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Mutants of *Actinomyces viscosus* T14V Lacking Type 1, Type 2, or Both Types of Fimbriae

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Mutants of *Actinomyces viscosus* T14V lacking type 1 or type 2 fimbriae or both were selected by their failure to react with rabbit antibodies against either or both fimbrial antigens. Immunospecific double labeling with iron dextran and ferritin-conjugated antibodies showed two types of fimbriae on individual cells of the parent organism, a single type on mutant strains with type 1^+2^- and type 1^-2^+ fimbriae and no labeled or unlabeled fimbriae on a type 1^-2^- fimbria-deficient strain. The mutational loss of one fimbrial antigen did not appear to affect expression of the other, since bacteria with one or two types of fimbriae bound similar amounts of a monoclonal antibody directed against the fimbrial antigen present on both bacterial phenotypes. The strong adsorption of strains with type 1^+2^+ or 1^+2^- fimbriae to saliva-treated hydroxyapatite and weak adsorption of those with type 1^-2^+ or no fimbriae was consistent with the known involvement of type 1 fimbriae in this attachment process. Similarly, the *A. viscosus* lectin was clearly associated with the expression of type 2 fimbriae, since only the strains with type 1^+2^+ or 1^-2^+ fimbriae participated in lactose-sensitive coaggregations with *Streptococcus sanguis* 34. Further studies using the fimbria-deficient mutant strains showed that aggregation of *A. viscosus* T14V in the presence of sialidase-treated human saliva involved both types of fimbriae, whereas neither type was required for the lactose-resistant coaggregation of the organism with certain streptococcal strains.

Colonization of teeth by the gram-positive oral bacterium *Actinomyces viscosus* involves the functional activities of antigenically distinct fimbriae. Thus, rabbit antibody against type 1 fimbriae specifically inhibited bacterial adsorption to saliva-treated hydroxyapatite (SHA) (9, 10, 27), while antibody against type 2 fimbriae blocked the lactose-sensitive coaggregation of *A. viscosus* with *Streptococcus sanguis* 34 (22). Both fimbria-mediated interactions may be important ecological determinants, since *A. viscosus* and *S. sanguis* are early colonizers of the tooth surface and remain numerically prominent in dental plaque (3, 20, 21, 24, 25).

An attractive approach to further defining the contribution of each fimbrial antigen to oral microbial adherence and colonization involves the use of specific fimbria-deficient mutant strains. Previous studies have shown that a spontaneous mutant of *A. viscosus* T14V selected by its failure to participate in lactose-sensitive coaggregation (19) expressed type 1 but not type 2 fimbriae (4). The present study was initiated to obtain additional mutants specifically lacking type 1 or both fimbrial antigens. These were isolated by their failure to react with specific antibodies.

MATERIALS AND METHODS

Bacterial strains. Wild-type *A. viscosus* T14V (1, 17) and mutant strains T14V-J1 (28) and PK256 and PK455 (19) have been described. Table 1 summarizes the relationship of these strains to each other and to the fimbria-deficient mutant strains isolated in this study.

Antibodies. Rabbit antisera R59 against type 1 fimbriae and R55 against type 2 fimbriae of *A. viscosus* T14V were prepared by immunization with each purified fimbrial antigen (4). Immune rabbit immunoglobulin G (IgG) was purified by DE-52 (Whatman, Inc., Clifton, N.J.) cellulose column chromatography (9). Monoclonal antibodies 8A against type 1 fimbriae (J. O. Cisar, E. L. Barsumian, R. P. Siraganian, W. B. Clark, M. K. Yeung, S. D. Hsu, S. H. Curl, A. E. Vatter, and A. L. Sandberg, manuscript submitted) and 2A against type 2 fimbriae (2) have been characterized.

Immunological methods. Bacterial agglutination assays were set up in Microtiter polyvinyl V-bottom plates (Dynatech Laboratories, Inc., Alexandria, Va.) as previously described (2). The procedure for indirect immunofluorescence labeling of bacteria involved incubation of the cells for 30 min with monoclonal antibody 8A or 2A (25 μg/ml) and 30 min with fluorescein-labeled goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). Primary and secondary antibodies were diluted in Tris-buffered saline (TBS; 0.15 M NaCl, 0.02 M Tris hydrochloride, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.02% sodium azide [pH 7.8]) containing 0.05% Tween 20 (TBS-Tween), and labeled cells were mounted in this buffer containing 10% glycerol for examination with a phase contrast microscope equipped with epifluorescence (Zeiss, Oberkochen, Federal Republic of Germany).

Binding studies with ^{14}C- or ^{3}H-labeled (18) monoclonal antibodies were performed with bacteria harvested from complex medium (6) during the exponential phase of growth. Approximately 10^8 bacteria (25 μg, dry weight) were incubated for 2 h at room temperature with excess radiolabeled antibody (5 μg) in 0.1 ml of TBS-Tween containing 2 mg of bovine serum albumin per ml. Bacteria with bound antibody were collected by vacuum filtration on a 0.2-μm-pore-size, polycarbonate membrane (Nuclepore Corp., Pleasanton, Calif.) and washed with 3 5-ml volumes of cold TBS-Tween

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TABLE 1. Mutant bacterial strains derived from *A. viscosus* T14V

Bacterial strain (fimbrial phenotype) of:		Mutant selection (reference)
Parent	Mutant	
T14V (1^+2^+)	PK256 (1^+2^+)	Resistance to streptomycin and rifamycin (19)
PK256 (1^+2^+)	PK455 (1^+2^-)	No coaggregation with *S. sanguis* 34 (19)
PK256 (1^+2^+)	256-1 (1^-2^+)	No agglutination with anti-type 1 rabbit IgG
PK455 (1^+2^-)	455-2 (1^-2^-)	No agglutination with anti-type 1 rabbit IgG
T14V (1^+2^+)	T14V-J1 (1^+2^+)	Resistance to streptomycin (28)
T14V-J1 (1^+2^+)	5519 (1^+2^-)	No agglutination with anti-type 2 rabbit IgG
T14V-J1 (1^+2^+)	5951 (1^-2^+)	No agglutination with anti-type 1 rabbit IgG
5519 (1^+2^-)	147 (1^-2^-)	No agglutination with anti-type 1 rabbit IgG

to remove unbound antibody. The amount of antibody bound was determined by scintillation counting of the washed membranes in Ultrafluor (National Diagnostics, Inc., Somerville, N.J.). Nonspecific binding of radiolabeled antibody to membranes was negligible.

Mutant isolation. The procedure outlined previously (5) in studies of *Actinomyces naeslundii* was used to isolate spontaneous fimbria-deficient mutants of *A. viscosus*. This involved the addition of filter-sterilized anti-type 1 or anti-type 2 immune rabbit IgG to log-phase cells of a fimbriated parent strain, the removal of agglutinated bacteria by low-speed centrifugation, and the inoculation of fresh medium with cells that remained in suspension. The enrichment procedure was repeated for 3 or 4 consecutive days to select for cells that failed to agglutinate in the presence of added antibody. At least 20 colonies from the final culture were screened for the expression of both fimbiral antigens to identify those of the desired phenotype.

Electron microscopy. Double labeling experiments were performed with anti-type 1 rabbit (R59) IgG conjugated to periodate-oxidized iron dextran (Nonemic; Burn-Biotec Laboratories, Omaha, Nebr.) (12) and anti-type 2 rabbit (R55) IgG that was biotinylated by using equal amounts of biotin-*N*-hydroxysuccinimide ester (Miles Laboratories, Inc., Elkhart, Ind.) and IgG by weight (16). Approximately 5×10^8 bacteria were incubated for 30 min with an excess of biotinylated R55 IgG and iron dextran-conjugated R59 IgG. The cells were washed by centrifugation in phosphate-buffered saline to remove unbound antibody, suspended, and incubated for 30 min with an excess of ferritin-avidin conjugate (EY Laboratories, Inc., San Mateo, Calif.) and washed with phosphate-buffered saline to remove unbound conjugate. Labeled bacteria were fixed in tannic acid (0.5%) and glutaraldehyde (2.5%), postfixed in OsO_4 (1%), and processed for electron microscopy as previously described (7). Lead aspartate stain was prepared by the method of Walton (26), stored frozen in small plastic tubes, and used in a modified procedure to stain sections. Each incubation step of the following procedure was performed in a humid chamber at 60°C. Sections mounted on collodion-coated grids were floated, face down, on a drop of cacodylate buffer (0.1 M, pH 5.5) for 10 to 15 s. The grids were transferred to a drop of buffered lead aspartate stain for 0.5 to 2 min, removed, rinsed by floating each grid on two successive drops of cacodylate buffer for 15 s each, rinsed on a drop of distilled water, removed, and dried. Specimens prepared by this procedure exhibited less contamination that did those stained with lead citrate (23).

Bacterial adherence. Adsorption of [^{3}H]thymidine-labeled actinomyces to SHA was performed as previously described (8, 9). Coaggregation of actinomyces with *S. sanguis* DL1, H1, 34, or J22 was determined by visual examination (6).

Assays for saliva-induced aggregation of *A. viscosus* were performed by mixing 10^8 bacteria in 50 μl of adsorption buffer (8) with 100 μl of whole saliva that was freshly collected from individual donors, clarified by centrifugation (30 min, 10,000 × *g*), and incubated for 30 min with sialidase (0.15 U of enzyme per ml of saliva) from *Clostridium perfringens* (neuraminidase type X; Sigma Chemical Co., St. Louis, Mo.) before the addition of bacteria.

RESULTS

Spontaneous mutants of *A. viscosus* PK256 expressing type 2 but not type 1 fimbriae were isolated after the immunospecific enrichment of this strain for the cells that were not agglutinated by rabbit antibody against type 1 fimbriae (Table 1). Of 20 colonies examined, 14, including strain 256-1, failed to react with monoclonal antibody 8A against type 1 fimbriae but reacted with antibody 2A against type 2 fimbriae. The other six isolates reacted with both antibodies. Similar enrichment of strain PK455, which has type 1^+2^- fimbriae (4), resulted in the isolation of strain 455-2, which failed to react with either monoclonal antibody. Fimbria-deficient mutant strains specifically lacking type 1, type 2, or both types of fimbriae were also isolated from enrichment cultures of *A. viscosus* T14V-J1 by using the enrichment procedures summarized in Table 1. Though not included, mutants lacking both fimbrial antigens were obtained from a type 1^-2^+ fimbria-deficient mutant strain when antibody against type 2 fimbriae was used in the enrichment step.

The presence of both fimbrial antigens on individual cells of parent strain *A. viscosus* PK256 was demonstrated at the ultrastructural level by immunospecific labeling of type 1 fimbriae with rod-shaped iron dextran particles and type 2 fimbriae with dot-shaped ferritin granules (Fig. 1A). Similar labeling and electron microscopic examination of strain PK455 showed iron dextran but not ferritin on the fimbriae of this type 1^+2^- organism (Fig. 1B). In contrast, only ferritin-labeled fimbriae were observed on the type 1^-2^+ mutant strain (Fig. 1C). Finally, mutant strain 455-2, lacking both fimbrial antigens, was not labeled with either marker, and in addition, unlabeled fimbriae were not observed (Fig. 1D).

The binding of a type-1-specific monoclonal antibody to each type 1^+2^- mutant strain or of a type-2-specific monoclonal antibody to each type 1^-2^+ mutant strain was comparable with the binding of each antibody by the respective type 1^+2^+ parent strains, i.e., PK256 and T14V-J1 (Table 2). Cells lacking either or both fimbrial antigens did not bind detectable amounts of the corresponding radiolabeled monoclonal antibodies, and in addition, indirect immunofluorescence of these reaction mixtures showed that the vast majority of cells were unreactive. However, the latter tech-

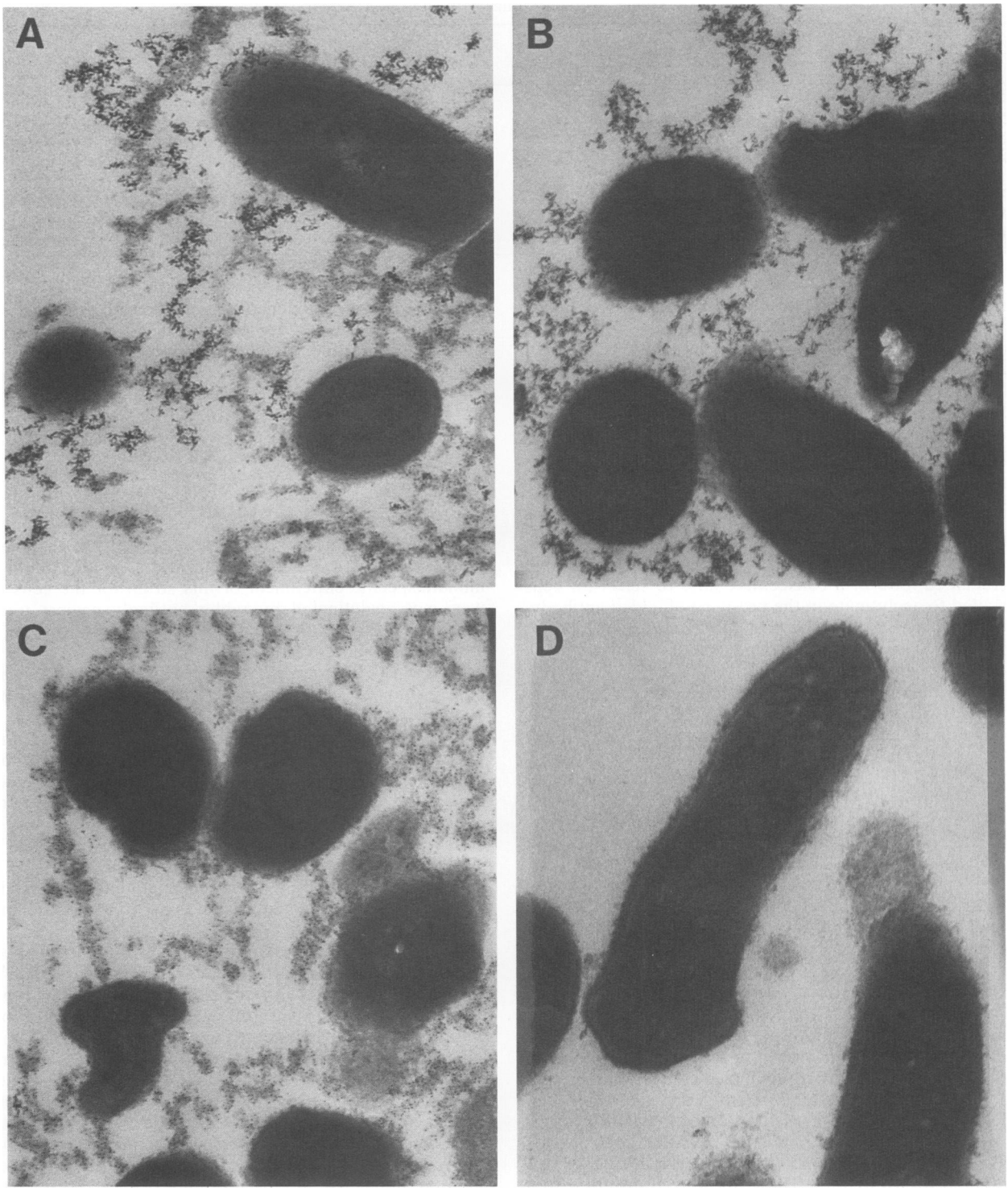

FIG. 1. Electron micrographs of thin sections of bacteria showing immunospecific labeling of type 1 and type 2 fimbriae with iron dextran and ferritin, respectively. *A. viscosus* strains (fimbriae) were as follows: A, PK256 (1^+2^+); B, PK455 (1^+2^-); C, 256-1 (1^-2^+); D, 455-2 (1^-2^-). Each bacterial strain was incubated with iron dextran-conjugated anti-type 1 and biotinylated anti-type 2 rabbit antibodies before reaction with ferritin-avidin conjugate. Magnification, ×44,000.

nique invariably revealed rare, fluorescently labeled bacteria within each mutant population (data not shown). These were seen at frequencies within the range expected for reversion of a mutation (less than 1 in 10^5).

Strains of *A. viscosus* with both types of fimbriae adsorbed well to SHA (>80% adsorption) and participated in lactose-sensitive as well as lactose-resistant coaggregations with various streptococci (Table 3). Strong adsorption to SHA

TABLE 2. Binding of type 1 or type 2 fimbria-specific radiolabeled monoclonal antibodies to *A. viscosus* T14V and mutant strains

Bacterial strain (fimbrial phenotype)	Amt of monoclonal antibody bound[a]	
	Anti-type 1 (8A)	Anti-type 2 (2A)
T14V (1^+2^+)	5.22	5.50
PK256 (1^+2^+)	4.20	3.52
PK455 (1^+2^-)	5.38	<0.28
256-1 (1^-2^+)	<0.14	5.20
455-2 (1^-2^-)	<0.14	<0.28
T14V-J1 (1^+2^+)	6.74	5.80
5519 (1^+2^-)	6.52	<0.28
5951 (1^-2^+)	<0.14	7.22
147 (1^-2^-)	<0.14	<0.28

[a] Micrograms of IgG per milligram of bacteria. Each value is the average of duplicate determinations.

was observed with mutant strains that expressed type 1^+2^- fimbriae but not with those expressing type 1^-2^+ or no fimbriae. In contrast, only strains with type 2 fimbriae participated in the lactose-sensitive coaggregation with *S. sanguis* 34. Lactose-resistant coaggregations of *A. viscosus* T14V with streptococcal strains such as DL1, H1, or J22 were unaffected by the mutational loss of either type 1 or type 2 fimbriae, and indeed, mutant strains lacking both fimbrial antigens participated in these interactions (Table 3).

The potential role of each fimbrial antigen in saliva-induced aggregation of *A. viscosus* T14V was revealed by mixing parent and fimbria-deficient mutant cells with sialidase-treated saliva (Fig. 2). Bacteria with type 1^+2^+ and type 1^-2^+ fimbriae formed large aggregates, those with type 1^+2^- fimbriae formed smaller aggregates, and those with no fimbriae failed to aggregate. Similar experiments performed in the presence of 0.12 M lactose showed that the inhibitory effect of this disaccharide on aggregation was greatest with the type 1^-2^+ cells, least with the type 1^+2^- cells, and intermediate with type 1^+2^+ cells. Thus, the interaction of *A. viscosus* T14V with sialidase-treated saliva involved both types of fimbriae.

DISCUSSION

Spontaneous mutants of *A. viscosus* T14V specifically lacking either type or both types of fimbriae were obtained

TABLE 3. Adherence properties of *A. viscosus* T14V and mutant strains

Bacterial strain (fimbrial phenotype)	Adsorption to SHA (% ± SE)[a]	Coaggregation score[b] with *S. sanguis*:			
		DL1	H1	34	J22
T14V (1^+2^+)	88.4 ± 0.7	3	3	4[c]	4
PK256 (1^+2^+)	82.7 ± 0.7	3	3	4[c]	4
PK455 (1^+2^-)	80.7 ± 1.0	3	3	0	3
256-1 (1^-2^+)	27.2 ± 1.4	3	3	4[c]	4
455-2 (1^-2^-)	23.3 ± 1.5	3	3	0	4
T14V-J1 (1^+2^+)	85.0 ± 0.8	3	3	4[c]	4
5519 (1^+2^-)	87.0 ± 2.1	3	3	0	4
5951 (1^-2^+)	9.6 ± 2.7	3	3	4[c]	4
147 (1^-2^-)	12.0 ± 2.2	3	3	0	4

[a] Percentage of 4×10^6 cells adsorbed to 10 mg of SHA in a 0.25-ml total volume. Each mean and standard error was calculated from results of at least six independent experiments.

[b] Scores: 0 for no coaggregation to 4 for maximum coaggregation.

[c] Coaggregation was reversed completely by 0.06 M lactose.

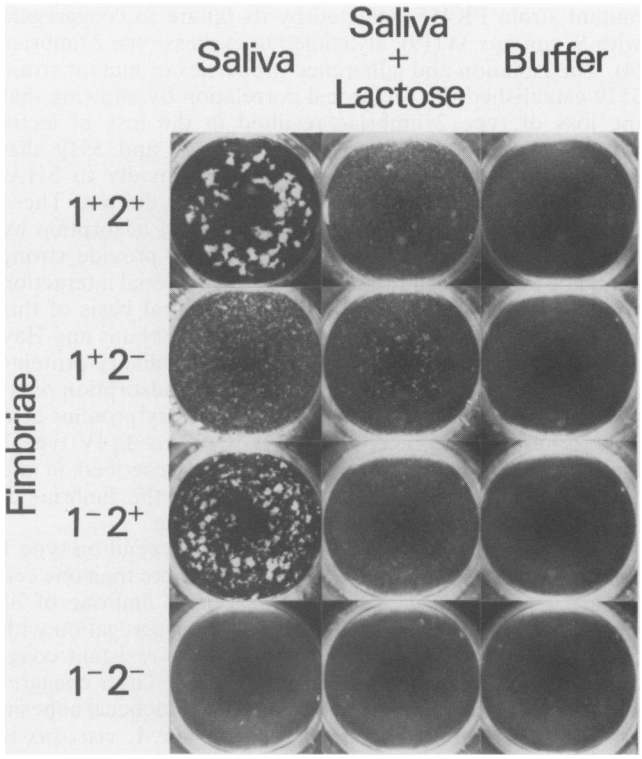

FIG. 2. Saliva-induced bacterial aggregation. *A. viscosus* T14V-J1 expressing type 1 and type 2 fimbriae (1^+2^+) and fimbria-deficient mutant strains 5519 (1^+2^-), 5951 (1^-2^+), and 147 (1^-2^-) were mixed for 1 h with sialidase-treated whole human saliva (Saliva), sialidase-treated saliva in the presence of 0.12 M lactose (Saliva + Lactose), or adsorption buffer (Buffer).

by an approach that was independent of the functional activities of these cell surface structures. This involved immunospecific enrichment of the type 1^+2^+ fimbriated parent to obtain cells lacking one fimbrial antigen and further enrichment of these by using a second antibody to select for cells with no fimbriae. The fimbriation of parent and mutant strains was confirmed at the ultrastructural level by specific labeling of type 1 and type 2 fimbriae with iron dextran and ferritin, respectively (Fig. 1). This technique revealed both fimbrial antigens on individual cells of a type 1^+2^+ parent strain, different fimbrial antigens on the type 1^+2^- and type 1^-2^+ mutant strains, and no labeled or unlabeled fimbriae on the type 1^-2^- fimbria-deficient mutant. In addition, the mutational loss of one fimbrial antigen did not appear to influence expression of the other, since strains with one or two types of fimbriae bound similar amounts of a radiolabeled monoclonal antibody directed against the shared antigen (Table 2). Each fimbria-specific monoclonal antibody used in these determinations reacts with the corresponding fimbrial subunit. These proteins have molecular weights near 60,000 (11, 29), and each has been detected by Western blotting (immunoblotting) of crude soluble extracts prepared from cells that express specific fimbriae but not from those that are fimbria deficient (results not presented). Thus, the genetic defects in the various mutant strains seem to affect the biosynthesis of either the type 1 or type 2 fimbrial subunit.

Various lines of evidence have previously associated the lactose-sensitive lectin of *A. viscosus* T14V with the type 2 fimbriae of this organism, including the demonstration that

mutant strain PK455, selected by its failure to coaggregate with *S. sanguis* 34 (19), also failed to express type 2 fimbriae (4). The isolation and adherence properties of mutant strain 5519 established the reciprocal correlation by showing that the loss of type 2 fimbriae resulted in the loss of lectin activity (Table 3). Mutant strains PK455 and 5519 that expressed only type 1 fimbriae adsorbed avidly to SHA, while those with only type 2 or no fimbriae did not. These findings, as well as the specific inhibition of adsorption by rabbit antibody against type 1 fimbriae (9), provide strong evidence that these fimbriae mediate the bacterial interaction with SHA. Further insight into the structural basis of this interaction has recently been provided by Gibbons and Hay (14), who identified the acidic proline-rich salivary proteins as likely receptors for the type-1-mediated adsorption of *A. viscosus* LY7 to SHA. These purified salivary proteins also function as specific receptors for *A. viscosus* T14V type 1 fimbriae, as shown clearly by the study described in the accompanying article (15) performed with the fimbria-deficient mutant strains described in this article.

Whereas certain adherence phenomena depend on type 1 or type 2 fimbriae, others are mediated by more than one cell surface structure. For example, the type 2 fimbriae of *A. viscosus* T14V mediate lactose-sensitive coaggregation with *S. sanguis* 34 and contribute to the lactose-resistant coaggregation with *S. sanguis* J22 (Table 3). The latter coaggregation also involves a lactose-resistant streptococcal adhesin (6). That the complementary receptor(s) on *A. viscosus* is distinct from either type of fimbriae is suggested by the lactose-resistant coaggregation of strain J22 with nonfimbriated cells of *A. viscosus* (Table 3). Saliva-induced aggregation of *A. viscosus* also involves a number of distinct interactions. These include the exposure of lectin receptors on salivary glycoproteins by the action of bacterial sialidase and the aggregation of cells by a mechanism that appears to involve both the type 2 fimbrial lectin and a component that is not inhibited by lactose or β-linked galactosides (13). With *A. viscosus* T14V, the latter interaction clearly depends on the presence of type 1 fimbriae, since cells with no fimbriae failed to aggregate (Fig. 2). Thus, the aggregation of this organism by saliva appears to be an additional adherence phenomenon that is fimbria dependent. Further studies are in progress to define the structural basis of these interactions and the role they play in oral microbial colonization.

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