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Hemagglutination Properties and Adherence Ability of *Aeromonas hydrophila*

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Human diarrheal isolates and enterotoxigenic strains of *Aeromonas hydrophila* are strong hemagglutinators with human blood cells. Sugar inhibition studies and yeast coagglutination tests with 11 selected strains revealed six different hemagglutination mechanisms for this species. These were characterized by inhibition by L-fucose, inhibition by D-galactose, inhibition by D-mannose, and two distinguishable mechanisms which were inhibited by either L-fucose or D-mannose, one being pilus mediated. Inhibition of hemagglutination by another strain required a combination of D-galactose and D-mannose. The hemagglutinating strains also attached well to human blood cells and buccal epithelial cells, with as many as 55% of the cells of a culture attaching successfully. In some cases the attachment to buccal epithelial cells appeared to involve mechanisms different from those used for hemagglutination.

Aeromonas hydrophila is recognized as an opportunistic pathogen in debilitated or immunologically compromised humans (14), and there are increasing numbers of reports of systemic infections in apparently normal people (4, 9, 22, 24). However, the most frequent reports concern the involvement of *A. hydrophila* in acute diarrheal disease. The organism has been described in this enteropathogenic role throughout the world, and it appears that its enteropathogenic potential is mediated by an enterotoxin (2, 3, 15).

Studies on the virulence mechanisms of other enteropathogenic bacteria have revealed another crucial factor: an organism must be able to attach to target cells of the host animal if it is to cause gastrointestinal disease. Enteric pathogens are commonly found in the upper respiratory tract and oral cavity during acute and convalescent stages of gastrointestinal disease (10, 21), and attachment of pathogens to cells of the gastrointestinal tract has been described elsewhere (8, 11, 13, 18). This attachment allows for maximal effect of any toxins that the organism may produce and is a prerequisite for successful invasion.

The ability of a variety of enteropathogenic bacteria to cause hemagglutination and to bind to buccal epithelial cells has been used to study the various mechanisms of their attachment to animal cells. However, the adhesive properties of *A. hydrophila* have not received this attention, although Tweedy et al. (23) described one example of mannose-sensitive hemagglutination by a strain of *Aeromonas liquefaciens*. Because of the numbers of reports of isolation of *A. hydrophila* from acute diarrheal disease and because of the importance of attachment in the pathogenesis of gastrointestinal disease, we have examined the ability of *A. hydrophila* to adhere to human cells by determining its hemagglutination characteristics and by quantitating its attachment to human blood cells and buccal epithelial cells. Here we report the occurrence of a variety of different attachment mechanisms for different strains of this species and the ability of a large proportion of a population to adhere to human cells.

MATERIALS AND METHODS

Bacterial strains. The bacteria used and their sources are shown in Table 1. All fecal strains were from human diarrhea. Strains Ah423, Ah424, Ah434, and Ah435 were received as enterotoxigenic. We did not attempt to confirm this. Strains were confirmed as *A. hydrophila* with API 20E multitest strips (Analytab Products, Plainview, N.Y.), resistance to 2,4-diamino-6,7-diisopropylpteridine phosphate (150-μg disk) (Sigma Chemical Co., St. Louis, Mo.), and cytochrome oxidase activity. All strains were motile. Stock cultures were lyophilized, and working cultures were maintained on Trypticase soy agar (TSA; BBL Microbiology Systems).

Culture conditions. Bacteria were grown on TSA, human blood agar (HBA) prepared from TSA with 5% citrated blood added, nutrient agar, MacConkey agar, brain heart infusion agar, Trypticase soy broth (TSB; BBL Microbiology Systems), and TSB without glucose or phosphate (TSB-GP) (Trypticase peptone, 15 g; phytone peptone, 5 g; NaCl, 5 g; distilled water, 1 liter; pH 7.4). Incubation was aerobic at 37°C. Dehy-

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TABLE 1. *Bacterial strains*

Culture no.	Species received as:	Source
A6	*A. hydrophila* A6	Human feces, H. M. Atkinson, Australia
A10	*A. hydrophila* A10	Human blood, H. M. Atkinson, Australia
A46	*A. hydrophila* A46	Human feces, H. M. Atkinson, Australia
A69	*A. hydrophila* A69	Human feces, H. M. Atkinson, Australia
Ah412	*A. sobria* 93VL2632[a]	Human feces, J. F. Lee, England
Ah413	*A. sobria* 61VL2685	Human feces, J. F. Lee, England
Ah416	*A. hydrophila* 52VL1362	Human feces, J. F. Lee, England
Ah423	*A. hydrophila* A54	Human feces, B. Dunsmore and M. Gurwith, Canada
Ah424	*A. hydrophila* A52	Human feces, B. Dunsmore and M. Gurwith, Canada
Ah434	*A. hydrophila* 593	Water, S. Sanyal, India
Ah435	*A. hydrophila* 167	Water, S. Sanyal, India

[a] *A. sobria* cannot be distinguished from *A. hydrophila* by DNA-DNA hybridization (16).

drated media were obtained from Difco Laboratories, Detroit, Mich. (MacConkey and brain heart infusion agars) and BBL Microbiology Systems, Cockeysville, Md.

Hemagglutination. Human group O blood was collected by venipuncture, immediately placed into Alsever solution (1), and stored at 4°C until required. Immediately before use, blood cells were washed three times in Dulbecco phosphate-buffered saline, pH 7.4 (PBS) (Oxoid, Basingstoke, England), and a 3% (vol/vol) suspension was prepared in this saline. Plate-grown culture suspensions of bacteria were prepared in PBS to yield approximately 1.5×10^9 bacteria per ml by McFarland turbidity standards. Broth-grown cultures were harvested and washed twice in PBS, and the cells were suspended in PBS to the same turbidity as plate-grown suspensions.

Slide hemagglutinations were performed at room temperature by mixing 20 μl of blood suspension with 20 μl of bacterial suspension on a slide and gently rocking the slide by hand. A PBS-blood cell control was always included. Strains were considered negative hemagglutinators if hemagglutination had not occurred within 10 min.

The minimum hemagglutinating dose (MHD) was measured as the smallest number of bacterial cells/ml that gave visible hemagglutination in 10 min and the hemagglutinating power of a culture was calculated as 10^{11}/MHD (5).

Inhibition of hemagglutination. Slide hemagglutination was performed as before with 20 μl of blood suspension added to 20 μl of a 1% (wt/vol) solution of the test sugar in PBS plus 20 μl of a suspension containing 10 MHD of bacteria. Reactions were compared with positive controls (20 μl of bacteria, 20 μl of PBS, and 20 μl of blood cells) and a negative control (40 μl of PBS and 20 μl of blood cells). All sugars were of the highest grade available (Sigma and Serva, Heidelberg, Germany). Any sugars which inhibited hemagglutination were tested in a series of two-fold decreasing concentrations until an end point was reached. The minimum hemagglutination inhibitory concentration of sugar was recorded as the lowest concentration of sugar in the reaction mixture which completely inhibited hemagglutination by 10 MHD of bacteria.

Yeast cell coagglutination. Dried bakers' yeast (Engendura, Edward Agencies Ltd., Winnipeg, Canada) was reconstituted with PBS, washed three times in PBS, and resuspended to a concentration of 3% (vol/vol) in PBS. Yeast coagglutination tests were performed in a similar manner to hemagglutination. Results were read after 10 min at room temperature and compared with positive and negative controls. Minimal yeast agglutinating concentration and yeast agglutinating power were determined in similar fashion to MHD and hemagglutinating power.

Inhibition of yeast coagglutination. Testing of the ability of sugars to inhibit yeast coagglutination was performed in a manner similar to hemagglutination inhibition.

Attachment. The ability of bacteria to attach to human group O blood cells and human buccal epithelial cells was quantitated by a modification of the method described by Lambden et al. (17). Bacteria were radiolabeled by growth in TSB-GP with added [1-^{3}H]glucose (2.5 μCi/ml) (Amersham Corp., Oakville, Canada), for 18 h at 37°C. The bacteria were then washed three times in PBS and resuspended in PBS to a concentration of 10^7/ml. Buccal cells were collected from a minimum of six adults, pooled, washed three times in PBS, and adjusted to a concentration of 4% (vol/vol) with PBS. For blood cell attachment a 10% (vol/vol) suspension was used. A 1-ml amount of buccal or blood suspension was added to 1 ml of tritiated bacteria, and the reaction was allowed to proceed with constant gentle mixing on a mechanical roller for 1 h at 37°C. Control tubes contained only the tritiated bacteria and 1 ml of PBS. After the incubation period, unattached bacteria were separated from bacteria attached to cells by layering 500 μl of reaction mixture onto a 10-cm column of Ficoll 400 (47.6 g/liter in PBS) (Sigma) in a Nunclon plastic tube (Nunc, Roskilde, Denmark), centrifuging at 500 × *g* for 3 min in a swing-out head, freezing the tube at −70°C, and recovering the cell pellet by cutting off the tip of the tube with a miniature circular saw. A second control was used to estimate the amount of extracellular radioactivity that would associate with the eucaryotic cells. In this control, the bacterial suspension was incubated by itself for 1 h under the test assay conditions. Bacterial cells were then removed by recentrifugation at 500 × *g* for 10 min. The cell-free supernatant was removed and added to a buccal or blood suspension, and the reaction was allowed to proceed as for the test assay. This control was then subjected to the Ficoll separation procedure. The radioactivity associated with the tip fractions was determined by scintillation counting with 10 ml of Aquasol-2, universal liquid scintillation counting cocktail (New England Nuclear Corp., Boston, Mass.), in a Beckman LS8100 counter with the automatic quench correction mode. Epithelial cell-containing fractions were counted without further treatment, but fractions con-

taining blood cells were first lysed with 100 μl of 1.0 M NaOH for 5 min and then decolorized for 3 h at 37°C after the addition of 100 μl of 30% H_2O_2. After the addition of scintillant, the tubes were placed in the dark for 18 h before counting. The radioactivity in samples of uncentrifuged reaction mixtures was also determined. The degree of attachment was calculated from the ratio of radioactivity associated with the Ficoll-pelleted eucaryotic cells to the total radioactivity in the reaction mixture.

Electron microscopy. The presence of pili on hemagglutinating plate cultures was determined by staining of unfixed specimens with uranyl acetate on Formvar-coated grids. Grids were examined in a Philips EM300 transmission electron microscope. Hemagglutination and the adherence of bacteria to buccal epithelial cells were observed by scanning electron microscopy. The cells were passed through a Ficoll gradient and fixed with 5% glutaraldehyde (Soquelec, Montreal, Canada). After washing in distilled water, the cells were progressively dehydrated through 30, 40, 50, 60, 70, 80, 90, and 100% (vol/vol) acetone. Cells were then critical-point dried (Omar SPC-1500 critical point dryer) and gold coated for 5 min at 10 mA (Technics Hummer III Sputter Coater).

RESULTS

Hemagglutination. A preliminary screening of 37 strains of *A. hydrophila* isolated from humans or known to be enterotoxigenic showed that hemagglutinating activity is a frequent property of this bacterium, because 22 strains gave strong agglutination with human group O blood when tested off HBA plates.

Once it had been established that hemagglutination could be demonstrated with *A. hydrophila*, experiments were performed to determine optimum conditions for the production and detection of hemagglutinins. Ca^{2+} (4.5 μmol) was required for maximal hemagglutination by some strains (A6, Ah416, Ah424), but no Mg^{2+} requirement was demonstrable. Hemagglutination occurred equally well at 4, 10, 16, 20, 30, 37, and 42°C and in the pH range 6.3 to 8.3. Below pH 6.3, agglutination was perceptibly weaker and was barely detectable at pH 4.5, the lowest pH tested. Henceforth all hemagglutination tests were performed in PBS (containing 4.5 μmol of Ca^{2+}), pH 7.4, at room temperature. When bacteria were grown on different agar media, it was found that HBA and TSA produced the best hemagglutinins for all strains tested. Hemagglutinins were produced on brain heart infusion, nutrient, and MacConkey agars, but they were weaker. Bacteria grown in TSB agglutinated poorly or not at all, but when grown in TSB-GP strong hemagglutinins were produced in static cultures. Hemagglutinins were not, however, produced when bacteria were grown in TSB or TSB-GP rotated at 80 rpm. Bacteria grown on HBA at 16, 20, 30, 37, and 42°C produced hemagglutinins. For all subsequent experiments, bacteria were grown on HBA or in static TSB-GP at 37°C for 18 h.

Further investigations with three sugars known to inhibit hemagglutination in other bacterial genera (7, 8, 10, 20) (namely, L-fucose, D-galactose, and D-mannose) revealed a variety of inhibition patterns. Eleven strains were chosen for further testing because their hemagglutination properties appeared to represent a spectrum of sugar sensitivities. The ability of these strains to hemagglutinate was quantitated, and the results are shown in Table 2.

The five patterns of sugar inhibition represented by these 11 strains are also seen in Table 2. These are inhibition by L-fucose or D-mannose (A6, A10, Ah416, Ah424), inhibition by D-galactose and D-mannose in combination (A46), inhibition by D-mannose (A69), inhibition by D-galactose (Ah412, Ah413, Ah423, Ah435), and inhibition by L-fucose (Ah434). The weakest hemagglutinators were Ah416, Ah423, and Ah424 with a hemagglutinating power of 10^3. The hemagglutination by Ah412, the strongest hemagglutinator, was 500 times stronger.

Table 2 also shows the ability of *A. hydrophila* to coagglutinate with yeast cells. Since yeast cells possess surface mannans, these coagglutination tests can be used to further quantitate and differentiate mannose-sensitive hemagglutinins. The yeast agglutinating power data suggest that the L-fucose- or D-mannose-sensitive hemagglutinins of A6, Ah416, and Ah424 are different from that of A10. The latter did not coagglutinate with yeast and appears to represent a sixth attachment mechanism. Strains which produce hemagglutinins sensitive to D-galactose only do not coagglutinate with yeast, nor did Ah434 which is an L-fucose-sensitive strain. The best agglutinators of yeast were strains A46 and A69.

To define the structural characteristics required by the various sugars to inhibit hemagglutination, the inhibitory properties of additional sugars were tested with selected strains. The results in Table 3 show the inhibition of hemagglutination by the various sugars with strains A6, A10, A69, and Ah434. The difference between the hemagglutinins of A6 and A10 that was revealed by yeast coagglutination was confirmed by the inability of yeast mannan to inhibit the hemagglutination of A10. In addition, A10 was not inhibited by D-mannose-6-phosphate and displayed marked differences in sensitivity to L-fucosides and α-D-mannosides. The inhibition patterns shown by A69 and Ah434 were simpler. In the case of A69, only D-mannose and its derivatives inhibit, whereas Ah434 is only inhibited by L-fucose and its derivatives. The

TABLE 2. *Hemagglutination and coagglutination properties of A. hydrophila*

Strain[a]	Hemagglutinating power	Minimal sugar concentration inhibiting hemagglutination[b] (mM)			Coagglutination with yeast	Yeast coagglutinating power
		L-Fucose	D-Galactose	D-Mannose		
A6	1×10^4	0.5	N[c]	0.5	+	1×10^4
A10	1×10^4	0.3	N	0.9	−	NT[d]
A46[e]	5×10^4	N	N	N	+	7×10^4
A69	2×10^4	N	N	0.5	+	2×10^4
Ah412	5×10^5	N	0.9	N	−	NT
Ah413	2×10^5	N	0.9	N	−	NT
Ah416	1×10^3	2.0	N	2.0	+	8×10^3
Ah423	1×10^3	N	0.9	N	−	NT
Ah424	1×10^3	2.0	N	2.0	+	8×10^4
Ah434	2×10^5	0.08	N	N	−	NT
Ah435	2×10^5	N	0.9	N	−	NT

[a] Cultures were grown on human blood agar at 37°C for 18 h.
[b] Determined with bacterial suspensions at 10 times the minimal hemagglutination dose.
[c] N, No inhibition by sugar at 17 mM final concentration.
[d] NT, Not tested.
[e] Strain 46 is inhibited by a mixture of D-galactose and D-mannose containing 0.5 mM concentration of each sugar.

TABLE 3. *Sugar inhibition of hemagglutination by A. hydrophila strains A6, A10, A69, and Ah434*

Sugar	Minimal sugar concentration inhibiting hemagglutination (mM)[a]			
	A6	A10	A69	Ah434
L-Fucose	0.5	0.3	N	0.08
D-Fucose	N[b]	N	N	N
p-Nitrophenyl-α-L-fucoside	0.04	0.07	N	1.0
p-Nitrophenyl-β-L-fucoside	0.6	0.009	N	0.1
L-Galactose	0.5	0.3	N	0.08
D-Galactose	N	N	N	N
D-Mannose	0.5	0.9	0.5	N
L-Mannose	N	N	N	N
p-Nitrophenyl-α-D-mannoside	1.1	10.0	0.3	N
α-Methyl-D-mannoside	1.0	9.0	0.3	N
D-Mannoheptulose	1.6	0.8	8.0	N
D-Mannosamine	N	N	N	N
D-Mannose-6-phosphate	0.3	N	N	N
Yeast mannan	0.2[c]	N[d]	0.03[c]	N[d]
D-Glucose	N	N	N	N
D-Altrose	N	N	N	N
D-Talose	N	N	N	N

[a] Determined with bacterial suspensions at 10 times the minimal hemagglutinating dose.
[b] N, Not inhibitory at 17 mM final concentration.
[c] Values given for mannan concentration are percents (wt/vol).
[d] Mannan not inhibitory at 0.5% (wt/vol) final concentration.

TABLE 4. *Sugar inhibition of hemagglutination by A. hydrophila strain Ah413*

Sugar	Minimal sugar concentration inhibiting hemagglutination (mM)[a]
L-Fucose	N[b]
D-Fucose	0.8
L-Galactose	N
D-Galactose	0.7
Lactose	5.0
D-Galactopyranosyl-β-thio-galactopyranoside	5.0
D-Galactosamine	1.8
N-Acetyl-D-galactosamine	0.7
p-Nitrophenyl-α-D-galactoside	11.0
p-Nitrophenyl-β-D-galactoside	0.2
D-Glucose	N
D-Altrose	N
D-Talose	1.8

[a] Determined with bacterial suspensions at 10 times the minimal hemagglutinating dose.
[b] N, Not inhibitory at 17 mM final concentration.

pattern displayed by Ah413 was also simple (Table 4). In this case only D-galactose and derivatives inhibited hemagglutination. Tables 3 and 4 also show that in no case did D-glucose or D-altrose inhibit. However D-talose, which was not inhibitory for the hemagglutinins of A6, A10, A69, or Ah434, inhibited hemagglutination by Ah413.

In addition to differences in sugar sensitivities, some differences were shown in the stability of the hemagglutinins between different strains. The hemagglutinins of A6, Ah416, and Ah424 were labile when the bacteria were suspended in PBS at 25°C for 10 min, whereas those of the other eight strains were stable for 1 h. All strains were stable after 1 h at 25°C in TSB-GP. The hemagglutinins of all strains were destroyed by heating at 55°C for 5 min in PBS or TSB-GP.

Attachment. Quantitative and qualitative

differences in the ability of strains to adhere to blood cells and to buccal epithelial cells were revealed by radiolabel assay. Table 5 shows that Ah434 and Ah435 attach very well to both cell types. Strain Ah416 attached at 31% to blood cells, but at 3% it was a very poor attacher to buccal cells. Strain A6 with the same hemagglutination sugar sensitivity as Ah416 attached in a different manner. Although its attachment to erythrocytes was less than that of Ah416, its attachment to buccal cells was markedly better.

Electron microscopy. Bacteria were examined for the presence of pili by transmission electron microscopy. Cells of strain A6, when stained from a hemagglutinating plate culture, had numerous pili (Fig. 1), and most cells were pilated. Cells from the same culture, suspended in PBS for 10 min before staining, had no observable pili. A colony variant of strain A6 (designated A6b) did not produce hemagglutinins and was not pilated. This strain gave only 6% attachment to buccal cells and <1% attachment to blood cells. Occasional cells of strain A46 had one or two extremely long pili (3 to 5 μm), but no pili were observed in the other nine strains.

TABLE 5. *Attachment of A. hydrophila to human blood group O cells and human buccal epithelial cells*[a]

Strain	% Attachment	
	Blood cells[b]	Buccal cells[c]
A6	15	19
A10	25	20
A46	36	19
A69	26	20
Ah412	36	16
Ah413	19	24
Ah416	31	3
Ah423	17	12
Ah424	12	3
Ah434	55	25
Ah435	53	23

[a] Means of three experiments performed on three different occasions.
[b] Final concentration was 5% (vol/vol).
[c] Final concentration was 2% (vol/vol).

Scanning electron microscopy was used to examine the association between bacterial and human cells upon hemagglutination and to confirm the ability of the various strains to attach to buccal epithelial cells. A typical result is shown in Fig. 2. In this case *A. hydrophila* strain Ah423 is shown after hemagglutination and after attachment to buccal epithelial cells. Typically there was considerable bacterial clumping seen in hemagglutination, whereas bacterial cells attaching to buccal cells were often more evenly distributed on the buccal surface. A good correlation was noted between attachment as assayed by radiolabel experiments and attachment to buccal cells as visualized in the electron microscope.

DISCUSSION

We have shown that many strains of *A. hydrophila* are strong hemagglutinators of human

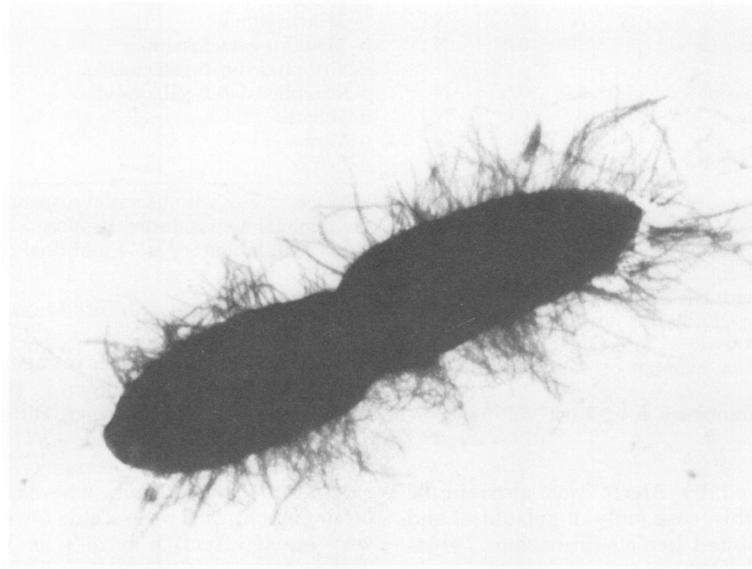

FIG. 1. *Electron micrograph of A. hydrophila strain A6 stained with uranyl acetate showing extensive pilation (×14,000).*

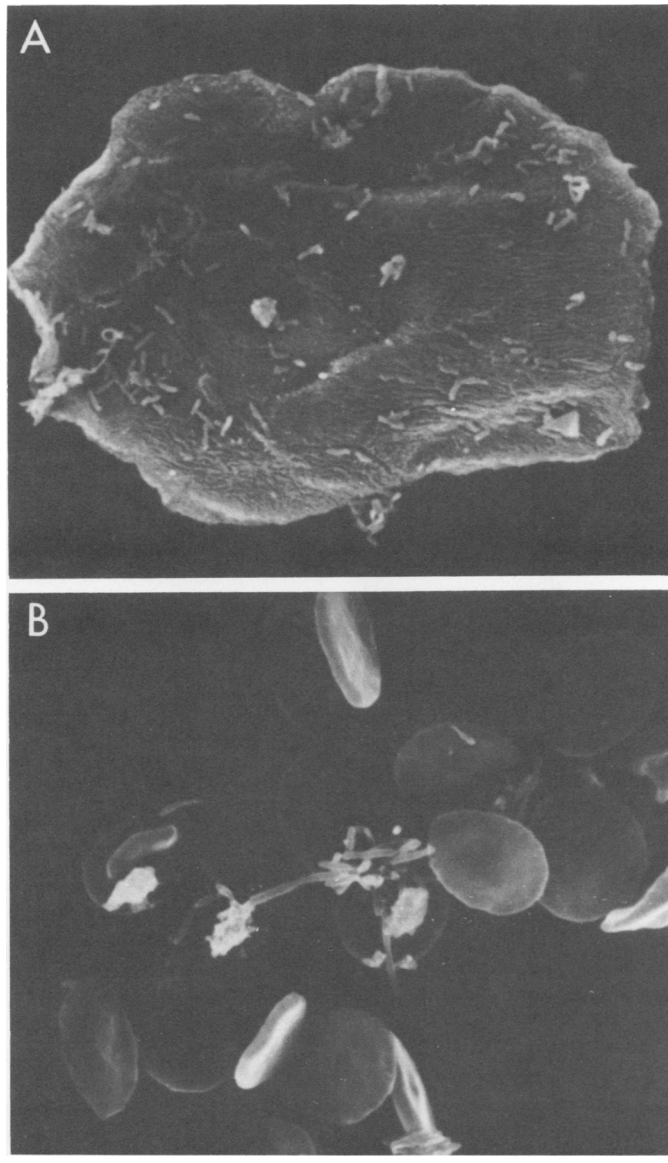

FIG. 2. *Scanning electron micrographs showing association of A. hydrophila strain Ah423 with human cells (A) attachment to buccal epithelial cells (×1,635); (B) hemagglutination with group O blood cells.*

blood cells, with hemagglutinating powers of the same order of magnitude as produced by other bacterial genera (6, 20). These hemagglutinins can be inhibited in a highly specific fashion by sugars in millimolar concentrations. The acuteness of this specificity can be adjudged by the ability of the bacterial hemagglutinin to recognize quite subtle structural differences in the positioning of single hydroxyl groups on hexoses as illustrated by the differences in activity displayed by D-galactose, D-glucose, and D-mannose. The hemagglutinins can be inactivated rapidly by heat (55°C for 5 min), indicating that they are probably protein. In view of the sensitivity and specificity and probable protein structure of the hemagglutinins, they may be ascribed a lectin-like nature. The concentrations of sugars required to inhibit *A. hydrophila* hemagglutination are similar to those described for other bacterial genera. Old (20) reported that the fimbrial hemagglutinin of *Shigella flexneri* could be inhibited by 1.1 mM D-mannose. This value is similar to the 0.5 to 2 mM range that we determined for 5 strains of *A. hydrophila*. In the

case of L-fucose sensitivity, the 1 mM value reported by Gones and Freter (13) for inhibition of the nonfimbrial hemagglutinin of *Vibrio cholerae* is similar to the 2 mM value that we obtained with Ah416 and Ah424. However, the fucose lectin of Ah434 is one order of magnitude more sensitive.

Sugar inhibition patterns indicate that a variety of lectins is produced by different strains of *A. hydrophila*. At least five lectins with different inhibition properties were recognized, and one strain (A46) produces two distinct lectins at once. Hemagglutination by this strain cannot be inhibited by any one sugar, but D-galactose and D-mannose combined (0.5 mM galactose + 0.5 mM mannose) are inhibitory. This strain also coagglutinated strongly with *Saccharomyces cerevisiae*, confirming that one of the lectins is a mannose (mannan)-recognizing lectin. This yeast coagglutination was inhibited by D-mannose or yeast mannan but not by L-fucose or D-galactose.

In contrast, strains A6, A10, Ah416, and Ah424 produce a single lectin which can be inhibited by either L-fucose or D-mannose. Jones and Freter (13), when examining the adherence of *V. cholerae* to isolated rabbit brush border membranes, made the similar observations that either L-fucose or D-mannose caused inhibition but one sugar did not augment the other. They suggested that D-mannose forms part of the L-fucose receptor. We believe that another explanation is possible. If one examines the structural formulas of L-fucose and D-mannose shown in Fig. 3, unexpected similarities are revealed. The spatial arrangement of the hydroxyl groups on carbon atoms 2, 3, and 4 of D-mannose relative to the pyranose ring is identical to the arrangement of hydroxyls on C-4, C-3, and C-2 of L-fucose if the molecules are viewed with C-1 of D-mannose and C-6 of L-fucose on the right. These three hydroxyl groups are very probably essential for lectin recognition. This is confirmed by the observation that D-glucose, which differs from D-mannose at C-2, and D-mannosamine do not inhibit. Similarly D-altrose and D-talose which differ from D-mannose at C-3 and C-4, respectively, are also not inhibitory, nor is D-galactose which differs at C-2 and C-4. However, the lectins of these four strains exhibit differences. The lectins of A6, Ah416, and Ah424 are not stable in PBS, require Ca^{2+} for maximal hemagglutination, are inhibited by yeast mannan or D-mannose-6-phosphate and coagglutinate with yeast. In respect of each of the above characteristics, A10 is different.

A6 was the only strain of the 11 strains examined which produced large numbers of pilated cells. A loss of pilation by A6 coincides with a loss of hemagglutinability as well as a marked reduction in attachment ability. Clearly in this case the hemagglutinin is also an adhesin and is a pilus. In the case of Ah416 and Ah424, hemagglutination occurs in the absence of pili, and the lectins in these cases would appear to be surface proteins. The presence of two mannose-sensitive systems has been reported in *Escherichia coli*, with one system involving pili and the other a surface lectin (6). Purification of the pili of A6 is currently in progress so that we can compare better the lectin's properties with those of other species.

For further studies, we chose A6 and A10 to represent two types of fucose/mannose lectins. It would appear that the different effect of yeast mannan on the lectins of A6 and A10 is central to our understanding of their specificity. Nakajima and Ballou (19) suggest that the mannan of *S. cerevisiae* consists of a polymer of D-mannose in which the terminal sugar groups are almost exclusively D-mannose linked to the rest of the polymer through C-1, with hydroxyls at C-2, C-3, C-4, and C-6 available for lectin recognition. The A10 lectin does not recognize mannose in the mannan polymer and is only poorly inhibited by *p*-nitrophenyl-α-D-mannoside or α-methyl-D-mannoside. In contrast to the α-D-mannosides, L-fucosides are excellent inhibitors of the A10

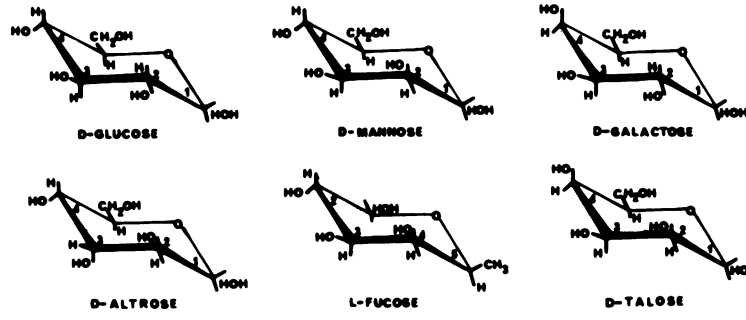

FIG. 3. *Structures of* D*-glucose,* D*-mannose,* D*-galactose,* D*-altrose,* L*-fucose, and* D*-talose.*

lectin, *p*-nitrophenyl-α-L-fucoside being 140 times more effective as an inhibitor and *p*-nitrophenyl-β-L-fucoside being more than 1,000 times more effective. These data suggest that the lectin of A10 recognizes a terminal L-fucose linked to a polymer on the surface of the receptor cell through a β-glucoside link and does not recognize D-mannose in polymeric form. In addition, D-mannoheptulose, which inhibits the lectin as well as does D-mannose, is structurally very similar to a β-L-fucoside. The type of lectin represented by A6 is obviously able to recognize D-mannose in the mannan polymer. However, the best inhibitor of this lectin is *p*-nitrophenyl-α-L-fucoside, which is 15 times more effective as an inhibitor than *p*-nitrophenyl-β-L-fucoside and 25 times more effective than α-D-mannosides. This suggests that the primary recognition structure for the A6 lectin is α linked L-fucose.

Another L-fucose-recognizing lectin is produced by Ah434. This lectin is very sensitive to L-fucose, but in contrast to A6 and A10 is not affected by D-mannose. L-Fucosides inhibit this lectin, but the β linkage is more effective than the α linkage, again suggesting that a β-linked terminal L-fucose may be the important recognition feature on the receptor cell.

Two other lectins require some discussion, these being typified by strains A69 and Ah413.

Strain A69 hemagglutinin can be inhibited by D-mannose or yeast mannan, and the strain coagglutinates strongly with *S. cerevisiae*. This lectin is not inhibited by L-fucose or L-fucosides, but α-D-mannosides are good inhibitors. D-Glucose, D-altrose, and D-talose do not inhibit, indicating that hydroxyls at C-2, C-3, and C-4 are concerned with lectin recognition, and D-mannose-6-phosphate does not inhibit, suggesting that the C-6 hydroxyl is probably involved in recognition also. This probably explains why L-fucose does not inhibit this lectin, because it has no hydroxyl group analogous to the C-6 hydroxyl of D-mannose. These data suggest that the A69 lectin recognizes terminal D-mannose α linked to the receptor cell surface polymer, and as such is very similar to the *S. flexneri* lectin described by Old (20).

The lectin of Ah413 has D-galactose specificity, and several D-galactosides inhibit. D-Glucose and D-altrose do not inhibit, but D-galactosamine has a moderate inhibitory effect. This suggests that C-4 and C-3 hydroxyls are required for lectin binding, but C-2 hydroxyl is not required. D-Talose, which differs from D-galactose at C-2, is also inhibitory, confirming the D-galactosamine result. *p*-Nitrophenyl-β-D-galactoside is a good inhibitor of hemagglutination and is 55 times more effective than *p*-nitrophenyl-α-D-galactoside. However, two other β-D-galactosides are relatively poor inhibitors (lactose and β-thiogalactopyranoside). This lectin probably recognizes β-linked D-galactose which may be further linked to other sugar moieties by way of glycosidic linkages through the C-2 position of D-galactose.

It is quite apparent that very subtle differences characterize the *A. hydrophila* lectins. The lectins of A6, A10, A46, and Ah434 recognize L-fucose but exhibit perceptible differences from one another, whereas the lectins of A6, A10, and Ah413 can be inhibited by D-mannose again in perceptibly different ways. A46 elaborates two different lectins, one of which is D-galactose sensitive, but Ah413 produces a single, D-galactose-sensitive lectin. What the differences between these lectins may be, at a molecular level, is not yet clear and awaits further investigation. However, it is apparent that there exists a spectrum of different attachment mechanisms, as demonstrated by hemagglutination inhibition.

Further evidence is provided by attachment assays. *A. hydrophila* attaches to buccal epithelial cells, but this attachment does not necessarily correlate with hemagglutination or attachment to blood cells. Clearly a variety of attachment mechanisms is available for *A. hydrophila* to attach to different cell types. Certainly those strains with hemagglutinins unstable in saline would appear to use other mechanisms to allow the amount of attachment shown to blood cells and, in the case of A6, to buccal cells. The individual specificity of the various attachment mechanisms may be very important for the virulence of an organism because it may determine how well and to which target cells it will adhere and may even recognize phenotypic differences in the surface receptors of different individuals or species.

A. hydrophila is obviously interesting in its variety of attachment mechanisms, because it covers the spectrum of sensitivities exhibited by other genera. It even elaborates at least two different lectins capable of recognizing L-fucose or D-mannose. This finding suggests that other species of bacteria producing L-fucose- or D-mannose-sensitive hemagglutinins should be reexamined to define properly their true sensitivity. Further studies are obviously needed to reveal how important these differences in attachment ability are in the natural history of *A. hydrophila* diarrheal disease and how enterotoxin production correlates with adhesive capacity.

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