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Electrophoretic and Immunochemical Analyses of the Lipopolysaccharides from Various Strains of *Aeromonas hydrophila*

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Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to analyze the lipopolysaccharides isolated from strains of *Aeromonas hydrophila* which exhibit virulence for fish and which autoaggregate during growth in static broth culture. The lipopolysaccharides contained O-polysaccharide chains of homogeneous chain length. Two of the strains produced a surface protein array, and immunofluorescence and phage-binding studies revealed that a number of these O-polysaccharide chains of homogeneous length traversed the protein array and were exposed on the cell surface. Immunochemical analyses by immunoblotting, enzyme-linked immunosorbent assay, immunofluorescence, and immunoprecipitation with both polyclonal and monoclonal antibodies revealed the presence of three epitopes on the polysaccharide moiety of this homogenous-chain-length lipopolysaccharide morphotype. One epitope was species serogroup specific and reactive by immunoblotting. This epitope was not present on the heterogeneous-chain-length O polysaccharides of non-autoaggregating strains of *A. hydrophila* examined. The second epitope was conformation dependent and cross-reactive with an epitope on the homogenous-chain-length O polysaccharides of *Aeromonas salmonicida* lipopolysaccharide. The third epitope was recognized by a monoclonal antibody and appeared to involve that region of the *A. hydrophila* and *A. salmonicida* lipopolysaccharide molecules which contained the O-polysaccharide–core oligosaccharide glycosidic linkage.

Aeromonas hydrophila is an important pathogen causing disease in humans and other animals, including fish. While many strains appear to be associated with opportunistic infections, especially in the case of salmonid fishes, one group of strains has been reported which exhibits enhanced virulence for salmonids (10). These strains exhibit serum resistance, autoaggregation, and serotypic properties which suggest that their surface structure may differ from that of other strains of *A. hydrophila*, especially with respect to lipopolysaccharide (LPS) structure.

The smooth LPS of the majority of gram-negative bacteria studied to date have had O-polysaccharide chains of heterogeneous chain length; this characteristic is exemplified by the *Enterobacteriaceae* (3, 6, 9, 11). Recently, however, an exception to this rule was reported when the LPS from the fish pathogen *Aeromonas salmonicida* was shown to contain O-polysaccharide chains of very similar length. This morphological feature was shown to be strongly conserved among strains of diverse origins, and the O-polysaccharide chains were also shown to be antigenically cross-reactive among these strains (1). These O-polysaccharide chains appear to play an important structural role with respect to the ability of virulent strains of *A. salmonicida* to produce the surface protein array known as A layer (E. E. Ishiguro, W. W. Kay, and T. J. Trust, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, D24, p. 55).

In this paper we report that the LPSs of the group of *A. hydrophila* strains with enhanced virulence for salmonids and of a number of other autoaggregating strains display distinctive morphological and antigenic properties. The LPSs of these virulent *A. hydrophila*, like those of *A. salmonicida*, contain O-polysaccharide antigens which are of very similar chain length, and in two strains this distinct O-polysaccharide morphology was associated with the production of a surface protein array. These constant-chain-length O-polysaccharides are strongly immunogenic and carry a serogroup-specific epitope, an epitope cross-reactive with an epitope on the homogeneous-chain-length O polysaccharide of *A. salmonicida*, and a binding site for an *A. salmonicida* bacteriophage. The polysaccharide moiety of the LPS of both species also carries a third epitope which binds a monoclonal antibody (MAb) raised to *A. salmonicida* LPS.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. Stock cultures were maintained at −70°C in 15% (vol/vol) glycerol-tryptic soy broth (GIBCO Diagnostics, Madison, Wisconsin). Cultures were grown on tryptic soy agar (GIBCO) at 20 or 37°C for *A. hydrophila* strains and at 20°C for *A. salmonicida*. *Salmonella typhimurium* LT2 was also grown on tryptic soy agar at 37°C.

For LPS purification, *A. hydrophila* LL1 (SJ-44) and *A. salmonicida* A438 (SJ14) were cultured in tryptic soy broth at 25°C with heavy aeration in a 25-liter fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.). The 2.5-liter inoculum for the final culture had itself been inoculated with 250 ml of a seeded inoculum. Inocula were grown for 10 h, and the final broth was grown for 18 h (*A. salmonicida*) or 24 h (*A. hydrophila*). The cells were killed with 0.3% (vol/vol) formaldehyde (18 h), collected by continuous centrifugation, and lyophilized. *A. hydrophila* TF7 was grown on tryptic soy agar at 37°C for 18 h.

Extraction of LPS. Freeze-dried cells were extracted by the aqueous phenol method of Westphal et al. (17) with two re-extractions of the combined phenol layer and cell debris. The aqueous layer and washings were combined and dialyzed for 48 h against cold tap water. The dialysate was

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TABLE 1. Strains of *Aeromonas* spp. used

Strain	Source[a]
A. hydrophila	
LL1 (SJ-44)[b]	Trout lesion, Quebec, R. Lallier
TF7[b]	Trout lesion, Quebec, R. Lallier
A80-140[b]	Aborted piglet liver, Quebec, R. Lallier
A80-160[b]	Bovine brain, Quebec, R. Lallier
A82-256[b]	Moribund trout, Quebec, R. Lallier
P77-115[b]	Otary, lung, Quebec, R. Lallier
Ah274	Sloth, septicemia, Australia, H. M. Atkinson
Ah423	Human feces, Manitoba, B. Dunsmore
Ah598	Human feces, this laboratory
Ba5	Eel, Quebec, R. Lallier
A80-199	R. Lallier, Quebec
Ah427	Tinned milk, ATCC 7966
TF2	Moribund trout, Quebec, R. Lallier
DVA9	Human feces, Australia, H. M. Atkinson
688	Human feces, Australia, H. M. Atkinson
220	Human feces, Australia, H. M. Atkinson
484	Human feces, Australia, H. M. Atkinson
355	Human feces, Australia, H. M. Atkinson
810	Human feces, Australia, H. M. Atkinson
Ah65	Trout kidney, this laboratory
A. salmonicida	
A440	Trout, ATCC 14174
A438 (SJ14)	Salmon, British Columbia, T. P. T. Evelyn strain 76/30
A438-R	Phage-resistant derivative of A438, this laboratory
A449	Trout, France, C. Michel, strain TF 36/75
A449-3	A^- derivative of A449, this laboratory
A449-3R	Phage-resistant derivative of A449-3, this laboratory
A449-TM4	Transposon TN*5*-induced LPS mutant of A449, this laboratory
A449-TM5	Transposon Tn*5*-induced LPS mutant of A449, this laboratory

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[b] Strains shown to exhibit enhanced virulence for fish (10).

concentrated by lyophilization and incubated at 37°C overnight with 0.1 mg of proteinase K (Boehringer Mannheim GmbH-Biochemica, Mannheim, Federal Republic of Germany) per ml. The LPS was again dialyzed for 24 h against cold tap water and then isolated from the dialysate by ultracentrifugation (repeated twice) at 105,000 × *g* for 3 h. The resulting sedimented gel was suspended in water and freeze-dried, giving LPS samples which were usually free of both DNA and RNA. The yield of LPS from strains LL1 and TF7 was 0.64 and 3.05%, respectively, based on dry weights. Protein content of the two LPS samples was 1.0 and 1.4%, respectively, by Folin assay, but no protein bands were detectable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) when stained by either Coomassie blue or silver.

SDS-PAGE. SDS-PAGE was carried out by the method of Laemmli (7). Outer membrane (50 μg) or whole cell mass (100 μg), solubilized in sample buffer, was stacked in 4.5% acrylamide (10 mA) and separated with 12.5% acrylamide (20 mA).

Silver staining of LPS. The LPS morphology in whole-cell lysates, outer membrane preparations, or purified LPS was determined by a modification of the procedure of Hitchcock and Brown (4). Cells were boiled in SDS-PAGE solubilization buffer for 5 min and subsequently digested with proteinase K at 60°C for 1 h with a ratio of 10 mg of bacterial cells to 1 mg of proteinase K. Cells (5 μg of original sample) or purified LPS samples (0.1 to 0.5 μg) were normally loaded onto each lane of SDS-PAGE gels. After electrophoresis, gels were stained for LPS by the silver staining procedure of Tsai and Frasch (16).

Re-electrophoresis of LPS. Following an initial SDS-PAGE separation with duplicate gels, one gel was fixed and rapidly silver stained for LPS. The duplicate unfixed gel was aligned with the stained gel, and areas containing the bands of interest were excised from the unfixed gel and placed in the wells of a fresh gel. After the addition of 20 μl of SDS-PAGE solubilization buffer, the samples were subjected to electrophoresis, and the gel was silver stained for LPS.

Polyclonal antisera. Adult New Zealand White rabbits were immunized with 50 μg of phenol-extracted LPS in Freund complete adjuvant. Identical booster doses were given 14 and 28 days after initial immunization. On day 42, the rabbits were bled, and the serum was either collected and stored at −20°C in 100-μl aliquots or lyophilized. Preimmune sera were used as control sera.

MAb to *A. salmonicida* LPS. MAb iiC_5 to *A. salmonicida* LPS was prepared as previously described (1, 12) and was used as a component of mouse ascites fluid.

Absorption of antisera. Cells were grown overnight on tryptic soy agar and harvested into cold (4°C) phosphate-buffered saline (pH 7.4). The cells were washed twice at 12,000 × *g* with phosphate-buffered saline, 10 mg of the cell pellet was suspended in 1 ml of antiserum, and the suspension was incubated at room temperature for 2 h. The cells were removed, fresh cells were added, and the incubation was repeated. The cells were finally removed by centrifugation at 12,000 × *g* for 10 min. Phenylmethylsulfonyl fluoride was added to a final concentration of 200 μg/ml.

ELISA. The enzyme-linked immunosorbent assay (ELISA) procedure was essentially that of Engvall and Perlmann (2). Antigens were tested, in triplicate, at 3 μg per well, and the developing antibody was alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse immunoglobulin (Sigma Chemical Co., St. Louis, Mo.).

Immunofluorescence. Indirect immunofluorescence antibody technique (IFAT) assays (1) with living cells employed MAb iiC_5 to *A. salmonicida* LPS and goat anti-rabbit or anti-mouse globulin conjugated to fluorescein isothiocyanate (GIBCO).

Immunoblot detection of LPS. After SDS-PAGE, separated LPS components were transferred from the slab gel to nitrocellulose paper (NCP, pore size, 0.45 μm) by the methanol-Tris glycine system described by Towbin et al. (15). Electroblotting was carried out in a Bio-Rad transblot apparatus (Bio-Rad Laboratories, Richmond, Calif.) for 3 h at 60 V. Unreacted binding sites were blocked by soaking the NCP sheet overnight in a solution of 0.25% gelatin (100 bloom; Fisher Scientific Co., Pittsburgh, Pa.) in 10 mM Tris hydrochloride (pH 7.4)–0.9% saline. The NCP was incubated with antiserum (final dilution of 10^{-3}) in Tris-saline-gelatin for 2 h at room temperature with gentle agitation. After being extensively washed, the NCP was incubated with ^{125}I-labeled *Staphylococcus aureus* protein A (Pharmacia, Inc., Piscataway, N.J.) in Tris-saline-gelatin at 5×10^5 cpm/ml. Detection of bound radiolabeled protein A was accomplished by autoradiography of washed and dried NCP sheets.

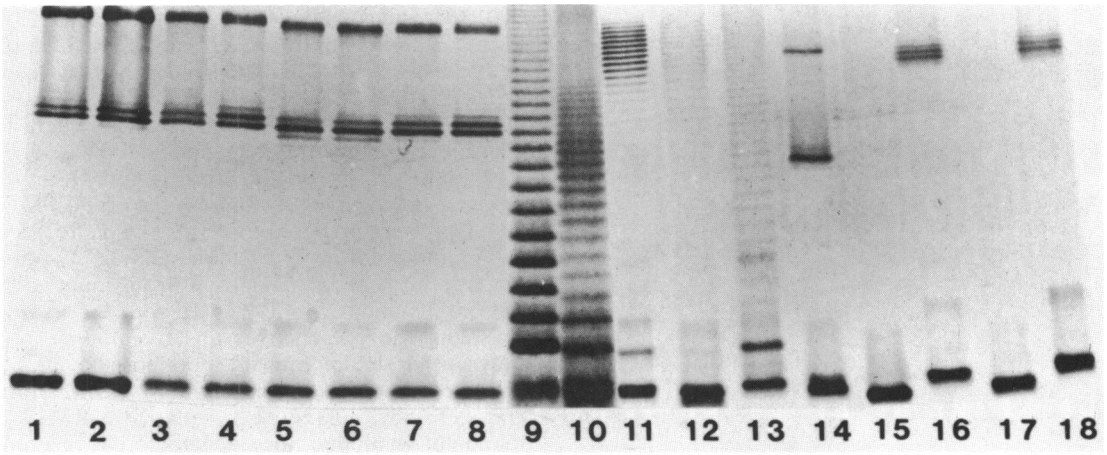


FIG. 1. Silver stain of SDS-PAGE of LPS of *A. hydrophila* (grown at 37°C) and *A. salmonicida* (grown at 20°C). Purified LPS (0.5 μg) of *A. hydrophila* TF7 (lane 1) and A80-160 (lane 2). Proteinase K-digested whole-cell lysates (10 μl) of *A. hydrophila* LL1 (lane 3), TF7 (lane 4), A80-160: (lane 5), A80-140 (lane 6), P77-115 (lane 7), A82-256 (lane 8), Ba5 (lane 9), A80-199 (lane 10), ATCC 7966 (lane 11), TF2 (lane 12), DVA9 (lane 13), Ah274 (lane 14), and Ah65 (lane 15) and *A. salmonicida* 449 (lane 16), 449-3R (lane 17), and 449-3 (lane 18).

Immunoprecipitation of purified LPS. Solutions of purified LPS were made up in 30 mM Tris hydrochloride (pH 8.0)–5 mM EDTA to a final concentration of 1 μg/μl. LPS solution (50 μl) was mixed with 25 μl of antibody (either polyclonal or monoclonal) in Tris-EDTA buffer (final antibody dilution, 1:20). The mixture was incubated at room temperature for 2 h. A 50-μl volume of a 50% suspension of protein A-Sepharose (Pharmacia) was added and incubated at room temperature for 30 min, with occasional gentle mixing. The protein A-Sepharose beads were collected by a brief centrifugation and washed three times with Tris-EDTA. SDS-PAGE solubilization buffer (35 μl per pellet) was added and boiled for 10 min. A 5-μl volume of a 1-mg/ml solution of proteinase K in 30 mM Tris hydrochloride (pH 8.0) was added, and digestion proceeded for 60 min at 60°C. The protein A-Sepharose beads were removed by centrifugation, and the supernatant was loaded onto an SDS-PAGE gel lane. After electrophoresis, the presence of LPS was detected by the silver staining procedure of Tsai and Frasch (16).

Assay of adsorption of bacteriophage to bacterial cells. Bacteriophage strain 55R-1 specific for the polysaccharide of *A. salmonicida* LPS (5) was obtained from W. D. Paterson, Connaught Laboratories Ltd., Willowdale, Ontario, Canada. The bacterial strains to be tested were grown to a density of 10^8 viable cells per ml in tryptic soy broth. To measure phage adsorption, 0.1 ml of a phage 55R-1 suspension (about 2×10^4 PFU) was mixed with 8 ml of bacterial suspension. A control consisting of phage 55R-1 in 8 ml of tryptic soy broth was also prepared, and the suspensions were incubated at room temperature (about 22°C). After 1 h of incubation, samples (1 ml) were taken, and the bacteria and adsorbed phage were removed by centrifugation at 12,000 × *g* for 5 min at 4°C. One drop of chloroform was added to the supernatant, and unadsorbed phage was assayed on *A. salmonicida* A440 by the agar overlay method as previously described (5).

RESULTS

Electrophoretic analysis of LPS. The structural morphology of *A. hydrophila* LPS was analyzed by SDS-PAGE. The strains initially chosen for analysis included six members of the enhanced-virulence group isolated from both diseased fish and diseased homeothermic-animal tissues as well as a range of additional strains from quite diverse sources including human feces. The electrophoretogram of the high-virulence group (Fig. 1, lanes 1 through 8) displayed a slow-migrating band, a small number of well-resolved faster-migrating bands, and the fast-migrating lipid-A–core oligosaccharide fraction. When the intermediate-migrating bands (Fig. 2, lane 1, arrow B) were subjected to re-electrophoresis (lane 3), bands which comigrated with bands B and C (lane 1) as well as some faint bands of higher M_r were seen, suggesting that bands with M_r greater than B in lane 1 were multimers or aggregates of B. Therefore, when the slow-migrating band (lane 1, arrow C) was subjected to re-electrophoresis, bands of intermediate mobility were obtained (lane 4, arrow B), confirming that band C was composed of aggregates of band B. The O-polysaccharide

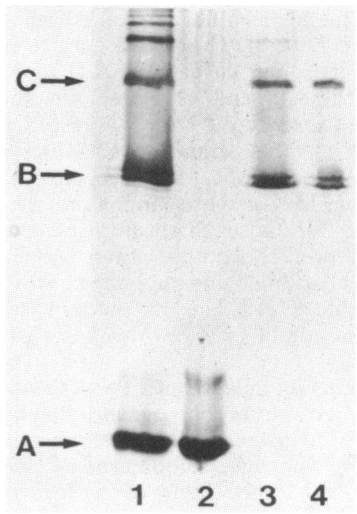


FIG. 2. Silver stain of SDS-PAGE of purified LPS of *A. hydrophila* TF7. Labeled arrows indicate bands excised from an unstained equivalent gel and subjected to a second SDS-PAGE separation. Lane 1, 10 μg of purified LPS; lane 2, re-electrophoresis of band A; lane 3, re-electrophoresis of band B; lane 4, re-electrophoresis of band C.

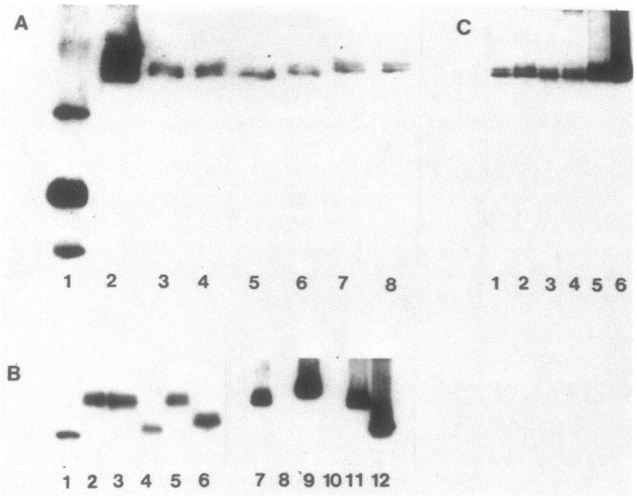


FIG. 3. Immunoblot of SDS-PAGE of *A. hydrophila* LPS reacted with polyclonal rabbit antisera produced to purified LPS from *A. hydrophila* TF7. (A) Reference ^{125}I-radiolabeled protein markers at 66,000, 45,000, 31,000, and 21,000 (lane 1); purified LPS (0.5 μg) from *A. hydrophila* TF7 (lane 2); proteinase K-digested whole-cell lysates (10 μl) of *A. hydrophila* TF7 (lane 3), LL1 (lane 4), A80-160 (lane 5), A80-140 (lane 6), P77-115 (lane 7), and A82-256 (lane 8). (B) Reference ^{125}I-radiolabeled protein marker at 45,000 (lane 1); proteinase-K-digested whole-cell lysates (10 μl) of *A. hydrophila* TF7 (lane 2), A80-160 (lane 3),Ah274 (lane 4), 598 (lane 5), Ah423 (lane 6),TF7 (lane 7), 688 (lane 8), 220 (lane 9), 484 (lane 10), 355 (lane 11), and 810 (lane 12). (C) Reaction after absorption of antisera against live cells of *A. salmonicida* A438. Proteinase-K-digested whole-cell lysates (10 μl) of *A. hydrophila* TF7 (lane 1); LL1 (lane 2); A80-160 (lane 3); A80-140 (lane 4); P77-115 (lane 5), and A82-256 (lane 6).

chains of this group of *A. hydrophila* appeared to be shorter than those of *A. salmonicida* (Fig. 1, lanes 16 and 18). Eight other *A. hydrophila* strains which were autoaggregating when grown in static broth culture also provided SDS-PAGE profiles characteristic of LPS with homogeneous O-polysaccharide chain length. In two of these strains (Ah423 and Ah598), the electrophoretic mobility was identical to that shown by the high-virulence group (data not shown); in one case (strain 220), the O polysaccharides showed longer chains; and in the other cases (Ah274 [Fig. 1, lane 14] and strains 688, 355, 810, and 423 [data not shown]), the O polysaccharides appeared to show shorter chains.

The LPSs of 11 other strains of *A. hydrophila* examined contained O polysaccharides of heterogeneous chain length. The results from five of these strains are shown in Fig. 1, lanes 9 through 13, and each strain displayed a distinctive electrophoretic profile. Another strain (Ah65) appeared to lack O-polysaccharide chains altogether, providing an electrophoretic profile (Fig. 1, lane 15) similar to that of the core oligosaccharide mutant of *A. salmonicida* (strain A449-3R [Fig. 1, lane 17]).

Polyclonal antibody analysis of LPS. The antigenic cross-reactivity between the morphologically homogenous-chain-length LPS of *A. hydrophila* was first examined by immunoblotting. The presence of a serodeterminant common to the O polysaccharide of this group of *A. hydrophila* was revealed when polyclonal antiserum prepared to *A. hydrophila* TF7 LPS gave a strong signal with the homogeneous O-polysaccharide fraction of all strains of the high-virulence group (Fig. 3A) and with strains Ah598 and Ah427 (Fig. 3B, lanes 5 and 6). The different-chain-length homogeneous O polysaccharides of strain Ah274 (Fig. 3B, lane 4) and strains 220, 355, and 810 (Fig. 3B, lanes 9, 11, and 12) also reacted with this antiserum. No reaction was obtained with the LPS of strain 688 or 484 (Fig. 3B, lanes 8 and 10, respectively) or of any other *A. hydrophila* which contained O polysaccharides of heterogeneous chain length (data not shown). Similarly, no reaction was obtained with *A. salmonicida* LPS (data not shown). Identical results were obtained with antisera prepared to *A. hydrophila* LL1 LPS. When polyclonal antisera prepared to *A. salmonicida* LPS was reacted by immunoblotting, the homogenous O-polysaccharide chains of *A. salmonicida* gave a strong immunoblot reaction, but no reaction was obtained with *A. hydrophila* LPS, revealing the presence of an *A. salmonicida*-specific O-polysaccharide determinant (data not shown).

Since antibody reaction with conformation-dependent epitopes may not be seen after SDS-PAGE by the immunoblot technique, the antigenic cross-reactivity between the homogeneous-chain-length O polysaccharides of *A. hydrophila* and *A. salmonicida* was then examined by ELISA. The extent of the cross-reactivity can be seen in Fig. 4. While the homologous LPS antigen-polyclonal antiserum reaction was strongest, polyclonal antiserum raised to *A. salmonicida* A438 LPS clearly bound to *A. hydrophila* TF7 LPS (Fig. 4A), and polyclonal antiserum raised to *A. hydrophila* TF7 LPS clearly bound to *A. salmonicida* A438 LPS (Fig. 4B). The two LPSs were also antigenically cross-reactive in immunoprecipitation experiments. Polyclonal antiserum raised to *A. salmonicida* A438 LPS was able to immunoprecipiate *A. hydrophila* TF7 LPS (Fig. 5, lane 2) and *A. salmonicida* A438 LPS (Fig. 5, lane 4). Similarly, antiserum raised in rabbits to *A. hydrophila* TF7 LPS immunoprecipitated both the TF7 and A438 LPSs (data not shown).

To confirm that the homogeneous-chain-length LPSs of *A. hydrophila* TF7 and *A. salmonicida* A438 carried cross-reactive determinants, as well as a species-specific immunoblottable determinant, absorption experiments were performed. With both polyclonal anti-LPS sera, cells of the homologous strain absorbed antibodies to homologous and

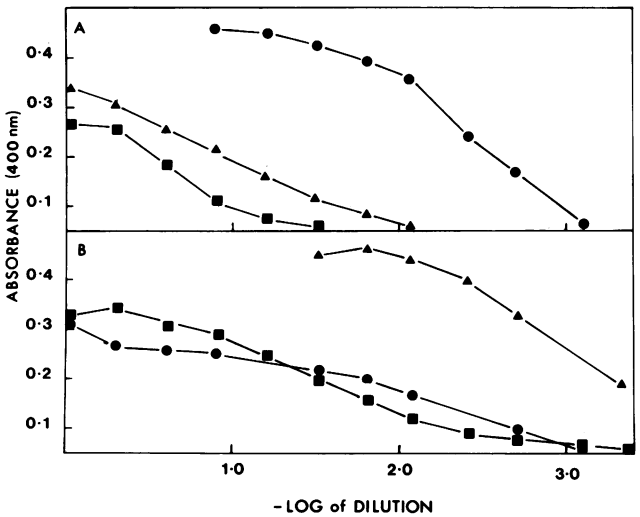


FIG. 4. ELISA of 3 μg of purified LPS of (A) *A. hydrophila* TF7 and (B) *A. salmonicida* A438 (SJ14) reacted with polyclonal rabbit antisera raised to purified LPS of *A. hydrophila* TF7 (●) and *A. salmonicida* A438 (SJ14) (▲) and to MAb iiC$_5$ (■).

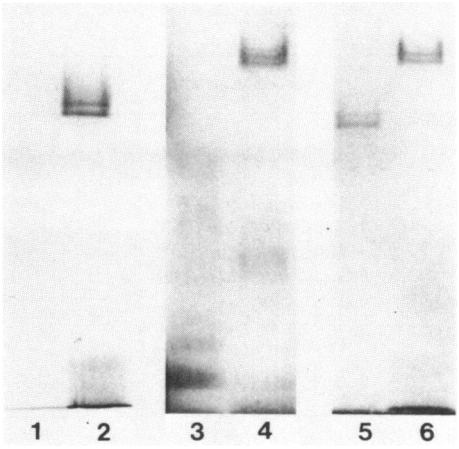


FIG. 5. Silver stain of SDS-PAGE of immunoprecipitation of *A. hydrophila* TF7 LPS (lanes 2 and 5) and *A. salmonicida* A438 LPS (lanes 4 and 6) by polyclonal rabbit antisera raised to purified LPS of *A. salmonicida* A438 (lanes 2 and 4) and MAb iiC_5 (lanes 5 and 6). Control non-immune rabbit antisera is shown in lanes 1 and 3.

heterologous LPS (Table 2). Cells of the heterologous strain also removed antibodies to heterologous LPS, but did not remove antibodies to homologous LPS. Therefore, antiserum prepared to *A. hydrophila* TF7 LPS still produced a strong immunoblot reaction with *A. hydrophila* cells (Fig. 3C), even after adsorption with *A. salmonicida* A438 cells. Control adsorptions with cells of the smooth-LPS-producing *S. typhimurium* LT2 failed to remove antibodies to the LPS of either *A. hydrophila* TF7 or *A. salmonicida* A438.

MAb analysis of LPS. MAb iiC_5, which had previously been shown to bind to a conformation-dependent epitope on the polysaccharide of *A. salmonicida* LPS (1), was also used to probe the antigenic cross-reactivity of *A. hydrophila* TF7 and *A. salmonicida* A438 LPS. MAb iiC_5 did bind to both species of LPS in ELISA experiments (Fig. 4), and this result was confirmed by immunoprecipitation assay (Fig. 5, lanes 5 and 6). Since parallel studies had shown that *A. hydrophila* TF7 cells produce a surface protein array, the ability of TF7 cells to bind MAb iiC_5 was examined by IFAT to determine whether the polysaccharide chains of the LPS penetrated to the external surface of the protein array. Surprisingly, MAb iiC_5 did not provide a positive IFAT reaction with TF7 or LL1 cells, but did so with A-layer-producing cells of *A. salmonicida*. Control assays with polyclonal antiserum to the LPS of strain TF7 gave a positive IFAT reaction with TF7 and LL1 cells, indicating that LPS antigenic determinants did penetrate the surface array and were exposed on the cell surface. This result suggested that the epitope for MAb iiC_5 was masked by the protein array on strains TF7 and LL1 and further implied that the epitope for this MAb was not carried by the O-polysaccharide repeat unit per se, but involved some other portion of the LPS molecule. Since transposon-induced A-layer-deficient (A^-) and O-polysaccharide-deficient mutants of *A. salmonicida* were available, immunofluorescence assays were performed to provide further information on the location of the epitope for MAb iiC_5. The LPSs of A^- mutants A449-TM4 and A449-TM5 lacked O-polysaccharide chains but contained a lipid-A–core oligosaccharide fraction with unaltered electrophoretic mobility (Fig. 6, lanes 2 and 3), suggesting that the core oligosaccharide of these mutants is unaltered. Both mutants gave a negative IFAT reaction with MAb iiC_5, as did the phage-resistant core oligosaccharide A^- mutant A449-3R (Fig. 6, lane 1), indicating that the determinant for this MAb was not carried by the core oligosaccharide itself, but suggesting that the epitope for MAb iiC_5 involved that portion of the LPS molecule carrying the O-polysaccharide–core oligosaccharide linkage.

Adsorption of bacteriophage. The structural and steric similarities between the homogeneous-chain-length O polysaccharides of *A. hydrophila* and *A. salmonicida* LPSs was next investigated with bacteriophage 55R-1, which has been shown to utilize *A. salmonicida* O polysaccharide as a receptor (5). Phage 55R-1 bound to cells of *A. hydrophila* TF7 and A80-160 and *A. salmonicida* A449 (Table 3), but failed to bind to the heterologous-chain-length O-polysaccharide-bearing cells of *A. hydrophila* Ba5 and Ah427 or to the control O-polysaccharide-deficient cells of *A. salmonicida* A449-3R. This ability of TF7 cells to bind bacteriophage 55R-1 confirmed that some O polysaccharide penetrated to the exterior surface of the protein array.

DISCUSSION

This study has shown that a group of strains of *A. hydrophila* which was first identified because of its enhanced ability to produce disease and by its autoaggregating growth characteristics produces an LPS with O-polysaccharide chains of homogeneous length. Other autoaggregating strains of *A. hydrophila* from diverse sources, including human isolates, also produced an LPS with O polysaccharides of homogeneous chain length. In the majority of autoaggregating strains examined, the chain length appeared to be very similar, and preliminary sizing experiments indicate this length to be approximately 10 repeat units (Shaw, unpublished data). This chain length was measurably shorter than the homogeneous-length O-polysaccharide chains produced by *A. salmonicida* (16 to 18 repeat units). However, like *A. salmonicida*, *A. hydrophila* TF7 and LL1, both of which contained this homogeneous O-polysaccharide mor-

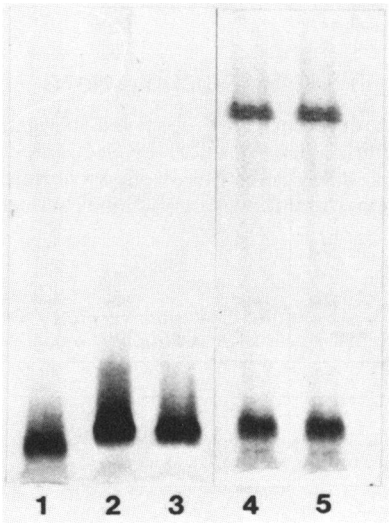


FIG. 6. SDS-PAGE of 10 μl of proteinase K-digested whole-cell lysates of *A. salmonicida* strains stained by LPS silver staining procedure. Lanes: 1, A449-3R; 2, A449-TM5; 3, A449-TM4; 4, A449-3; and 5, A449.

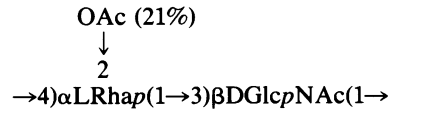
TABLE 2. Absorption of antibodies to LPS of *A. hydrophila* TF7 and *A. salmonicida* A438(SJ14) by cells of *A. hydrophila* TF7 and *A. salmonicida* A438

Polyclonal rabbit anti-LPS antisera[a]	LPS antigen	ELISA[b] (A_{405})	Absorbing strain	% Reduction in ELISA[b]
A. hydrophila TF7	*A. hydrophila* TF7	0.6 ± 0.03	*A. hydrophila* TF7	97 ± 3
			A. salmonicida A438	8 ± 1
			S. typhimurium LT2	3 ± 1
	A. salmonicida A438	0.47 ± 0.02	*A. hydrophila* TF7	75 ± 3
			A. salmonicida A438	76 ± 3
			S. typhimurium LT2	2 ± 1
A. salmonicida A438	*A. salmonicida* A438	0.58 ± 0.03	*A. salmonicida* A438	100 ± 4
			A. hydrophila TF7	11 ± 2
			S. typhimurium LT2	3 ± 1
	A. hydrophila TF7	0.38 ± 0.03	*A. salmonicida* A438	66 ± 5
			A. hydrophila TF7	76 ± 4
			S. typhimurium LT2	3 ± 2

[a] Antiserum was reacted with antigen for 2 h at a dilution of 1:160.
[b] Mean ± standard error of three determinations.

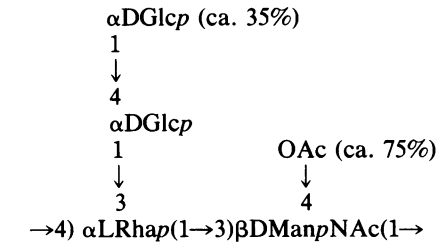
phology, have also been shown to produce a surface protein array (Dooley, Murray, and Trust, unpublished data). Immunofluorescence analysis with polyclonal antisera to LPS, as well as phage-binding studies, showed that a number of these O-polysaccharide chains of *A. hydrophila* penetrate the protein array and are exposed on the cell surface. Since other studies in this laboratory (8) have shown that a third protein array-producing organism, *Campylobacter fetus*, also exhibits homogenous-length O-polysaccharide chains, a possible structural role for this O-polysaccharide morphotype is indicated. Certainly mutants and strains of *A. salmonicida* which still produce the protein subunits of the array but lack O polysaccharides appear to be unable to assemble these subunits on the cell surface (E. E. Ishiguro et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1984), and it may be that the constancy of the O-polysaccharide chain structure permits them to serve as a framework around which the array is assembled.

Chemical analysis of the O polysaccharide of *A. hydrophila* LL1 (14) has revealed that the repeating unit of the O chain has the following structure:



and the minor differences we observed in relative electrophoretic mobilities may possibly be accounted for by the differences in O-acetyl substitution shown in this repeat unit. Immunoblot experiments with polyclonal antisera prepared to the LPS of *A. hydrophila* LL1 and TF7 showed that this O-polysaccharide structure contains a serogroup-specific epitope. This serogroup comprises a diverse range of *A. hydrophila* strains, all producing an LPS with O polysaccharides of homogeneous chain length. The epitope was not detected on the heterogeneous-chain-length O-polysaccharides of the other strains of *A. hydrophila* tested or on *A. salmonicida* A438 LPS.

The O-polysaccharide structures of *A. hydrophila* LL1 and TF7 were also shown to carry an additional epitope which displayed antigenic cross-reactivity with an epitope on the O polysaccharide of *A. salmonicida* A438. Therefore, polyclonal antisera prepared to the LPS of *A. hydrophila* TF7 contained antibodies which were bound by both *A. hydrophila* TF7 and *A. salmonicida* A438 cells, and antisera prepared to the LPS of *A. salmonicida* A438 contained antibodies which were bound by the cells of both species. This shared determinant was not reactive by immunoblotting but was reactive in ELISA and immunoprecipitation assays. Both O polysaccharides also carried a spatial grouping recognized by bacteriophage 55R-1. As the repeating unit of the *A. salmonicida* O chain (13) has the following structure:



it is possible that the conformational group(s) recognized by the polyclonal antisera or the bacteriophage or both involve the rhamnose-amino sugar backbone, since, apart from the configuration of the C-2 hydroxyl group in the amino sugar, the two O-antigen repeat units have identical backbone structures.

Immunochemical analysis with MAb iiC$_5$ revealed a third epitope on the polysaccharide of *A. hydrophila* TF7 LPS and on *A. salmonicida* LPS. This epitope was conformation dependent and not detectable by immunoblotting. Immunofluorescence assays with intact cells of *A. hydrophila* TF7 and LPS mutants of *A. salmonicida* provided evidence that this third epitope requires an intact linkage between the core

TABLE 3. Absorption of *A. salmonicida* LPS-specific bacteriophage 55R-1 cells of *A. hydrophila* and *A. salmonicida*

Strain	% Phage binding
A. hydrophila	
TF7	53 ± 3
A80-160	45 ± 4
Ba5	5 ± 4
ATCC 7966	5 ± 4
A. salmonicida	
A449	77 ± 2
A449-3R	7 ± 3

oligosaccharide and the O-polysaccharide chain. Methylation analysis has demonstrated that in the case of both *A. salmonicida* A438 and *A. hydrophila* LL1, attachment of the O chain to the core oligosaccharide is always through C-3 of the nonreducing terminal galactose unit on the core (Shaw, unpublished data). Thus there is an essentially similar conformational arrangement in the area of the galactose-to-amino-sugar glycosidic bond making antigenic cross-reactivity possible. This same structure has also been demonstrated for the linkage region of a human strain of *A. hydrophila* LPS (F. Michon, Ph.D. thesis, Memorial University of Newfoundland, St. John's, Newfoundland, Canada, 1983). In *A. salmonicida* cells, this epitope for MAb iiC_5 is surface exposed even in the presence of the surface protein array, but in *A. hydrophila* TF7, this epitope is masked by the surface protein array.

In summary, electrophoretic analysis of LPS in whole-cell lysates has shown that while many strains of the species *A. hydrophila* produce an LPS with O-polysaccharide chains of heterogeneous lengths, as demonstrated by electrophoretic profiles, one group of strains isolated from a variety of clinical sources displayed an LPS with O polysaccharides of homogeneous chain length. Two of these strains also produced a surface protein array, and in these cases, O-polysaccharide chains were shown to traverse the protein array. Antigenic analysis revealed that the polysaccharide moiety of the LPS of this group of *A. hydrophila* strains carried three antigenic determinants. One of these determinants was species serogroup-specific and reactive by immunoblotting. The second determinant was conformation dependent and cross-reactive with an epitope on the homogeneous chain length O polysaccharide of *A. salmonicida* LPS. Both polysaccharides also carried a binding site for an *A. salmonicida* bacteriophage. The third epitope on the polysaccharide of both *A. hydrophila* and *A. salmonicida* LPSs was recognized by MAb iiC_5, was conformation dependent, and appeared to involve that region of the LPS molecule bearing the O-polysaccharide–core oligosaccharide glycosidic linkage. In the case of surface array-producing strains of *A. hydrophila*, this epitope was masked by the protein array.

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