-mediated killing of *Aeromonas* sp. strains from serogroup O:11 were investigated by using different wild-type strains (with an S-layer characteristic of this serogroup) and their isogenic mutants characterized for their surface components (S-layer and lipopolysaccharide [LPS]). All of the *Aeromonas* sp. serogroup O:11 wild-type strains are unable to activate complement, which suggested that the S-layer completely covered the LPS molecules. We found that the classical complement pathway is involved in serum killing of susceptible *Aeromonas* sp. mutant strains of serogroup O11, while the alternative complement pathway seems not to be involved, and that the complement activation seems to be independent of antibody. The smooth mutant strains devoid of the S-layer (S-layer isogenic mutants) or isogenic LPS mutant strains with a complete or rather complete LPS core (also without the S-layer) are able to activate complement but are resistant to complement-mediated killing. The reasons for this resistance are that C3b is rapidly degraded, and therefore the lytic membrane attack complex (C5b-9) is not formed. Isogenic LPS rough mutants with an incomplete LPS core are serum sensitive because they bind more C3b than the resistant strains, the C3b is not completely degraded, and therefore the lytic complex (C5b-9) is formed.**

Mesophilic aeromonads are increasingly being reported as important pathogens of humans and lower vertebrates, including amphibians, reptiles, and fish (14). Infections produced by mesophilic aeromonads in humans can be classified into two major groups, i.e., noninvasive disease, such as gastroenteritis, and systemic illnesses (12). Recently, a group of virulent *Aeromonas hydrophila* and *Aeromonas veronii* strains isolated from humans (13) and fish (16) have been described; these strains are serologically related by their O-antigen lipopolysaccharide (LPS) (serogroup O:11), with a known chemical structure (40), and by having a surface array protein with a molecular weight of ca. 52,000 (termed the S-layer) (4, 5, 28, 32). The strains from this serogroup are the most frequently isolated from septicemia caused by mesophilic *Aeromonas* spp. (15).

The complement system plays a crucial role in the humoral defense against microbial pathogens and has been extensively reviewed (43). This group of serum proteins, which are sequentially activated, produces two major effects in terms of the host defense: 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) 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 (the classical pathway of complement [CPC] and the alternative pathway of complement [APC]), 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 LPS, outer membrane (OM) proteins, and capsules (11, 29, 42), have been identified. A recent study on *A. salmonicida* strains published by us established the role of the S-layer, O-antigen LPS, and LPS core in complement activation and resistance (19).

In this study we obtained both isogenic mutants devoid of the S-layer with complete LPS and isogenic LPS mutants (some lacking the O-antigen LPS and others LPS core mutants). By using these isogenic mutants, we investigated the mechanism of complement activation by *A. hydrophila* strains of serogroup O:11, as well as the roles of LPS and the S-layer in the susceptibility of these strains to the bactericidal activity of nonimmune serum (NIS). Furthermore, we also investigated the roles of high-molecular-weight LPS (HMW-LPS) (O-antigen-enriched fraction) and low-molecular-weight LPS (LMW-LPS) (core and lipid A fraction) isolated by LPS fractionation.

### MATERIALS AND METHODS

**Bacterial strains.** The bacterial strains used in this study are listed in Table 1. *A. hydrophila* TF7 (5) and ATCC 9071 and *A. veronii* AS-28 (16) from serogroup O:11 were previously characterized. Cultures were maintained and grown as previously described (23). Isogenic mutants with mutations in surface components (S-layer and LPS) from the wild-type strains were obtained after diethyl sulfate mutagenesis and double immunoscreening with anti-S-layer and anti-O:11 LPS sera. Colonies negative in screening with anti-S-layer serum or anti-O:11 LPS serum were rescreened with the same serum and assayed by different techniques: by LPS gels (in order to determine the presence or absence of the O-antigen LPS, as well as the relative moiety of the LPS core), by Western blotting (immunoblotting) with antiserum against the S-protein (presence or absence of the S-protein), and finally by electron microscopy (EM) (negative-staining of immuno-EM with antiserum against the S-protein or antiserum against LPS) (in order to determine the presence or the absence of the S-layer).

**LPS and S-layer isolation.** LPS from mesophilic *Aeromonas* sp. serogroup O:11 strains was purified by the method of Westphal and Jann (51) as modified

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TABLE 1. Strains of *Aeromonas* sp. serogroup O:11 used and some of their relevant characteristics

Strain <sup>a</sup>	S-layer	HMW-LPS	LMW-LPS	Serum resistance
TF7	+	+	+	R <sup>b</sup>
AH-45	-	+	+	R
AH-21 <sup>c</sup>	-	-	+	R
AH-24	-	-	+	S
AH-26	-	-	+	S
AH-27	-	-	+	R
ATCC 9071	+	+	+	R
AH-48	-	+	+	R
AS-28	+	+	+	R
AH-50	-	+	+	R

<sup>a</sup> Strains AH-45, AH-48, and AH-50 are isogenic mutants, devoid of the S-layer, selected from wild-type strains TF7, ATCC 9071, and AS-28, respectively. Strains AH-21, AH-24, and AH-26 are LPS mutants selected from TF7. Strain AH-27 is a spontaneous serum-resistant mutant selected from AH-26.

<sup>b</sup> R, resistant; S, sensitive.

<sup>c</sup> Strain AH-21 produces the S-protein but is unable to assemble the S-layer (S<sup>-</sup>).

by Osborn (31). Purified S-layers were isolated from different mesophilic *Aeromonas* sp. strains of serogroup O:11 (S<sup>+</sup>) as previously described (4).

**Subfractionation of LPS by column chromatography.** Lyophilized LPS from *A. hydrophila* AH-45 (serum resistant, O<sup>+</sup>:S<sup>-</sup>) 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 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 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.

The presence of LPS in column fractions was monitored by the determination of total carbohydrates and 3-deoxy-D-manno-octulosonic acid (Kdo) and by SDS-PAGE. Fractions containing HMW-LPS showed a high ratio of total carbohydrates to Kdo (15:1), and fractions containing LMW-LPS showed a low ratio of total carbohydrates to Kdo (2:1) as can be expected from the fact that these fractions are free of O-antigen LPS.

**Electrophoretic techniques.** SDS-PAGE was performed by the procedure of Laemmli (17). Samples were mixed 1:1 with sample buffer (containing 4% SDS) and boiled for 5 min, and 10- $\mu$ l portions were applied to the gel. LPS bands were detected by the silver stain method of Tsai and Frasch (49).

**Analytical procedures.** Total carbohydrates were measured by the phenol procedure (10) with glucose as a standard. Kdo was measured by the thiobarbituric acid method after hydrolysis of samples in 4 M HCl for 30 min (15). Monosaccharides were also analyzed to their alditol acetate derivatives by gas-liquid chromatography on a 3% SP-3840 column (Supelco) as previously described (47).

**Antisera.** Anti-O:11 LPS serum was obtained and assayed as previously described for other LPSs (25). Anti-purified-S-layer antiserum was obtained and assayed as previously described by us (23).

**Western immunoblotting.** After SDS-PAGE, immunoblotting was carried out by transfer to polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.) at 1.3 A for 1 h in the buffer of Towbin et al. (48). The membranes were then incubated sequentially with 1% bovine serum albumin (BSA), anti-S-layer serum (1:500) or anti-O:11 LPS serum (1:100), alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (IgG), and 5-bromo-4-chloro-3-indolylphosphate disodium-nitroblue tetrazolium (BCIP-NBT) (2). Incubations were carried out for 1 h, and washing steps with 0.05% Tween 20 in phosphate-buffered saline (PBS) were included after each incubation step.

**Immunoscreening of colonies.** After growth of the colonies, a nitrocellulose filter (Millipore Corp.) was placed on the surface of the plate for 5 min, air dried, and blocked with 1% BSA. The immunological procedure was identical to that described for the Western immunoblotting, and the positive colonies gave a dark violet color. Replica plating was previously performed in order to recover the colonies.

**EM.** A solution containing 2% paraformaldehyde and 0.5% glutaraldehyde in 0.075 M sodium cacodylate was cooled at 4°C. Specimens were dissected into pieces of <1 mm<sup>3</sup>, fixed for 1 h at 4°C, and washed overnight in 0.075 M sodium cacodylate-0.2 M sucrose at 4°C. The sample was dehydrated at room temperature with a graded series of ethanol up to 95% before infiltration overnight with LR White resin (Agar Scientific). The sample was embedded in fresh resin in

gelatin capsules and polymerized by incubation at 55°C for 24 h. Ultrathin, 90-nm-thick sections were prepared and mounted on Formvar-coated gold grids. Sections were negatively stained as previously described by us (23). Immuno-EM on sections with specific anti-S-layer serum was performed as described by McPhail et al. (18).

Immuno-EM of whole cells was performed with anti-O:11 LPS serum and anti-S-layer serum as previously described by us (21).

**Bacterial survival in fresh NIS.** The survival of exponential-phase bacteria in fresh NIS was measured as previously described (44). NISs were obtained from a pool of healthy animals (rainbow trout or rabbits) or healthy human volunteers. Human NIS diluted 1/100 did not react with whole cells of *A. hydrophila* AH-24 or AH-26 or with purified OM proteins of the same strains in enzyme-linked immunosorbent assay (ELISA) or immunoblot experiments. Control measurements with bacteria in PBS (containing 0.15 M sodium chloride and 0.15 M sodium phosphate, pH 7.2) or heat-inactivated (56°C for 30 min) NIS were performed. Serum-resistant mutants were obtained as previously described (24).

**Treatment of serum.** CPC activity in serum was selectively inhibited by chelation with 20 mM ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) plus 2 mM MgCl<sub>2</sub> as previously described (7). Serum was treated with inulin (2 mg/ml) by the method of Gotze and Muller-Eberhard (9) or heated at 50°C for 20 min to inactivate factor B, which is required for APC activity in serum (6). After each serum treatment, we assayed the anticomplement activity of the treated serum by the method of Morrison and Kline (27) as modified in a microtiter assay by Vukajlovich et al. (50) to be sure that the treatment was correct. Both pathways were inhibited either by treatment of serum with 20 mM EDTA or by heating the serum at 56°C for 30 min. EGTA, EDTA, and inulin at the concentrations mentioned above had no effect on the survival of mesophilic *Aeromonas* sp. serogroup O:11 strains in PBS during 3 h of incubation.

Agammaglobulinemic (agamma) serum was obtained from patients receiving chemotherapy for non-Hodgkin's lymphoma, depleted of C1q, and reconstituted with purified C1q, as we previously described (1). This serum contained 0.38, 0.07, and 0.08 mg of IgG, IgM, and IgA, respectively, per ml; the total content of proteins in this serum was 57.40 mg/ml; and the undiluted serum was completely unable to cross-react with either whole cells or purified OM proteins from serum-sensitive strains of *A. hydrophila* serogroup O:11 (AH-24 and AH-26) in ELISA or immunoblot experiments.

**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) **Whole cells.** NIS (1.5 ml) was incubated at 37°C for 1 h with 10<sup>8</sup> cells and centrifuged for 5 min in an Eppendorf microfuge. The supernatant was filtered through a 0.45- $\mu$ m-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  $\times$  10<sup>7</sup> CFU) of *A. hydrophila* AH-26 (serum sensitive) or a similar strain and incubated at 37°C for 3 h. Samples were taken hourly, and bacterial concentrations were determined by dilution plating.

(ii) **Purified or fractionated LPS.** Complete LPS or fractionated LPS was suspended in PBS to a final concentration of 1 to 5 mg/ml and briefly sonicated at 4°C until the solution cleared. LPS solutions in the concentration range of 0.01 to 0.2 mg/ml were added to 1 ml of NIS and incubated for 30 min at 37°C. After this treatment, the bactericidal activity of the serum was determined with strain AH-26 or a similar strain as described above.

(iii) **Purified S-layer.** Purified S-layer was suspended in PBS at a final concentration of 5 mg/ml and 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 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 S-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 S-layer was measured by the method of Shafer et al. (39). 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 (21). Briefly, specific anti-C1q or anti-C3 antisera (Sigma) were applied to a microtiter plate, incubated overnight at 4°C, washed, and incubated for 1 h at 37°C with 1% BSA. Meanwhile, NIS was treated with *Aeromonas* sp. serogroup O:11 whole cells, purified LPS (complete or fractionated), or purified S-layer for 30 min at 37°C. Untreated NIS was used as a standard with the same incubation period. After the plates were washed, the treated or untreated NIS was added and 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 were washed, the color reaction was developed with 4-nitrophenyl phosphate (1 mg/ml) and the A<sub>405</sub> was recorded.

**Binding of C3b, C5b, and C5b-9 to whole cells.** The interaction between *Aeromonas* sp. serogroup O:11 whole cells and complement components C3b, C5b, and C5b-9 was quantified by an enzyme immunoassay as previously described by us (19). Briefly, 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 (but able to react with iC3b, C3c, and C3d), anti-C5b, or anti-C5b-9 (Calbiochem) (1:100 dilution in PBS plus 1% BSA), and washed again by microcentrifugation. Next, the bacteria were incubated with protein A-alkaline phosphatase (1:1,000 dilution in PBS) at 37°C for 45 min, and after washing, the color reaction was developed as described above and the  $A_{405}$  was recorded. Controls consisted of cells treated with protein A-alkaline phosphatase in the absence of specific antibodies.

**Analysis of bound C3 fragments.** An *Aeromonas* sp. serogroup O:11 cell suspension ( $2 \times 10^8$  CFU/ml) was opsonized with NIS diluted in PBS (final concentration, 25%). Opsonization was carried out at 37°C for 0 to 90 min, and the reaction was stopped by adding ice-cold PBS. Serum-sensitive strains were opsonized for only 0 to 20 min. The tubes were centrifuged, and the pellets were washed three times with PBS. The pellet were resuspended in 1 M hydroxylamine in 50 mM carbonate-bicarbonate buffer (pH 9.0) to disrupt ester bonds between the complement fragments and the bacterial surface (8). 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 (8). 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 (48), and the membranes were blocked overnight with PBS plus 1% BSA. After being washed, 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 (2). Two purified preparations of C3 (kindly provided by F. Vivanco, Fundación Jimenez Diaz, 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, were used as controls. The Western blots were scanned with a Bio-Image densitometer (Millipore), as we previously described (20).

## RESULTS

**Isolation and characterization of mutants.** After diethyl sulfate mutagenesis of the *Aeromonas* sp. serogroup O:11 wild-type strains (TF7, ATCC 9071, and AS-28;  $O^+ : S^+$ ), colonies were immunoscreened with anti-S-layer serum and anti-O:11 LPS serum. Some of these colonies (strains AH-45, AH-48, and AH-50, derived, respectively, from TF7, ATCC 9071, and AS-28) were positive with anti-O:11 LPS serum and negative with anti-S-layer serum. These strains showed LPS profiles identical to those of their wild-type strains, their purified OMs were unable to react with anti-S-layer serum in Western blotting, EM sections negatively stained or treated with anti-S-layer serum linked to gold particles showed a lack of the S-layer, immuno-EM of whole cells with both antisera confirmed the immunoscreening results, and the purified LPSs showed chemical compositions identical to those of the LPSs purified from the wild-type strains (Fig. 1 to 3 and Table 2 show the data for TF7 and mutant strain AH-45). We characterized these strains as  $O^+ : S^-$ . They also were sensitive to bacteriophage PM2 (22), while their respective wild-type strains were resistant.

We also obtained colonies unable to react with anti-O:11 LPS serum after mutagenesis. We characterized those from strain TF7 as isogenic LPS mutants always lacking the O:11 antigen LPS ( $O^-$ ) (strains AH-21, AH-24, and AH-26) (Fig. 1A). Strain AH-21 is an LPS mutant with a complete or rather complete LPS core, showing an LPS lacking only hexosamines and mannose in comparison with the LPS from wild-type strain TF7 (Table 2). In the purified OM of this strain, some S-protein could be detected by Western blotting (Fig. 1B), and some patches of S-protein but not a regular S-layer surface could be detected by immuno-EM of sections with anti-S-layer serum ( $S^-$ ) (data not shown). Strain AH-24 is an LPS core mutant because its purified LPS showed a lack of galactose in comparison with purified LPS of strain AH-21, indicating an incomplete LPS core (Fig. 1 and Table 2). Strain AH-26 is an LPS core mutant, probably a deep rough mutant, because a reduction in the glucose content of its purified LPS is observed in comparison with the purified LPS of strain AH-24 (Fig. 1 and Table 2). No S-protein or S-layer could be detected for

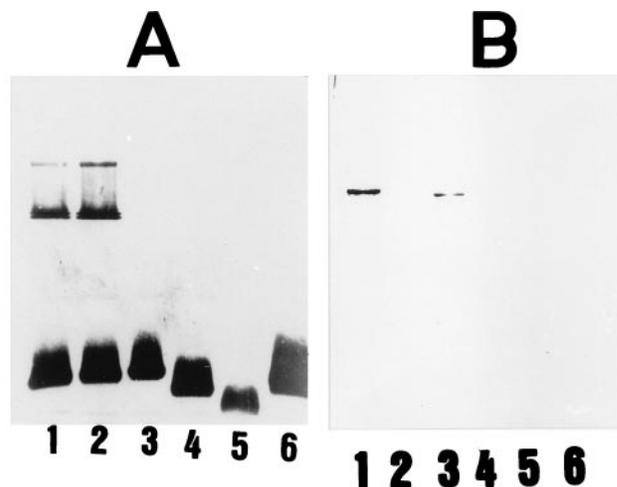


FIG. 1. (A) SDS-PAGE of LPSs from *Aeromonas* sp. strains of serogroup O:11 (wild-type TF7 and isogenic mutants). (B) Western blot analysis of purified OMs of *A. hydrophila* wild-type strain TF7 (serogroup O:11) and isogenic mutants with anti-S-layer antiserum as described in Materials and Methods. Lanes: 1, strain TF7; 2, strain AH-45 ( $O^+ : S^-$ ); 3, strain AH-21 ( $O^- : S^-$ ); 4, strain AH-24 ( $O^- : S^-$ ); 5, strain AH-26 ( $O^- : S^-$ ); and 6, strain AH-27 ( $O^- : S^-$ ). Strain AH-27 is able to synthesize the S-protein but unable to assemble the S-layer ( $S^-$ ).

LPS mutants AH-24 and AH-26 by any method described ( $O^- : S^-$ ).

*Aeromonas* sp. strains of serogroup O:11 were tested for their ability to resist the bactericidal activity of NISs from different sources (rabbits, trout, and humans). The results obtained for rabbit and trout sera were similar to the ones shown for human serum (Fig. 4).  $O^+ : S^+$  and  $O^+ : S^-$  strains were resistant to human, rabbit, and trout NISs.  $O^- : S^-$  strains showed resistance or sensitivity to NIS depending on their LPS core.  $O^-$  strains with a complete or rather complete LPS core, like strain AH-21, were resistant to NIS, while  $O^-$  strains, like AH-24 and AH-26, were sensitive to NIS; the deep rough mutant (AH-26) was the most sensitive to NIS (Fig. 4). Mutants selected by serum resistance from AH-26 (like strain AH-27) showed a purified LPS similar to that of strain AH-21 (Fig. 1 and Table 2), no S-protein by Western blotting (Fig. 1), and no S-layer by EM (data not shown). Thus, strain AH-27 seems to recover part of the LPS core lost from mutant strain AH-26 after selection by serum resistance. All of the following experiments on complement-mediated killing were done with human NIS.

**Mechanism of complement activation by *A. salmonicida* strains.** The mechanism of complement activation by serum-sensitive *A. hydrophila* strains (serogroup O:11) was examined. These strains were rapidly killed in untreated serum. Serum treated with  $Mg^{2+}$ -EGTA (which selectively inhibits the classical complement pathway) was nonbactericidal for strain AH-26, while serum pretreated with inulin or heated at 50°C for 20 min (which depletes the alternative complement pathway) was bactericidal for the same strain (Fig. 5). However, serum treated with EDTA or heated at 56°C for 30 min (which inhibits both complement pathways) was nonbactericidal for strain AH-26. Similar results were obtained with other serum-sensitive *A. hydrophila* mutant strains (data not shown). Furthermore, these strains were killed by complement factor B-deficient serum (Sigma) but not by complement component C1q-deficient serum (Sigma). These results suggested that serum killing of strains of serogroup O:11 is mediated by the classical complement pathway. Also, agamma serum depleted of C1q

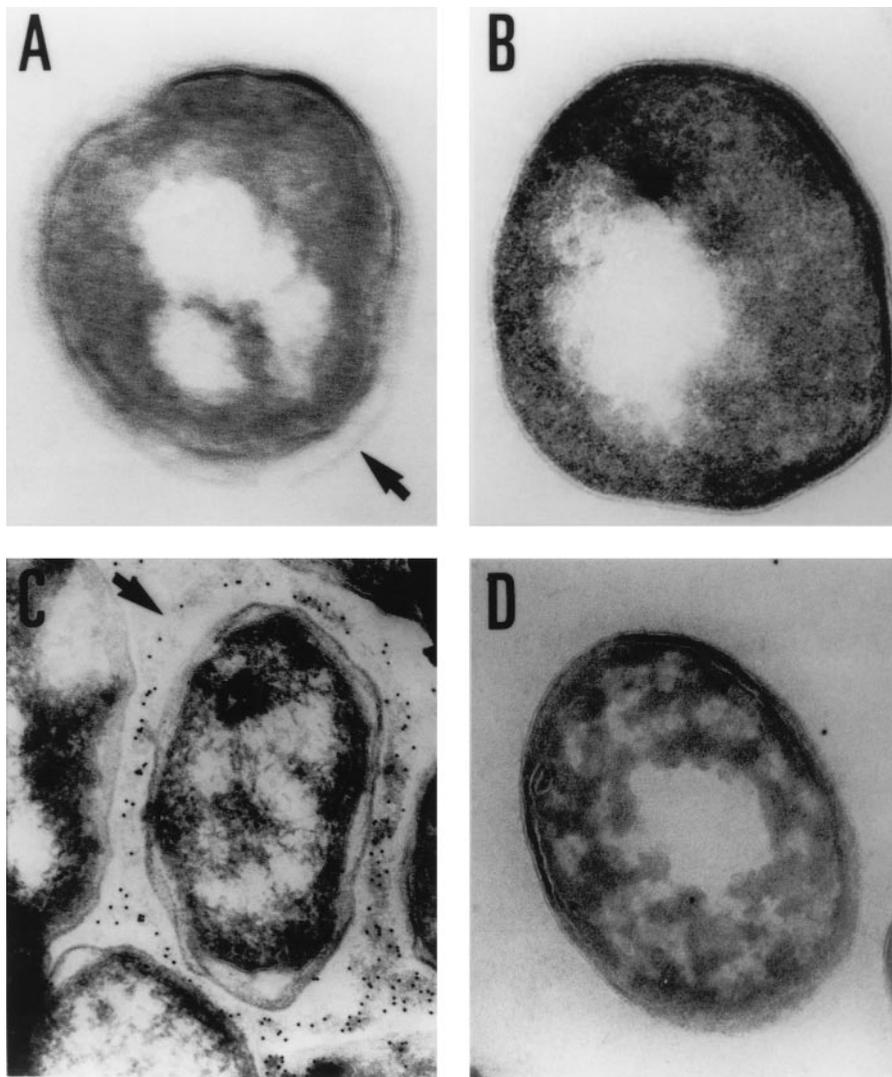


FIG. 2. (A and B) Negatively stained EM sections of *A. hydrophila* TF7 ( $O^+:S^+$ ) (A) and the isogenic mutant AH-45 ( $O^+:S^-$ ) (B). (C and D) Sections of strain TF7 (C) and the isogenic mutant AH-45 (D) immunolabelled with anti-S-layer serum linked to protein A-gold particles (10 nm). Bar, 0.2  $\mu\text{m}$ ; arrows, S-layer.

and reconstituted with purified C1q, as we previously described (1), was incubated with purified OM from strain AH-26. A dose-dependent reduction of the total hemolytic activity of this serum was observed, reaching a 50% reduction after incubation with approximately 2  $\mu\text{g}$  of purified OM from strain AH-26. This reduction of the total hemolytic activity in this reconstituted agamma serum suggested an antibody-independent activation of the CPC.

**Inhibition of serum bactericidal activity by whole cells or purified surface molecules.** Preincubation of NIS with whole cells of *Aeromonas* sp. serogroup O:11 strains ( $O^+:S^+$ ) was unable to inhibit the serum bactericidal activity, while  $O^+:S^-$  or  $O^-:S^-$  strains inhibited serum bactericidal activity when tested against serum-sensitive strain AH-24 or AH-26 (LPS core mutants without the S-layer).

Various concentrations of purified whole LPS (a mixture of O-antigen-containing and O-antigen-deficient LPS molecules) from *Aeromonas* sp. serogroup  $O^+:S^+$  or  $O^+:S^-$  strains, or various concentrations of purified LPS from *Aeromonas* sp.

serogroup  $O^-$  strains (with only O-antigen-deficient LPS molecules), inhibited the bactericidal activity of NIS in a dose-dependent manner when tested against strain AH-26 (data not shown). For instance, these purified LPSs at 0.1 mg/ml inhibited the bactericidal activity of NIS against strain AH-26. This fact prompted us to examine the interaction of HMW-LPS and LMW-LPS with NIS. HMW-LPS pooled fractions from strain AH-45 ( $O^+:S^-$ ) were unable to inhibit the bactericidal activity of NIS, except that some residual activity was found, perhaps because a minor amount of LPS core and lipid A is still present in this pooled fraction. By contrast, LMW-LPS pooled fractions from the same strain were able to completely inhibit the bactericidal activity of NIS.

Finally, purified S-layers (with LPS contamination of <1%, as determined with specific antibodies and gels) were obtained from strains TF7, ATCC 9071, and AS-28. None of these S-layers, even at high concentrations (0.2 or 0.4 mg/ml), were able to inhibit the bactericidal activity of NIS when tested against the serum-sensitive strains.

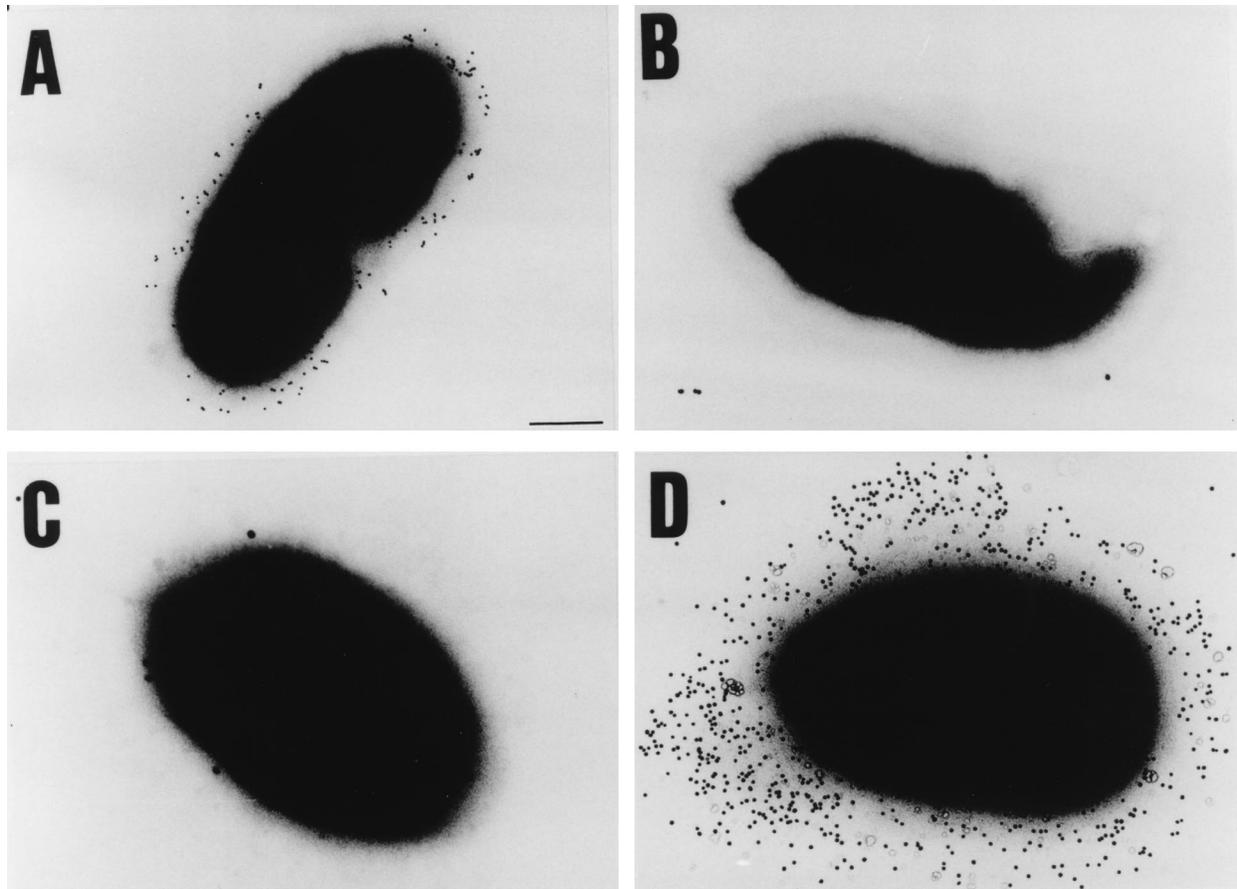


FIG. 3. Immuno-EM of whole cells of *A. hydrophila* TF7 (O<sup>+</sup>:S<sup>+</sup>) with anti-S-layer serum (A) and with anti-O:11 LPS serum (B) and of the isogenic mutant AH-45 (O<sup>+</sup>:S<sup>-</sup>) with anti-S-layer serum (C) and with anti-O:11 LPS serum (D). Bar, 0.4  $\mu$ m.

**Complement consumption by whole cells and purified surface molecules.** The complement-absorbing activity of whole cells and purified surface molecules was measured to determine whether inhibition of serum bactericidal activity was due to the depletion of serum complement. Whole cells of O<sup>+</sup>:S<sup>-</sup> and O<sup>-</sup>:S<sup>-</sup> strains inhibited complement-mediated hemolysis of sensitized sheep erythrocytes, while whole cells of O<sup>+</sup>:S<sup>+</sup> strains were unable to do so (Table 3). Furthermore, complement components C1q and C3 were depleted when NIS was treated with O<sup>+</sup>:S<sup>-</sup> or O<sup>-</sup>:S<sup>-</sup> whole cells; no C1q or C3 depletion was observed when NIS was treated with whole cells of O<sup>+</sup>:S<sup>+</sup> strains (Table 3).

The ability to inhibit complement-mediated hemolysis of sensitized sheep erythrocytes by purified LPSs from all of the strains was dose dependent and is shown in Table 4. Also, the C3 concentration was depleted when NIS was treated with these purified LPSs (Table 4). Furthermore, HMW-LPS pooled fractions from AH-45 (O<sup>+</sup>:S<sup>-</sup>) at the concentration examined were unable to deplete C3 from NIS, while LMW-LPS pooled fractions from the same strain were able to do so (Table 4).

When we tested purified S-layers from different O:11 strains, we found that none of them were able to inhibit the complement-mediated hemolysis of sensitized sheep erythrocytes or to deplete C3 from S-layer-treated NIS (Table 4).

**Binding of C3b, C5b, and C5b-9 to whole cells.** As shown in Table 5, whole cells of serum-resistant *Aeromonas* sp. serogroup O<sup>+</sup>:S<sup>-</sup> strains (AH-45, AH-48, and AH-50) or a

serogroup O<sup>-</sup>:S<sup>-</sup> strain (AH-21) bound less C3b than serum-sensitive O<sup>-</sup>:S<sup>-</sup> strains (AH-24 and AH-26). Also, serum-resistant mutants like AH-27 (O<sup>-</sup>:S<sup>-</sup>) were practically unable to bind C3b. Furthermore, serum-resistant strains did not bind C5b or C5b-9, while a high level of binding of these complement components (C3b, C5b, and C5b-9) was observed for the serum-sensitive strains. Strain TF7, as well as other wild-type strains (O<sup>+</sup>:S<sup>+</sup>), is unable to activate complement or, of course, to bind any of the complement components (Table 5).

**Analysis of bound C3 fragments.** The results of analysis of bound C3 fragments are shown in Table 6. As can be observed,

TABLE 2. Chemical compositions of purified LPSs from *Aeromonas* sp. strains of serogroup O:11

Strain	Content ( $\mu$ mol/mg of LPS) of <sup>a</sup> :					
	Kdo	Heptose	Glucose	Galactose	Mannose	Hexosamines
TF7	0.033	0.32	1.52	1.27	1.22	0.57
AH-45	0.034	0.31	1.55	1.29	1.24	0.60
AH-21	0.063	0.72	1.73	1.22	0.17	<0.05
AH-24	0.112	0.98	2.12	<0.01	<0.01	<0.05
AH-26	0.228	1.41	0.57	<0.01	<0.01	<0.05
AH-27	0.061	0.69	1.82	1.15	<0.01	<0.05

<sup>a</sup> Kdo was assayed by a colorimetric method (15), and the other monosaccharides were assayed by gas-liquid chromatography as described in Materials and Methods.

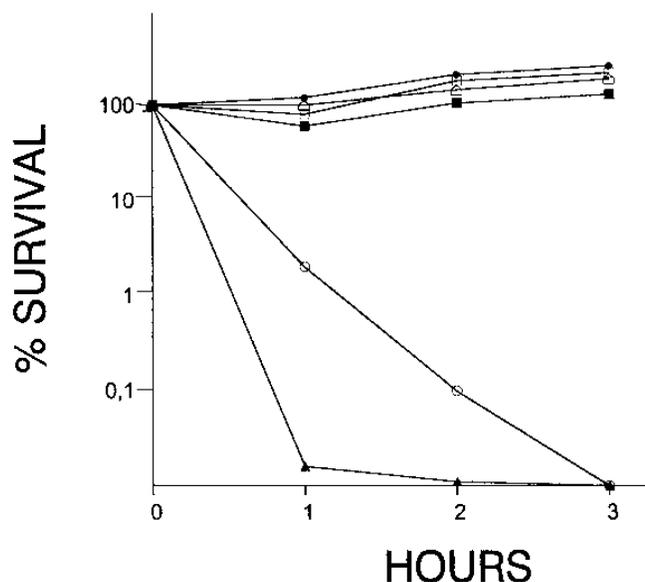


FIG. 4. Bactericidal effects of human NIS on *A. hydrophila* serogroup O:11 strains TF7 (O<sup>+</sup>:S<sup>+</sup>) (●), AH-45 (O<sup>+</sup>:S<sup>-</sup>) (□), AH-21 (O<sup>-</sup>:S<sup>-</sup>) (■), AH-24 (O<sup>-</sup>:S<sup>-</sup>) (○), AH-26 (O<sup>-</sup>:S<sup>-</sup>) (▲), and AH-27 (O<sup>-</sup>:S<sup>-</sup>) (◻).

the serum-resistant strain AH-45 (O<sup>+</sup>:S<sup>-</sup>) showed a large decrease and finally absence of the 105-kDa band (which represents C3b, the  $\alpha'$  chain of C3b [8, 34]) over time, as well as large amounts of the 68-kDa band (which represents iC3b, a degraded form of C3b [8, 34]) and the 43-kDa band (which represents C3c, another C3b degradation product [8, 19]). Similar results were observed for strain AH-21 (O<sup>-</sup>:S<sup>-</sup>, serum resistant). However, the serum-sensitive strain AH-26 (O<sup>-</sup>:S<sup>-</sup>) showed a large amount of the 105-kDa band and, by contrast, the 68- and 43-kDa bands were reduced in comparison with the

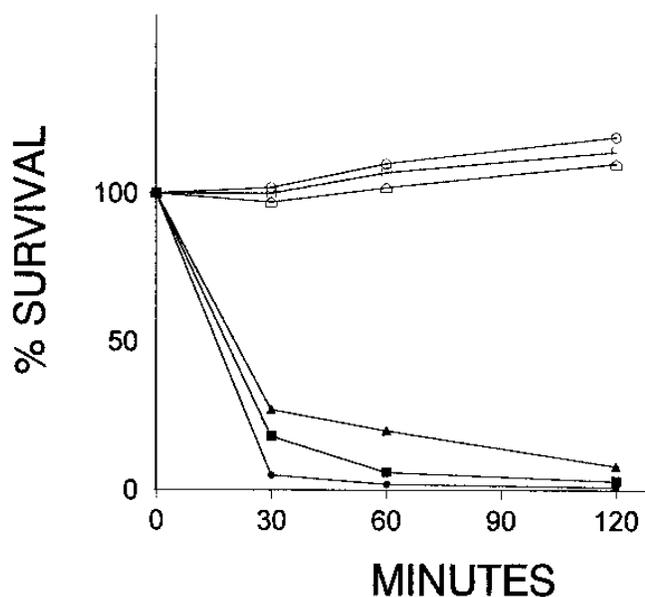


FIG. 5. Bactericidal effects of NIS on the serum-sensitive strain *A. hydrophila* AH-26. Control serum (●) was unaltered prior to the addition of cells; otherwise, NIS was pretreated as follows: 56°C for 30 min (○), 20 mM EDTA (□), 20 mM EGTA plus 2 mM Mg<sub>2</sub>Cl (◻), 2 mg of inulin per ml (▲), or 50°C for 20 min (■).

TABLE 3. Concentrations of complement components C1q and C3 in untreated NIS and NIS treated with whole *Aeromonas* sp. cells (serogroup O:11) and inhibition of complement-mediated hemolysis

Strain used for treatment	Concn <sup>a</sup> of:		% Inhibition of complement-mediated hemolysis
	C1q	C3	
None	1.31	1.95	<5
TF7 (O <sup>+</sup> :S <sup>+</sup> )	1.28	1.87	<5
ATCC9071 (O <sup>+</sup> :S <sup>+</sup> )	1.27	1.89	<5
AS-28 (O <sup>+</sup> :S <sup>+</sup> )	1.30	1.91	<5
AH-45 (O <sup>+</sup> :S <sup>-</sup> )	0.72	0.81	93
AH-48 (O <sup>+</sup> :S <sup>-</sup> )	0.71	0.89	94
AH-50 (O <sup>+</sup> :S <sup>-</sup> )	0.72	0.85	93
AH-21 (O <sup>-</sup> :S <sup>-</sup> )	0.67	0.77	94
AH-24 (O <sup>-</sup> :S <sup>-</sup> )	0.41	0.60	97
AH-26 (O <sup>-</sup> :S <sup>-</sup> )	0.38	0.55	98
AH-27 (O <sup>-</sup> :S <sup>-</sup> )	0.69	0.71	95

<sup>a</sup> Concentrations were determined by ELISA and are given in arbitrary A<sub>405</sub> units. Results are means from experiments done in triplicate at least twice. Standard deviations were all <0.1.

same bands in serum-resistant strains. No significant differences were observed for the 75-kDa band, the common band for C3b and iC3b, over the incubation time.

## DISCUSSION

The bactericidal effects of immune sera and NISs are mediated by activated components of the CPC or APC (33, 43). Activation of either pathway can lead to membrane damage culminating in cell death (43). Our study of the mechanism of

TABLE 4. Concentrations of complement component C3 in untreated NIS and NIS treated with purified *Aeromonas* sp. (serogroup O:11) surface molecules and inhibition of complement-mediated hemolysis

Surface molecules used for NIS treatment	Concn of C3 <sup>a</sup>	% Inhibition of complement-mediated hemolysis
None	1.95	<5
LPS <sup>b</sup> from strain:		
TF7 (O <sup>+</sup> :S <sup>+</sup> )	0.64	86
ATCC9071 (O <sup>+</sup> :S <sup>+</sup> )	0.69	85
AS-28 (O <sup>+</sup> :S <sup>+</sup> )	0.66	84
AH-45 (O <sup>+</sup> :S <sup>-</sup> )	0.62	86
AH-48 (O <sup>+</sup> :S <sup>-</sup> )	0.71	87
AH-50 (O <sup>+</sup> :S <sup>-</sup> )	0.65	85
AH-21 (O <sup>-</sup> :S <sup>-</sup> )	0.57	92
AH-24 (O <sup>-</sup> :S <sup>-</sup> )	0.46	97
AH-26 (O <sup>-</sup> :S <sup>-</sup> )	0.42	98
AH-27 (O <sup>-</sup> :S <sup>-</sup> )	0.53	93
HMW-LPS from strain AH-45 <sup>c</sup>	1.87	<5
LMW-LPS from strain AH-45 <sup>c</sup>	0.45	97
Purified S-layer <sup>d</sup> from strain:		
TF7	1.92	<5
ATCC9071	1.91	<5
AS-28	1.89	<5

<sup>a</sup> Concentrations were determined by ELISA and are given in arbitrary A<sub>405</sub> units. Results are means from experiments done in triplicate at least twice. Standard deviations were all <0.09.

<sup>b</sup> Purified LPS at a concentration of 0.1 mg/ml.

<sup>c</sup> Purified LPS at a concentration of 0.05 mg/ml.

<sup>d</sup> Purified S-layer at a concentration of 0.2 mg/ml.

TABLE 5. Interaction of complement components C3b, C5b, and C5b-9 with *Aeromonas* sp. serogroup O:11 whole cells

Strain	Relative concn (mean $\pm$ SD) <sup>a</sup> of:		
	C3b	C5b	C5b-9
TF7 (O <sup>+</sup> :S <sup>+</sup> ) <sup>b</sup>	<0.1	<0.1	<0.1
AH-45 (O <sup>+</sup> :S <sup>-</sup> )	0.35 $\pm$ 0.05	<0.1	<0.1
AH-48 (O <sup>+</sup> :S <sup>-</sup> )	0.38 $\pm$ 0.04	<0.1	<0.1
AH-50 (O <sup>+</sup> :S <sup>-</sup> )	0.34 $\pm$ 0.05	<0.1	<0.1
AH-21 (O <sup>-</sup> :S <sup>-</sup> )	0.42 $\pm$ 0.09	<0.1	<0.1
AH-24 (O <sup>-</sup> :S <sup>-</sup> )	1.37 $\pm$ 0.18	1.22 $\pm$ 0.13	1.25 $\pm$ 0.12
AH-26 (O <sup>-</sup> :S <sup>-</sup> )	1.72 $\pm$ 0.19	1.45 $\pm$ 0.16	1.48 $\pm$ 0.16
AH-27 (O <sup>-</sup> :S <sup>-</sup> )	0.15 $\pm$ 0.03	<0.1	<0.1

<sup>a</sup> Results are given in arbitrary  $A_{405}$  units and are 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.

<sup>b</sup> Strain TF7, like the other wild-type strains, is unable to activate complement.

complement activation by *Aeromonas* sp. serogroup O:11 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<sup>2+</sup>-EGTA abolished serum bactericidal activity. Sera treated with inulin or heated at 50°C for 20 min, which depletes them of the APC, were bactericidal. Thus, the APC is probably not involved in the serum killing of serum-sensitive *Aeromonas* sp. strains of serogroup O:11. Other gram-negative bacteria, such as *Haemophilus influenzae* (37), *Salmonella* spp. (46), *Escherichia coli* (46), and *Klebsiella pneumoniae* (3), are known to activate both complement pathways, but *Neisseria gonorrhoeae* (39), *Pseudomonas aeruginosa* (38), *Haemophilus ducreyi* (30), *A. hydrophila* serotype O:34 (24), and *A. salmonicida* (19) activate mainly the CPC. The CPC activation in NIS and agamma sera (depleted of C1q and reconstituted with purified C1q), by either whole cells or purified OMs of susceptible *A. hydrophila* serogroup O:11 strains, suggested that this is an antibody-independent phenomenon. A similar situation has been described by us for *K. pneumoniae* (1) and also by other authors for different bacteria (26, 41).

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 (43). We clearly demonstrate that all of the *Aeromonas* sp. serogroup O:11 wild-type strains tested, which have the S-layer exposed only at the cell surface, resist complement-mediated killing by impeding complement activation. It is also clear that purified S-layers from different wild-type strains were unable to activate complement. This situation is similar to that observed with the K1 capsule of *E. coli* or the capsular polysaccharide of *K. pneumoniae*, where a nonactivating complement structure impedes the exposure of the LPS (which is the complement-activating molecule) at the cellular surface (21, 36, 45).

However, *Aeromonas* sp. strains of serogroup O:11 lacking the S-layer are able to activate complement, and the resistance to complement-mediated killing of the serum-resistant strains should be explained by the second reason (failure of activated complement to exert its effect). We clearly showed that these strains (O<sup>+</sup> or O<sup>-</sup>) 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. Furthermore, HMW-LPS (a fraction high-

ly enriched in O antigen) is unable to activate complement, while LMW-LPS (a fraction containing lipid A and LPS core) is able to do so, as shown by different techniques (for instance, directly measuring C3 complement component depletion). Our study clearly shows that cells of *Aeromonas* sp. serum-resistant strains are able to bind C3b but are unable to form C5b or C5b-9. These cells, which have smooth LPS or a complete LPS core and are serum resistant, bind less C3b than cells of serum-sensitive strains (with rough LPS devoid of O antigen with incomplete LPS core) (Table 5). This fact can be easily observed for strain AH-27, selected by serum resistance from sensitive strain AH-26; strain AH-27 showed a complete LPS core similar to that of strain AH-21, while the serum-sensitive strain (AH-26) showed the most incomplete LPS core of the LPS mutants selected. Furthermore, these serum-resistant strains rapidly degraded C3b to iC3b and other C3 degradation fragments, and no bound C5b can be detected on these cells because no C3b is found (Tables 5 and 6). We have thus explained the defect of activated complement that renders the *Aeromonas* sp. serogroup O:11 cells with smooth LPS or with complete LPS core without the S-layer also resistant to complement-mediated killing.

However, the serum-sensitive strains (with incomplete LPS core) bind more C3b than the serum-resistant strains (Table 5); also, this bound C3b is only partially degraded to iC3b and other C3 degradation fragments, and some of the C3b is not degraded. Because not all of the C3b is degraded, a large amount of C5b and C5b-9 could be easily observed on the cell membrane of the serum-sensitive strains (with rough LPS) (Tables 5 and 6). The formation of C5b-9 in the critical sites of the bacterial cell membranes causes membrane damage and cell death, i.e., sensitivity to complement.

This study has more closely defined the roles of the different *Aeromonas* sp. serogroup O:11 surface molecules in the resistance of these bacteria to complement and provides additional evidence about the structural distribution of the LPS and the S-layer on the bacterial surface. The S-layer seems to completely cover the LPS molecules in these strains (no complement activation), a situation different from that in *A. salmonicida*, where some LPS molecules are also exposed at the cell surface and activate complement (19, 35).

TABLE 6. C3 fragments linked to the bacterial cell surface after NIS opsonization<sup>a</sup>

Strain	Incubation time (min)	Relative amounts of C3 fragment <sup>b</sup>			
		105 kDa	75 kDa	68 kDa	43 kDa
AH-45 (O <sup>+</sup> :S <sup>-</sup> ) (serum resistant)	5	5.2	31.6	31.8	30.7
	10	2.7	32.7	32.9	31.8
	20	<1.0	33.0	33.1	31.7
AH-21 (O <sup>-</sup> :S <sup>-</sup> ) (serum resistant)	5	6.5	29.7	30.2	31.4
	10	3.1	33.9	32.1	31.9
	20	<1.0	35.0	34.8	32.3
AH-26 (O <sup>-</sup> :S <sup>-</sup> ) (serum sensitive)	5	19.0	46.3	11.3	12.0
	10	21.1	48.5	11.0	13.4
	20	24.2	49.7	10.9	13.6

<sup>a</sup> The 105-kDa band represents C3b. The 68- and 43-kDa bands represent iC3b and C3c, respectively. The 75-kDa band is a common band for C3b and iC3b (8, 19, 34). The results are averages from at least three independent experiments.

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