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Production and Characterization of Monoclonal Antibodies Specific for *Shewanella colwelliana* Exopolysaccharide

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Six monoclonal antibodies were produced to whole cells of *Shewanella colwelliana* (Aco1 to Aco6) and two (Aco22 to Aco23) to purified exopolysaccharide (EPS). Aco1, -4 to -6, -22, and -23 bound to both the cell surface and the purified EPS, while Aco2 and -3 bound to cells only. The EPS of *S. colwelliana* was antigenically unique from those of nine other species of marine bacteria that were tested. Mapping studies revealed that all of the EPS-specific monoclonal antibodies bound to the same epitope. This EPS epitope was sensitive to cleavage of ester bonds, but neither pyruvate, acetate, nor terminal nonreducing sugars were required for antigenicity. When *S. colwelliana* was grown on rich media, most of its EPS was loosely associated with the cell surface.

Shewanella colwelliana is a gram-negative, aerobic, periphytic, rod-shaped bacterium originally isolated in association with the marine bivalve *Crassostrea virginica*, the Eastern Oyster (36, 37). During late logarithmic or early stationary stages of growth, it undergoes a morphological shift from a motile rod (ca. 1 μm in length) to an elongated, 3- to 40-μm, nonmotile, tightly coiled helix (37). It is also during the late stages of growth that *S. colwelliana* increasingly adheres to surfaces and produces melanin (25) and a high-molecular-weight (<1,000,000) acidic exopolysaccharide (EPS) (1, 3).

The EPS is composed of mannose, glucose, galactose, and pyruvate in a 1:2:2:4 molar ratio (28). Molecular weight analysis by gel filtration demonstrates that the EPS is polydispersive and composed of a major high-molecular-weight species and lesser amounts of smaller-molecular-weight species (22). Although regulation of EPS synthesis has not been conclusively demonstrated, *S. colwelliana* produces significantly more EPS in brain heart infusion broth amended with 2.5% NaCl than in marine broth 2216 (22, 23). *S. colwelliana* forms large flocs at late stages of growth in brain heart infusion broth, which are not observed in marine broth.

Since the recognition of capsules as an important factor in the pathogenicity of bacteria, the immunochemistry of polysaccharides has received much attention (4, 17, 21, 26, 27, 33, 38, 39). Polyclonal antibodies and monoclonal antibodies (MAbs) have proven useful in elucidating structural features of polysaccharides (2, 12, 15, 17, 18, 29).

As part of a project to study the structure and regulation of *S. colwelliana* EPS(s), we generated MAbs specific to the major surface polysaccharide of *S. colwelliana*. Here we report the production and characterization of those MAbs.

(A preliminary account of this work has been published [32].)

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MATERIALS AND METHODS

Unless otherwise noted, all chemicals were purchased from either VWR (Baltimore, Md.) or Sigma Chemical Co. (St. Louis, Mo.) and were of reagent grade or higher.

Preparation of whole-cell antigen. *S. colwelliana* was grown at 25°C on marine agar 2216 (Difco Laboratories, Detroit, Mich.) plates. After 72 h, cells were scraped from the plates and resuspended at a concentration of approximately 10^{10}/ml in sterile 0.85% phosphate-buffered saline (PBS; 7.65 g of NaCl per liter, 1.36 g of $Na_2PO_4 \cdot 7H_2O$ per liter, 0.22 g of KH_2PO_4 per liter [pH 7.4]) plus 0.337% formaldehyde. The cells were incubated for 30 min at 25°C, washed three times in PBS, and resuspended in PBS to a concentration of approximately 10^8 cells per ml. Formalinized *S. colwelliana* cells were prepared fresh on the day of injection.

Preparation of EPS antigen. EPS antigen was purified as described previously (22, 30). Briefly, *S. colwelliana* was aerobically grown to the early stationary phase in marine broth 2216 at 25°C. Cells were removed by centrifugation and discarded. Three volumes of ice-cold isopropanol was added to the supernatant, and after 12 h at 4°C the precipitate was collected, dialyzed extensively (molecular weight cutoff, 12,000), against distilled water, and lyophilized. The crude EPS was redissolved in a minimal volume of 50 mM $MgCl_2$ and digested at 25°C for 2 h with 100 μg of DNase and RNase per ml. It was treated twice with proteinase K (100 μg/ml), first for 4 h at 25°C and then for 12 h at 25°C. This purified preparation of EPS was dialyzed (molecular weight cutoff, 12,000) against distilled water and lyophilized.

Immunization of mice. Four- to six-week-old BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) were injected intraperitoneally with either 0.5 ml of formalinized *S. colwelliana* (5×10^7 cells per ml; see above) mixed 1:1 with complete Freund's adjuvant or 0.5 ml of 0.1% purified EPS in sterile 0.85% PBS mixed 1:1 with complete Freund's adjuvant. Higher concentrations of EPS were tested (2.0 and 1.0%) but led to abdominal swelling and increased mouse mortality. The mice were boosted intraperitoneally with antigen mixed 1:1 with incomplete Freund's adjuvant on days 21 and 28. On day 31, a tail bleed was performed on

each mouse, and the blood was tested for antibody titer by using an enzyme-linked immunosorbent assay (ELISA; see below). If the antibody titer was sufficient (>2,000), the mice were boosted a final time on day 31 and sacrificed on day 36; otherwise the mice were boosted weekly until the antibody titer was sufficient.

Production of MAbs. The weak immunogen method of Lane et al. (24) was used for the cell fusions. Hybridomas were grown, screened, cloned, and stored in liquid nitrogen as described previously (10, 13).

The isotype of each MAb was determined by using a Screentype Isotyping kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

ELISA. Initially, hybridomas were screened by using an ELISA (8, 9, 35) with each well of a 96-well Falcon flexible microtiter plate (VWR) coated overnight at 4°C with 100 μl of formalinized *S. colwelliana* cells (1 mg of dry cell weight per ml, prepared as described above) in coating buffer (10.6 g of Na_2CO_3 per liter, pH 9.6).

For hybridoma screening, wells were scored by visual examination as strong positive, weak positive, or negative in comparison with the controls lacking supernatant. In most cases, only strong positive scores were examined further. As more hybridoma colonies became visible, the tissue culture plates were screened at least once more.

Characterization of MAbs. The specificities of the MAbs were determined by cross-reactivity with species of *Shewanella*, *Alteromonas*, and other selected marine bacterial genera. All bacteria were grown on marine agar plates at 25°C, except for *Shewanella hanedai* and *Shewanella benthica*, which were grown at 15 and 4°C, respectively. Cells were collected and bound to ELISA plates as described above. Twofold serial dilutions of MAb were used to determine the titer of cross-reacting antigen for each species. A well was scored negative if its ELISA absorbance was <0.1. A bacterium was scored cross-reactive if its titer (1 divided by the dilution of MAb that gave a positive ELISA reading) was greater than 1/100 the titer of the homologous reaction.

Biotinylation of the MAbs. MAbs were conjugated to NHS-LC biotin (Pierce, Rockford, Ill.) as described in the manufacturer's instructions. Briefly, 0.4 mg of NHS-LC biotin was added to 20 mg of antibody in 50 mM bicarbonate buffer (pH 8.5), and the mixture was incubated on ice for 2 h. The unreacted biotin was removed by gel filtration with 0.1 M PBS (pH 7.0) and stored at 4°C with 0.1% thimerosal. This protocol yields approximately two biotin molecules per antibody molecule.

Peroxidase labeling of the MAbs. Antibodies were labeled with ImmunoPure activated peroxidase (Pierce) as described in the manufacturer's instructions. Briefly, 0.3 mg of lyophilized, salt-free antibody was dissolved in 50 μl of conjugation buffer (1 M $NaHCO_3$ [pH 9.5], 0.9% NaCl), mixed with 1 mg of activated peroxidase dissolved in 50 μl of distilled water, and incubated overnight at 4°C. Quench buffer (30 μl of 0.2 M lysine) was added, and after 2 h at 25°C, 350 μl of stabilizing solution (1% bovine serum albumin in distilled water) was added. The mixture was dialyzed (50 mM sodium phosphate [pH 6.8], 0.9% NaCl, 0.2% thimerosal) extensively and stored at 4°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting). EPS was prepared, electrophoresed, electroblotted, and immunostained as described previously (31).

Competitive ELISA assay. Microtiter plates were coated at 4°C overnight with 150 ng (dry weight) of formalinized *S. colwelliana* cells in coating buffer per well. Plates were washed three times and incubated for 1 h at 25°C with 100 μl of unlabeled MAb diluted in BLOTTO (unlabeled MAbs were ammonium sulfate concentrated). Without removing the unlabeled antibody, 3 μl of labeled MAb was added, and the plates were incubated for 1 h. Plates were washed three times, incubated for 1 h with 100 μl of goat anti-mouse peroxidase-labeled antibody (diluted 1:1,000 in BLOTTO), washed five times, and incubated for 10 to 15 min with substrate buffer. Reactions were stopped by the addition of 50 μl of 2.5 N H_2SO_4. Absorbances were read on a Mikroscan microplate reader (Titertek, McLean, Va).

For sugar inhibition studies, an anti-*S. colwelliana* EPS MAb (Aco1, diluted 1:5,000) was preincubated for 1 h with either 0.1 mM fucose, 10 mM glucose, 0.146 M sucrose, 0.1 μM galactose, 0.01 μM mannose, 25 mM fructose, 0.01 mM 2-deoxy-D-galactose, 1 mM ribose, 1 μM arabinose, 1 μM xylose, or saturated solutions of xanthan gum, alginate, or chitin. The titer of the preincubated antibody was compared with that of a control incubated for 1 h in diluent alone.

Sodium hydroxide treatment of EPS. *S. colwelliana* EPS (0.125 mg in 0.25 ml of distilled water) was mixed with an equal volume of 0.5 M NaOH and heated at 56°C for 0, 1, 2, 4, 6, and 8 h. The samples were neutralized with 0.5 ml of 0.25 M HCl, dialyzed extensively against 0.1 M PBS (pH 7.0), mixed with an equal volume of 2× SDS-PAGE sample buffer, and boiled for 5 min. Samples were then electrophoresed and immunoblotted.

Reducing sugar assay. Polysaccharide (400 μl) was mixed with 200 μl of stop solution (1% [wt/vol] $Na_2HPO_4 \cdot H_2O$, 1% [wt/vol] NaOH [pH 12.5]) and 300 μl of color solution (0.116% [wt/vol] K_3FeCN_6, 0.22% [wt/vol] NaOH [pH 11.8]). The samples were boiled for 3 min and cooled to 25°C, and absorbances were read at 420 nm within 1 h. Sample concentrations were determined by comparison to a glucose standard curve.

Depyruvalation of EPS. *S. colwelliana* EPS was depyruvalated by the method of Duckworth et al. (7). Briefly, 1.5 ml of 0.08 N oxalic acid was added to 5 mg of purified EPS in 1.5 ml of dH_2O and boiled for 1, 2, 4, 6, and 8 h. After cooling, 0.23 g of $CaCO_3$ was added, the mixture was vortexed for 2 min, and the precipitate was removed by centrifugation. Half of the supernatant was dialyzed (molecular weight cutoff, 12,000) and stored. Antigenicity was assayed by Western blots. The remaining half of the supernatant was assayed for released pyruvate by using lactate dehydrogenase (7) (Sigma).

Deacetylation of EPS. EPS was deacetylated by the method of Hungerer et al. (15). Purified EPS (5 mg) was dissolved in 0.687 ml of $NaBH_4$ solution (15 mg of $NaBH_4$, 62.5 μl of glycerol, and 0.625 ml of distilled water) and incubated for 24 h at 4°C. The EPS was then dialyzed extensively against distilled water and lyophilized. Antigenicity was assayed by Western blot analysis.

Periodate oxidation of EPS. ELISA plates coated with formalinized *S. colwelliana* were rinsed once with 200 μl of 50 mM sodium acetate (pH 4.5). Each well was incubated with 200 μl of 10 mM periodic acid (pH 4.5) for 1 h at 25°C and washed five times with washing buffer. Wells were blocked with BLOTTO containing 1% glycine for 30 min at 25°C and washed five times. Incubation with MAbs, secondary antibody, and substrate was done as described previously (8, 9, 35).

Determination of carbohydrate concentration. Five-tenths milliliter of phenol reagent (25 g of double distilled phenol in 500 ml of distilled water) and 2.5 ml of sulfuric acid reagent (2.5 g of hydrazine in 500 ml of reagent-grade concentrated

TABLE 1. Specificity of MAbs against *S. colwelliana*

Antigen	Reaction to MAb[a]							
	Aco1 (IgM)[b]	Aco2 (IgG2b)	Aco3 (IgG2a)	Aco4 (IgG2b)	Aco5 (IgG2a)	Aco6 (IgG1)	Aco22 (IgG3)	Aco23 (IgG1)
Shewanella colwelliana D	+	+	+	+	+	+	+	+
Shewanella colwelliana W	+	+	+	+	+	+	+	+
Shewanella hanedai	−	−	−	−	−	ND	−	−
Shewanella putrifaciens	−	−	−	−	−	ND	−	−
Shewanella benthica	−	+	+	−	−	ND	−	−
Purified EPS	+	−	−	+	+	+	+	+
Deleya marina	−	−	−	−	−	−	−	−
Hyphomonas jannaschiana	−	−	−	−	−	−	−	−
Hyphomonas neptunium	−	−	−	−	−	−	−	−
Alteromonas nigrifaciens	−	−	−	ND	ND	ND	−	−
Alteromonas macleodii	−	−	−	ND	ND	ND	−	−
Alteromonas atlantica	−	−	−	−	−	−	−	−
Pseudomonas aeruginosa	−	−	−	ND	ND	ND	−	−

[a] +, strong reaction; −, no reaction; ND, not done.
[b] Immunoglobulin isotype is shown in parentheses.

sulfuric acid) was added to 0.5 ml of sample in a test tube (100 by 15 mm). The test tubes were covered with aluminum foil and allowed to stand for 1 h in the dark. Absorbances (at 490 nm) were compared with those of a glucose standard curve.

Immunofluorescence microscopy of *S. colwelliana*. Cells were harvested during the log phase of growth and gently heat fixed to a slide. The slides were incubated for 30 min with a 1:1,000 dilution of MAb Aco5, washed, and incubated with a 1:1,000 dilution of fluorescein-labeled goat anti-mouse immunoglobulin G (IgG) antibody (Pierce). The slides were rinsed with 0.1 M PBS (pH 7.0) and photographed with a Zeiss Axiophot microscope (Baltimore Instrument Co., Baltimore, Md.).

EDTA extraction of EPS from *S. colwelliana* cells. Lawns of *S. colwelliana*, grown for 72 h on brain heart infusion plates, were resuspended in 20 mM EDTA–2.5% NaCl, vortexed for 2 min, pelleted, and lyophilized. The supernatants were dialyzed extensively (molecular weight cutoff, 12,500) against distilled water and lyophilized. Both cells and supernatants were assayed for total carbohydrate.

RESULTS

Production of hybridomas. From 10 formalinized cell fusions and 3 purified EPS fusions, a total of eight hybridoma cell lines that produced MAbs reactive against whole cells of *S. colwelliana* were isolated. Of these, six (Aco1, -4, -5, -6 [cell fusions] and -22 and -23 [purified EPS fusions]) bound to purified *S. colwelliana* EPS, while two (Aco2 and -3) did not (Table 1). None of the six putative anti-EPS MAbs cross-reacted with any of the bacteria tested (Table 1). Aco2 and -3 cross-reacted with the closely related species *S. benthica* (6) but not with any of the other species from the *Shewanella-Alteromonas* group. All of the MAbs had kappa light chains and were IgGs except for Aco1, which was an IgM.

Indirect immunofluorescence microscopy of *S. colwelliana*. The EPS antigen evenly surrounds the outer surface of *S. colwelliana*, with the largest accumulations being around cell aggregates (Fig. 1, compare the mid-log-phase cells on the edge of the aggregate with those in the center of the aggregate). Similar results were observed with cells harvested during the early log and stationary phases.

Labeling of the MAbs. MAbs were labeled by conjugation with biotin and horseradish peroxidase (HRP). However, titers of the biotinylated MAbs were too low to be of use in epitope mapping studies (Table 2). In each case, the titer of the biotinylated MAbs (detected by avidin-conjugated peroxidase or peroxidase-labeled goat anti-mouse secondary antibody) was 1.5 to 2 orders of magnitude lower than the titer of the nonbiotinylated MAbs (detected by HRP-labeled goat anti-mouse secondary antibody). Treatment of MAbs with biotin conjugation buffer without the addition of NHS-LC biotin had no effect on antibody titer (data not shown).

A better method for labeling the anti-EPS MAbs was direct coupling of the antibodies to HRP. Except for Aco22, this method yielded antibody titers equivalent to those of unconjugated MAbs (Table 2). The inability to label Aco22 to a higher specific activity made it unsuitable for antibody competition studies (see below).

Competitive ELISA analysis of EPS epitopes. To determine

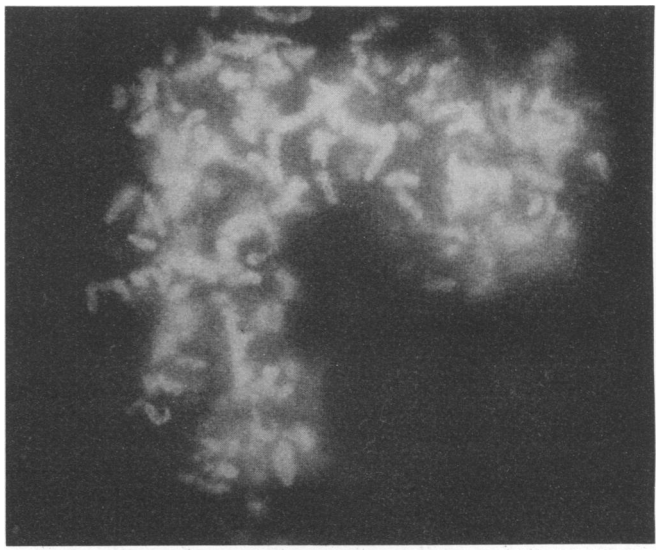

FIG. 1. Indirect immunofluorescence microscopy of *S. colwelliana* cells by using MAb Aco5. Magnification, ×1,000.

TABLE 2. Titer of labeled MAbs

MAb	Titer[a]			
	Not labeled[b]	HRP[c]	Biotinylated MAb detected by:	
			Avidin HRP[d]	Goat anti-mouse HRP[e]
1	3.80	4.31	2.36	2.10
5	4.21	4.01	3.02	2.35
6	3.80	4.10	ND[f]	ND
22	2.96	<1.0	1.78	<1.0
23	3.60	3.81	2.46	2.10

[a] Titer equals log(1/dilution) at an A_{490} of 1.5.
[b] Titer of unlabeled antibodies detected with goat anti-mouse peroxidase-labeled secondary antibodies.
[c] Titer of antibodies conjugated with HRP.
[d] Biotinylated antibodies detected with HRP-labeled avidin.
[e] Biotinylated antibodies detected with peroxidase-labeled goat anti-mouse antibodies.
[f] ND, not done.

whether the five anti-EPS MAbs recognized identical epitopes, each was tested for the ability to interfere with the binding of every other MAb. Antigen-coated microtiter plates were preincubated with unlabeled MAbs. Subsequently, a labeled MAb was added. If the MAbs bound the same epitopes, the ELISA absorbance of the labeled MAb would decrease compared with that of the controls (34). Each of the anti-EPS MAbs interfered with the binding of every other anti-EPS MAb (Table 3). Therefore, it was concluded that each MAb recognized mutually exclusive, or even identical, epitopes. Aco2, a protein-specific MAb, did not interfere with the binding of any of the anti-EPS MAbs.

MAb specificity was also examined in competition studies with potentially cross-reacting antigens. Ten sugars (fucose, glucose, sucrose, galactose, mannose, fructose, 2-deoxy-D-galactose, ribose, arabinose, and xylose) and three polysaccharides (chitin, xanthan gum, and alginate) were tested for their ability to interfere with the binding of Aco1 to *S. colwelliana* EPS. None of the tested antigens interfered with such binding (data not shown).

SDS-PAGE and Western blot analysis of EDTA-extractable material from *S. colwelliana*. The polymer released from approximately 10^9 *S. colwelliana* cells reacted strongly with each of the anti-EPS MAbs (Fig. 2, lane 2; only the Aco5 blot is shown), while an equal number of EDTA-washed cells retained very little MAb-reactive EPS (Fig. 2, lane 1). Both cell-bound and EDTA-extracted EPS form a ladder-like banding pattern during SDS-PAGE, possibly reflecting degrees of partial polymerization of the EPS. At lower concentrations, a single high-molecular-weight EPS band predominates (31). Interestingly, the highest-molecular-weight cell-bound EPS appears to migrate slightly faster than the EDTA-extracted EPS.

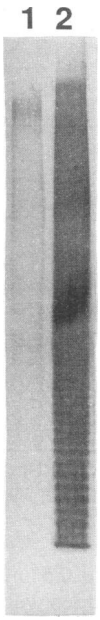

FIG. 2. SDS-8% PAGE and Western blot analysis of cell-bound and EDTA-extracted EPSs from *S. colwelliana*. Lanes: 1, *S. colwelliana* cell-bound EPS (from approximately 10^9 cells); 2, *S. colwelliana* EDTA-extracted EPS (from approximately 10^9 cells). The blot was immunostained with MAb Aco5.

SDS-PAGE and Western blot analysis of Aco2 and Aco3 MAbs. Aco2 and -3 did not bind to purified EPS (Table 1). In a Western blot, they bound a protein of ca. 100,000 molecular weight in the wild-type lysates and a slightly smaller protein (ca. 96,000 molecular weight) in *S. colwelliana* D lysates (Fig. 3).

Effects of depyruvalation, deacetylation, and periodate oxidation of EPS on antigenicity. NaOH treatment degrades polysaccharides by breaking *O*-acetyl and intrachain ester bonds (15). During 4 h of treatment, *S. colwelliana* EPS became progressively less antigenic (Fig. 4; Table 4). Cleavage of the EPS backbone by NaOH would cause an increase in the amount of terminal reducing sugars. Since the amount of reducing sugar did not increase (Table 5), it was con-

TABLE 3. Competitive ELISA analysis of the epitope specificity of the anti-EPS MAbs

Unlabeled MAb	% Inhibition by unlabeled MAb							
	1/100 Labeled MAb[a]				1/200 Labeled MAb			
	Aco1	Aco5	Aco6	Aco23	Aco1	Aco5	Aco6	Aco23
Aco1	40	66	62	39	63	72	68	57
Aco5	52	64	71	34	57	69	88	58
Aco6	51	49	53	46	63	56	63	54
Aco23	40	48	54	45	48	68	70	69
Aco22	55	58	64	47	61	63	68	60
Aco2	0	0	0	0	0	0	0	0

[a] Peroxidase-labeled MAbs were added to ELISA plates which were coated with *S. colwelliana* cells and preincubated with unlabeled MAb.

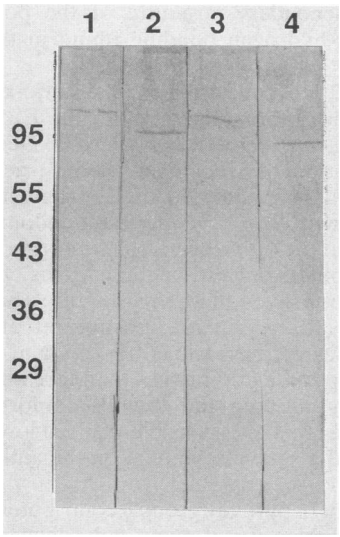

FIG. 3. SDS-PAGE and Western blots of Aco2 and Aco3 MAbs. Lanes: 1 and 2, Aco2 bound to *S. colwelliana* wild type and strain D, respectively; 3 and 4, Aco3 bound to *S. colwelliana* wild type and strain D, respectively. The numbers on the left indicate approximate molecular weights (10^3).

cluded that NaOH did not decrease the antigenicity of *S. colwelliana* by degrading intrachain ester bonds.

$NaBH_4$ (15) specifically removes *O*-acetyl groups from EPS but has no effect on *S. colwelliana* EPS antigenicity (Table 4). Although NaOH sensitivity suggested the involvement of ester bonds, $NaBH_4$ treatment showed that the ester bonds were not *O*-acetyl ester bonds.

The pyruvate content of polysaccharides was assayed by cleaving the pyruvic acid from the polymer with dilute oxalic acid (7). Free pyruvate was then measured by a lactate dehydrogenase assay (7). Such treatment of *S. colwelliana* EPS released pyruvate (Table 6). However, the recovered high-molecular-weight EPS retained most of its antigenicity

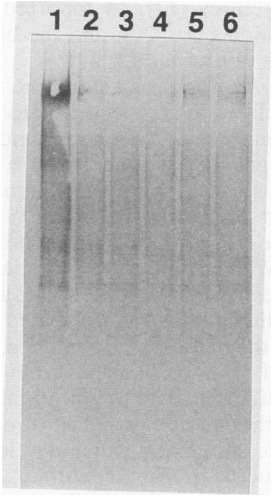

FIG. 4. SDS-PAGE and Western blot analysis of NaOH-treated *S. colwelliana* EPS. Lanes: 1 to 6, samples of *S. colwelliana* EPS that were incubated with 0.25 M NaOH for 0, 1, 2, 4, 6, and 8 h, respectively.

TABLE 4. Effects of various chemical treatments on *S. colwelliana* EPS antigenicity

Treatment	Effect	Antigenicity[a]
NaOH	Breakage of all ester bonds	−
$NaBH_4$	Breakage of *O*-acetyl ester bonds	++
Oxalic acid	Depyruvalation	+
Periodic acid	Oxidation of uronic acids and terminal nonreducing sugars	++

[a] Antigenicity was assayed by SDS-PAGE and Western blots for NaOH, $NaBH_4$, and oxalic acid. Periodic acid was assayed with an ELISA. Symbols: ++, no effect; +, small loss; −, total loss.

(Table 4). The loss of antigen was attributed to low pH rather than oxalic-acid-specific depyruvalation because treatment of the EPS with other acids yielded the same results (data not shown). Periodic acid treatment had no effect on MAb binding (Table 4), suggesting that terminal nonreducing sugars were not important for antigenicity (15).

DISCUSSION

MAbs were made against formalinized cells and purified EPS of *S. colwelliana*. The rationale for using formalinized cells was that they would elicit a better immune response than pure EPS, that all potential EPS epitopes would be present in a natural state, and that surface-bound EPS would mask other surface components, e.g., lipopolysaccharide. After 13 fusions, eight anti-*S. colwelliana* hybridoma cell lines were obtained. Of these, six reacted to purified EPS. None of the anti-EPS MAbs reacted to other *Shewanella* species or other species of marine bacteria. However, the two protein-binding MAbs (Aco2 and -3) did cross-react with *S. benthica*. Like *S. colwelliana*, *S. benthica* was originally isolated from a marine bivalve and serologically was most closely related to *S. colwelliana* (6).

Bacterial capsules (polysaccharides) tend to promote a T-independent antigenic response characterized by high titers of IgM antibodies (11). *S. colwelliana* EPS is unusual in that respect, since of the six anti-EPS MAbs, only Aco1 was identified as an IgM. It is possible that the epitopes recognized by the antibodies include the less-common highly charged pyruvate, sulfate, or melanin groups, covalently bound to (or integrally associated with) *S. colwelliana* EPS (1, 23).

Previous work has shown that Aco5 binds a protease-resistant, high-molecular-weight antigen that migrates differently in an SDS-polyacrylamide gel than *S. colwelliana* LPS (31). Here we have shown that the anti-EPS MAbs react with purified *S. colwelliana* EPS and bind to the cell surface. It is interesting that the most-intense fluorescence occurs among cell aggregates. This could be attributed to an intercellular EPS matrix which binds the cells together. It is not clear, however, whether excess EPS causes *S. colwelliana* to

TABLE 5. Reducing sugar of *S. colwelliana* EPS remaining after treatment with NaOH

Length of treatment (h)	EPS (μg/ml)	Reducing sugar (μg/ml)	% Reducing sugar
0	264	37	14.0
1	264	36	13.6
4	264	29	11.0

TABLE 6. Pyruvate content of selected polysaccharides

Polysaccharide	Pyruvate content (μg of carbohydrate/mg)	
	Measured	Reported[a]
Xanthan gum	24	10–36
Alginic acid	<0.01	0.0
S. colwelliana	2.6	3.7
Hyphomonas sp. strain MHS-3	0.4	Unknown
Hyphomonas sp. strain V6	33	Unknown

[a] The amounts of pyruvate in xanthan gum (12), alginic acid (16), and *S. colwelliana* EPS (28) were within the expected range.

aggregate or aggregated cells make more EPS. It is possible that aggregates are remnants of biofilms that have sloughed off the surface of the growth flask into the medium.

EDTA was shown to release peripherally associated EPS from *Pseudomonas* cell surfaces by chelating divalent cations (5). Anti-*S. colwelliana* EPS MAbs reacted most strongly with the material released by EDTA treatment and weakly with the treated cells, suggesting that most of the EPS is only loosely bound to the *S. colwelliana* cell surface when grown on brain heart infusion agar plates.

Pseudomonas sp. strain S9 integral EPS was reported to be extracellularly polymerized or cross-linked during starvation, and these larger, peripheral EPS were shown to slough off the cell (41). By using different methods, we show in this article that *S. colwelliana* also synthesizes a peripheral EPS with a higher molecular weight than the cell-bound EPS.

Biotinylation of the antibodies through covalent attachment to activated amines (e.g., lysine) decreased antibody titers by 1.5 to 2 orders of magnitude as detected by both avidin-peroxidase and goat anti-mouse peroxidase. Either biotinylation stearically interfered with labeled secondary antibody binding or biotin coupling destroyed an active amine residue in the variable, antigen-binding region. Since the biotin and associated linker chain were small (molecular weight, 556; ca. two biotin molecules per antibody molecule) and the secondary antibody was polyclonal, stearic hindrance would not be predicted to significantly reduce titers. More likely, the biotinylation reaction interfered with the antigen-binding region of the antibody molecule. Activated biotin reacts with lysines, suggesting that positively charged residues are important components of the variable region of the anti-EPS MAbs. This is consistent with the negative charge of the EPS.

The selective removal or destruction of polysaccharide substituents (*O*-acetyl and pyruval groups) as well as uronic acids and nonreducing terminal sugars was used to probe the antigenic structure of *Escherichia coli* K-1 EPS (14, 15). The only treatment that significantly destroyed *S. colwelliana* EPS antigenicity was prolonged (>4 h) heating in NaOH. *E. coli* K-1 EPS antigen was also sensitive to this treatment, exhibiting altered physical (15) and immunological properties but not a loss of antigenicity (14).

The antigenicity of *S. colwelliana* EPS was not lost after cleavage of *O*-acetyl groups by $NaBH_4$. Two lines of evidence argue against the notion that NaOH destroyed antigenicity by cleaving the EPS backbone. The ladder-like pattern of the EPS on Western blots did not shift towards lower-molecular-weight components with increasing incubation time, and incubation with NaOH did not increase total reducing sugar. It is possible that NaOH cleaved an ester bond involved in the binding of unidentified substituents or affected the secondary structure of the polymer (14) by disrupting the hydrogen bonding found in the native EPS (15).

Oxalic acid (depyruvalation) and sulfuric acid treatments slightly decreased the antigenicity of the EPS as measured by SDS-PAGE and Western blot analysis. Neither treatment affected migration of EPS in an electric field. Therefore, pyruvate could contribute to the antigenicity of the EPS epitope; however, it is not the immunodominant epitope. Depyruvalation of *Xanthomonas campestris* EPS (xanthan) abolished the binding of anti-xanthan MAbs, yet, even in this case, it was not proven that pyruvate directly interacts with the antibody (12). Pyruvate substitution of hexoses frequently imposes conformational rigidity and change on the sugar (17). Depyruvalation might therefore abolish antibody recognition by altering the overall conformation of the polysaccharide. It is also possible that the low pH of 0.04 M oxalic acid had a secondary effect on the antigenicity of the polymers.

At neutral pH, pyruval groups contribute to the overall negative-charge density of many polysaccharides (17). The molar ratio of pyruvate to carbohydrate in *S. colwelliana* EPS is 4:5 (28). Since depyruvalation did not affect the migration of *S. colwelliana* EPS in SDS-polyacrylamide gels, the molecular weight of EPS could be a more important factor than charge density. Sufficient charge density could be supplied by other substituents (e.g., sulfate). An analogous situation exists with proteins, which vary widely in their net negative charge (pI), but under nondenaturing conditions, their migration rate through a polyacrylamide gel is dominated by molecular weight. It is possible that native and depyruvalated EPS could be separated by isoelectric focusing or Western blotting.

On the basis of work with mouse anti-dextrans, anticarbohydrate antibodies can be classified into two groups, those having groove-type antigen-binding sites which bind to nonterminal locations along linear chains and those having cavity-type binding sites that react at the nonreducing termini of chains (18). Since the *S. colwelliana* EPS epitopes are not sensitive to periodate treatment, the anti-EPS MAbs may bind to nonterminal locations. Thus, Aco1, -5, -6, -22, and -23 are atypical since most anticarbohydrate MAbs are directed against periodate-sensitive nonreducing terminal carbohydrate structures (17), as reported for *E. coli* (40), anti-dextran antibodies (19, 20), and the branched mannans of *Saccharomyces cerevisiae* (2, 29).

The *S. colwelliana* EPS was among the first marine EPSs studied antigenically. This report provides evidence that the molecule is antigenically homogeneous and phylogenetically unique like the more-well-characterized EPSs of other bacterial genera. Each anti-EPS MAb bound depyruvalated, deacetylated, and periodate-treated EPS but not NaOH-treated EPS. The ladder-like EPS pattern detected with SDS-PAGE and Western blots was identical for each MAb. Also, the four labeled MAbs (Aco1, -5, -6, and -23) competed for the same binding site as the unlabeled MAbs in ELISA competitions. On the basis of this evidence, it was concluded that all six anti-EPS MAbs (isolated from four independent fusions and two different EPS preparations) recognize identical epitopes. Although composed of three different sugars (glucose, galactose, and mannose) and at least three noncarbohydrate substituents (pyruvate, sulfate, and acetate [28]), *S. colwelliana* EPS is an antigenically simple molecule, i.e., it is composed of a single epitope. The antigenic simplicity of *S. colwelliana* EPS is common among capsular polysaccharides such as the K antigens of *E. coli* and *Klebsiella*

pneumonia and the capsular antigens of *Haemophilus influenza* and *Pseudomonas aeruginosa* (11) and is one reason they provide such a good defense against the mammalian immune system (11).

Also like the EPSs of many pathogens, the EPS of *S. colwelliana* is species specific, i.e., the MAbs did not cross-react with any of the related or unrelated bacteria tested. The marine bacteria that have been studied have very complex, opportunistic life cycles within the diverse environment of marine waters. Cell surface diversity would provide an efficient means for the successful colonization of greatly different sites as well as avoidance of a multitude of predators and parasites. We are currently using these MAbs to quantitate the synthesis and probe the function of *S. colwelliana* EPS.

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