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Structural and Immunochemical Homogeneity of *Aeromonas salmonicida* Lipopolysaccharide

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Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to analyze the lipopolysaccharides of typical and atypical strains of the fish pathogen *Aeromonas salmonicida*. ^{32}P intrinsically radiolabeled lipopolysaccharide in sarcosinate-extracted outer membrane preparations, lipopolysaccharide stained by silver in proteinase K-digested outer membrane preparations and whole cell lysates, as well as purified lipopolysaccharide, displayed *O*-polysaccharide chains which were unusually homogeneous with respect to chain length. Chemical analysis further revealed that the sugar composition of the smooth lipopolysaccharide purified from three typical strains was very similar. Immunoblotting and immunofluorescent staining with both polyclonal and monoclonal antibody showed that the *O*-polysaccharide chains were strongly immunogenic and were antigenically cross-reactive on typical and atypical strains from diverse origins. Immunofluorescence analysis and phage binding studies demonstrated that a number of these *O*-polysaccharide chains traversed the surface protein array of virulent strains of *A. salmonicida* and were exposed on the cell surface.

Gram-negative bacteria are defined by an outer membrane containing lipopolysaccharide (LPS). In the case of members of the *Enterobacteriaceae*, the smooth LPS has *O*-polysaccharide chains of heterogeneous lengths extending from the cell surface into the surrounding mileau (6, 8a, 11, 15, 17). An increasing number of bacteria have been found to produce superficial layers largely composed of protein subunits assembled with a high degree of structural regularity (24, 25). These are known as surface protein arrays, and little is known of the organization of these layers, or of their topological relationship to the LPS.

The gram-negative fish pathogen *Aeromonas salmonicida* (7) produces a surface protein array known as the A-layer, the principal component of which is a tetragonally arrayed protein known as A-protein (12, 20). This superficial structure is a primary virulence property, and mutants lacking the A-layer are avirulent (9). Monoclonal and polyclonal antibody analysis of *A. salmonicida* strains isolated from diverse geographic locations, from different fish species, and from different fish diseases and differing widely with respect to their physiology has shown that virulent strains appear to have this surface layer in common and that surface-exposed regions of the structure are antigenically cross-reactive (W. W. Kay et al., submitted for publication).

In this communication, we report that the *O*-antigenic polysaccharides of the LPS of *A. salmonicida* are also antigenically cross-reactive. We show that at least some of these *O*-polysaccharide chains traverse the A-layer and are exposed on the cell surface. We also demonstrate that, in contrast to members of the *Enterobacteriaceae*, the *O*-antigen polysaccharides of *A. salmonicida* are of very similar chain length and that this morphologically unique feature is strongly conserved.

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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, Wis.). Cultures were grown on tryptic soy agar (GIBCO) at 20°C.

For LPS analysis, strains A438 and SJ-15 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.). Strain A449 was grown at 20°C to prevent loss of A-layer (9). The inoculum for the final culture was 2.5 liters which had itself been inoculated with 250 ml of a seeded inoculum. Inocula were grown for 10 h and the final broth for 18 h. The cells were killed with 0.3% (vol/vol) formaldehyde (18 h), collected by continuous centrifugation, and lyophilized.

Isolation of outer membranes. Cells were harvested from agar plates or broth cultures, washed in 20 mM Tris-hydrochloride (pH 7.2; Sigma Chemical Co., St. Louis, Mo.), and suspended in this buffer containing 10 mM EDTA (Sigma), 0.1 μg of DNase per ml, and 0.1 μg of RNase per ml. After disruption of cells by three passages through a precooled French pressure cell (16,000 lb/in^2), unbroken cells were removed by centrifugation at 4,000 × *g* for 30 min. Cytoplasmic membranes were selectively solubilized with sodium lauryl sarcosinate by the method of Filip et al. (5), and the remaining outer membrane (OM) fraction was sedimented by centrifugation at 40,000 × *g* for 1 h. The OM was washed in 20 mM Tris-hydrochloride (pH 7.2) and stored at −20°C until required.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (13). OM (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). Carbohydrate was stained with Schiff's reagent (22).

TABLE 1. Strains and mutants of *A. salmonicida* examined, immunoblot reaction of LPS, and IFAT staining reactions

Strain	Source[a]	Presence of O-polysaccharide chains[b]		IFAT reaction[c]		
		Silver stain	Immunoblot	A-layer	LPS Polyclonal	LPS Monoclonal
Typical						
A449	Brown trout, France, C. Michel, strain TG36/75	+	+	+	+	+
A449-3	A^- derivative of A449	+	+	−	+	+
A449-11	Phage-resistant derivative of A449-3	−	−	−	±	−
A438(SJ-14)	Coho salmon, Canada, T. P. T. Evelyn, strain 76-30	+	+	−	+	+
A438-1	Phage-resistant derivative of A438	−	−	−	±	−
A440	ATCC 14174	+	+	−	+	+
A450	Brown trout, France, C. Michel, strain TG72/78	+	+	+	+	+
A450-1	A^- derivative of A450	−	−	−	±	−
A450-2	A^- derivative of A450	+	+	−	+	+
A450-3	A^- derivative of A450	+	+	−	+	+
A450-14	Phage-resistant derivative of A450-3	−	−	−	±	−
A451	Rainbow trout, France, C. Michel, strain TG51/79	+	+	+	+	+
A451-2	A^- derivative of A451	+	+	−	+	+
A451-25	A^- derivative of A451	−	−	−	±	−
A474	Atlantic salmon, Canada, J. Corninck, strain 81377	+	+	−	+	+
SJ-15	Sockeye salmon, Canada, T. P. T. Evelyn, strain B1-2-399	+	+	−	+	+
Atypical						
A206	*A. salmonicida* var. *masoucida* Japan, T. Kimura, strain NCMB 2020	+	+	−	+	+
A400	Goldfish, Australia	+	+	+	+	+
A401	Goldfish, Australia	+	+	−	+	+
A403	Goldfish, Australia	−	−	−	±	−
A419	Goldfish, United States	+	+	+	+	+
A430	*Haemophilus piscium*[d] ATCC 10801	−	−	+	±	−
A442	*H. piscium* ATCC 15711	+	+	−	+	+
A444	*H. piscium* ATCC 14361	+	+	−	+	+
A445	*H. piscium* ATCC 14362	+	+	−	+	+
A460	Atlantic salmon, W. D. Paterson, strain SL1	+	+	+	+	+
A461	Atlantic salmon, W. D. Paterson, strain SK2	+	+	+	+	+
A462	Atlantic salmon, W. D. Paterson, strain SK3	+	+	+	+	+
A463	Atlantic salmon, W. D. Paterson, strain P1K1d	+	+	+	+	+
A479	European carp, The Netherlands, D. Evenberg, strain V76/59	+	+	+	+	+

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[b] High molecular-weight LPS in SDS-PAGE of proteinase K-digested whole cell lysates, silver stained and immunoblotted with rabbit anti-SJ-15 LPS as per the text.

[c] Reaction with rabbit anti-*A. salmonicida* A450 A-protein immunoglobulin G diluted 1:30, with rabbit anti-*A. salmonicida* A440 and SJ-15 LPS diluted 1:100 (identical results were obtained with each antiserum), and with anti-LPS monoclonal antibody iiC5 diluted 1:1,000 (IFAT of LPS was performed with both living and fixed cells, and identical results were obtained). +, Strong fluorescence; ±, weak fluorescence; −, no fluorescence.

[d] *H. piscium* is now recognized as an atypical strain of *A. salmonicida* (18, 28).

^{32}P radiolabeling of LPS. LPS was intrinsically radiolabeled by growth on tryptic soy agar containing carrier-free $^{32}P_i$ (120 μCi/9-cm-diameter plate; Amersham, Oakville, Ontario, Canada) (16). OM was prepared from harvested cells and separated by SDS-PAGE. ^{32}P-containing components were visualized by autoradiography.

Silver staining of LPS. LPS was detected in SDS-PAGE profiles by the method of Tsai and Frasch (27). Proteins in whole cell lysates were enzymatically removed by digestion with proteinase K (E. Merck AG, Darmstadt, West Germany), using essentially the method of Hitchcock and Brown (8). Cell preparations were reacted at a cell mass/enzyme ratio (wt/wt) of 20:1 in 20 mM Tris-hydrochloride (pH 7.2). When OM was analyzed, a protein/enzyme ratio of 10:1 was used. After incubation (60°C, 1 h), digestion was terminated by the addition of SDS-PAGE sample buffer (20 μl of 62.5 mM Tris-hydrochloride [pH 6.8]–5% [vol/vol] 2-mercaptoethanol–3% [wt/vol] SDS) and incubation at 100°C (5 min). Standard controls included laboratory strains of *Salmonella typhimurium* with defined LPS structures (8).

Polyclonal antisera. Antiserum against the A-protein was obtained from adult New Zealand white rabbits previously injected with 50 μg of purified strain A450 A-protein (20) in Freund complete adjuvant, followed by two successive injections at 2-week intervals. After 2 weeks, the animals were bled and serum was collected. The immunoglobulin G

fraction was prepared by $(NH_4)_2SO_4$ precipitation and DEAE-Sepharose chromatography (20). Antisera against the LPS were obtained in the same manner by immunizing rabbits with 50 μg of phenol-extracted LPS from *A. salmonicida* A440 or SJ-15.

Monoclonal antibody to LPS. Two BALB/c mice were injected intraperitoneally with 10^7 Formalin-fixed cells of *A. salmonicida* A450. Three weeks later they were given an identical injection and 10 days after this an intravenous injection. Three days after the final injection, the mice were sacrificed, the spleens were taken, and cell suspensions were prepared. The protocol employed and media formulations for fusion and cloning were essentially those of Pearson et al. (19). The nonsecreting murine myeloma cell line used in the fusion was X63-Ag 8.653, and antibody production by hybridomas was assayed by an enzyme-linked immunosorbent assay (4) against LPS purified from strain SJ-15. Positive hybridoma iiC5 was doubly cloned by limiting dilution and grown as ascites, and cells from the ascites were frozen in liquid nitrogen. Mouse ascites fluid containing monoclonal antibody iiC5 was used for immunofluorescence testing.

Immunofluorescence. The A-layer and LPS of *A. salmonicida* strains were observed by the indirect fluorescent antibody staining technique (IFAT). Twenty microliters of a bacterial suspension in phosphate-buffered saline (10^9/ml) was air dried on acid-cleaned glass slides, fixed with methanol, and again air dried. Smears were then overlaid with rabbit antisera or monoclonal antibody as mouse ascites fluid, incubated for 30 min in a moist environment, washed in a large volume of phosphate-buffered saline, and overlaid with a 1:35 dilution of fluorescein-labeled goat anti-rabbit or anti-mouse globulin (GIBCO Laboratories, Grand Island, N.Y.). After incubation for a further 30 min, slides were washed, mounted in buffered glycerol, and observed under a Zeiss fluorescent microscope fitted with an epifluorescence attachment.

Results obtained with fixed preparations were confirmed by IFAT of living cells. Twenty microliters of a bacterial suspension in phosphate-buffered saline (10^9/ml) containing 10% (vol/vol) fetal calf serum was added to 50 μl of antibody and incubated at 4°C for 60 min. After two washes, 20 μl of a 1:85 dilution of fluorescein-labeled goat anti-rabbit or anti-mouse globulin was added and incubated at 4°C for 60 min. Excess antibody was removed by being washed in phosphate-buffered saline-fetal calf serum at 4°C, the pellets were suspended, and 10 μl of cell suspension was observed immediately for immunofluorescence.

Immunoblot detection of LPS. *A. salmonicida* fractions resolved on polyacrylamide gels were transferred directly to sheets of nitrocellulose paper by electrophoresis (60 V, 3 h) in cooled buffer containing 25 mM Tris-hydrochloride (pH 8.3), 192 mM glycine, and 20% (vol/vol) methanol (26). After transfer, the nitrocellulose paper was reacted overnight at 4°C in 10 mM Tris-hydrochloride (pH 7.4) containing 0.9% (wt/vol) NaCl and 2% (wt/vol) bovine serum albumin (TNB buffer). Rabbit antiserum raised against purified LPS from strain SJ-15 was applied (10 μl) to the nitrocellulose paper in 100 ml of the TNB buffer and allowed to react for 3 h. After at least three washes in Tris-saline, peroxidase-conjugated goat anti-rabbit serum (Miles Laboratories, Inc., Elkhart, Ind.) (50 μl) was applied in TNB buffer (100 ml). After 3 h, the paper was washed as above, followed by a final wash in 10 mM Tris-hydrochloride (pH 7.4). Antigenic components were visualized with dianisidine. Stock solutions of freshly prepared dianisidine (1% [wt/vol] in methanol) and 0.3% (vol/vol) hydrogen peroxide were diluted in 10 mM Tris-hydrochloride (pH 7.4) (0.125 ml of dianisidine and 1.67 ml of H_2O_2 in 50 ml of Tris-hydrochloride). After 30 min, the reaction was stopped by rinsing the paper in distilled water.

Assay of adsorption of bacteriophage to bacterial cells. Bacteriophage strain 55R-1 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 (ca. 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. The suspensions were incubated at room temperature (about 22°C). At predetermined times, 1-ml samples were taken, and the bacteria and adsorbed phages were removed by centrifugation at 12,000 $\times$ *g* for 5 min at 4°C. One drop of chloroform was added to the supernatant, and the unadsorbed phages were assayed on strain A440 by the agar overlay method as previously described (10).

Extraction of LPS. Freeze-dried cells were extracted by the aqueous phenol method of Westphal et al. (29) with two water washes of the phenol layer. The aqueous layer and washings were combined and dialyzed for 48 h against cold tap water. The LPS was isolated from the dialysate by ultracentrifugation (repeated twice) at 105,000 $\times$ *g* for 3 h. The resulting sedimented gel was resuspended in water and freeze-dried, giving an LPS free from both DNA and RNA.

Mild acid hydrolysis of the LPS. Purified LPS was dissolved in 1% acetic acid and hydrolyzed (120 min, 100°C) (21). After cooling to −20°C overnight and thawing, the precipitated lipid A was removed by centrifugation, washed with 1% acetic acid, and freeze-dried. The supernatant, plus washings, was freeze-dried, dissolved in 47 mM pyridinium acetate buffer (pH 4.26), centrifuged to remove traces of lipid A, and separated into component fractions by elution from Sephadex G-50 with the same buffer. Components were visualized with a differential refractometer.

Analysis of the hydrolysis fragments. Monosaccharide analysis was performed on the fractions isolated by Sephadex chromatography by hydrolysis in $N-H_2SO_4$ for 4 h at 100°C, reduction with $NaBH_4$, and subsequent gas-liquid chromatography of the derived alditol acetates (14). Final identification of the alditol acetates was by a combination of their relative retention times and mass spectra.

Gas-liquid chromatography was performed on columns of 1.5% Silar 7CP on Gas Chrom Q 100/120 mesh (180 cm by 2 mm) at 210°C (for the alditol acetates) (1, 2). Gas chromatography-mass spectrometry of the above was conducted on a Hewlett-Packard model 5981A gas chromatograph-mass spectrometer controlled by a 5934A data system, with membrane separator, a source temperature of 160°C, and ionizing voltage of 70 eV. Confirmation of the sugars in the *O*-polysaccharide was as previously reported (23). Phosphorus was analyzed by the method of Chen et al. (3).

RESULTS

Electrophoretic analysis of LPS. The structural morphology of *A. salmonicida* LPS was analyzed by SDS-PAGE. The strains chosen for analysis were from diverse geographic origins, differed markedly in physiological properties (so called typical and atypical *A. salmonicida* [17, 26], were isolated from different fish species, and produced different pathogenesis. Initial experiments with intrinsically ^{32}P-radiolabeled LPS in OM isolated from typical strains demonstrated that *A. salmonicida* LPS migrated with a high-

molecular-weight O-polysaccharide-core oligosaccharide-lipid A fraction covering an apparent molecular weight range (with respect to protein standards) of 45 to 65 kilodaltons and a fast-migrating low-molecular-weight core oligosaccharide-lipid A fraction. Both of these ^{32}P-radiolabeled fractions stained positive for carbohydrate by the periodate-Schiff's procedure (data not shown).

When LPS in proteinase K-digested whole cell lysates (8) was stained by the silver staining method of Tsai and Frasch (27), the increased sensitivity of this staining procedure together with lighter gel loadings allowed the high-molecular-weight fraction to be resolved into a small number of distinct bands, indicating a very high degree of homogeneity in O-polysaccharide chain length (Fig. 1). This was true for both typical (Fig. 1A) and atypical (Fig. 1B) strains. This electrophoretic profile was further confirmed by examination of purified LPS as shown in Fig. 1A, lanes 5 and 6, where the effect of gel loading on the electrophoretic profile is also well illustrated.

Immunochemical analysis of SDS-PAGE. The antigenic cross-reactivity between the morphologically homogeneous LPS of diverse strains of *A. salmonicida* was then examined by immunoblotting and IFAT. The results in Fig. 2 show an immunoblot reaction obtained when SDS-PAGE-separated LPS purified from typical strain SJ-15 (lane 1), OM from typical strains (lanes 2 through 4), and whole cell lysates of selected atypical strains (lanes 5 through 13) were reacted with antiserum prepared in rabbits to SJ-15 LPS. Atypical strain A403 (lane 12) was included in the example shown because its LPS was lacking in O-polysaccharide chains (Table 1). With the exception of strain A403, a strong immunoblot reaction was obtained with the O-polysaccharide-containing high-molecular-weight LPS fraction of both typical and atypical strains. This immunoblot profile was also obtained with the O-polysaccharide chains of all other smooth strains examined, whereas no reaction was seen in the case of any mutant or strain lacking O-polysaccharide chains (Table 1). Antisera prepared in rabbits to the LPS of typical strain A440 gave identical results (data not shown). The anti-LPS monoclonal antibody iiC5 was unreactive in immunoblot assays.

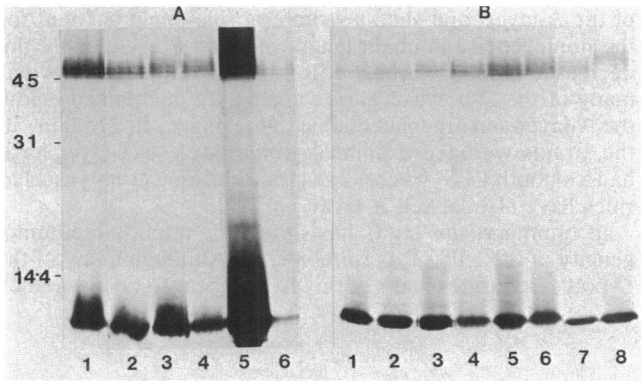

FIG. 1. Silver stain of SDS-PAGE of LPS of *A. salmonicida*. (A) Proteinase K-digested whole cell lysates of typical strains: lane 1, A449; lane 2, A438; lane 3, A450; lane 4, A451. Purified LPS from typical strain SJ-15 is shown at heavy loading (2 μg) (lane 5) and light loading (0.1 μg) (lane 6). (B) Proteinase K-digested whole cell lysates of atypical strains: lane 1, A400; lane 2, A401; lane 3, A419; lane 4, A445; lane 5, A444; lane 6, A442; lane 7, A463; lane 8, A206. Molecular weight (×1,000) on left is based on protein standards.

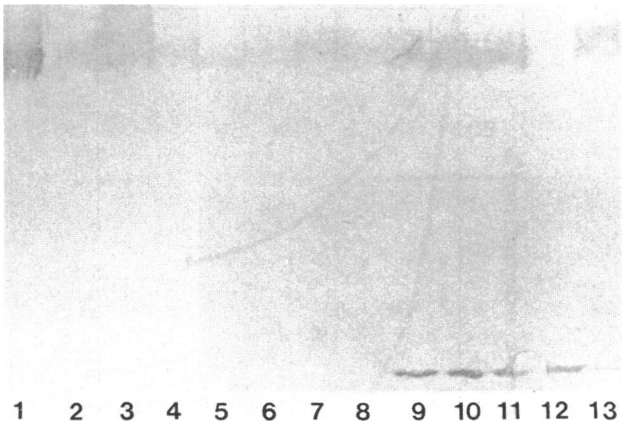

FIG. 2. Immunoblot of SDS-PAGE of *A. salmonicida* LPS reacted with polyclonal rabbit antisera produced to purified LPS from typical strain SJ-15. Purified LPS from typical strain SJ-15 (lane 1); OM isolated from typical strains A449 (lane 2), A438 (lane 3), A450 (lane 4); whole cell lysates of atypical strains A400 (lane 5), A401 (lane 6), A419 (lane 7), A445 (lane 8), A444 (lane 9), A442 (lane 10), A460 (lane 11), 403 (lane 12), A462 (lane 13).

The results in Table 1 show that rabbit antisera to the LPS of two typical strains of *A. salmonicida* also gave a positive fluorescence reaction with all of the strains tested, with the LPS visible as a continuous ring of fluorescence around the entire cell. In those strains and mutants lacking O-polysaccharide chains, the intensity of fluorescence was perceptibly reduced. When monoclonal antibody iiC5 was used, the results in Table 1 show that it also gave a positive IFAT reaction with both typical and atypical strains of *A. salmonicida* having O-polysaccharides, but failed to react with those mutants and isolates deficient in O-polysaccharides. No fluorescence was seen in control assays with cells of *Escherichia coli*, *S. typhimurium*, or *Aeromonas hydrophila*.

Arrangement of LPS and A-layer. The presence of A-layer on the strains tested was determined by IFAT with rabbit immunoglobulin G prepared to the A-protein of strain A450 (Table 1). The ability of both anti-LPS polyclonal antiseras tested and the anti-O-polysaccharide monoclonal antibody to react by IFAT with the LPS on both fixed and unfixed A^+ cells (Table 1) suggested that some O-polysaccharide penetrated to the exterior surface of the A-layer. This was confirmed by using bacteriophage 55R-1, which has been shown to utilize *A. salmonicida* LPS as a receptor (8a). The results in Fig. 3 show that phage 55R-1 adsorbed to A^+ strain A451, albeit at a slower rate than to A451-3 (A^-), confirming that LPS phage receptor was exposed on the surface of A^+ cells. Similar results were obtained with A^+ strains A449 and A450 and their isogenic A^- mutants (data not shown).

Chemical analysis. The structural analysis of *A. salmonicida* LPS by SDS-PAGE was extended by chemical analysis of smooth LPS purified from three representative typical strains. Mild acetic acid hydrolysis of the three LPS, gave precipitated lipid A and a soluble polysaccharide fraction. Sephadex chromatography of the soluble fraction afforded O-polysaccharide with $K_{av} = 0$, core oligosaccharides with K_{av} of ca. 0.64 (Table 2), and a salt peak containing no carbohydrate. From the yields given in Table 2, it is evident that the LPS isolated from the virulent A-layer-containing strain A449 had the largest amount of O-chain. Since SDS-PAGE indicated that O-chains from these different strains

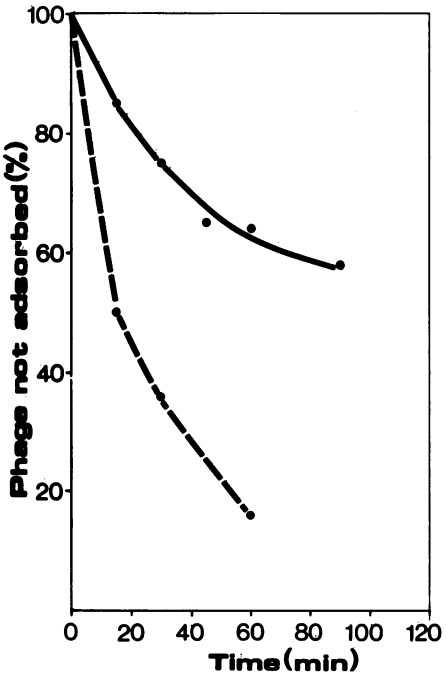

FIG. 3. Absorption of *A. salmonicida* LPS-specific bacteriophage 55R-1 to *A. salmonicida* A451 (——) and A451-3 (- - -) as a function of time.

migrated at the same speed and were thus of approximately equal molecular weight, it is clear that the quantitative differences in *O*-chains seen in Table 2 are due to differences in the degree of substitution of the core stubs in the native LPS rather than inherent differences in the degree of polymerization of the *O*-chain. In all strains examined, the ratio of core to lipid A remained constant for different batches of each individual strain.

Sulfuric acid hydrolysis of the whole LPS and the *O*-polysaccharide fractions of the three strains gave the quantitative monosaccharide analysis shown in Table 3. In keeping with the structural homogeneity seen upon SDS-PAGE, the LPS monosaccharide compositions also displayed pronounced similarities.

DISCUSSION

LPS components migrate during SDS-PAGE, owing to their content of lipid A and phosphate, and in the case of smooth LPS, the relative mobility of the individual molecular species depends on the degree of polymerization of the *O*-chain (6, 8, 11, 17). By this technique, *A. salmonicida* LPS appears to have an *O*-polysaccharide chain which is remarkably homogeneous with respect to length of chain, when compared with similar experiments on smooth LPS for *E. coli* and *S. typhimurium* (6, 8, 11, 17). On gels stained with silver, the LPS with *O*-chains consisted of finely divided bands very closely spaced. These minor differences in relative mobility were considered to be a consequence of incomplete sugar (glucose) or *O*-acetyl substitution in the *O*-chain as demonstrated for strain SJ-15 (23), rather than a variation in the number of *O*-chain repeating units, which are responsible for the much more marked heterogeneity and distinct banding observed in SDS-PAGE profiles of LPS from members of the *Enterobacteriaceae*.

By chemical analysis, the smooth LPS of three typical strains, A438, A449, and SJ-15, all contained rhamnose, glucose, galactose, L-glycero-D-mannoheptose, and *N*-acetyl mannosamine in ratios which indicate that the structures of the LPS from A438 and A449 probably do not differ chemically from the structure recently reported for SJ-15 *O*-polysaccharide (23). In this strain glucose residues substituting a rhamnose-*N*-acetyl mannosamine backbone have been shown to constitute the repeating unit of the *O*-polysaccharide (23), and all evidence suggests that this structure is common to other strains in which *O*-polysaccharide is present. This *O*-polysaccharide was clearly the predominant immunogenic portion of the *A. salmonicida* LPS, and immunofluorescence studies suggest that the epitope recognized by monoclonal antibody iiC5 is on this *O*-polysaccharide. The *O*-polysaccharides on diverse strains displayed antigenic cross-reactivity both by immunoblotting and fluorescent staining with both polyclonal and monoclonal antibodies, further suggesting that the sugars in the *O*-polysaccharide chains share a similar conformation. Interestingly, the conformation of the SJ-15 *O*-polysaccharide molecule would indicate that glucose is far more likely to be exposed and accessible to antibody than the rhamnose and *N*-acetyl mannosamine. However, enzyme-linked immunosorbent assay with competitive rhamnose, glucose, galactose, and mannosamine at 1% (wt/vol) concentrations failed to inhibit the binding of the anti-LPS monoclonal antibody to purified LPS (Chart and Trust, unpublished observation), suggesting that rather than a single sugar serving as the antigenic determinant for this monoclonal antibody, a spatial grouping of sugars is being recognized. Certainly this particular sugar grouping appears to be conserved in the *O*-polysaccharide structure from different strains of *A. salmonicida* LPS.

One difference that was apparent among the smooth LPS of the three typical strains examined by chemical analysis was in the number of core stubs substituted by *O*-chains, with the greatest degree of substitution existing in the A-layer-producing strain A449. Some of these *O*-chains clearly penetrate the A-layer. Since the surface of this A-layer on diverse strains is also antigenically cross-reactive (Kay et al., submitted for publication), this means that no matter which strain of *A. salmonicida* infects a fish, the fish is presented with a very similar antigenic profile. The LPS may in fact play an important role in assembly and maintenance of the A-layer, and such a structural role might account for the homogeneity in chain length of this LPS. Certainly the decreased binding of phage 55R-1 to A^+ strains suggests that many of the *O*-polysaccharide chains are maintained below the A-layer and are inaccessible to the phage. In addition, all the strains we have examined which possess A-layer have had a smooth LPS, whereas all strains lacking *O*-polysaccharides have also lacked A-layer.

In summary, this study has shown the remarkable homogeneity of the LPS of *A. salmonicida*. Although many of the *O*-polysaccharide chains of this LPS appear to be masked by

TABLE 2. Sephadex chromatography of acetic acid hydrolysate of *A. salmonicida* LPS

Strain	% *O*-polysaccharide (K_{av} = 0.0)	% Core oligosaccharide (K_{av} = 0.64)
A449	84.7	15.3
SJ-15	60.9	39.1
A438	58.0	42.0

TABLE 3. Carbohydrate composition of LPS and O-polysaccharide from typical strains of *A. salmonicida*

Strain	Mol%					
	Rhamnose	Galactose	Glucose	L-glycero-D-mannoheptose	Mannosamine	Galactosamine
A449 LPS	24.0	4.0	39.8	8.8	16.8	tr
SJ-15 LPS	20.2	7.2	36.3	15.3	19.6	tr
A438 LPS	22.6	2.8	52.8	5.5	16.3	tr
A449 O-polysaccharide	24.0	4.0	39.8	8.8	16.8	
SJ-15 O-polysaccharide	27.8	2.5	42.7	3.8	23.2	
A438 O-polysaccharide	24.2	1.0	48.3	4.3	22.2	

the A-layer on the surface of virulent strains and may play an important role in the assembly and maintenance of this surface protein array, many of the O-polysaccharides penetrate the A-layer and are exposed on the cell surface. The antigenic determinants of these strongly immunogenic polysaccharide chains appear to be shared among diverse strains. Since the surface-exposed antigenic domains of the A-layer are also shared by diverse strains, the surface of *A. salmonicida* is rather unique in that the two predominant surface-exposed structures in contact with the host defense mechanisms display considerable antigenic conservation. This clearly must have important implications in the virulence of this pathogen and must be an important consideration in the design of an effective vaccine.

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