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Exclusive Presence of Lactose-Sensitive Fimbriae on a Typical Strain (WVU45) of *Actinomyces naeslundii*

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Lactose-sensitive fimbriae were identified as the only fimbriae present on *Actinomyces naeslundii* WVU45 (ATCC 12104). A single antigen reactive with antiserum against WVU45 cells was detected by cross immunoelectrophoresis of isolated fimbriae, and a monospecific antiserum against this antigen reacted with all fimbriae observed on the bacterial surface by immunoelectron microscopy. Moreover, the loss of one cell surface antigen by a spontaneous mutant of *A. naeslundii* WVU45 (WVU45M), isolated by its failure to react with a monospecific antibody against the fimbriae, was associated with the loss of all fimbriae. The functional involvement of the fimbriae in lactose-sensitive bacterial adherence was demonstrated by the ability of WVU45, but not WVU45M, cells to agglutinate neuraminidase-treated erythrocytes and by the lactose-sensitive hemagglutinating activity of immune complexes formed with isolated fimbriae and monospecific antibody. Bacterial agglutination assays with different monospecific antibodies revealed an antigenic similarity between the fimbriae of *A. naeslundii* WVU45 and the lactose-sensitive fimbriae (type 2) of *Actinomyces viscosus* T14V. In contrast, cross-reactivity was not observed between the WVU45 fimbriae and type 1 fimbriae, the structures involved in lactose-resistant adherence of strain T14V to saliva-treated hydroxyapatite. Functional differences between the fimbriae of *A. naeslundii* and *A. viscosus* strains may be correlated with well-established differences in the in vivo distribution of these organisms: namely, the preference of typical *A. naeslundii* for epithelial surfaces and of *A. viscosus* for tooth surfaces.

Actinomyces viscosus and *Actinomyces naeslundii* are closely related species (11, 18) with different patterns of adherence and oral colonization (13, 14). *A. viscosus* has been identified as an initial colonizer of teeth (34), a property attributed to its relatively high affinity for tooth surfaces as indicated experimentally by studies of bacterial adherence to saliva-treated hydroxyapatite (S-HA) (8, 29). In contrast, typical strains of *A. naeslundii* adhere poorly to S-HA (8, 29) and are not prominent colonizers of the tooth surface but exhibit an affinity for epithelial surfaces as shown by their appearance in the mouths of predentate infants (13, 14).

After the initial description of fimbriae on *A. viscosus* and *A. naeslundii* (19), subsequent studies (2, 7, 17, 27, 35) associated these structures with bacterial adherence. A structural basis for selective adherence was suggested when the fimbriae of *A. viscosus* T14V were found to be of two antigenic types (i.e., types 1 and 2) each with distinct functional properties. Type 1 fimbriae have been identified as the principal structures mediating the adsorption of bacteria to S-HA (9), an interaction that is unaffected by various simple sugars (29). In contrast, type 2 fimbriae are the sites of a lactose-sensitive lectin activity detected by the coaggregation of actinomyces with certain plaque streptococci (3, 5, 30) and by the hemagglutination of neuraminidase-treated erythrocytes (4, 10, 16).

The findings with *A. viscosus* T14V raise the possibility that differences in oral distribution of *A. viscosus* and *A. naeslundii* isolates may be related to the presence of different fimbriae on these organisms. In this regard, virtually all isolates of both species are thought to possess type 2 fimbriae (4), a finding consistent with the general ability of these bacteria to participate in lactose-sensitive adherence. The strain distribution of type 1 fimbriae has not been established although the presence of these structures on certain isolates of both species has been suggested (8). Correlation of colonization patterns with the expression of specific fimbriae may be further complicated by the possible existence of additional types of fimbriae that are antigenically and functionally distinct from those identified on *A. viscosus* T14V. These considerations emphasize the need for analyses and comparison of the fimbriae on strains of *A. naeslundii* with those on strains of *A. viscosus*.

MATERIALS AND METHODS

Bacterial strains. *A. naeslundii* strains WVU45 (ATCC 12104), W1096 (ATCC 27040), WVU398A, I, and W1544 and *A. viscosus* strain T14V were those studied previously (6, 10). *A. viscosus* strain WVU627 was provided by M. A. Gerencser, West Virginia University, Morgantown, W. Va. Bacteria were cultured in complex medium (6) unless indicated otherwise.

Mutant selection. The spontaneous mutant of *A. naeslundii* WVU45 lacking fimbriae was obtained by enriching for cells unable to agglutinate in the presence of antibody against the fimbriae. WVU45 cells grown overnight in complex medium (10 ml) were washed three times with cold Dulbecco modified Eagle medium with glutamine (HEM Research Inc., Rockville, Md.) and suspended in 3 ml of this medium. Filter-sterilized immunoglobulin G (IgG) from antiserum R61 (see below) against WVU45 fimbriae was added to a final concentration of ca. 200 μg/ml. After 15 min in the cold with occasional gentle mixing, agglutinated bacteria were pelleted by low-speed centrifugation (5 min, 200 × *g*). The entire supernatant was transferred to a separate tube and centrifuged (5 min, 200 × *g*), and the upper 1 ml was used to inoculate 10 ml of complex medium for overnight growth at 37°C. Three cycles of enrichment yielded a population of

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cells not agglutinated by antibody, which was then cloned on brain heart infusion agar (Difco Laboratories, Detroit, Mich.). Of 20 clones examined for agglutination by the monospecific antibody, 14 reacted weakly, whereas 6 gave no reaction. One of the latter isolates (designated WVU45M) was selected for further characterization.

Bacterial antigens. Crude extracts of bacterial antigens were prepared by the Lancefield procedure as outlined by Powell et al. (28).

Fimbriae were isolated from *A. naeslundii* WVU45 by methods previously described for the isolation of *A. viscosus* T14V fimbriae (3–5, 9, 30). Briefly, bacteria in Tris-buffered saline (TBS; 0.15 M NaCl, 0.02 M Tris-hydrochloride [pH 7.8], 10^{-4} M $CaCl_2$, 10^{-4} M $MgCl_2$, 0.02% sodium azide) were cycled through a continuous-flow sonifier under conditions which removed fimbriae with a minimum (<1%) of cell lysis. Intact cells and cell wall fragments were pelleted by centrifugation, and the clear supernatant, containing fimbriae, was concentrated by ultrafiltration, applied to a column of Agarose 5m (exclusion limit, 5×10^6 daltons for globular proteins; Bio-Rad Laboratories, Richmond, Calif.) equilibrated with TBS, and eluted with this buffer. Fimbriae and other high-molecular-weight components emerged in fractions collected at the void volume and were stored at 4°C. Protein determinations were performed by the method of Lowry et al. (22) with bovine serum albumin as a standard.

The WVU45 fimbrial antigen was further purified on an affinity column prepared by coupling monospecific IgG from antiserum R61 (see below) to CNBr-activated (12) Sephacryl S1000 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). Fimbriae isolated by gel filtration chromatography were applied in excess to an affinity column, and unbound material was removed by extensive washing with TBS containing 0.05% Tween 20 (TBS-Tween). Bound antigen was eluted with 6 M guanidine hydrochloride in TBS-Tween and dialyzed against TBS-Tween to remove guanidine.

Rabbit antisera. The monospecific antisera against types 1 and 2 fimbriae of *A. viscosus* T14V were those described previously (5).

Antiserum R57 against *A. naeslundii* WVU45 and antiserum R64 against strain WVU45M, a mutant lacking fimbriae, were prepared by immunization of rabbits with whole bacteria. The cells used for immunization were cultured in Dulbecco modified Eagle medium with glutamine, washed with saline containing 0.01% merthiolate, and adjusted to about 2×10^9 bacteria per ml. The immunization schedule consisted of 20 intravenous injections administered three times weekly with 0.5 ml of bacterial suspension for each of the first 4 injections and 1.0 ml for each remaining injection. Serum was obtained 2 to 4 weeks after completion of the series.

A monospecific rabbit antiserum (R61) was prepared against the fimbriae of *A. naeslundii* WVU45 by immunization with the immunoprecipitated fimbrial antigen isolated by cross immunoelectrophoresis (30). Separations (e.g., see Fig. 1A) were each performed with 25 μg of antigen, and plates were rinsed 3 days with six changes of cold saline to remove soluble protein. Immunoprecipitate from six plates was harvested as indicated in the legend for Fig. 1, brought to 1 ml with saline, emulsified with an additional 1 ml of Freund complete adjuvant (Difco Laboratories), and injected subcutaneously at multiple sites. Additional injections of immunoprecipitate were administered with incomplete adjuvant at weeks 4, 6, and 8, and antisera was obtained 2 to 8 weeks after the final injection.

A second monospecific rabbit antiserum (R70) was produced by immunization with WVU45 fimbriae purified by affinity chromatography. The immunization schedule has been described previously (5) and consisted of two subcutaneous injections of 50 to 100 μg of affinity-purified fimbriae with adjuvant at multiple sites.

Immunological methods. The isolation of rabbit IgG (9, 30), the assay for bacterial agglutination (4), and the conditions for cross immunoelectrophoresis (4) have been described. Immunoelectron microscopy was performed as previously described (4, 7) except that the reaction of biotin with avidin was used to link horseradish peroxidase with antibody bound on the bacterial surface. Rabbit IgG against the fimbriae of *A. naeslundii* WVU45 was biotin-labeled as described by Guesdon et al. (20) with biotin-*N*-hydroxysuccinimide (Miles Laboratories, Inc., Elkhart, Ind.). Bacteria cultured anaerobically on Columbia blood agar (England Laboratories, Beltsville, Md.) and washed with TBS were incubated 30 min with biotin-labeled antibody (100 μg/ml) and 30 min with avidin-horseradish peroxidase conjugate (50 μg/ml) (E-Y Laboratories, Inc., San Mateo, Calif.), and were fixed 10 min with 2% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.3). The reaction for peroxidase labeling was performed as previously described (4, 7). Labeled specimens were fixed with 1% OsO_4, dehydrated, embedded in Epon, sectioned, lead stained (31), and examined with a Philips 300 electron microscope.

Adherence assays. Bacteria-mediated hemagglutination and the reversal of this interaction by lactose were measured with a Dual Channel Aggregation Module (Payton Associates, Inc., Buffalo, N.Y.). Cell mixtures for aggregometry contained 1×10^7 neuraminidase-treated (10) human O or B

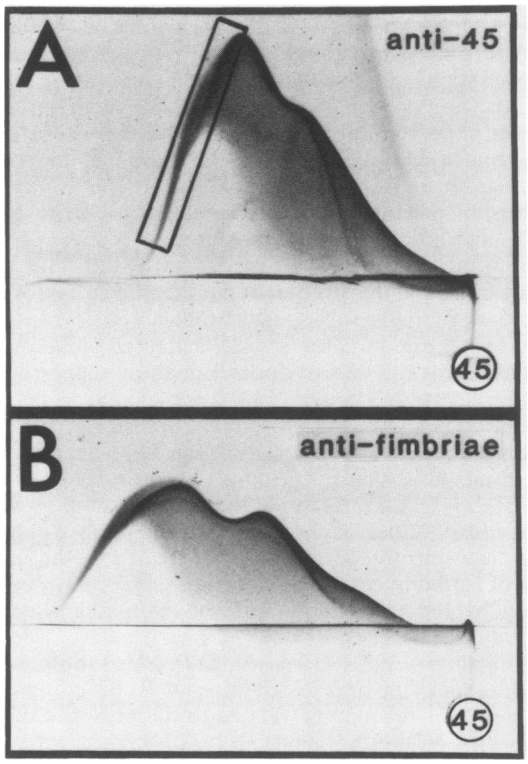

FIG. 1. Cross immunoelectrophoresis of cell surface antigens (9 μg) from *A. naeslundii* WVU45 with (A) antiserum R57 against WVU45 cells and (B) monospecific antiserum R61 against WVU45 fimbriae. The monospecific antiserum (B) was prepared by immunization with immunoprecipitate from the region outlined in (A). The anode was to the left for electrophoresis in the first dimension.

erythrocytes and 2.5×10^8 bacteria (determined with a Petroff-Hausser bacteria counter) in 0.5 ml of phosphate-buffered saline with 2 mg of bovine serum albumin per ml.

The assay to detect fimbrial lectin activity has been described (3, 4). Briefly, isolated fimbriae and monospecific rabbit IgG were used to form immune complexes with hemagglutinating activity for neuraminidase-treated erythrocytes. The inhibition of lectin activity by various commercially available sugars was examined by incubating fimbriae-antibody complexes with a sugar for 30 min before the addition of neuraminidase-treated erythrocytes. Control experiments to assess the agglutinating activity of fimbriae and antibody alone also were performed.

RESULTS

A single antigenic type of fimbriae on *A. naeslundii* WVU45. One prominant antigen was detected by cross immunoelectrophoresis when the high-molecular-weight cell surface structures of *A. naeslundii* WVU45 reacted with antibody against bacterial cells (Fig. 1A). Immunization with the immunoprecipitated antigen harvested from agarose gels (Fig. 1A, outlined region) resulted in the production of a specific rabbit antiserum against this component (Fig. 1B). The use of this antibody as a reagent in immunoelectron microscopy resulted in the specific labeling of fimbriae on the bacterial surface, and unlabeled fimbriae were not observed (Fig. 2A). The presence of a single antigenic type of fimbriae on strain WVU45 was investigated further by the selection of a spontaneous mutant of strain WVU45 by its failure to agglutinate in the presence of antifimbriae IgG (see above). The examination of mutant strain WVU45M by immunoelectron microscopy with antifimbriae IgG showed no evidence of reaction with antibody and the complete absence of fimbriae on the mutant cell surface (Fig. 2B).

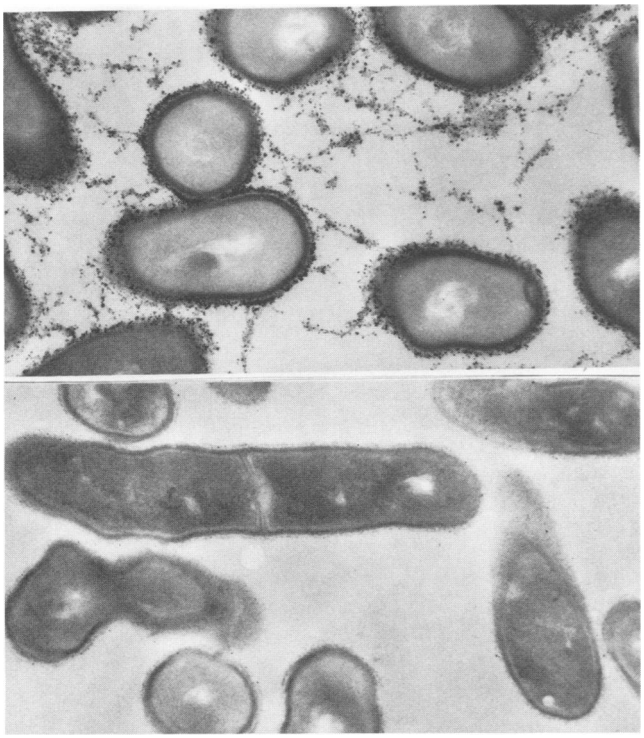

FIG. 2. Electron micrographs of thin sections of *A. naeslundii* WVU45 showing fimbriae on this organism (upper panel) and *A. naeslundii* WVU45M, a mutant lacking fimbriae (lower panel). Bacteria were incubated with biotin-labeled antibody (R61) against the fimbrial antigen, washed, and reacted with an avidin-peroxidase conjugate. Immunochemical labeling occurred only on the fimbriae of *A. naeslundii* WVU45 (×35,000).

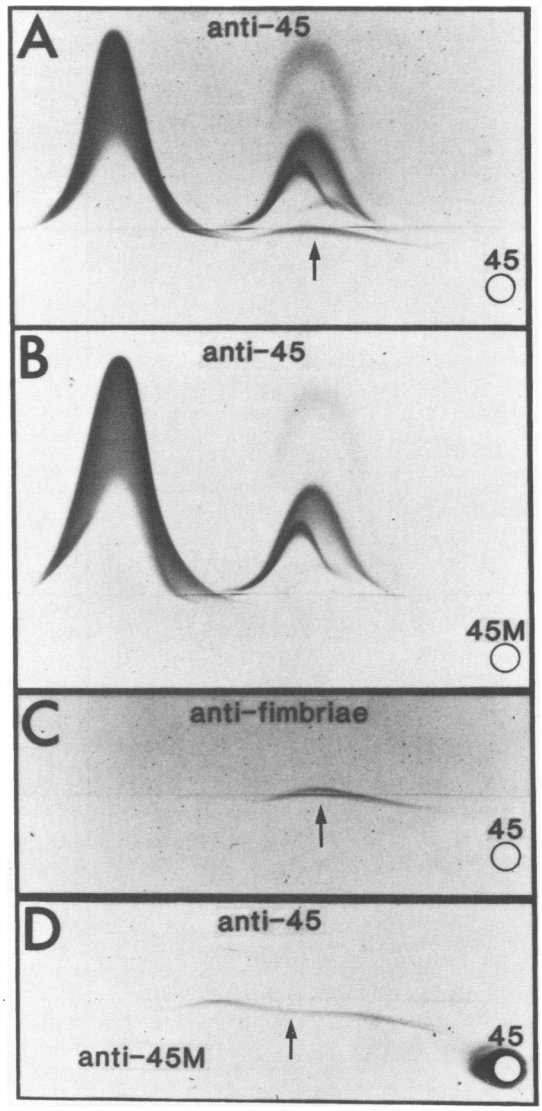

FIG. 3. Cross immunoelectrophoresis of bacterial antigens extracted by the Lancefield procedure from *A. naeslundii* WVU45 (45) and mutant strain WVU45M (45M). Antisera were R57 against *A. naeslundii* WVU45 (anti-45), R61 against WVU45 fimbriae (anti-fimbriae), and R64 against *A. naeslundii* WVU45M (anti-45M). All antigen wells contained 1 μg of protein. In (D), anti-45M was present in the agarose gel for electrophoresis in the first dimension (anode to the left), and anti-45 was present in the gel for electrophoresis in the second dimension. Arrows in (A, C, and D) identify the immunoprecipitated fimbrial antigen.

The antigenic difference between the fimbriated parent and nonfimbriated mutant strain was analyzed by cross immunoelectrophoresis with different antisera and antigens extracted from bacteria by the Lancefield procedure (Fig. 3). Antiserum R57 against the parent strain (i.e., WVU45) reacted with a number of antigens shared by parent and mutant cells and with one additional antigen unique to the parent strain (compare Fig. 3A and B). The unique component was identified by its reaction with specific antibody (R61) against the WVU45 fimbriae (Fig. 3C). In contrast,

antiserum R64 against the nonfimbriated mutant (strain WVU45M) reacted identically with the antigens extracted from parent and mutant cells (results not shown) and thus did not reveal any antigens unique to strain WVU45M. When the extract of the parent was subjected to electrophoresis through gel containing antiserum R64 against the mutant, all shared antigens precipitated, whereas the unique fimbrial antigen migrated and was detected by its reaction with antibody against WVU45 cells (Fig. 3D) or fimbriae (results not shown). Thus, the antigenic difference between the fimbriated parent, *A. naeslundii* WVU45, and nonfimbriated mutant, strain WVU45M, was restricted to a single antigenic component.

Lactose-sensitive adherence mediated by WVU45 fimbriae. The lactose-sensitive lectin activity of *A. naeslundii* WVU45 (6, 10), was detected by the agglutination of neuraminidase-treated erythrocytes by bacteria. The nonfimbriated mutant cells (WVU45M) lacked this activity (Fig. 4). The association of lectin activity with the fimbriae was further established by the lactose-sensitive hemagglutinating activity of immune complexes formed with WVU45 fimbriae and monospecific antibody (Table 1). As was found in studies of the lactose-sensitive fimbriae of *A. viscosus* T14V (3, 4), agglutinating activity was not a property of isolated WVU45 fimbriae, even at concentrations of 500 μg/ml, but resulted when concentrations as low as 2 μg/ml were cross-linked by antibody to form multivalent complexes with enhanced binding affinity (2). The specificities of fimbriae-mediated hemagglutination (Table 1) and bacteria-mediated hemagglutination (10, 16) were similar since both were inhibited more effectively by methyl-β-D-galactoside than methyl-α-D-galactoside.

Antigenic comparison of the fimbriae on *A. naeslundii* and *A. viscosus* strains. The fimbriae of *A. naeslundii* were compared with those of *A. viscosus* by bacterial agglutination with various monospecific antibodies (Table 2). Agglutination of strain T14V was not observed with IgG from antiserum (R61) prepared against immunoprecipitated

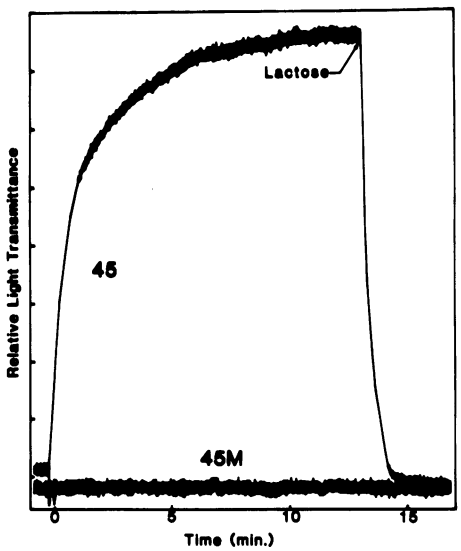

FIG. 4. Bacteria-mediated agglutination of neuraminidase-treated erythrocytes determined by the continuous recording of turbidity with an aggregometer. The hemagglutinating activity of *A. naeslundii* WVU45 (45) was reversed by the addition of lactose (0.02 M final concentration). The *A. naeslundii* mutant (45M) without fimbriae lacked hemagglutinating activity.

TABLE 1. Sugar inhibition of the agglutination of neuraminidase-treated erythrocytes by immune complexes containing *A. naeslundii* WVU45 fimbriae and antifimbriae antibody[a]

Inhibitor	Concn for complete inhibition (mM)[b]
Lactose	5
Methyl-β-D-galactoside	10
N-Acetyl-D-galactosamine	10
D-Galactose	20
Methyl-α-D-galactoside	160
Methyl-β-D-glucoside	>200

[a] Complexes contained 6 μg of fimbriae per ml and 40 μg of antifimbriae IgG from antiserum R61 per ml.
[b] Endpoint concentration determined from serial twofold dilution of inhibitors. Similar results were obtained in five independent experiments.

WVU45 fimbriae. However, IgG from a more potent antiserum (R70) raised against affinity-purified WVU45 fimbriae caused agglutination of strain T14V (Table 2) but not strain T14V(PK455) (results not shown), a mutant specifically lacking type 2 fimbriae (5). Likewise, monospecific IgG against the type 2 fimbriae of *A. viscosus* T14V agglutinated strain WVU45 (Table 2) but not strain WVU45M (results not shown), the nonfimbriated mutant. These antigenic cross-reactions are consistent with the previous finding that one of nine monoclonal antibodies against the type 2 fimbriae of strain T14V caused agglutination of strain WVU45 (4). In contrast to the cross-reactivity of anti-type 2 IgG, monospecific antibody against the type 1 fimbriae of strain T14V failed to agglutinate WVU45 cells.

Further studies with other strains of *A. naeslundii* indicated that those regarded as typical (18) (i.e., W1096 and WVU398A) reacted like strain WVU45, whereas two other *A. naeslundii* strains (i.e., I and W1544) and a strain of *A. viscosus*, WVU627 (23, 24), were agglutinated by antibodies against both type 1 and type 2 fimbriae. Thus, the typical strains of *A. naeslundii* appeared to have only type 2 fimbriae, whereas the other strains of *A. naeslundii*, like *A. viscosus*, possessed fimbriae of both types.

DISCUSSION

The present findings firmly establish an antigenic and functional relationship between the fimbriae on a typical strain of *A. naeslundii* and the type 2 fimbriae on *A. viscosus*. The type 2 fimbriae of *A. viscosus* T14V have previously been shown to mediate lactose-sensitive adherence between actinomyces and specific plaque streptococci (3–5, 30) or other cells with lectin receptors (2). *A. viscosus* T14V also possesses type 1 fimbriae which are antigenically distinct from type 2 and function in the adsorption of bacteria to S-HA (9, 35). The presence of both fimbrial components appears to be a general property of *A. viscosus* strains and one that has often gone undetected. For example, Masuda et al. (23, 24) reported a single fimbrial antigen from strain WVU627 and from other strains of *A. viscosus* and *A. naeslundii*. In our studies, both fimbrial antigens have been detected on a number of *A. viscosus* strains, including WVU627, and on certain isolates of *A. naeslundii* such as strains I and W1544 (Table 2).

In contrast to findings with *A. viscosus* T14V, fimbriae of only one antigenic type appear to be present on *A. naeslundii* WVU45 (ATCC 12104), a strain designated as typical by numerical taxonomy (18). The presence of a single type of fimbriae on this organism was shown by the isolation of one fimbrial antigen (Fig. 1A), by the reaction of all fimbriae on

TABLE 2. Agglutination of *A. naeslundii* and *A. viscosus* strains by monospecific antibodies against the fimbriae of strains WVU45 and T14V

Antibody specificity	Minimum antibody concn (μg of IgG per ml) for agglutination[a]						
	A. naeslundii strain:					*A. viscosus* strain:	
	WVU45	W1096	WVU398A	I	W1544	WVU627	T14V
Anti-type 2 (WVU45)[b]	0.8	0.8	0.2	—[c]	—	—	—
Anti-type 2 (WVU45)[d]	0.02	0.006	0.006	6	6	6	6
Anti-type 2 (T14V)	6	3	3	12	0.2	0.05	0.02
Anti-type 1 (T14V)	—	—	—	0.8	3	0.2	0.2

[a] Endpoint concentration determined from serial twofold dilution of antibodies. Similar results were obtained in three independent experiments.
[b] Prepared from antiserum R61 against immunoprecipitated fimbriae.
[c] —; No agglutination with 1,000 μg of IgG per ml.
[d] Prepared from antiserum R70 against affinity-purified fimbriae.

the bacterial surface with a monospecific antibody (Fig. 2A), and by the absence of fimbriae on a spontaneous mutant lacking the one antigenic component (Fig. 2B). Bacterial agglutination assays (Table 2) revealed a relatively weak but significant cross-reaction between the fimbriae on typical *A. naeslundii* strains (i.e., WVU45, W1096, and WVU398A) and the type 2, but not type 1, fimbriae of *A. viscosus* T14V. Moreover, like the type 2 fimbriae of strain T14V, the fimbriae of *A. naeslundii* WVU45 mediate lactose-sensitive adherence, a property demonstrated by the failure of nonfimbriated mutant cells to agglutinate neuraminidase-treated erythrocytes (Fig. 4) and by the hemagglutinating activity of isolated WVU45 fimbriae in immune complexes formed with monospecific antibody (Table 1). Thus, by antigenic as well as functional criteria, the fimbriae of typical *A. naeslundii* are related to the type 2 fimbriae of *A. viscosus* strains.

The exclusive presence of type 2 fimbriae on a typical strain of *A. naeslundii* suggests that lactose-sensitive adherence may be a major determinant of its colonization of oral sites. Thus, it is of interest that typical *A. naeslundii* inhabits epithelial surfaces and is abundant in saliva and that, whereas it can be found in dental plaque, it is not among the organisms regarded as primary colonizers of the tooth surface (34). These observations favor a role for the fimbrial lectin in bacterial adherence to epithelial cells (1, 17, 32, 33) and to specific plaque bacteria such as strains of *Streptococcus sanguis* (6, 15, 25–27). They also suggest that lactose-sensitive adherence may not be a mechanism of primary importance in the adsorption of bacteria to the acquired pellicle of teeth. This is supported by the failure of lactose to specifically inhibit the weak interaction of *A. naeslundii* 12104 with S-HA (29) and also by the identification of type 1 rather than type 2 fimbriae as the principal structures mediating adsorption of *A. viscosus* T14V to S-HA (9). These considerations clearly point to the type 1 fimbriae as an important ecological determinant and suggest that their presence on certain strains of *A. naeslundii* such as I and W1544 might enhance the affinity of these organisms for tooth surfaces.

The in vivo distribution of actinomyces may also be attributed to strain-specific variations of the type 2 fimbriae which influence lectin binding to receptors on plaque or epithelial surfaces. This possibility is suggested by differences between *A. viscosus* and *A. naeslundii* strains in their lactose-sensitive coaggregations with specific streptococci (6) and also by obvious differences in the extent to which certain *Actinomyces* strains cause agglutination of neuraminidase-treated erythrocytes (10, 16). These findings may reflect subtle differences in the affinity and fine specificity of the lectin combining sites (25) or in the regulation of fimbriation by environmental factors. Since the unmasking of receptors on mammalian cells by neuraminidase significantly enhances bacterial adherence (1, 10, 16, 21, 33), the production of this enzyme by bacteria and the susceptibility of various mammalian cells to neuraminidase represent other variables that could influence binding of the fimbrial lectin to different tissue surfaces. Thus, further studies may well reveal additional molecular determinants that influence the in vivo distribution of *A. naeslundii*, *A. viscosus*, and other oral bacteria.

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