

## Chemical and Immunological Comparison of Surface Fibrils of Strains Representing Six Taxonomic Groups of *Actinomyces viscosus* and *Actinomyces naeslundii*

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Human isolates of *Actinomyces viscosus* and *Actinomyces naeslundii* have been divided into six clusters in a numerical taxonomy study. Surface fibrils of strains representing these clusters were isolated and purified. Chemical analyses revealed that the major component of all fibrils was protein and that although differences in percentages of specific amino acid residues were found, the relative proportions of basic, acidic, polar uncharged, and nonpolar amino acids were rather similar among clusters. All of the fibrils except those from strain B236 (cluster 2) either failed to migrate or penetrated only slightly into gels during sodium dodecyl sulfate-polyacrylamide gel electrophoresis, even after boiling, reduction, or alkylation. Immunological studies by electron microscopic examination of fibril-antibody immunocomplexes, whole bacterial cell agglutination, inhibition of hemagglutination, and immunofluorescence by using antifibril antisera and antibodies demonstrated that strains of typical *A. naeslundii* (cluster 5) have a specific fibril-associated antigen(s) distinct from those of strains of other clusters. Cross-reactions for atypical *A. naeslundii* (cluster 3) were few. The fibrils from *A. viscosus* clusters 1, 2, 4, and 6 demonstrated several cross-reactions. By absorbing antifibril antibodies with cross-reactive strains it was possible to obtain cluster-specific antibodies, as determined by whole cell agglutination, only for cluster 5. Absorbed antifibril antisera for both *A. naeslundii* clusters 3 and 5 were specific by indirect immunofluorescence, whereas anti-cluster 1 fibril antisera cross-reacted only with other *A. viscosus* cluster representatives. Purification of *Actinomyces* fibrils by methods used for appendages of other species yields preparations containing common antigens among taxonomic groups. However, absorbing antifibril antisera, gamma globulin, or both has promise for producing cluster-specific reagents useful in identification.

Surface fibrils of *Actinomyces viscosus* and *Actinomyces naeslundii* have been implicated in attachment functions such as adherence to epithelial cells and hydroxyapatite (8, 14, 30), bacterial coaggregation (4, 5, 18, 25), and hemagglutination (9, 13). Because the fibrils of only *A. viscosus* strain T14V have been studied previously (6, 7, 30), we decided to undertake a comparative study of the chemical and antigenic properties of several strains of *A. viscosus* and *A. naeslundii*. Human isolates of these species have been divided by Fillery et al. into six clusters according to their overall phenotypic similarities (15). Strains representing these six clusters differ in hemagglutinating activity, suggesting different characteristics for their surface fibrils (13).

In a recent study of ours, the fibrils of strain WVU627, a representative strain of cluster 1-typical *A. viscosus*, were purified and characterized (24). These fibrils contained mainly protein. Antiserum raised against the purified fibrils revealed some common antigens in extracts of strains representing all of the other clusters except cluster 5-typical *A. naeslundii*. The aims of the work reported herein were (i) to purify the fibrils of representative strains of the remaining clusters by methods and criteria often used for fimbriae of other species, (ii) to compare their chemical properties, and (iii) to use antisera raised against the fibrils of all six cluster representatives to either confirm or negate observations of their antigenic relationships made previously with sera against only one of them (24).

### MATERIALS AND METHODS

**Cultures and cultural conditions.** The representative strains of each numerical taxonomy cluster of *Actino-*

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*myces* used in this study were WVU627 (cluster 1), B236 (cluster 2), B74 (cluster 3), 8A06 (cluster 4), TF11 (cluster 5), and W1053 (cluster 6), all of which possess numerous fibrils on their cell surfaces. Cells were maintained by monthly transfer on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) slants. Bacterial cultures used for fibril preparation were grown in a partially defined medium as described previously (24). For serological studies, other *Actinomyces* strains which have been classified by Fillery et al. (15) were selected from the stock culture collection of our laboratory (see Table 3).

**Preparation of fibrils.** Detachment of the fibrils from the cells was performed as described previously (24). Cells were harvested, washed three times with phosphate-buffered saline (PBS; 0.01 M, pH 7.0), suspended (4 g of packed cells per 100 ml of PBS), and homogenized at full speed for 5 min (type 23; The VirTis Co., Inc., Gardiner, N.Y.). After centrifugation at  $12,000 \times g$  for 30 min, supernatants were collected. The cells were homogenized a second time in the same manner. After ultrafiltration, fibrils were concentrated by three cycles of 50% ammonium sulfate precipitation. The fibrils of WVU627 (cluster 1) and B236 (cluster 2) were purified as previously described (24). Concentrated fibril suspensions were subjected to gel filtration chromatography on Sepharose 6B columns, and fibril-rich fractions were further purified by DEAE-cellulose ion-exchange chromatography with NaCl gradient fractionation. The yield of fibrils in these preparations was approximately  $1.3 \mu\text{g}/100 \text{ mg}$  (wet weight) of cells. Fibrils of other clusters showed strong nonspecific adsorption to DEAE-cellulose, DEAE-sephadex (Pharmacia Fine Chemicals, Piscataway, N.J.), and Ecteolacellulose (Schleicher & Schuell Inc., Keene, N.H.) and had to be purified by one of the following two methods.

Purification of *A. naeslundii* B74 (cluster 3) and TF11 (cluster 5) fibrils was performed by the method used by Korhonen et al. (19) to purify *Escherichia coli* pili. After dialysis against water, the fibril suspension was solubilized in Tris-hydrochloride buffer (0.01 M, pH 7.5) containing 0.5% sodium deoxycholate (Sigma Chemical Co., St. Louis, Mo.) and dialyzed against the same buffer for 48 h. After centrifugation at  $10,000 \times g$  for 30 min, the supernatant was concentrated by ultrafiltration. This sample was ultracentrifuged at an average speed of  $52,800 \times g$  (model L2-65B; Beckman Instruments, Inc., Fullerton, Calif.) for 24 h in a 10 to 60% sucrose gradient. The top layer yielding a single protein peak measured by UV absorption was dialyzed against Tris-hydrochloride buffer and concentrated in a rotary evaporator. The sample was applied to Sepharose 6B and eluted with Tris buffer with or without 6 M crystalline urea. Electron microscopy revealed fibrils only in the first of the two peaks (see Fig. 1). Fibril-rich fractions were combined and dialyzed against distilled water. The final yield of purified fibrils was approximately  $1.7 \mu\text{g}$  (strain B74) and  $1.0 \mu\text{g}$  (strain TF11) per 100 mg (wet weight) of cells.

Fibrils of *A. viscosus* 8A06 (cluster 4) and W1053 (cluster 6) were prepared by a modification of the method of Wheeler and Clark (30) for the isolation of *A. viscosus* T14V fibrils. After detachment from cell surfaces, fibrils were purified by repeated cycles of ammonium sulfate precipitations, the last being at 10% saturation. After dialysis against water, the concen-

trated fibril suspension was applied to a Sepharose 6B column and eluted with PBS. Fractionation was performed as previously described (24). The maximum yield was only  $0.5 \mu\text{g}/100 \text{ mg}$  (wet weight) of cells.

**Characterization of fibrils.** Polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (21). Fibril preparations containing  $50 \mu\text{g}$  of protein were treated with 1.2% sodium dodecyl sulfate (SDS)-1.3% 2-mercaptoethanol, boiled for 3 min, and applied to 5 and 7% polyacrylamide gels. Fibril preparations which showed no migration into the gel were boiled for 60 min or treated with 40 mM dithiothreitol for reduction or 100 mM iodoacetamide for alkylation, or with both, as described by Newhall et al. (26). SDS-PAGE gels with known molecular weight standards were run in parallel as described previously (24). Protein was measured by the method of Lowry et al. (23), using bovine serum albumin as the standard. Total carbohydrate was measured by both the phenol sulfuric acid method (11) and gas liquid chromatography of trimethylsilyl derivatives as described previously by Sweeley et al. (29), using Sigma-Sil-A (Sigma) as the silylating agent. Amino acids were analyzed as described previously (24). The UV absorption spectrum was measured (Sp800A; Unicam, Cambridge, England). Nucleic acid content was assayed indirectly by monitoring the effects of RNase and DNase on fibril morphology and antigenicity (24).

**Preparation of antifibril (anti-F) antisera.** Rabbit antisera against all of the fibril preparations were raised as described previously (24). Antibody titers were quantified by homologous whole cell agglutination. Gamma globulin fractions of anti-F antisera were prepared by repeated ammonium sulfate precipitations. In an attempt to obtain cluster-specific anti-F antisera, the gamma globulin solutions were absorbed with cross-reacting strains by mixing equal volumes of antibody solution and packed cells at  $37^\circ\text{C}$  for 2 h followed by overnight storage at  $4^\circ\text{C}$ . Cells with absorbed antibodies were removed by centrifugation at  $10,000 \times g$  for 30 min.

**Serological procedures.** Attempts to solubilize the fibrils to enhance migration in immunoelectrophoresis gels failed in five of the six cases. Because the whole fibril preparations barely migrated and because some *Actinomyces* antigens are known not to precipitate in gels (15), four other methods were used to study antigenic relationships among the fibrils from the six cluster strains.

(i) **Direct observation by electron microscopy.** A solution containing  $50 \mu\text{g}$  of fibril protein per ml of PBS was prepared as an antigen. Equal volumes ( $30 \mu\text{l}$ ) of antigen solution and anti-F gamma globulin solution diluted either 1/5, 1/10, or 1/50 in PBS were mixed in a capillary tube. After incubation at  $37^\circ\text{C}$  for 2 h and storage at  $4^\circ\text{C}$  overnight, the mixture was centrifuged at  $12,000 \times g$  for 30 min. The sediment was washed and resuspended with a drop of distilled water and prepared for electron microscopic observation.

(ii) **Whole cell agglutination.** Agglutination of bacterial cells by the anti-F gamma globulin was measured in microtiter plates as reported previously (24). Cells were grown in a chemically defined medium. Washed cells were dispersed by sonication for 30 s to prevent self-agglutination and adjusted to an optical density of 1.0 at 550 nm. Cell agglutination reactions were graded as 3+ (maximum agglutination) to 0 (no agglutination).

TABLE 1. Amino acid composition

Amino acid	No. of residues per 1,000 <sup>a</sup>					
	WVU627 (1) <sup>b</sup>	B236 (2)	B74 (3)	8A06 (4)	TF11 (5)	W1053 (6)
<b>Acidic</b>	(20) <sup>c</sup>	(22)	(24)	(14)	(22)	(11)
Aspartic acid	103	96	96	48	91	27
Glutamic acid	101	129	147	96	132	70
<b>Basic</b>	(13)	(10)	(13)	(17)	(11)	(14)
Lysine	67	54	87	39	55	52
Arginine	36	23	25	44	24	34
Histidine	16	23	21	65	27	43
Ornithine	12	ND <sup>d</sup>	ND	ND	ND	ND
<b>Polar uncharged</b>	(29)	(40)	(36)	(46)	(40)	(52)
Threonine	91	63	58	43	66	49
Glycine	107	154	158	196	158	182
Serine	65	158	132	223	156	223
Tyrosine	24	20	8	9	21	19
Cysteine	4	ND	ND	ND	ND	ND
<b>Nonpolar</b>	(38)	(28)	(27)	(22)	(27)	(22)
Alanine	96	101	97	105	105	138
Leucine	58	46	42	13	6	48
Valine	72	46	45	100	54	45
Isoleucine	38	27	25	9	30	29
Phenylalanine	27	20	17	ND	21	21
Methionine	9	3	4	9	4	20
Proline	75	37	39	ND	49	ND

<sup>a</sup> Not corrected for losses. Number in parentheses after strain designation shows cluster number.

<sup>b</sup> Based on reference 24.

<sup>c</sup> Percentage of total amino acids rounded to nearest whole number.

<sup>d</sup> ND, Not detected.

(iii) **Hemagglutination inhibition.** Anti-F antisera were tested for their ability to inhibit hemagglutination by homologous and heterologous strains. Each cell suspension was mixed with each of several diluted anti-F antisera. After incubation at 37°C for 2 h, the cells were harvested, washed, sonicated, and suspended to the original volume. With a macroscopic scoring method (13), the degree of agglutination of neuraminidase-treated human type AB erythrocytes was scored as 8+ (maximum agglutination) to 0 (no agglutination detected).

(iv) **Immunofluorescence microscopy.** Anti-F antisera for strains WVU627 (cluster 1), B74 (cluster 3), and TF11 (cluster 5) were absorbed with heterologous strains as listed in Table 5. Bacterial smears were prepared on standard microscope slides. Absorbed anti-F antisera were applied at predetermined working dilutions, and the slides were incubated in a moist chamber for 30 min. After washing for 15 min with three changes of 0.001 M PBS at pH 7.2, fluorescein isothiocyanate-labeled goat anti-rabbit gamma globulin (Cappel Laboratories, Cochranville, Pa.) at a dilution of 1/50 was applied for 30 min and then washed in the same manner. The slides were examined with a microscope (Dialux; Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, N.J.) equipped with a Ploemopak 2.3 fluorescence vertical illuminator with a 420 Wild Leitz filter system and an FL 40/0.85 objective lens.

## RESULTS

**Characterization of fibrils.** The major fibril component of all of the cluster representatives appeared to be protein. All preparations showed maximum UV absorbance at approximately 278 nm. Neither the colorimetric nor the gas chromatographic method detected more than minute traces of carbohydrate. Nuclease treatment changed neither the morphology nor the antigenicity of the fibrils. During SDS-PAGE, all of the fibrils except those from B236 (cluster 2) either failed to migrate (B74, 8A06, W1053) or migrated only slightly beyond the bovine serum albumin (66,000 daltons) standard (WVU627, TF11) into either the 5% or the 7% polyacrylamide gels, even after boiling in the presence of SDS and 2-mercaptoethanol. B236 yielded two protein bands, one of which migrated beyond other fibril bands to the ovalbumin (45,000 daltons) standard. Only TF11 fibrils migrated further after boiling for 60 min. Treatment of fibrils from all strains by either dithiothreitol or iodoacetamide did not produce detectable subunits.

Considering groups of amino acids by polarity and charge, the fibril amino acid composition

was broadly similar among the six representative strains (Table 1). The sum of polar uncharged and nonpolar amino acids comprised 62 to 74% of the total amino acids. Glycine, serine, glutamic acid, aspartic acid, and alanine were the most plentiful. Fibrils of strains B236 (cluster 2), B74 (cluster 3), and TF11 (cluster 5) were almost identical, with the exceptions being a high lysine value for B74 and low leucine value for TF11. The amino acid composition of these strains was somewhat similar to the composition previously reported by us for strain WVU627. However, fibrils of WVU627 contained fewer serine and glycine residues, resulting in the lower value for polar uncharged amino acids, and more residues of ornithine, cysteine, and nonpolar amino acids. Amino acid analyses of strains 8A06 and W1053 fibril preparations were grossly different from the others.

The stability of the fibrils from all six cluster strains was rather similar. Boiling (30 min) and treatment with base (0.1 M NaOH at 37°C for 60 min) destroyed both the antigenicity and morphology so that the fibrils were undetectable by the usual EM methods. Acid treatment (0.1 M HCl at 37°C for 60 min) diminished the antigenicity and disrupted the structural integrity of intact fibrils except in the cases of strains 8A06 (cluster 4) and W1053 (cluster 6), which were less affected by this treatment.

**Immunological studies.** Figure 1A shows an example of an electron micrograph of the fibril preparation of TF11 (cluster 5) which was used for a direct reaction with homologous anti-F antibody. Figure 1B illustrates the resulting immunocomplex. Fibrils were coated with anti-

body and were aggregated. Figure 1C demonstrates the homologous reaction with WVU627 (cluster 1) in which thickened fibrillar structures are evident. Preimmune rabbit sera neither coated nor aggregated the fibrils of any of the six cluster strains. Table 2 summarizes cross-reactions of the fibrils among the six representative strains. Fewer cross-reactions with anti-F gamma globulin were observed at a dilution of 1/10 than 1/5. At 1/10, antibody for TF11 (cluster 5) reacted only with homologous fibrils, and fibrils of TF11 reacted only with homologous antibody. Fibrils of strains from clusters 1, 2, 4, and 6 possessed a number of cross-reacting antigens in addition to some specific antigens. B74 (cluster 3) cells reacted only with homologous gamma globulin at 1/10 dilution. Antibody directed to B74 fibrils reacted with cells of strain 8A06 (cluster 4) in addition to the homologous reaction. All reactions with the 1/50 dilution of anti-F antibodies were negative in this precipitation test.

The results of the effects of anti-F antisera on hemagglutination are summarized in Table 3. All untreated strains except B25 (cluster 6) showed strong control hemagglutination reactions with neuraminidase-treated human erythrocytes. In all instances, treatment of bacterial cells with homologous anti-F antibodies prevented hemagglutination. The inhibiting homologous reaction was also observed for a second strain representing each cluster. Agglutination of neuraminidase-treated human erythrocytes by TF11 and WVU398A (cluster 5) was not strongly inhibited by the anti-F antiserum for any other cluster. Hemagglutination by cluster 1, 2, 4, and 6 strains

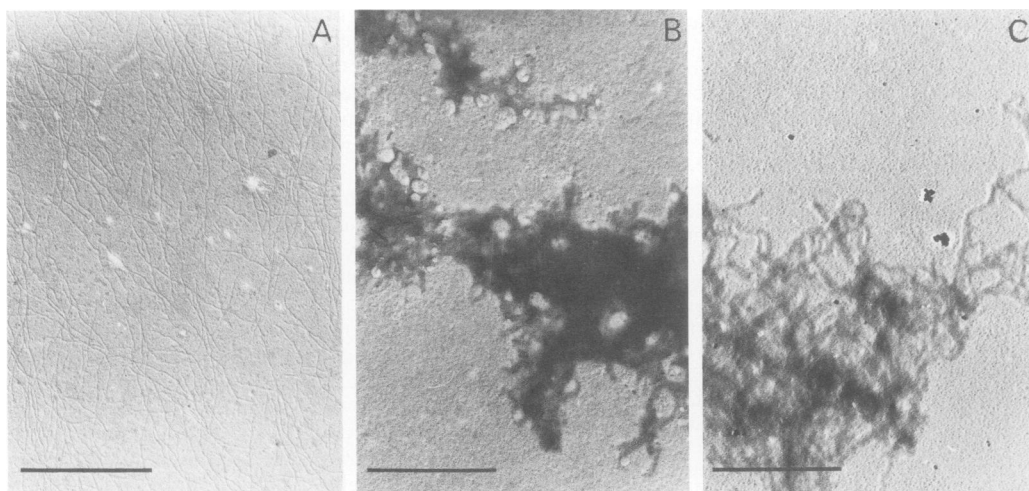


FIG. 1. Electron micrographs of shadowed fibrils. (A) Purified fibrils of strain TF11. (B) Immunocomplex of the fibrils of strain TF11 and homologous anti-F antibody. (C) Immunocomplex of the fibrils of WVU627 and homologous anti-F antibody. (Bar, 1  $\mu$ m)

TABLE 2. Direct immune reactions of *A. viscosus* and *A. naeslundii* anti-F gamma globulin with fibrils of each cluster strain<sup>a</sup>

Source of fibrils (cluster)	Reaction with anti-F gamma globulin to: <sup>b</sup>											
	WVU627 (1)		B236 (2)		B74 (3)		8A06 (4)		TF11 (5)		W1053 (6)	
	1/5 <sup>c</sup>	1/10	1/5	1/10	1/5	1/10	1/5	1/10	1/5	1/10	1/5	1/10
WVU627 (1)	⊕	⊕	+	+	+	-	+	+	-	-	+	-
B236 (2)	+	+	⊕	⊕	+	-	+	-	-	-	+	-
B74 (3)	+	-	+	-	⊕	⊕	+	-	+	-	+	-
8A06 (4)	+	-	+	-	+	+	⊕	⊕	+	-	+	+
TF11 (5)	-	-	+	-	+	-	+	-	⊕	⊕	+	-
W1053 (6)	+	-	+	-	+	-	+	+	+	-	⊕	⊕

<sup>a</sup> Detected by electron microscopy.  
<sup>b</sup> Homologous reaction circled.  
<sup>c</sup> Dilution (vol/vol).

was inhibited by antisera to the fibrils of these same four clusters, but not always strongly. Hemagglutination by B74 cells was inhibited by the anti-F antisera of all clusters except cluster 5, which did inhibit hemagglutination by strain B120, the other cluster 3 strain. Treatment with the anti-F antiserum for B74 only weakly interfered with the hemagglutination of other strains.

With the above results as a guide, absorption of the anti-F antibodies with cross-reactive strains was attempted to determine the feasibility of producing solutions of cluster-specific anti-F antibodies. Absorbed immunoglobulin solutions were examined for cross-reactivity by the whole cell agglutination test run in microtiter plates (Table 4). Absorption of anti-TF11 fibril antibodies with B74 (cluster 3) cells removed all cross-reactions, but other antibody solutions still continued to cross-react among the six clusters. Antibodies against B236 (cluster 2)

absorbed with WVU627 (cluster 1) whole cells decreased its homologous reaction and still cross-reacted with cluster 3, 4, 5, and 6 strains to virtually the same degree. Absorption of anti-B236 fibril antibodies with additional strains did not yield cluster-specific anti-F antibodies. This might suggest that the fibrils of cluster 2 have no cluster-specific antigen. This was also suggested by the finding that unabsorbed anti-B236 fibril antibodies showed positive cell agglutination with cluster 1 and 6 strains at almost the same maximum dilution as the homologous reaction (data not shown). Anti-WVU627 fibril antibodies absorbed with B236 (cluster 2) cells showed strong cross-reactions with cluster 6 strains, but absorption with W1053 (cluster 6) yielded more specific results. The reactions of anti-8A06 (cluster 4) fibril antibodies revealed that strains 8A06 and Be32 were antigenically quite different even though the two strains belong to cluster 4. This

TABLE 3. Effects of anti-F antisera on hemagglutination by *Actinomyces* strains

Strain (cluster)	Hemagglutination score with anti-F antisera to: <sup>a</sup>						
	Control	WVU627	B236	B74	8A06	TF11	W1053
WVU627 (1)	7	⊕	0	5	2	7	0
11B2 (1)	7	0	1	5	2	7	0
B236 (2)	8	0	⊕	5	0	5	0
B74 (3)	8	2	0	⊕	0	7	0
B120 (3)	7	2	0	0	0	0	0
8A06 (4)	7	3	2	3	⊕	0	0
Be32 (4)	7	0	0	1	0	0	0
TF11 (5)	8	6	5	8	5	⊕	5
WVU398A (5)	8	6	8	8	7	0	7
W1053 (6)	8	0	0	3	3	5	⊕
B25 (6)	2	0	0	2	2	2	0

<sup>a</sup> 8+, Maximum, to 0, no hemagglutination detected. Reaction with homologous strain is circled.

TABLE 4. Bacterial cell agglutination titers of anti-F gamma globulin absorbed with closely related strains

Strain (cluster)	Agglutination titer						
	Anti-WVU627 (1) absorbed with:		Anti-B236 (2) absorbed with	Anti-B74 (3) absorbed with	Anti-8A06 (4) absorbed with W1053	Anti-TF11 (5) absorbed with B74 (3)	Anti-W1053 (6) absorbed with 8A06 (4)
	B236 (2)	W1053 (6)	WVU627 (1)	WVU627 (1)			
WVU627 (1)	7 <sup>a</sup>	6	0	2	4	0	5
11B2 (1)	8	6	0	2	3	0	5
B236 (2)	0	3	4	2	5	0	5
B74 (3)	2	3	3	5	6	0	5
B120 (3)	2	3	2	5	6	0	5
8A06 (4)	3	1	3	3	8	0	0
Be32 (4)	4	1	4	4	5	0	2
TF11 (5)	0	3	4	2	2	6	4
WVU398A (5)	0	1	4	0	3	6	4
W1053 (6)	5	2	3	4	3	0	8
B25 (6)	6	3	3	2	3	0	8

<sup>a</sup> Minimum dilution (log<sub>2</sub>) yielding 1+ positive reaction. Titer for homologous reactions of unabsorbed gamma globulin solutions: WVU627, 8; B236, 10; B74, 9; 8A06, 9; TF11, 10; W1053, 11.

was also observed in a homologous reaction, using the unabsorbed anti-8A06 fibril antibodies. Strain 8A06 agglutinated at the maximum dilution of 2<sup>-12</sup>, but strain Be32 agglutinated at only 2<sup>-7</sup>, a titer similar to that of many strains from heterologous clusters.

Table 5 summarizes results of immunofluorescence studies with absorbed anti-F antisera. At the working dilutions, these antisera showed no cross-reactions among strains from clusters 1, 3, and 5. Anti-F antisera against B74 (cluster 3) and TF11 (cluster 5) reacted only with strains from the homologous cluster even though both clusters are composed of strains designated *A. naeslundii*. Anti-F antiserum against WVU627 (cluster 1) reacted with other *A. viscosus* strains, even those classified in clusters 2, 4, and 6. Strain B25 (cluster 6) reacted with none of the sera at their working dilutions.

#### DISCUSSION

Antigenic differences are partly responsible for the classification of *A. viscosus* and *A. naeslundii* human isolates into six groups by numerical taxonomy (15). Differences between *A. viscosus* and *A. naeslundii* intraoral colonization patterns (12) and their abilities to attach to saliva-coated hydroxyapatite (8), to agglutinate with saliva (R. P. Ellen, D. Bratthall, M. Borgström, and T. P. Howley, Scand. J. Dent. Res., in press), to hemagglutinate, and to prime erythrocytes for hemagglutination via sialidase (13) further suggest that some of the serological specificities among clusters may be accounted for by antigens located in their surface fibrils. In

our previous investigation of fibrils of an *A. viscosus* cluster 1 strain (24) and in this study of representative strains of all clusters, we have

TABLE 5. Immunofluorescence reactions of absorbed anti-F antisera

Strain (cluster)	Immunofluorescence with: <sup>a</sup>		
	Anti-WVU627 (1) ab- sorbed with B74 (3) or TF11 (5)	Anti-B74 (3) ab- sorbed with WVU627 (1), B236 (2), and 8A06 (4)	Anti-TF11 (5) ab- sorbed with WVU627 (1) and B74 (3)
WVU627 (1)	4	0	0
11B2 (1)	4	0	0
B236 (2)	4	0	0
B74 (3)	0	4	0
B120 (3)	0	4	0
8A06 (4)	1	0	0
Be32 (4)	4	0	0
TF11 (5)	0	0	4
WVU398A (5)	0	0	4
W1053 (6)	4	0	0
B25 (6)	0	0	0

<sup>a</sup> Working titer. Highest dilution yielding bright 4+ homologous reaction: anti-WVU627 (1) = 1/128; anti-B74 (3) = 1/16; anti-TF11 (5) = 1/90. 4+, Bright fluorescence equivalent to homologous reaction, to 0, no fluorescence observed.

approached this question by trying to purify fibrils by methods established for appendages of other species and by testing a variety of serological reactions with antibodies raised against the fibril preparations. Although purified fibrils from representative strains of some of the groups were found to be broadly similar as regards chemical and amino acid analyses, stability during attempts at physiochemical degradation, and serological cross-reactivity, the antigenic distinctiveness of one group, typical *A. naeslundii* (cluster 5), was strikingly evident.

Isolating acceptably pure fibrils of all strains by identical procedures proved difficult. Therefore, a major emphasis in this work was placed on the purification of fibrils by whatever means would achieve apparent purity by the criteria of electron microscopy, SDS-PAGE, and amino acid analysis and still provide reasonable yields for further chemical and serological studies. These are standard criteria which have been used previously for coliforms, gonococci, corynebacteria, and actinomyces. Fibrils of *A. viscosus* strains could be purified by a combination of repeated ammonium sulfate precipitations and gel filtration as used previously by both Wheeler and Clark (30) and us (24). The cleanest preparations were obtained by an additional step of ion-exchange chromatography. These preparations were free of amino acids indicative of cell wall and membrane contamination. Much difficulty was encountered with strains 8A06 (cluster 4) and W1053 (cluster 6). Yields after gel filtration were very low, casting some doubt as to whether the purified fibril preparation was truly representative of all fibrils found on the intact bacterial cell. Moreover, their amino acid compositions were grossly different from the other strains.

Additional technical difficulties plagued attempts to use similar methods for cluster 3 and 5 fibrils. Amino acid analysis of fibrils prepared by our usual methods demonstrated an elevated concentration of ornithine which could have been derived from contaminating cell wall material. Furthermore, fibrils of strain TF11 (cluster 5) were irreversibly retained by all three ion exchangers employed. Instead, a method previously used for separating *E. coli* pili from outer membrane proteins and lipopolysaccharide was chosen for *A. naeslundii* fibril preparation. Unlike *E. coli* pili, which are totally solubilized by the deoxycholate and thus completely separable from insoluble material, SDS-PAGE revealed an *Actinomyces* fibril-specific protein band in the deoxycholate-insoluble fraction (data not shown). However, the final preparation from the soluble material which had been separated from the rest by sucrose gradient centrifugation was found to be highly pure by the morphological and chemical criteria. Furthermore, the use of 6

M urea did not alter the elution profile during gel filtration, suggesting that fibrils prepared in this manner are stable in concentrated urea.

Despite differences used to purify fibrils, many chemical characteristics seem to be shared by fibrils of all strains. As we found previously for *A. viscosus* WVU627 fibrils (24), fibrils of most strains resisted solubility in SDS and hardly penetrated even 5% polyacrylamide gels. Fibrils of strain B236 (cluster 2) were the exceptions; two protein bands were detected, one well within the gel. After boiling for 60 min, only TF11 (cluster 5) fibrils migrated further into the gel. Furthermore, attempts at reduction and alkylation both failed to yield subunits detectable by PAGE. These findings differ greatly from those concerning pili of gram-negative bacteria (1-3, 20) and indicate that the *A. viscosus* fibril molecules are not easily degraded.

Wheeler and Clark (30) also reported that *A. viscosus* T14V fibrils failed to penetrate polyacrylamide gels. However, using the same strain, Cisar et al. (6, 7) were able to obtain an immunodiffusion reaction midway between antigen and antibody wells, indicating that fibril antigens could at least migrate in agarose. In our attempts, none of the fibril preparations, except for strain B236 again, migrated freely into 1% agarose gels, even after 6 h (data not shown). During immunoelectrophoresis, these fibrils migrated only slightly anodally. This may be attributed to both a weak charge and a cumbersome molecular size or configuration. These findings raise the interesting possibility that strains T14V and B236 may be somewhat similar. T14V was not among the strains classified phenotypically by Fillery et al. (15). Its low DNA homology with WVU627, the typical *A. viscosus* cluster 1 standard (10), suggests that its taxonomic relationship to clusters 2, 4, and 6 should be investigated, especially since T14V is often used as representative of the species.

Amino acid analysis revealed some differences in composition among strains of the six clusters. Disregarding 8A06 and W1053, the fibrils of the remaining strains were rather similar, except that WVU627 showed a lower percentage of polar uncharged amino acids. When the relative contents of acidic (11 to 24%), basic (10 to 17%), and sum of polar uncharged and nonpolar (62 to 74%) amino acids were calculated, the values for the clusters were reasonably similar. Because of the numerous serological cross-reactions among the fibrils, it is also possible that they share long polypeptide segments with similar sequences. If this were so, the greatest deviation would be expected for cluster 5 strains. Unfortunately, the low yield of purified fibrils precludes sequencing at this time.

Fibrils of most cluster representatives were

also found to be similar in their structural and antigenic sensitivity to heat, acid, and alkali. We have also recently found that hemagglutination by whole bacteria can likewise be inhibited by these treatments (unpublished data). The exception is acid treatment of 8A06 and W1053, those strains with fibrils found to retain morphological and antigenic integrity after extraction with acid. These findings and those demonstrating hemagglutination inhibition by anti-F antisera add further direct support to the compelling evidence of Cisar and coworkers (4) with strain T14V that the *Actinomyces* hemagglutinin, a  $\beta$ -galactoside-specific lectin, is fibril borne.

Negligible migration in gels of fibrils from all clusters precluded immunoelectrophoresis as a method to study cross-reactivity among strains. Direct studies by electron microscopy and whole cell agglutination suggest that the fibrils of all representative strains carry more than one antigen, some demonstrating strong cross-reactivity among clusters. However, typical *A. naeslundii* strains such as TF11 and WVU398A carry an antigen or antigens seemingly specific to cluster 5. Immunological and genetic differences between typical *A. viscosus* and typical *A. naeslundii* have been reported before (10, 16, 17, 22, 28), but atypical strains falling into the other clusters of Fillery's classification scheme (15) have not been studied in detail. The finding in this study and our previous study (24) of more than one antigen specificity per fibril preparation can be interpreted either as multiple types of determinants located along individual, identical fibrils or as single strains elaborating heterogeneous fibril populations bearing distinct antigenic determinants. Recent demonstrations by Cisar and coworkers (4, 27) that both monoclonal antibodies and rabbit Fab fragments specific by immunoelectrophoresis for one of the precipitable fibril antigens of T14V fail to react with some of the fibrils on the whole cells strongly supports the second viewpoint. Moreover, their finding of differences in "fine specificity" toward heterologous strains among the various monoclonal antibodies prepared against this same precipitable T14V antigen (Ag2) (4) agrees with our viewpoint that each *Actinomyces* strain, especially those exclusive of cluster 5, may carry several fimbrial determinants which cross-react to various degrees. Since the  $\beta$ -galactoside-specific lectin is common to most *A. viscosus* and *A. naeslundii* strains, including cluster 5 (5, 13, 18), it is possible that this fimbrial component does not account for the antigenic specificity which discriminates cluster 5 strains from the others in this study but might account for cross-reactions of anti-cluster 5 fibrils antiserum seen at low dilutions.

Only antiserum to cluster 2 strain B236

showed no remaining homologous agglutinating activity after absorption with one cross-reacting strain, WVU627. This suggests that its fibrils contain no real cluster-specific antigen which may be helpful for identification of clinical isolates. Furthermore, the fibrils of B236 were a bit odd in that they were the only ones to easily penetrate polyacrylamide and immunoelectrophoresis gels. Their serological cross-reactions with fibrils of *A. viscosus* clusters 1, 4, and 6 suggest that fibril antigen determinants can be expressed on smaller fragments than achievable by our purification methods.

In some instances, the immunological results for the strains from the same cluster were different. The same trend was reported by Coykendall in his DNA homology study (10). Generally, the DNA homology as determined by hybridization was similar but not completely homologous within clusters. We had hoped that anti-F antibody would provide a better means to rapidly identify strains in clinical material by using cluster-specific anti-F antibodies directly in agglutination tests or by labeling them with either fluorescent or enzyme markers. Our absorption studies with anti-F antisera suggest that this is indeed feasible, with some limitations. Our antisera cannot distinguish among *A. viscosus* strains of the various clusters. However, by indirect immunofluorescence, these antisera can distinguish between the *A. viscosus* and *A. naeslundii* strains used and, more significantly, between cluster 3 and cluster 5 *A. naeslundii*. We are currently testing these antisera with a great variety of laboratory strains and recent oral isolates. If successful, they should facilitate studies of the relationship of fibril antigenicity to the natural acquisition and ecology of the major *A. viscosus* and *A. naeslundii* clusters.

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