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# NOTES

## Carbohydrate-Reactive, Pore-Forming Outer Membrane Proteins of *Aeromonas hydrophila*

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**Two outer membrane proteins of *Aeromonas hydrophila* A6, isolated in a one-step affinity chromatography process based on carbohydrate reactivity, were found to be pore-forming molecules in artificial planar bilayer membranes. These carbohydrate-reactive outer membrane proteins (CROMPs; $M_r$s, 40,000 and 43,000) were subjected to amino acid analysis. The amino acid profiles for these two outer membrane proteins were almost identical. A partial protein sequence of a 14-amino-acid fragment of the 40,000-Da protein revealed homology with outer membrane porins of *Escherichia coli* and *A. hydrophila*. CROMPs were compared with carbohydrate-reactive porins also extracted from outer membranes of *A. hydrophila* A6. These porins were isolated by using standard porin purification techniques (insolubility in 2% sodium dodecyl sulfate, solubility in 0.4 M NaCl, and Sephacryl S-200 gel filtration), and then Synsorb H type 2 affinity chromatography was done. The physical and functional properties of the carbohydrate-reactive porins and CROMPs were found to be identical. On the basis of pore-forming properties in planar lipid bilayers and channel inhibition with maltotriose solutions, a nonspecific, general diffusion porin and a LamB-like maltoporin were identified in both CROMP and carbohydrate-reactive porin preparations. To our knowledge, the use of carbohydrate reactivity to isolate channel-forming proteins from bacterial outer membranes has not been reported previously.**

*Aeromonas* spp. have been shown to be important gastrointestinal pathogens, particularly in young children (2, 3, 9). However, characterization of the pathogenicity of this bacterium and identification of virulent strains remain problematic. Virulence factors in other gastrointestinal pathogens, such as *Salmonella* spp., *Vibrio* spp., and *Escherichia coli*, include fimbrial adhesins, lipopolysaccharides, exotoxins, and outer membrane adhesins (32). The principal mechanism of virulence in *Aeromonas*-associated gastrointestinal disease is believed to adhesive-enterotoxic (12), and this virulence-related adhesion is mediated by adhesins on the outer surface of the bacteria.

Detection of an outer membrane hemagglutinin of *Aeromonas hydrophila* which is sensitive to specific sugars has been reported (5). It is thought that this lectinlike reactivity may be associated with the ability of this organism to adhere to carbohydrate-rich surfaces such as the human gut, thereby contributing to colonization of this organism in enteric disease (4, 29). With affinity chromatography, several outer membrane proteins have been isolated from *A. hydrophila* on the basis of carbohydrate reactivity (29). In this study, we report further characterization of these carbohydrate-reactive outer membrane proteins (CROMPs) of *A. hydrophila* A6, using amino acid analysis, protein sequencing, and planar lipid bilayer analysis, and demonstrate that these proteins have the properties of outer membrane porins and specific channels. The possibility that there is identity between pore-forming CROMPS and adhesins of *Aeromonas* spp., which contribute to the virulence of the organism, is discussed.

CROMPs were isolated from outer membranes of *A. hydrophila* A6 by the one-step affinity chromatography technique of Quinn et al. (29). Outer membranes of *A. hydrophila* A6 were prepared by pressure disruption and differential centrifugation. No contamination of this preparation by cytoplasmic membrane, as assessed by NADH oxidase activity (31), was detectable. The membranes were then solubilized in *n*-octyl-β-D-thioglucopyranoside and adsorbed to Synsorb H type 2 (Chembiomed). The ligand of this affinity matrix is a trisaccharide which resembles the terminal sugars of the H type 2 antigen (Fig. 1). Following adsorption, the nonadsorbed fraction was collected. The components of this fraction were found to be indistinguishable from the solubilized outer membrane starting material when analyzed by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot techniques with serum 3/83, as previously described (29). This indicated that saturation of the available trisaccharide sites on the affinity column was achieved. To remove unadsorbed material, the column was repeatedly washed with 50 mM phosphate-buffered saline (pH 7.4) until no protein was detectable in the column eluate ($A_{280}$). Buffer containing soluble H substance isolated from saliva was applied, and 0.6 mg of protein per g of dry Synsorb affinity matrix was recovered. Elution with the H oligosaccharides from saliva was shown to be specific, as elution with 60 mM α-L-fucose in phosphate buffer (pH 7.5), glycine buffer (pH 3.0), or SDS-containing phosphate buffer (pH 7.4) failed to yield detectable protein when analyzed by SDS-PAGE and immunoblot techniques as described above. However, when columns were

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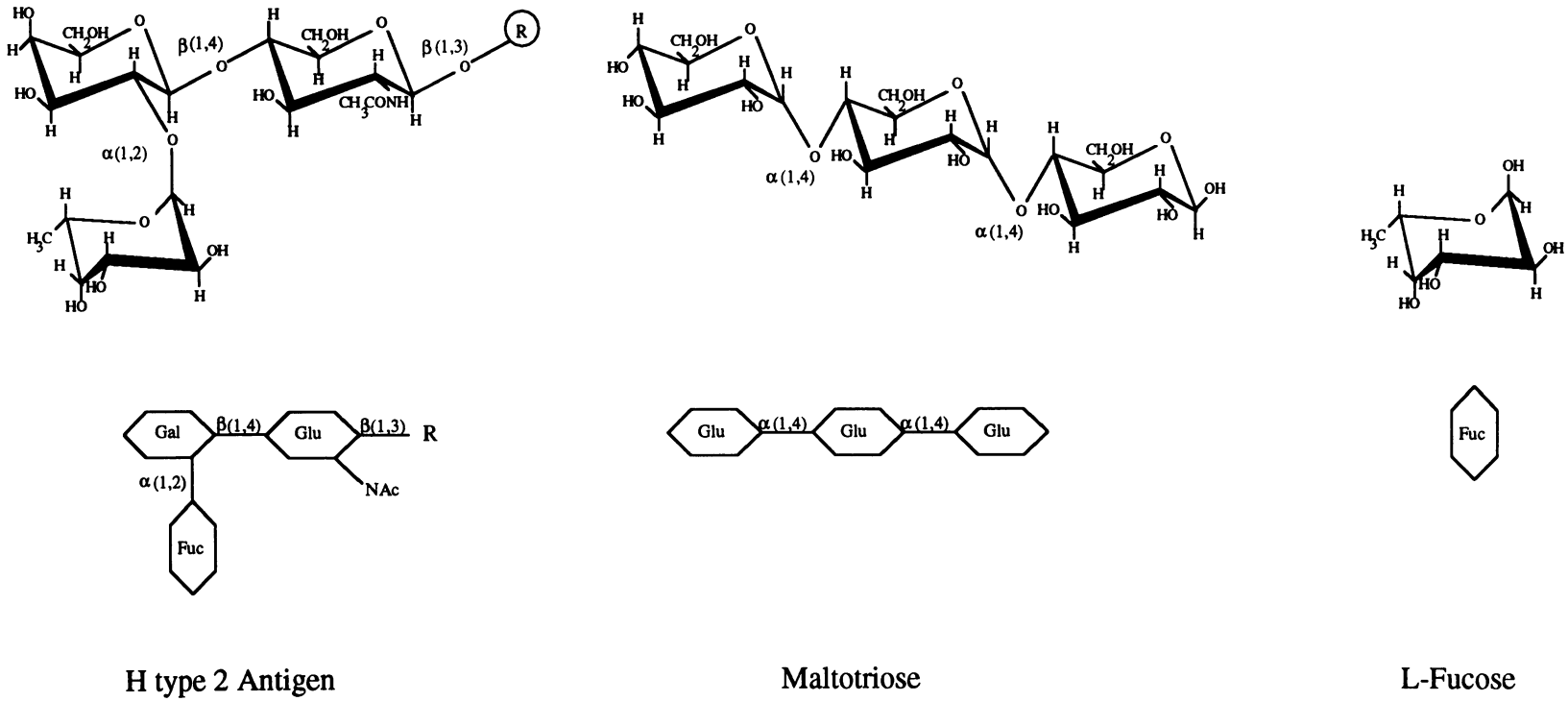


FIG. 1. Structures of carbohydrates discussed; terminal trisaccharide of the H type 2 antigen (affinity ligand for the purification of CROMPs), β-D-maltotriose (inhibits activity of channel B), and α-L-fucose (inhibits adhesion of *A. hydrophila* to human erythrocytes, rabbit brush borders, and human colonic cells). Gal, D-galactose; Fuc, L-fucose; NAc, *N*-acetyl group; Glu, D-glucose, R, core sugar chain.

eluted with 1% $NH_4OH$ buffer (pH 11.0), a small amount of protein was recoverable from the eluate (0.1 mg of protein per g of dry Synsorb). Elution under alkaline conditions is consistent with the presence of some hydrophobic interactions between the outer membrane receptor proteins and Synsorb H type 2. Lectins commonly bind to their complementary carbohydrate by hydrophobic bonding (20).

The H-substance-eluted fraction was separated by SDS-PAGE (12.5%) by the method of Laemmli (16) and found to contain two protein components ($M_r$s, 43,000 and 40,000). To characterize these proteins further, amino acid analysis and partial protein sequencing were performed. Preparative SDS–15.0% polyacrylamide gels of isolated CROMPS were transferred to Immobilon membranes (Millipore) (18). Blotted proteins were localized by staining with Ponceau S (30) and excised. For amino acid composition analysis, the proteins were hydrolyzed by 6 N HCl and analyzed in an Applied Biosystems model 420 amino acid analyzer, using procedures described by the manufacturers. The two proteins were found to be similar in composition, the ratio of hydrophobic/neutral/hydrophilic amino acid residues being 32.4:33.7:33.9 for the 40,000-Da protein and 31.2:35.6:33.4 for the 43,000-Da protein. Amino acid N′-terminal sequencing was performed on a 14-amino-acid fragment of the purified 40,000-Da protein, using an Applied Biosystems model 470A gas phase sequenator running a standard operating program. A GenBank search using the Fasta program (28) showed that the fragment shared identity with DNA sequences of genes coding for precursors of the *E. coli* porin proteins, 58% with OmpC (21) and PhoE (27) and 50% with OmpF (10). Substantial homology was found with an *A. hydrophila* outer membrane protein, protein II, as described by Jeanteur et al. (13). Only the third amino acid differed, which was a valine in the 40,000-Da protein and isoleucine in protein II. Although we were unable to obtain good N′-terminal sequence data for the 43,000-Da CROMP, these results suggested that these CROMPs isolated from *A. hydrophila* A6 could be porins or porinlike proteins which compare well to known outer membrane porin proteins of *Aeromonas* spp. and *E. coli.*

In an investigation of their potential to be pore-forming proteins, CROMPs were compared with proteins isolated from *A. hydrophila* A6, using standard procedures for bacterial outer membrane porins. Techniques described by Nikaido (23) were used to isolate peptidoglycan-associated porins on the basis of insolubility in SDS and solubility in high NaCl concentrations. From this preparation, a carbohydrate-reactive fraction was isolated by affinity chromatography on Synsorb H type 2 with specific oligosaccharide elution (29) and analyzed by SDS-PAGE. Two proteins ($M_r$s, 40,000 and 43,000) which were indistinguishable from CROMPs were detected in the carbohydrate-reactive porin eluate (Fig. 2). In comparison, Jeanteur et al. (13), using detergent solubilization (Nonidet P-40) and ion-exchange chromatography, detected six different outer membrane proteins in *A. hydrophila*. Our affinity-purified proteins appear to be a subpopulation of the known outer membrane proteins of *A. hydrophila*.

Determination of the pore-forming ability of the CROMP and carbohydrate-reactive porin fractions was performed by using modifications of the black lipid bilayer procedure of Benz et al. (6) and the planar bilayer procedure of Knowles et al. (14). CROMPs or carbohydrate-reactive porins were diluted in Tris-SDS solution (final concentration; 2 μg of protein per ml, 1.25 mM Tris HCl, 0.025% SDS, 0.75 mM $NaN_3$). Bilayers were formed by painting a mixture of lipids dispersed in *n*-decane, (17.5 mM 1-palmitoyl-2-oleoylphosphatidyl ethanolamine, 16.5 mM 1-palmitoyl-2-oleoylphosphatidyl choline,

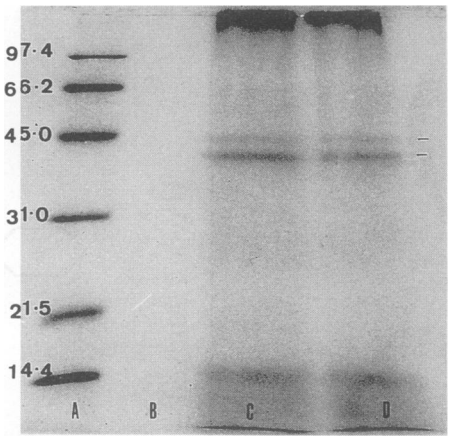


FIG. 2. Comparison of CROMPs and carbohydrate-reactive porin proteins of *A. hydrophila* A6, using SDS-PAGE: Coomassie blue-stained SDS–12.5% polyacrylamide gel of CROMPs and carbohydrate-reactive porins. Lane A, prestained low-molecular-mass markers (in kilodaltons; Bio-Rad); lane B, H-substance elution buffer prepared from secretor saliva (29); lane C, CROMPs; lane D, carbohydrate-reactive porins. No differences were seen in the protein bands of the CROMPs and carbohydrate-reactive porin fractions as revealed by Coomassie blue staining.

and 1.0 mM 1-palmitoyl-2-oleoylphosphatidyl serine; Avanti Polar Lipids, Pelham, Ala.) across a 0.3-mm hole separating two chambers containing unbuffered 200 mM KCl in typical voltage clamp bilayer equipment. A KCl gradient (450 mM *cis*–200 mM *trans*) was established to encourage incorporation of the proteins into the bilayer and thus to enable detection of ionic currents in the presence of a transmembrane holding potential. Incorporation of channel-forming molecules was observed as steplike changes in transmembrane current amplitude. Single-channel currents were filtered (frequency devices, low-pass filter, 902), displayed on a digital oscilloscope (Gould DS0 400), and stored on a digital audio tape recorder (Sony 750 ES). Recorded data were filtered at 100 Hz and analyzed after digitization at 300 Hz on a personal computer, using Axopatch (version 5.5) software (Axon instruments).

The pore-forming ability of CROMPs in planar lipid bilayers was compared with that of carbohydrate-reactive porins. When 5 ng of CROMPs was added *cis* to the bilayer, average single-channel current fluctuations of 27.8 and 1.8 pA were

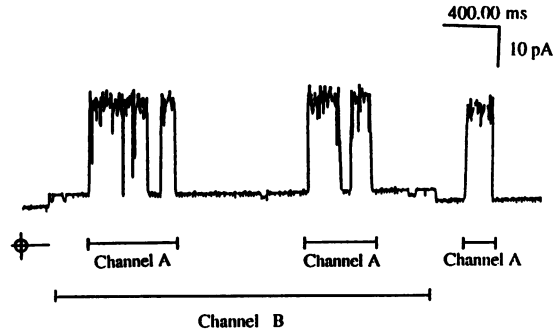


FIG. 3. Sample current trace from CROMPs of *A. hydrophila* A6 in a planar lipid bilayer. The conductivities of channels A and B are 278 and 18 pS, respectively. (+100 mV; KCl gradient, 450 mM: 200 mM *cis/trans*).

detected (KCl, 450:200 mM *cis/trans*; +100 mV applied to the bilayer, *cis* with respect to *trans*) (Fig. 3). Currents recorded in the presence of our carbohydrate-reactive porins were identical in amplitude and mean open times to those seen in the presence of CROMPs (data not shown). The two different amplitude current steps and gating were consistent with two different types of ion channel rather than substates within a single channel. One, designated channel A, had an average conductance of 278 pS and the second, designated channel B, had an average conductance of 18 pS (KCl, 450:200 mM *cis/trans*). When the protein samples were preheated at 100°C for 10 min, no current fluctuations could be detected following addition *cis* to the bilayer (data not shown). It is known that some pore-forming porins dissociate into nonfunctional monomers and lose their trimer structure when heated to 100°C (25).

As these pore-forming proteins were isolated on the basis of carbohydrate reactivity, it was predicted that the channel activity might be inhibitable with sugar solutions. Carbohydrate-inhibitable channels have been described in *E. coli* (7), *Salmonella typhimurium* (34), and *A. hydrophila* (13), all of which can be inhibited by maltose and, to a greater extent, by maltotriose. The activity of channel A from the CROMPs preparation was unaffected by the addition of up to 0.1 M α-L-fucose (Sigma), 0.1 M maltose (Sigma), or 0.1 M maltotriose (Sigma) *cis* to the bilayer. By contrast, maltotriose inhibition of channel B (10 mM maltotriose in 200 mM unbuffered KCl added *cis* to the bilayer) was observed when either a single or multiple (10 to 20) channels were induced in the bilayer and was reversible by perfusing the *cis* chamber with fresh 200 mM KCl (data not shown). This effect may have been due to either pore blockage or an inhibition of the channel opening. Studies of inhibition of channel B activity with other sugars are in progress. The LamB porin of *E. coli* has a specific binding site for maltose and maltotriose, as demonstrated by these ligands' ability to block the channel in black lipid bilayers (7).

Our results suggest that in addition to a large conductance porin (channel A) both the CROMPs and carbohydrate-reactive porin fraction contain a LamB-like maltoporin (channel B). This supports the work of Jeanteur et al. (13), who reported on the physical and functional properties of several outer membrane proteins including a general diffusion porin (protein II) and a maltoporin (protein I) from *A. hydrophila* Ah 65. Channel A and protein II may be similar channels, as their amino acid N′-terminal sequences share differ by only one residue. Channel B and protein I share similar maltoporin properties. The conductances observed for channels A and B do differ from those reported for proteins II (850 ± 50 and 50 pS) and I (150 ± 25, 50, and 1 nS), but this may be explained by the different ionic conditions employed: symmetrical 1 M NaCl by Jeanteur et al. (13) in contrast to 450:200 mM *cis/trans* KCl in the experiments reported here. Another possible explanation of the differences in the reported conductances is that channels A and B represent a carbohydrate-reactive subset of proteins II and I which have slightly different structures and properties.

The carbohydrate-reactive properties of channel B protein have been demonstrated by using sugar inhibition in planar lipid bilayer experiments; however, similar preliminary experiments on channel A do not demonstrate this carbohydrate reactivity. A possible explanation is that the two proteins are closely associated with each other within the outer membrane, but only one possesses an H-reactive, lectinlike domain. When this protein was isolated by carbohydrate affinity chromatography, the other may have copurified.

Outer membrane pore-forming proteins previously described in various *A. hydrophila* strains are of molecular weights 43,000 (8) and 27,000, 36,000, 39,000, and 47,000 (13). Interestingly, a 43,000-Da outer membrane hemagglutinin of *A. hydrophila* which is sensitive to α-L-fucose has also been detected (4). The molecular weights of our CROMPs and carbohydrate-reactive porins as analyzed by SDS-PAGE were identical. In addition to molecular weight, they are known to have other physical and functional properties in common. For example, outer membrane porin molecules are resistant to trypsin (24, 33), and the hemagglutinin of *A. hydrophila* A6 is also resistant to proteases (1).

Gram-negative bacteria have been shown to have different types of sugar-reactive proteins present on their outer surfaces, including sugar-binding toxins, fimbrial adhesins, outer membrane adhesins, and sugar transport proteins (20). Outer membrane porins of gram-negative bacteria have been previously associated with virulence in gastrointestinal infections (11, 19, 22, 35), although the exact mechanism of their contribution to virulence is unclear.

The outer surface of *A. hydrophila* has been shown to be carbohydrate reactive. Its adherence to human erythrocytes (in vitro hemagglutination, which is reversible with L-fucose) (5), rabbit brush borders (reversible with L-fucose) (17), and cultured human colonic cancer cells (also reversible with L-fucose) (26) is dependent on ligands expressed on external surfaces. The lectinlike outer membrane hemagglutinin of *A. hydrophila* may play a role in the colonization strategies of this organism (4) by facilitating adhesion to carbohydrate-rich surfaces of the human gut.

*Aeromonas* is a common aquatic organism which is often present in drinking water supplied for human consumption. However, it is probable that not all strains of this bacterium have a sufficient array of virulence factors to be able to cause human disease. Therefore, the detection of relevant virulence factors in strains isolated from drinking water, or in clinical material, may provide useful information regarding the significance of those isolates. If CROMPs do prove to be a factor associated with the virulence of this organism, then it is useful to note that the carbohydrate-reactive proteins described are not uniformly distributed among the DNA hybridization groups of *Aeromonas* spp. (15). Expression of these proteins in *Aeromonas* spp. may therefore assist in the differentiation of virulent from avirulent strains.

We have detected two types of porins in a carbohydrate-reactive fraction of outer membranes isolated from a virulent strain of *A. hydrophila* and demonstrated their physical and functional relatedness to known outer membrane proteins of *Aeromonas* spp. and *E. coli*. Further experiments to characterize these channel-forming proteins and to determine their relationship to virulence are required. Adhesion to host cell surfaces, mediated by outer membrane porins of these types in gram-negative bacteria, may prove to be an important step in the initial colonization of bacterial pathogens of the human enteric tract.

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