Isolation of Carbohydrate-Reactive Outer Membrane Proteins of Aeromonas hydrophila

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Outer membrane proteins of Aeromonas hydrophila A6 were isolated by affinity chromatography on the basis of their reactivity with trisaccharide structures analogous to the terminal trisaccharide of the H antigen of the human ABO(H) blood group system and were characterized by using antisera raised against the isolate. The outer membrane extract for affinity chromatography was prepared from pressure-disrupted outer membranes by differential centrifugation, followed by solubilization of outer membrane components in a non-denaturing, nonionic detergent. Carbohydrate-reactive outer membrane proteins (CROMPs) were then purified by affinity chromatography on two different affinity matrices composed of trisaccharides resembling the terminal trisaccharide of the H antigen, attached to inert silica beads. The relative efficiencies of H type 1 and 2 terminal trisaccharides as affinity adsorbents were established. Reactive proteins were eluted under alkaline conditions (pH 11.0) and in the presence of soluble H substance prepared from group O secretor saliva, but not by 60 mM α-L-fucose or under acid conditions (pH 3.0). The eluate contained at least three components (Mr, 43,000, 40,000, and <14,000), as detected by immunoblot analysis with a polyclonal, polyspecific rabbit antiserum to A. hydrophila A6 (serum 3/83). A specific antiserum (serum 3/91) prepared in a rabbit by repeated immunizations with nitrocellulose containing the 43,000- Da band reacted with three bands (Mr, 43,000, 40,000, and <14,000) in immunoblot analysis of solubilized outer membranes of A. hydrophila A6, suggesting that the 40,000- and <14,000-Da elements are immunologically related to components of the 43,000-Da protein. Furthermore, pretreatment of A. hydrophila A6 with serum 3/91 reduced the strength of bacterial hemagglutination. The purified CROMPs did not agglutinate human group O erythrocytes. The reactivity of isolated CROMPs with a second CROMP-specific antibody (lipopolysaccharide-absorbed serum 3/83) was investigated. CROMPs, proteinase K-treated CROMPs, and bovine serum albumin were bound to latex beads and reacted with lipopolysaccharide-absorbed serum 3/83. Antibodies eluted from CROMP-latex inhibited hemagglutination of human erythrocytes by A. hydrophila A6 to a titer of 4. Antibody eluted from proteinase K-treated CROMP-latex beads showed hemagglutination inhibition activity only when undiluted. There was no hemagglutination inhibition antibody activity detectable in the eluate from bovine serum albumin-latex beads. These results show that antibodies which react with the isolated CROMPs also react with an H-antigen-reactive hemagglutinin of A. hydrophila A6. The possibility that CROMPs act as an adhesin, or adhesins, and contribute to the virulence of this organism is discussed.

There are many reports of the isolation of Aeromonas species (aeromonads) from human diarrheal feces (2, 3, 6, 13–15, 24, 29), but some authors doubt whether aeromonads cause enteric disease (30). Nevertheless, some aeromonads have properties similar to other organisms well recognized as enteric pathogens. They elaborate toxins analogous to those produced by Vibrio cholerae and enteropathogenic Escherichia coli (11, 28, 34, 35, 43) and produce filamentous and nonfilamentous adhesins (4, 5, 17–19). Furthermore, strains isolated from diarrheal feces coproduce cytotoxins and hemagglutins significantly more frequently than strains isolated from nondiarrheal feces (4). Thus, there is circumstantial evidence that aeromonads, also, are capable of causing enteric disease by an adhesive and enterotoxic mechanism.

To date, the adhesins of aeromonads have not received the same attention as those of many other adhesive bacteria. To elucidate the pathogenesis of aeromonad-induced diarrhea, the relative contributions of filamentous and outer membrane (OM) adhesins to the colonization process will need to be studied. While methods for the purification of filamentous adhesins of aeromonads have been published (17–19), this is the first report of methods for the isolation of OM adhesins of aeromonads. One aeromonad, Aeromonas hydrophila A6, produces a hemagglutinin which is inhibited by α-L-fucose (5) and which is reported to be a 43,000-Da OM protein (4). Strains which produce this adhesin attach to the H-bloodgroup antigen of human erythrocytes (4). We used this property of A. hydrophila A6 as the basis for development of a protocol for preparation of carbohydrate-reactive components from solubilized OM by affinity chromatography. The affinity adsorbent used was composed of a trisaccharide, resembling the terminal trisaccharide of the H antigen, attached to inert silica beads. We present experimental evidence showing that this procedure resulted in the production of material which had retained a degree of biological activity associated with 40,000- and 43,000-Da proteins of the OM. We have coined the acronym CROMP (carbohydrate-reactive outer membrane protein) to distinguish this class of carbohydrate-reactive protein from other carbohydrate-reactive adhesins such as fimbrial adhesins (9, 17, 18), which may also contribute to Aeromonas adhesive mechanisms.

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MATERIALS AND METHODS

Bacterial strain. *A. hydrophila* A6, a strain isolated from human diarrheal feces, was obtained from the culture collection of the School of Medical Sciences, University of South Australia. The stock culture was stored in Trypticase soy broth (BBL) at −60°C (1) or at room temperature in a semisolid maintenance medium (10). The identity of strain A6 as *A. hydrophila* was confirmed by using the criteria of Popoff and Véron (33).

OM preparation. *A. hydrophila* A6 was grown in a stationary culture to late logarithmic phase in 15 liters of tryptonesoyabroth without glucose or phosphate (tryptone, 15.0 g; soya peptone, 5.0 g; NaCl, 5.0 g; distilled water, 1 liter; pH 7.4). Cells were harvested by centrifugation (10,000 × g for 10 min at 4°C), washed once in ice-cold Tris (20 mM Tris-HCl buffer, pH 7.4), and resuspended in 20 ml of ice-cold Tris buffer containing 1 mM diithiothreitol (Sigma Chemical Co., St. Louis, Mo.), 0.1 mM phenylmethylsulfonyl fluoride, and 20 μg of DNase (Sigma) per ml. The suspension was then frozen, thawed, and passed twice through a French pressure cell (Amicon) at 110,000 kPa. Unbroken cells were removed by centrifugation at 10,000 × g for 10 min at 4°C, and the supernatant was centrifuged at 140,000 × g for 45 min at 4°C to sediment the membrane fraction. The pellet was then washed once in ice-cold Tris buffer containing 1 mM diithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride and resuspended in Tris buffer, and a portion was taken for protein determination. Following resuspension of the pellet to a concentration of 20 mg/ml in 50 mM potassium phosphate buffer (pH 7.5) containing 50% (vol/vol) glycerol, the membranes were dispersed in 1-ml portions and stored at −60°C until required. Prior to use, OM preparations were thawed and glyceroled was removed by dialysis for 1 h at 4°C against imidazole-buffered normal saline (0.5 M imidazole, 0.145 M NaCl, pH 7.5).

Protein estimation. Absorbance of the sample at 280 and 260 nm was recorded by using bovine serum albumin (BSA) standards. Protein concentration was then calculated, correcting for nucleic acid, by the method described by Layne (26).

Solubilization of the OM fraction. The solubilization technique of Tsuchiya et al. (41) was used, replacing the detergent, octyl glucoside, with *n*-octyl-β-D-thioglucopyranoside (OTG). All procedures were performed with ice-cold reagents. Briefly, 4.09 ml of 50 mM potassium phosphate (pH 7.5), 0.5 ml of dialyzed membranes, and 50 μl of 100 mM diithiothreitol were mixed on a Vortex mixer. Stock OTG (15%, wt/vol), dissolved in 50 mM potassium phosphate buffer (pH 7.5), was added to bring the final detergent concentration to 25 mM, and the solution was mixed. The mixture was incubated on ice for 10 min and centrifuged at 140,000 × g for 60 min at 4°C. The supernatant was removed and dialyzed against 50 mM potassium phosphate buffer (pH 7.5) for 24 h at 4°C, with three changes of the dialysis fluid. Solubilized membranes were assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblot analysis.

Affinity chromatography. The carbohydrate-reactive components of the solubilized preparation were purified by column affinity chromatography at 4°C with Synsorb H type 1 and H type 2 columns (Chembiomed). Synsorb H type 1 was composed of a trisaccharide of the same structure as the H type 1 antigen (also known as Lewis d) of the human ABO(H) blood group system, covalently attached to inert silica beads via a nine-carbon-chain spacer (39). Similarly, the reactive site of Synsorb H type 2 was a trisaccharide resembling the structure of the terminal trisaccharide of the H type 2 antigen. A sample of the solubilized material was loaded onto the column and allowed to adsorb for 2 h at 4°C. Following washing of the column with 50 mM potassium phosphate buffer (pH 7.5), the column was eluted first with 60 mM α-L-fucose (Sigma) in phosphate buffer (pH 7.5), then with 0.1 M glycine buffer (pH 3.0), and finally with 1% NH₄OH (pH 11.0). Fractions of 3 ml were collected from the column, and the protein concentration of each was assayed as described previously. The protein-containing fractions obtained from each set of elution conditions were pooled, neutralized, dialyzed for 18 h against 50 mM potassium phosphate buffer (pH 7.5), and concentrated by reverse dialysis. Briefly, reverse dialysis was performed by transferring the samples to dialysis tubing, which was then covered with granules of polyethylene glycol, and the samples were incubated at room temperature until the desired volume was reached. Immunoblot analysis of SDS-PAGE gels was used to analyze these concentrated samples.

H-substance elution of CROMPs. H substance was prepared from group O secretor saliva as follows: approximately 10 ml of saliva was collected and boiled for 10 min. The boiled saliva was centrifuged at 5,000 × g for 10 min at room temperature, and the supernatant was collected (27). Titrations of hemagglutination inhibition (HI) activity of this preparation were performed. H-substance elution buffer was prepared by mixing 2 parts clarified saliva with 3 parts buffer (2.5 mM Tris-HCl, 0.05% SDS, 1.5 mM NaN₃, 25 mM phosphate-buffered saline [PBS]; pH 7.4). Batch affinity chromatography on Synsorb H type 2 was used to purify CROMPs. Briefly, 10 ml of OTG-solubilized *A. hydrophila* A6 OMs (0.4 mg/ml; A₂₈₀) was added to 2 g of H type 2 Synsorb. Carbohydrate-reactive components of the OM were allowed to adsorb onto the affinity matrix for 90 min at 4°C with mixing on a rotating blood mixer. The supernatant was removed, and the Synsorb was washed five times in 15-ml volumes of 50 mM PBS (pH 7.4). There was no detectable (A₂₈₀) protein in the last wash. The carbohydrate-reactive material was eluted with 10 ml of H-substance elution buffer at room temperature for 30 min with mixing as described before. The eluate was then adjusted to pH 11.0 with 1 M NaOH and concentrated by using Centricon-10 (Amicon), and the filtrate was discarded. The concentrated eluate was neutralized and dialyzed overnight at 4°C against three changes of imidazole-buffered saline (pH 7.5). The protein content of the eluate was determined (A₂₈₀). The H-substance-eluted CROMPs were assayed by SDS-PAGE.

SDS-PAGE and immunoblot analysis. SDS-PAGE (25) was performed with 12.5% acrylamide gels in a mini-gel apparatus (Mighty Small II, SE250; Hoeffer Scientific). Samples were prepared by mixing equal volumes of sample and sample buffer and boiling for 10 min. Unsolubilized material was pelleted by centrifugation (10,000 × g, 10 min). Gels were run at 100 V until optimal separation of prestained molecular weight markers (Bio-Rad) was achieved (approximately 90 min). For immunoblotting, separated components were electrophoretically transferred from acrylamide gels to nitrocellulose membranes (25 mM Tris-HCl, 192 mM glycine, 20% methanol; 50 V, 45 min). The membranes were blocked in 5% skim milk (1 h, room temperature). Immunoblot analysis was performed by overnight incubation (4°C) of membranes with dilutions of primary antibody followed by incubation (2 h, room temperature) with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (heavy and light chain) (Bio-Rad) and development with horseradish...
peroxidase color development reagent (Bio-Rad). $M_s$ of detected bands were determined by comparison of the electrophoretic mobilities of prestained molecular weight markers. $R_f$ values were determined and plotted against log molecular weight.

**Antiserum production.** (i) **Serum 3/83.** Serum 3/83 was obtained from a serum collection at the School of Medical Sciences, University of South Australia. It was produced by the immunization of an adult New Zealand White rabbit with *A. hydrophila* A6, as reported previously (4). Serum 3/83 inhibited the hemagglutination of *A. hydrophila* A6 with human type O erythrocytes (4).

(ii) **Serum 3/91.** CROMPs were separated by SDS-PAGE and transferred onto nitrocellulose membranes, and the proteins were localized by staining with Ponceau S (37). The nitrocellulose membrane containing the 43,000-Da protein band was excised and injected subcutaneously into a male New Zealand White rabbit. Immunizations were performed weekly for 4 weeks. The terminal bleed was performed on day 35.

**HI assays.** HI tests were performed by methods described previously (4). Bacteria grown on horse blood agar plates for 18 h at 37°C were suspended in Dulbecco PBS, pH 7.4 (Oxoid, Basingstoke, England), to a concentration equivalent to $10^8$ cells per ml and mixed with 50 $\mu$l of inhibitor (i.e., antiserum or carbohydrate). To this mixture was added 50 $\mu$l of washed human group O erythrocytes, and the slide was rotated on an orbital plate rotator for 10 min. The tests were then observed macroscopically for agglutination of erythrocytes. Inhibitors were serially diluted. The HI titer was taken as the inverse of the first dilution of inhibitor which did not show HI activity.

**Proteinase K treatment of CROMP.** Equal volumes of CROMP eluate and a 2-mg/ml aqueous solution of proteinase K (activity, 20 U/mg; Boehringer Mannheim) were mixed, and the mixture was incubated for 60 min at 60°C and then for 15 min at 95°C.

**Partial purification of immune serum.** Partial purification of serum 3/83 was performed by absorbing 3/83 with proteinase K-treated OMs of *A. hydrophila* A6 to remove anti-lipopolysaccharide (LPS). Briefly, 4.0 ml of proteinase K-treated *A. hydrophila* OMs (20 mg/ml) was incubated with 200 $\mu$l of serum 3/83 at room temperature with shaking for 10 min. The mixture was then centrifuged for 60 min at 140,000 $\times$ g and 18°C, and the supernatant was collected and concentrated to a volume of 2 ml, using polyethylene glycol as described previously.

**Latex adsorption of immune serum.** To prepare CROMP-specific antibody from absorbed serum 3/83, the following procedure was used. Six 1-ml portions of latex beads (10%, 0.8 mm; Sigma) were pelleted by centrifugation (10,000 $\times$ g, 5 min, room temperature), and the supernatant was discarded. The beads were then washed three times at room temperature by resuspending the beads in 1 ml of glycine-buffered saline (GBS; 0.1 M glycine, 0.17 M NaCl, pH 8.3) and pelleting the beads by centrifugation (10,000 $\times$ g, 5 min). The washed beads were then mixed with 1 ml of CROMP eluate, and the mixture was incubated at 37°C for 60 min. The beads were pelleted by centrifugation (10,000 $\times$ g, 10 min) and resuspended in GBS containing 1% (wt/vol) BSA. This reagent was designated CROMP-latex. To absorb anti-CROMP antibodies from partially purified serum 3/83, 1 ml of resuspended CROMP-latex beads and 200 $\mu$l of serum were mixed, and the mixture was incubated for 30 min at 37°C. To remove unabsorbed antibodies, the CROMP-latex-serum complex was washed three times in GBS. To

elute adsorbed antibody, the washed CROMP-latex(serum) beads were resuspended in 1 ml of GBS (adjusted to pH 3.0 with 0.1 M HCl), and the mixture was incubated on ice for 15 min. The beads were pelleted by centrifugation (10,000 $\times$ g, 10 min), and the supernatant was collected, neutralized with 0.1 M NaOH, and dialyzed against imidazole-buffered saline. The antibody adsorption and elution procedure was also applied to latex beads incubated with proteinase K-treated CROMPs (PK-CROMP-latex) and latex beads incubated with GBS containing 1% (wt/vol) BSA (BSA-latex). The HI titers of these eluates were determined.

**RESULTS**

**Affinity purification.** No protein, detectable by $A_{280}$ or immunoblot, was eluted by 60 mM $\alpha$-l-fucose or 0.1 M glycine buffer (pH 3.0) from Synsorb H type 1 and 2 affinity columns to which solubilized OM had been applied. Elution under alkaline conditions (1% NaOH, pH 11.0) resulted in recovery of 4% of the applied protein from Synsorb H type 1 columns and 14% of the applied protein from Synsorb H type 2 columns (Fig. 1). Elution with H-substance buffer resulted...
in a yield of 1.2 mg of protein, which was a recovery of 30% of the protein originally applied to 2 g of Synsorb H type 2.

**SDS-PAGE and immunoblot.** There was more intense staining of immunoblot bands of CROMPS, eluted under alkaline conditions, from Synsorb H type 2 columns than from Synsorb H type 1 columns (Fig. 2). In the solubilized crude OM preparation, at least 30 bands in the Mr range 83,000 to <14,000 were detected by immunoblot with serum 3/83 (Fig. 2). The CROMP eluates from both Synsorb H columns contained three components which reacted in immunoblot with serum 3/83 (Fig. 2). Two components (M, 43,000 and 40,000) stained with Coomassie brilliant blue and Ponceau S and were destroyed by proteinase K treatment, indicating that they were protein (data not shown). The third low-molecular-mass component did not stain with Ponceau S and was not destroyed by proteinase K treatment (data not shown). This component was detected in immunoblot and had an indicated molecular mass less than that of the lowest molecular size marker used (i.e., Mr, <14,000) (Fig. 2).

H-substance-eluted CROMPs contained two protein components (M, 43,000 and 40,000) (Fig. 3).

Serum 3/91, which was raised against the 43,000-Da CROMP protein of *A. hydrophila* A6, also reacted with these components (M, 43,000, 40,000, and <14,000) of *A. hydrophila* A6 in immunoblot analysis (Fig. 4).

**Investigation of hemagglutination properties of isolated CROMPS.** Isolated CROMPS did not hemagglutinate human type O erythrocytes.

Serum 3/91 inhibited the hemagglutination of *A. hydrophila* A6 with human erythrocytes to an HI titer of 8; serum 3/83 did so to an HI titer of 160 (where HI titer is the first dilution of inhibitor not showing HI activity). No HI activity was seen with PBS as the inhibitor.

The CROMP eluate from the H type 2 affinity column was attached to latex beads (CROMP-latex) and used to adsorb the HI activity from LPS-absorbed serum 3/83. The HI titer of LPS-absorbed serum 3/83 was 4. Antibody eluted from the CROMP-latex also inhibited hemagglutination of human erythrocytes by *A. hydrophila* A6 to a titer of 4. The titer with LPS-absorbed antiserum was also 4. When PK-CROMP-latex beads were used to absorb the LPS-absorbed serum 3/83, only the next antibody elution fraction from these beads inhibited hemagglutination (HI titer of 2). There was no hemagglutination-inhibiting antibody detected in the eluate from BSA-latex beads or when PBS was the inhibitor.

**DISCUSSION**

The best-known adhesins of gram-negative bacteria are those associated with fimbrilae or pili (8, 9, 12, 17-19, 22, 23, 38). OM nonfimbrial protein adhesins have also been described in gram-negative bacteria (4, 16, 21, 31), although some authors have expressed reservations, particularly in reference to aeromonads (20). The major functions that have been associated with OM proteins are cell architecture and transport of ions and molecules across the OM (16). In this paper, evidence which supports the notion of Atkinson et al.
(4) that OM proteins contribute to the adhesive properties of aeromonads is presented.

In the present study, we used a mild, nonionic detergent, OTG, to solubilize the OM of *A. hydrophila* A6, a strain which has been shown previously to produce an OM adhesin thought to be a 43,000-Da protein (4). OTG was chosen because it is non-denaturing and has a high critical micelle concentration (9 mM), which facilitates its removal by dialysis (36). Effective solubilization of *E. coli* OMs by OTG has been achieved at a final detergent concentration of 35 mM (42). OMs of *A. hydrophila* A6, solubilized in 35 mM OTG, contained carbohydrate-binding proteins which bound to, and could be eluted from, affinity columns of H-bloodgroup terminal trisaccharide. This demonstrates that OM components solubilized in this way retain their carbohydrate-binding activity.

Two different columns were used for affinity sorption; one column carried a trisaccharide analogous to the terminal trisaccharide of the H type 1 antigen as the target carbohydrate, and the other carried an H type 2-like trisaccharide. These represent the two major forms of H antigen in humans (32). They differ in the site of attachment of the subterminal D-galactose to the N-acetyl glucosamine residue. For H type 1, the linkage is β(1→3); for H type 2, it is β(1→4), as shown.

\[
\alpha\text{L-Fuc}(1,2) \downarrow \alpha\text{L-Fuc}(1,2) \\
\beta\text{DGal}(1,3)\beta\text{DGlcNAc-O-R} \downarrow \beta\text{DGal}(1,4)\beta\text{DGlcNAc-O-R}
\]

H type 1  H type 2

These arrangements result in considerable conformational difference between the trisaccharides. For H type 2, the β(1→4) linkage exposes the 2′ hydroxyl group of the terminal fucose; however, this reactive site is shielded when the linkage is β(1→3), as in the H type 1 antigen (27). Differences were observed in protein recovery from the two columns; considerably more protein was eluted from the H type 2 column than from the H type 1 column. This provides evidence that the spatial conformation of the H-antigen trisaccharide is an important factor in receptor recognition by the CROMP. This could have in vivo consequences, as the different H antigens are not evenly distributed in the body. H type 2 antigen is expressed on erythrocytes, and H types 1 and 2 are both found on the epithelial surface of the gastrointestinal tract (32). In individuals who are secretors of blood group substances, H antigens are also found in soluble form in saliva and other secretions (27, 32). Thus, different tissues and different individuals may exhibit different susceptibility to aeromonad attachment through H-specific adhesins.

When solubilized OMs were subjected to SDS-PAGE and immunoblot analysis with serum 3/83, approximately 30 bands were visualized (Fig. 2). However, when CROMPs which were eluted from the H type 2 affinity column were subjected to similar analysis, three bands (Mr, 43,000, 40,000, and <14,000) were visualized (Fig. 2). The two larger components (Mr, 43,000 and 40,000) were determined to be protein. The smaller component (Mr, <14,000 Da) is probably copurified LPS, which was associated with CROMPs. Thus, the affinity column afforded a considerable degree of purification of proteins from the original preparation of solubilized OM.

H substance, crudely purified from group O secretor saliva, was the basis of an elution buffer used for affinity purification of CROMPs from the OMs of *A. hydrophila* A6 on Synsorb H type 2. The eluate was alkali treated and concentrated with Centricon-10 to dissociate and remove the ligand. The protein yield achieved with H-substance elution buffer was 30% of the protein originally applied to the Synsorb. This compares to 14% recovery with alkali-eluted CROMPs. The Coomassie blue-stained protein component of H-substance-eluted CROMPs had Mr similar to those of alkali-eluted CROMPs (Mr, 43,000 and 40,000). Thus, ligand elution of CROMPs provided CROMPs identical to alkali-eluted CROMPs with more than 100% of the recovery achieved by using alkali elution.

Adhesins, primarily associated with fimbiae or pili, have been described in aeromonads (17-19). *A. hydrophila* A6 can be grown in conditions which do not favor the production of pili; these bacteria are still able to hemagglutinate human erythrocytes. The OM hemagglutinin can be blocked by serum 3/83 (4). Filamentous adhesins were eliminated during the purification procedure because they are insoluble in OTG and are sedimented in the centrifugation step following solubilization (21).

Although the CROMP retained its carbohydrate-binding capacity, as shown by its ability to bind to the affinity columns and to be specifically eluted by H-substance elution buffer, it was unable to function as a hemagglutinin. A possible explanation for this is that the linking of adhesin (hemagglutinin) and receptor (H trisaccharide) may require a higher affinity between the ligand and substrate when the substrate is located on the surface of negatively charged erythrocytes than when it is located on the surface of a neutral matrix such as Synsorb. Another possible explanation for this anomaly includes partial denaturation of the CROMP resulting from preparative procedures, such as detergent treatment. In another case, that of isolated fimbiae, monovalency has been proposed as an explanation for their inability to cause hemagglutination (7). Similarly, it is possible that CROMPs are monovalent and unable to crosslink erythrocytes unless a number are attached, and appropriately oriented, on the surface of a bacterium.

To investigate whether antiserum to isolated CROMPs also reacted with the intact hemagglutinin, two experimental strategies were employed. Serum 3/91 was produced by immunizing a rabbit with the 43,000-Da protein of the CROMP eluate. This protein was chosen because previous work had indicated that *A. hydrophila* A6 produces an OM hemagglutinin (Mr, 43,000) (4). This serum inhibited hemagglutination of *A. hydrophila* A6, indicating that a component of the CROMP fraction has immunological similarity to the hemagglutinin. Conclusive evidence that the antiserum to isolated OM proteins also reacted with H-reactive hemagglutinin components in intact bacteria was the recovery of HI activity from CROMP-latex beads following adsorption with serum 3/83 (see Results).

Antiserum 3/91, produced against the 43,000-Da CROMP, reacted in immunoblot assays with three OM components (Mr, 43,000, 40,000, and <14,000) (Fig. 3). These had the same Mr as the components of the original CROMP preparation. The molecular mass of the CROMP is similar to that suggested for the hemagglutinating adhesin of *A. hydrophila* A6 (Mr, 43,000) (4). The reaction of this serum with 40,000- and <14,000-Da components of the OM suggests that the 43,000-Da protein may have been partially degraded or incompletely disrupted by component subunits during the experimental procedures. The exact relationship of the smaller components to the 43,000-Da protein has not been resolved and will be the subject of further investigations.

The OM of a gram-negative organism is its primary link with the environment, providing the bacterial cell with a
regulatory system that determines what may affect, enter, or leave the bacterium (16). Porins, which constitute a major group of proteins in the OM, contribute significantly to this regulatory function and typically have a molecular size in the range of 30,000 to 48,000 Da (16). The functions of various porins include transport of small hydrophilic molecules, ions, and sugars (16). The CROMP described in this report is in this size range, an association which led us to speculate whether the adhesion might be a porin or a porinlike molecule. The H antigen, to which the bacterium attaches, has L-fucose as a terminal sugar (Fig. 4). The CROMPs we isolated also have H reactivity. A hypothesis of considerable interest is that CROMPs may contain a porin responsible for L-fucose transport (or that they have developed from such a porin). When this putative porin encounters a cell with fucose as the terminus of the carbohydrate component of a surface polymer (e.g., H antigen), it would attach to, but be unable to transport, the fucose across the OM. The binding site of this porin would then remain attached to the fixed fucose site (e.g., erythrocyte or intestinal epithelium) and thus act as an adhesin. Further studies on the physical and biological properties of these CROMPs to examine this proposition and to develop an understanding of the origins and roles of these molecules, particularly with regard to the colonization strategies of aeromonads associated with enteric disease, are in progress.

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