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Effects of Nutrients on Exopolysaccharide Production and Surface Properties of *Aeromonas salmonicida*

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Extracellular polysaccharide (EPS) and capsular polysaccharide (CPS) production by *Aeromonas salmonicida* A450 and the influence of the capsule on cell surface properties were studied. *A. salmonicida* did not produce CPS or EPS when glucose, phosphate, magnesium chloride, or trace mineral components were absent from the medium. The addition of yeast extract improved capsule production. Neither EPS nor CPS formation depended on the C/N ratio, although it appeared to be influenced by the level of carbon and nitrogen in the culture. Both EPS and CPS production started at the end of the logarithmic growth phase. The amounts of EPS and CPS produced were not influenced by temperature changes between 15 and 20°C and was maximal from pH 7 to 7.5. Cell surface properties were strongly influenced by capsule production; high CPS production was associated with enhanced cell hydrophilicity and autoagglutination. The effect of CPS on cell surface properties was independent of the presence of the surface protein array (A-layer).

Aeromonas salmonicida causes fish furunculosis, an infectious disease principally of salmonid fish (16, 17). Virulent strains produce a surface protein array (A-layer) (9), which is crucial to the virulence of this organism (6) and has been found to be associated with an increase in cell surface hydrophobicity (25).

Interactions between bacteria and their environment, including pathogenic phenomena, normally involve specific interactions between macromolecules found on bacterial and host cell surfaces (19). The macromolecular components of bacterial surfaces, e.g., lipopolysaccharide (LPS), protein, and exopolymers, have been shown to vary in quantity and composition with growth conditions (3). Changes in cell wall or polymer composition have a strong influence on the physicochemical properties of the cell surface, e.g., via differences in hydrophobicity (13).

Recently, Garrote et al. (5) have demonstrated the production by *A. salmonicida* of completely cell-detached extracellular polysaccharide (EPS) and cell-bound capsular polysaccharide (CPS). Since capsular polysaccharide is found on the bacterial surface, we proposed to assess the influence of this CPS on *A. salmonicida* cell surface properties. The aim of our work was also to study the effects of growth medium composition and environmental conditions on the formation of exopolysaccharides (sum of EPS and CPS) by *A. salmonicida*.

MATERIALS AND METHODS

Organisms. *A. salmonicida* strains used in this study were A450, which has an A-layer (A^+) and LPS O antigen (O^+), and its attenuated derivatives A450-3, which has an A-layer (A^+) but lacks LPS O antigen (O^-), and A450-1, which lacks an A-layer (A^-) and LPS O antigen (O^-) (9). Cultures were maintained as previously described (5).

Serological and microscopic analysis. The presence or absence of an A-layer, LPS O antigen, and capsule was demonstrated as previously described (5). Monoclonal antibodies against the A-layer and the LPS O antigen of *A. salmonicida* were kindly provided by W. W. Kay, University of Victoria, Victoria, Canada.

Media and culture conditions. Bacteria were usually cultured on Trypticase soy agar slants at 20°C for 48 h. For exopolysaccharide production, Trypticase soy broth, brain heart infusion broth, and the previously described (5) yeast extract-peptone-glucose-mineral salts (YPGS) medium were used. YPGS medium was composed of (per liter of distilled water) glucose, 20 g; peptone, 10 g; yeast extract, 5 g; KH_2PO_4, 0.25 g; $MgCl_2$, 0.01 g; and mineral salts solution, 6 ml. Mineral salts solution contained (per liter of distilled water) $CaCl_2 \cdot 2H_2O$, 0.1 g; $MnSO_4 \cdot 7H_2O$, 0.075 g; and $FeSO_4 \cdot 7H_2O$, 0.4 g. The pH of each medium was adjusted to 7 with 0.1 N NaOH before sterilization at 120°C for 20 min. The composition of this medium was varied to study the influence of the individual medium components on exopolysaccharide production by strain A450.

For inocula, cells harvested from 48-h cultures in YPGS medium without glucose were washed and resuspended in physiological saline (0.85% sodium chloride). In all cases, 250 ml of each modified YPGS medium assayed in 500-ml flasks was inoculated to give an initial viable count of 10^6 CFU/ml. The flasks were incubated at 20°C for 72 h with shaking. Growth was routinely monitored by measuring viable counts on Trypticase soy agar plates. Biomass was determined gravimetrically as dry-cell weight after drying twice-washed samples for 18 h at 110°C. When required, the glucose concentration remaining in the medium was determined by the glucose oxidase method, with the GOD/PERID test combination (Boehringer GmbH, Mannheim, Germany). Total nitrogen was determined by Kjeldahl digestion (1).

Isolation and purification of polysaccharides. Samples were centrifuged at high speed to separate cells and supernatant. EPS was isolated by precipitation with 3 volumes of ethanol added to the cell-free supernatant with stirring, dissolving the precipitate in water, reprecipitating with ethanol, and freeze-drying. To solubilize CPS, the cell pellet was suspended in 1 N NaOH and extracted at room temperature with stirring for 4 h. The cell residue was separated by centrifugation, the clear NaOH extract was precipitated by

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TABLE 1. Effect of yeast extract concentration on polysaccharide production by *A. salmonicida* grown in YPGS medium

Amt of yeast extract added (g/liter)	Initial C/N ratio	Final biomass (CFU/ml)[a]	Residual glucose concn (g/liter)	$Y_{P/S}$ (m/g of glucose)		Y_{CPS} (mg/g of cell dry wt)
				$Y_{CPS/S}$	$Y_{EPS/S}$	
0.00	5.08	5.12 (0.3) × 10^8	15.9	10.0	95.4	2.01
0.25	5.80	6.15 (1.7) × 10^8	15.6	16.5	84.8	3.39
0.50	5.28	6.24 (1.1) × 10^8	15.5	17.1	87.8	3.56
1.00	4.92	8.46 (1.8) × 10^8	15.3	37.2	80.4	4.53
2.00	4.39	1.06 (0.7) × 10^9	14.9	49.4	63.9	10.55
3.00	3.77	1.12 (0.7) × 10^9	14.7	50.2	58.9	10.61
4.00	3.71	1.14 (0.9) × 10^9	14.7	43.4	55.5	10.45
5.00	3.20	7.13 (1.4) × 10^9	14.6	41.7	55.2	10.40

[a] Values are the mean of measurements done in triplicate. Standard deviations are given in parentheses.

the addition of 3 volumes of ethanol, and the CPS was purified as described previously (5). Polysaccharides were quantified by the phenol-sulfuric acid procedure (2) with D-glucose as the standard.

Characterization of autoagglutination phenotypes. Strains were evaluated for their ability to autoagglutinate in optimized YPGS medium. Evidence of self-pelleting (SP) and precipitation after boiling (PAB) were tested as described by Janda et al. (7). The relative degree of precipitation (RDP) was calculated by measuring the turbidity of each culture (A_{540}) in a spectrophotometer and applying the formula RDP = A_{540}(untreated) − A_{540}(heated).

Surface hydrophobicity characterization. Encapsulated and unencapsulated strains were generated by growing cells in the optimized YPGS medium with and without glucose, respectively. CPS-positive and CPS-negative strains were evaluated for their relative cell surface hydrophobicity. For salt aggregation test studies, the concentration of $(NH_4)_2SO_4$ required to cause aggregation of bacterial cells was determined by the procedure of Rozgonyi et al. (15).

For hydrophobic interaction chromatography, the ability of bacteria to adsorb to octyl-Sepharose CL-4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was determined by the procedure of Smyth et al. (20). The turbidity (A_{520}) of the eluate was compared with that of an appropriately diluted volume of the bacterial suspension added to the column.

For bacterial adherence to hydrocarbons, a modification of the method of Rosenberg and Kjelleberg (14) was used with cyclohexane, xylene (8), and *n*-octane (4) as assay hydrocarbons. Hydrophobicity was then expressed as the percentage of cells in the hydrophobic phase by the formula percent adhesion = $[1 - (A/A_0)] \times 100$ (11), where A_0 is the initial absorbance.

Net electrostatic charges of cells. Net surface electrostatic charge was determined by electrostatic interaction chromatography. This technique is a measure of the affinity of bacteria for cation-exchange or anion-exchange resins, as described previously by Sakai (17), who used Amberlite IRA 410 and IR 120B (Sigma Chemical Co., St. Louis, Mo.) as anion-exchange and cation-exchange resins, respectively.

RESULTS

Development of basal-level experimental conditions. Trypticase soy broth and brain heart infusion broth supported good growth of *A. salmonicida* but not CPS production (final bacterial concentration, 8.1 × 10^9 and 9.3 × 10^9 CFU/ml, respectively; μ, 0.318 and 0.348/h, respectively). However, YPGS supported good growth of *A. salmonicida* strains and CPS production (Table 1).

Effect of yeast extract concentrations. The yeast extract concentration was varied between 0 and 5 g/liter in YPGS medium. The concentrations of the remaining components were not changed. The C/N ratios ranged between 5.08 and 3.20. When yeast extract was omitted, the biomass concentration decreased and EPS production was stimulated (Table 1). However, the yield of CPS (Y_{CPS} [milligrams of CPS per gram of cell dry weight]) was significantly lower. To maintain optimal Y_{CPS}, subsequent experiments were carried out in the presence of 2 g of yeast extract per liter.

Effect of peptone concentration. To evaluate the influence of peptone concentration, YPGS media with 3 to 10 g of peptone per liter were prepared, covering the C-to-N ratio range from 13.28 to 4.38 (Table 2). Yeast extract was used at 2 g/liter throughout the experiment, and the concentrations of the rest of the YPGS components remained constant. Reducing the peptone concentration resulted in lower biomass, indicating a medium limitation by the nitrogen content.

TABLE 2. Effect of peptone concentration on polysaccharide production by *A. salmonicida*[a]

Amt of peptone added (g/liter)	Initial C/N ratio	Final biomass (CFU/ml[b])	Residual glucose concn (g/liter)	$Y_{P/S}$ (mg/g of glucose)		Y_{CPS} (mg/g of cell dry wt)
				$Y_{CPS/S}$	$Y_{EPS/S}$	
3.0	13.28	1.76 (0.8) × 10^8	17.1	0.7	0.3	0.1
4.0	10.56	2.44 (1.3) × 10^8	16.3	10.5	9.7	2.2
5.0	8.53	2.97 (1.2) × 10^8	16.7	18.8	29.7	3.4
6.0	7.25	4.26 (1.7) × 10^8	16.3	20.3	47.3	4.0
7.0	6.04	8.44 (2.1) × 10^9	15.7	43.9	73.0	7.7
8.0	5.46	1.14 (0.7) × 10^9	15.1	41.6	87.1	8.1
9.0	5.03	6.14 (2.6) × 10^9	14.5	46.9	95.4	10.7
10.0	4.38	7.21 (2.8) × 10^9	14.1	46.7	95.8	10.6

[a] Optimized YPGS medium with a yeast extract concentration of 2 g/liter was used.
[b] Values are the mean of measurements done in triplicate. Standard deviations are given in parentheses.

TABLE 3. Effect of glucose concentration on polysaccharide production by *A. salmonicida*[a]

Amt of glucose added (g/liter)	Initial C/N ratio	Final biomass (CFU/ml)[b]	Residual glucose concn (g/liter)	$Y_{P/S}$ (mg/g of glucose)		Y_{CPS} (mg/g of cell dry wt)
				$Y_{CPS/S}$	$Y_{EPS/S}$	
0.0	0.00	5.62 (0.3) × 10^9	0.0	0.0	0.0	0.0
5.0	0.49	6.15 (1.7) × 10^9	1.1	3.1	4.1	0.8
7.5	1.77	7.25 (1.1) × 10^9	3.5	13.7	19.7	3.2
10.0	2.56	7.94 (1.8) × 10^9	5.1	20.4	25.7	5.7
12.5	2.99	8.53 (0.7) × 10^9	7.9	28.5	46.1	7.4
15.0	3.55	9.40 (0.7) × 10^9	10.3	46.0	90.4	11.0
17.5	4.33	9.41 (0.9) × 10^9	12.9	45.6	93.3	11.0
20.0	4.82	9.63 (1.4) × 10^9	14.1	46.8	93.1	11.1
30.0	7.34	9.36 (2.1) × 10^9	25.3	46.7	95.6	11.0

[a] Optimized YPGS medium with of 2 g of yeast extract per liter and 9 g of peptone per liter was used.
[b] Values are the mean of measurements done in triplicate. Standard deviations are given in parentheses.

The total exopolysaccharide (P) yield ($Y_{P/S}$ [milligrams per gram of glucose used]) increased with increasing peptone concentration. This yield was a linear function of peptone concentration between 3 and 9 g of peptone per liter (r^2 = 0.97; $P < 0.0001$). At high peptone concentrations, the yield of total exopolysaccharide obtained did not increase, which showed saturation of exopolysaccharide production. Because the optimal total exopolysaccharide yield ($Y_{CPS/S}$ [milligrams of CPS per gram of glucose used] [$Y_{CPS/S}$ = 46.9] plus $Y_{EPS/S}$ [milligrams of EPS per gram of glucose used] [$Y_{EPS/S}$ = 95.4]) was found to be 9 g of peptone per liter, subsequent experiments were carried out at this peptone concentration.

Effects of glucose concentration. Strains were grown in YPGS medium containing 0 to 20 g of glucose per liter, 2 g of yeast extract per liter, and 9 g of peptone per liter. YPGS without glucose supported good growth, but the cells did not produce exopolysaccharides (neither CPS nor EPS) (Table 3). Yields of bacteria and polysaccharides rose with increasing glucose concentrations up to 15 g of glucose per liter but did not increase above this concentration. Subsequent experiments were carried out at a glucose concentration of 15 g/liter.

Influence of potassium phosphate concentration. To evaluate the influence of phosphate concentration, YPGS containing 2 g of yeast extract per liter, 9 g of peptone per liter, and 15 g of glucose per liter was supplemented with 0 to 1 g of KH_2PO_4 per liter. After 72 h of incubation, the biomass concentration had not changed significantly (Fig. 1A). Both EPS and CPS yields were maximal at 0.25 g of KH_2PO_4 per liter. At lower KH_2PO_4 concentrations, a decrease in polysaccharide yield was observed. A certain surplus of phosphate was apparently required for *A. salmonicida* total exopolysaccharide formation. Lowering the amount of phosphate in the medium did not enhance polysaccharide (EPS and CPS) production, because it reduced the buffering capacity. Subsequent experiments were carried out with a medium containing 0.25 g of KH_2PO_4 per liter.

Influence of magnesium chloride concentration. YPGS medium optimized for yeast extract, peptone, glucose, and KH_2PO_4 content was used in the determination of the $MgCl_2$ concentration. The magnesium chloride concentration was varied between 0 and 0.1 g/liter. The highest total exopolysaccharide yield was reached at 0.01 g of $MgCl_2$ per liter (Fig. 1B). The biomass concentration decreased slightly when no magnesium chloride was added. Subsequent experiments were carried out at 0.01 g of $MgCl_2$ per liter.

Influence of trace elements. To investigate the influence of the concentration of the pool of trace elements on total exopolysaccharide production, we performed assays with the optimized YPGS medium. Different amounts of a solution containing a defined concentration of trace elements (see Materials and Methods) were added to the medium. Optimized EPS and CPS production was observed when 4 ml of the trace-elements solution per liter was added to the medium (data not shown).

Effect of culture conditions on total exopolysaccharide production. An optimized YPGS39 medium was defined as follows (per liter of distilled water): glucose, 15 g; peptone, 9 g; yeast extract, 2 g; KH_2PO_4, 0.25 g; $MgCl_2$, 0.01 g; and mineral salts solution, 4 ml. The influence of pH and temperature on polysaccharide production was studied by using this medium. The final biomass in YPGS39 medium did not change significantly between 10 and 30°C, but the CPS and EPS yields were optimal between 15 and 20°C (Fig. 2A). An optimum initial pH range for exopolysaccharide (CPS plus EPS) production was found between pH 7 and 7.5 (Fig. 2B). Lower or higher pH values caused a decrease in total exopolysaccharide yield.

Kinetics of growth and exopolysaccharide formation by

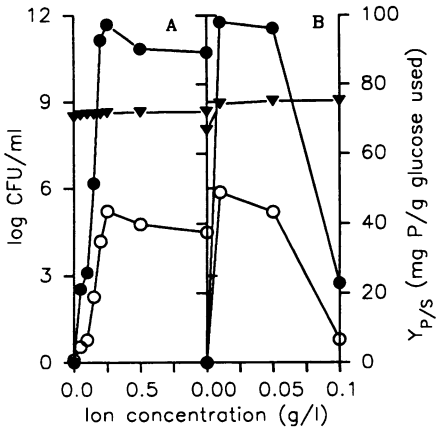

FIG. 1. Effect of initial potassium phosphate (A) and magnesium chloride (B) concentrations on final biomass (▼), CPS (○), and EPS (●) production by *A. salmonicida* A450. Media used were optimized YPGS medium with 2 g of yeast extract per liter, 9 g of peptone per liter, and 15 g of glucose per liter (panel A) and optimized YPGS medium with 2 g of yeast extract per liter, 9 g of peptone per liter, 15 g of glucose per liter, and 0.25 g of KH_2PO_4 per liter (panel B).

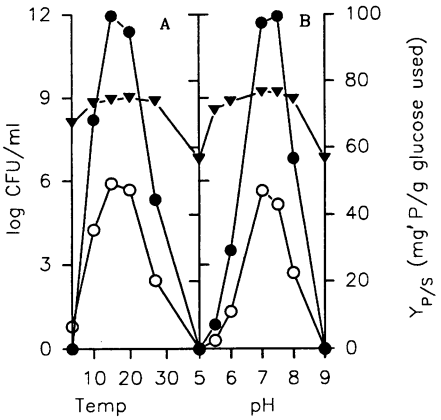

FIG. 2. Effect of temperature (A) and pH (B) on final biomass (▼), CPS (○), and EPS (●) production by *A. salmonicida* grown in YPGS39 medium.

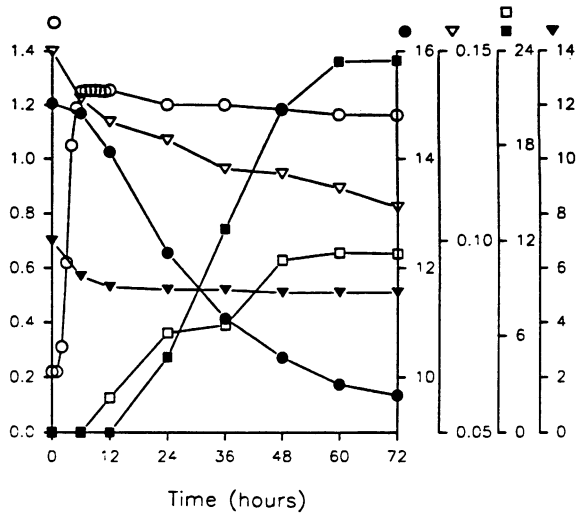

FIG. 3. Capsule production by *A. salmonicida* A450. Symbols: ○, time courses of growth (A_{520}); □, Y_{CPS}; ■, Y_{EPS}; ●, residual glucose concentration; ▽, residual nitrogen concentration; ▼, pH. YPGS39 medium was used for this experiment.

strain A450. To study the synthesis of CPS and EPS as a function of the growth phase, we grew *A. salmonicida* in YPGS39 at 20°C and initial pH 7. The growth rate of *A. salmonicida* A450 was 0.463/h, and the cells reached the stationary phase after 6 h. However, glucose consumption continued far beyond this time (72 h), as shown in Fig. 3. Exopolysaccharide production did not begin before the end of the logarithmic phase of growth. During the first 12 h of incubation, microscopic analyses of the culture showed that the polysaccharide was attached to the microbial cell as CPS. After that time, polysaccharide was also found in the extracellular fluid.

Influence of CPS production on cell surface hydrophobicity. To determine the effect of CPS on cell surface hydrophobicity, we studied three *A. salmonicida* strains: an A^+ O^+ strain (A450), the isogenic A^- O^+ strain (A450-3), and the A^- O^- strain derived from the A^- strain (A450-1). In each case the presence or absence of two major surface-exposed compounds, A-protein and LPS O-antigen polysaccharide chains was determined by enzyme-linked immunosorbent assay with monoclonal antibodies. The presence of CPS was monitored by optical microscopy.

The ability of the three isogenic strains to undergo autoagglutination in YPGS39 medium with or without added glucose is shown in Table 4. All strains tested showed similar surface phenotype (SP^+ PAB^+) when they were grown in presence of glucose.

When strain A450 was grown in YPGS39 medium without glucose, it developed a pronounced hydrophobic surface, but A450-1 and A450-3 showed an intermediate hydrophobicity (Table 4). When *A. salmonicida* strains were grown in YPGS medium, the hydrophobicity values decreased with time, being lowest at 48 to 72 h of incubation. Table 4 shows the hydrophobicity values after 72 h of incubation, when the amounts of capsule synthesized are the largest. In all *A. salmonicida* strains, cell surface hydrophobicity was inversely correlated to the amount of capsule production.

DISCUSSION

It has been reported that the production of EPS (27) and CPS (21) is a response to the nutrient composition of the growth medium. In our study, *A. salmonicida* EPS and CPS production was influenced by the growth conditions. We found that the initial concentration of available carbon and nitrogen, rather than the absolute C/N ratio, affected polymer production. In media with similar C/N ratios but different carbon and nitrogen concentrations, different $Y_{P/S}$ were obtained, as shown in Tables 2 and 3. Similar effects have been reported for *Zoogloea* strain MP6 (24) and *Butyrivibrio fibriosolvens* (26) exopolysaccharide production.

From our experiments, it follows that the yeast extract supply has a pronounced effect on the nature of the polysaccharide formed, EPS or CPS. Total exopolysaccharide pro-

TABLE 4. Influence of capsule on the surface properties of *A. salmonicida* strains after 72 h of incubation[a]

Strain	Capsule	Phenotype	RDP	BATH (% of cells absorbed)			% Cells retained in:			
				Xylene	Octane	Cyclohexane	HIC assay	Anion ESIC	Cation ESIC	SAT
A450	−	SP^+ PAB^+	0.20 (±0.01)	67.16 (±2.50)	51.99 (±1.01)	61.35 (±1.68)	93.87 (±0.29)	69.95 (±0.48)	26.12 (±0.13)	>0.01
A450	+	SP^+ PAB^+	0.81 (±0.01)	24.85 (±2.06)	6.68 (±0.83)	7.58 (±0.73)	13.42 (±0.77)	76.78 (±0.21)	47.86 (±1.70)	1.2
A450-1	−	SP^- PAB^-	0.00 (±0.00)	49.50 (±1.84)	36.30 (±1.43)	49.60 (±2.68)	42.55 (±0.46)	31.37 (±1.58)	28.23 (±1.45)	1.0
A450-1	+	SP^+ PAB^+	0.59 (±0.02)	26.12 (±2.43)	6.51 (±1.88)	17.56 (±1.17)	13.03 (±0.60)	74.18 (±0.09)	44.44 (±1.73)	1.2
A450-3	−	SP^- PAB^-	0.03 (±0.01)	45.10 (±2.60)	35.51 (±1.92)	43.70 (±2.05)	40.94 (±0.52)	33.01 (±0.47)	37.63 (±0.31)	1.1
A450-3	+	SP^+ PAB^+	0.81 (±0.03)	26.58 (±2.15)	6.04 (±1.58)	15.85 (±1.27)	11.96 (±0.43)	75.98 (±1.44)	47.69 (±1.28)	1.2

[a] Strains were grown in YPGS39 medium with or without glucose (capsule positive and capsule negative, respectively). Abbreviations: SP, self-pelleting ability; PAB, precipitation after boiling; BATH, bacterial adherence to hydrocarbons; HIC, hydrophobic interaction chromatography; ESIC, electrostatic interaction chromatography; SAT, salt-aggregating test. Standard deviations are given in parentheses.

duction remained constant throughout the experiment ($Y_{P/S}$ was around 100 [Table 1]), but the relative yields of EPS and CPS were strongly associated with the yeast extract concentration. Therefore, by altering the yeast extract concentration of the medium, it is possible to direct exopolysaccharide synthesis toward one product or another.

The kinetics of polysaccharide production by *A. salmonicida* A450 showed that EPS and CPS were produced at the end of the logarithmic phase of growth. This kinetic pattern could be significant because, in natural ecosystems, bacterial populations are influenced by changes in the microenvironment. Bacterial populations go through several periods of growth and nongrowth in response to these changes, with nongrowing bacteria using their resources for the production and release of exopolysaccharides (10).

The importance of cell surface chemical and physical properties in bacterial interactions and pathogenicity is well known (4, 12, 23). Production of CPS decreased the cell surface hydrophobicity of all *A. salmonicida* strains tested (Table 4). The production of capsule may mask the A-layer, effectively blocking the hydrophobic interactions of this protein layer. This masking effect has been reported by Trust et al. (22) for the increase in surface hydrophobicity shown by A^+ cells of *A. salmonicida*, which was due to the physical masking by the A-layer of the hydrophilic LPS O-antigen polysaccharide chains.

It has been reported that differences in adhesion between *A. salmonicida* strains are related to net electrical charges. Sakai (17) suggested that autoagglutinating A^+ *A. salmonicida* cells (whose net charges are negative) adhere to host tissue cells (whose net charges are positive) by surface charge interactions (18). Since the presence of CPS increased the electronegativity of the cell surface (Table 4), CPS production could enhance the autoagglutination and adhesiveness of A^- *A. salmonicida* strains.

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