Deoxyribonucleic Acid Hybridization Among Strains of *Actinomyces viscosus* and *Actinomyces naeslundii*

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Deoxyribonucleic acids (DNA) were extracted from strains of *Actinomyces viscosus* and *Actinomyces naeslundii* and were compared by DNA-DNA hybridization. When their DNAs were compared by hybridization on membrane filters, *A. viscosus* strains from humans were barely separable from *A. naeslundii* strains, but *A. viscosus* strains from hamsters were distinctly different. *A. viscosus* strains from veterinary infections were identical to human strains. With the use of the more sensitive S_1 nuclease hybridization method with DNA from a set of strains that had been used in a numerical taxonomy study, typical *A. viscosus* strains from humans could be distinguished from typical *A. naeslundii* strains. A group of atypical *A. naeslundii* strains could also be delineated. The results generally parallel the taxonomic divisions of these organisms based on serology or numerical taxonomy. *A. viscosus* strains from humans and *A. naeslundii* strains are similar but distinguishable, but *A. viscosus* strains from hamsters are clearly separable.

Howell (12) and Howell and Jordan (13) described a filamentous organism which had been found associated with periodontal disease in laboratory hamsters (16). It closely resembled *Actinomyces naeslundii* but was catalase positive and hydrolyzed starch. Since catalase-positive organisms were not included in the genus *Actinomyces* at that time, the "hamster organism" was placed in a new genus and species, *Odontomyces viscosus* gen. nov. (14).

When it was recommended that catalase-positive bacteria be accepted in the genus *Actinomyces*, *Odontomyces viscosus* was renamed *Actinomyces viscosus* (8). Gerencser and Slack (9) isolated *A. viscosus* strains from human mouths and found that they differed serologically from the hamster organism. Hamster strains were designated serotype 1, and human strains were designated serotype 2. Georg et al. found both *A. viscosus* serotypes 1 and 2 in veterinary infections (7). Both hamster and human *A. viscosus* strains as well as *A. naeslundii* strains will cause periodontal disease in experimental animals (15, 16, 21). However, there are distinct differences between *A. viscosus* and *A. naeslundii*. They can be distinguished by their catalase reaction, cell wall carbohydrates, and serological properties (20). However their overall phenotypic similarity is great, and when compared by numerical taxonomic methods the two species are similar (6, 11). To clarify the relationships among *A. naeslundii*, human *A. viscosus*, and animal *A. viscosus* strains, deoxyribonucleic acids (DNAs) from several strains of these or-

ganisms were compared based on their ability to form hybrid duplexes.

MATERIALS AND METHODS

Bacterial strains. The hybridization experiments were carried out by two methods and on two groups of strains. The first study was a comparison of several strains by hybridization of their DNA on membrane filters (Table 1). Later, a number of strains which had been extensively characterized for a numerical taxonomy study (6) became available through the kindness of E. D. Fillery, University of Toronto, Toronto, Canada (Table 2). This second group was compared by DNA hybridization in free solution, using single-strand-specific nuclease (1). This method was considered more precise (2) and was employed to increase the discrimination between *A. naeslundii* and *A. viscosus* serotype 2 because filter experiments indicated that although they were not identical, the "border" between *A. viscosus* and *A. naeslundii* was indistinct. Furthermore, we wished to discriminate, if possible, Fillery's cluster 3 strains which resemble *A. naeslundii* but are biochemically and serologically different from both cluster 1 ("typical" *A. viscosus* serotype 2) and cluster 5 ("typical" *A. naeslundii*). Thus the results of a numerical taxonomic study would be directly compared with those of a DNA hybridization study. Two additional strains, T14V and T14AV, were included. T14V is said to be periodontopathic in gnotobiotic animals, whereas the substrain T14AV is not (10). A colony from each strain was picked from a CNAC-20 agar plate (5), and pure cultures were obtained. The cultures were Gram stained and tested for the following: catalase activity; nitrate and nitrite reduction; starch and lactose fermentation; gelatin liquefaction; methyl red reaction; urease and indole production; esculin hydrolysis; and the Voges-Proskauer reaction.

TABLE 1. *Strains used in filter hybridization experiments*

Strain	Origin/reference	Donor ^a
<i>A. viscosus</i> strains from animals		
(serotype 1)		
WVU745 (= T6 = ATCC 15987) ^b	Hamster mouth (12)	Gerencser
WVU440 (= X601 = HS2)	Hamster mouth (12)	Gerencser
A1231	Dog abdomen abscess (7)	Fillery
A1351 ^c	Pig lung (7)	Fillery
A1353 ^c	Goat lymph node (7)	Fillery
W1308 ^c	Dog liver abscess (7)	Fillery
<i>A. viscosus</i> strains from humans		
(serotype 2)		
WVU845	Human dental plaque	Ellen
P1	Human dental plaque	Ellen
P2	Human dental plaque	Ellen
34a ₁	Human dental plaque	Ellen
M100	Human dental plaque (15)	Tanzer
UHC-BIB	Human dental plaque (22)	Tanzer
UHC-HAs	Human dental plaque (22)	Tanzer
UHC-R3A	Human dental plaque (22)	Tanzer
UHC-TO4	Human dental plaque (22)	Tanzer
<i>A. naeslundii</i> strains		
C2	Human dental plaque	Ellen
N9	Human dental plaque	Ellen
43e ₁	Human dental plaque	Ellen
WVU820 (= N16)	Human dental plaque (15)	Gerencser
WVU45 (= ATCC 12104) ^d	Human sinus (9)	Gerencser
WVU398A	Human dental calculus (9)	Gerencser
WVU631	Human dental calculus (9)	Gerencser

^a M. A. Gerencser, West Virginia University, Morgantown; E. D. Fillery, University of Toronto, Toronto, Ontario, Canada; R. P. Ellen, University of Toronto; J. M. Tanzer, University of Connecticut, Farmington.

^b Type strain of *A. viscosus*. ATCC, American Type Culture Collection, Rockville, Md.

^c Serologically similar to *A. viscosus* serotype 2 (7).

^d Reference strain; candidate for designation as type strain.

All strains conformed to the descriptions of *A. viscosus* and *A. naeslundii* as reported by Slack and Gerencser (20).

Isolation of DNA. Cells of these species are difficult to lyse, and upon lysis they seem to release a deoxyribonuclease (DNase) which rapidly decreases the viscosity of the suspension, and DNA cannot be recovered. The following method gave moderate lysis and milligram quantities of DNA. Cells were grown in 2 to 4 liters of Trypticase soy broth (BBL, Cockeysville, Md.) supplemented with 10 mmol of NaHCO₃ per liter. For radioactive DNA (index DNA), 1 or 2 mCi of [*methyl*-³H]thymidine was added to the broth. Bacteria were cultured for 24 to 36 h in air plus 10% CO₂ at 37°C. Samples were taken to monitor growth turbidimetrically. Before growth stopped, 2.5 U of penicillin per ml was added, and the cells were incubated for an additional 1 to 2 h. The cells were then harvested, washed with water, suspended in 25 ml of 0.15 M tris(hydroxymethyl)aminomethane buffer (pH 8.0) containing 50 mg of lysozyme (2 mg/ml) and incubated at 37°C for 2 h. The cells were then removed from lysozyme and suspended in 25 ml of 1 × SSC (0.15 M NaCl + 0.015 M Na citrate) and heated to 65°C, at which time 1 to 2 ml of 25% sodium lauryl sulfate was added. This treatment lysed the cells, and

the suspension became quite viscous. When this viscosity reached a maximum, and before it started to decrease (2 to 4 min), an equal amount of water-saturated phenol was added, and this mixture was shaken gently for 30 min at room temperature. The phenol appeared to halt most DNase activity. The DNA was purified by deproteinization with this phenol and then with chloroform; it was then treated with ribonuclease as described by Marmur (18). The final ethanol-precipitated product was dissolved and reprecipitated with isopropyl alcohol, which was added slowly to the DNA solution while it was blended in a Vortex mixer. DNA was stored frozen in 0.01 × SSC (1.5 mM NaCl + 0.15 mM sodium citrate) for use in filter hybridizations and in 0.42 M NaCl for S₁ hybridization.

Determination of DNA base contents. The DNA base contents (as moles percent guanine plus cytosine [mol% GC]) of the first group of strains were determined by thermal denaturation (19) in 0.25 × SSC. With the use of *Escherichia coli* DNA as a standard it was found that, in 0.25 × SSC, DNA denatured at 8.6°C below its normal denaturation point (*T_m*) in normal-strength SSC. This correction factor was applied to all *Actinomyces* DNA thermal denaturation temperatures, and the mol% GC was then calculated

TABLE 2. *Strains used in S₁ nuclease hybridization experiments*

Strain	Origin ^a
Cluster 1 (typical <i>A. viscosus</i> strains)	
WVU626	Human mouth
WVU627	Human mouth
AN6	Human dental plaque
Be66	Human dentin
H103	Human dental plaque
W752 ^b	Wound abscess
A1353	Goat lymph node
Cluster 3 (atypical <i>A. naeslundii</i> strains)	
B120	Human dental plaque
B74	Human dental plaque
H272	Human dental plaque
Cluster 5 (typical <i>A. naeslundii</i> strains)	
W1096	Human mouth
W1048 (= ATCC 27039) ^c	Human infection
4A05	Human dental plaque
X600 (= WVU447, ATCC 19039)	Human dental plaque
WVU398A	Human dental plaque
WVU45 (= ATCC 12104) ^d	Human sinus
Cluster 6 (atypical <i>A. viscosus</i> strains)	
W1053 (= ATCC 27044 = WVU474)	Human sputum
Unclassified strains (<i>A. viscosus</i> strains)	
T14V	Human mouth
T14AV	Avirulent form of T14V

^a All strains were from E. D. Fillery, except *A. viscosus* strains T14V and T14AV, which were from D. Birdsell, University of Florida, Gainesville.

^b Catalase negative.

^c ATCC, American Type Culture Collection, Rockville, Md.

^d Candidate for designation as type strain.

by the formula mol% GC = $(T_m - 69.3)/0.41$ (19). The high guanine plus cytosine content of the DNA of actinomyces necessitated the use of this dilute solvent.

Filter hybridizations. Quantities (50 µg each) of single-stranded DNA were applied to 25-mm Millipore type HAWP filters (Millipore Corp., Bedford, Mass.) and dried at 75°C for 4 h. Filters were then immersed in vials containing 1.2 ml of dimethyl sulfoxide (DMSO)-2 × SSC (0.3 M NaCl plus 0.03 M sodium citrate in 30% vol/vol, aqueous DMSO [3, 17]). After 30 min at 62°C, 1.0 or 0.5 µg of single-stranded labeled index [³H]DNA was added to each vial, and the filters were incubated at 62°C for 18 h. The filters were then rinsed extensively with DMSO-2 × SSC at 62°C and dried, and the hybridized [³H]DNA was quantitated by liquid scintillation. The extent of hybridization was determined by comparing the reassociation of index [³H]DNA with unlabeled DNA of the same strain (the homologous control) to all other (heterologous) reassociations. *E. coli* DNA was used as a negative control. These experiments were done in duplicate.

S₁ hybridizations. When DNA was to be used for hybridization by the S₁ method, it was sheared by ultrasound for 1 min (four 15-s periods with interven-

ing ice water cooling) and dialyzed for at least 48 h against cold 0.42 M NaCl. For hybridization, 25 µg of DNA was mixed with 0.05 µg of index [³H]DNA in 1.25 ml of 0.42 M NaCl and denatured by heating to 115 to 120°C for 20 min in an ethyleneglycol-water bath. The mixture was allowed to reassociate at 70°C for 6 to 8 h, after which unrenatured DNA was digested with 20 U of S₁ nuclease for 20 min at 70°C. The details of the S₁ method were described earlier (2). The method was adapted from Barth and Grinter (1). Remaining hybridized DNA was precipitated with 10% trichloroacetic acid, collected on glass-fiber filters (Whatman GF/C), and quantitated by liquid scintillation. Controls included a homologous reassociation as the positive control, *E. coli* DNA as a negative control, and controls of enzyme activity against double- and single-stranded DNA (2). For all S₁ experiments, the unlabeled DNAs were drawn from single batches isolated from each strain.

RESULTS

Hybridization on filters. Hybridizations on membrane filters showed that *A. viscosus* serotype-2 strain WVU845 [³H]DNA hybridized extensively with the DNAs of some other serotype-2 strains and somewhat less (70 to 80%) with the DNAs of *A. naeslundii* strains and serotype-2 strains (experiment 1, Table 3 and Fig. 1). When *A. naeslundii* strain WVU398A [³H]DNA was the index DNA (experiment 2), more separation between *A. naeslundii* and *A. viscosus* could be seen. DNAs from strains P1 and P2, which have characteristics of *A. viscosus*, did not hybridize well with index DNA. Taken together, experiments 1 and 2 indicate that *A. viscosus* and *A. naeslundii* are genetically somewhat different, but these experiments did not show two unequivocally distinct species. However, it is clear that the veterinary strains of *A. viscosus* (A1351, A1353, and A1231) were related to *A. viscosus* serotype 2 and that the hamster strains WVU745 and WVU440 are not closely related to either *A. viscosus* serotype 2 or *A. naeslundii*. The small experiment 3 confirmed that the two hamster strains were related to each other but not to any other strain from animal infections or humans. Experiment 4 showed that the three veterinary strains were related to each other and to *A. viscosus* serotype-2 strain WVU845 but not to strain W1308.

Hybridization using S₁ nuclease. The single-strand-specific nuclease S₁ digests DNA base sequences which are not accurately paired with complementary bases. Thus, nonspecific, spurious duplexes are eliminated and the method emphasizes differences between microorganisms. The method successfully discriminated the three "clusters" detected by Fillery et al. (6).

Experiment 5 (Table 4, Fig. 2) showed that cluster 1 strains (*A. viscosus*) were homologous

TABLE 3. *Extent of DNA hybridization of Actinomyces strains as determined by the membrane filter method*

Source of unlabeled DNA	% of homologous reaction attained with the following reference strains:			
	WVU845 ^a	WVU398A ^b	WVU745 ^c	A1353 ^d
<i>A. viscosus</i> strains from humans (serotype 2)				
WVU845	100	73	— ^e	93
34a ₁	106	59	—	—
M100	102	51	29	—
UCHC-BIB	86	67	—	—
UCHC-HAs	71	66	—	—
UCHC-R3A	78	71	—	—
UCHC-TO4	107	86	—	—
P1	42	5	—	—
P2	36	2	—	—
<i>A. naeslundii</i> strains				
WVU398A	82	100	—	—
WVU631	35	98	—	—
43e ₁	76	98	—	—
WVU820	85	79	46	50
WVU45	81	106	—	—
N9	78	85	—	—
C2	69	82	—	—
<i>A. viscosus</i> strains from animals (serotype 1)				
WVU745	53	54	100	68
WVU440	76	53	100	70
A1231	87	68	42	89
A1351	101	53	45	95
A1308	—	—	—	43
A1353	87	73	—	100
<i>E. coli</i>	15	2	4	8

^a Experiment 1.^b Experiment 2.^c Experiment 3.^d Experiment 4.^e —, Not done.

and were distinct from cluster 5 strains (*A. naeslundii*) and cluster 3 strains (atypical *A. naeslundii*). Similarly, experiment 6 showed that cluster 3 is distinct from clusters 1 and 5. (We suspect that the high degree of hybridization with strain WVU626 in this experiment is an artifact.) Experiment 7 indicated that cluster 5 strains were not all homologous with the index strain WVU398A. However, the lack of relationship between clusters 1 and 5 and between 3 and 5 was confirmed. Another experiment (not shown) using *A. naeslundii* strain WVU45 [³H]DNA as the index DNA produced similar results. DNA from strains of clusters 1 and 3 hybridized little with strain WVU45 [³H]DNA, but DNA from cluster 5 strains hybridized only moderately (65 to 70%) with the index [³H]DNA. Thus, although the *A. naeslundii* strains of cluster 5 may not be genetically homogeneous, they

are certainly distinct from *A. viscosus* strains (cluster 1) and atypical "*A. naeslundii*" strains (cluster 3), as indicated by experiments 5 and 6.

The DNAs from *A. viscosus* strains T14V and T14AV hybridized moderately with the DNAs of *A. viscosus* cluster 1 strains, indicating that they may not be typical of *A. viscosus*. However, small experiments using [³H]DNA from T14V as the index DNA showed that T14V and T14AV are indeed closely related to each other. In two experiments, T14AV DNA hybridized at 99 and 103% with T14V DNA.

DNA base contents. The DNA base contents of strains used in the filter hybridizations are listed in Table 5. Nearly all strains had base contents between 66 and 68 mol% GC. There was no significant difference in base content between *A. viscosus* and *A. naeslundii*. However, the somewhat higher guanine plus cytosine

FIG. 1. *Extent of DNA hybridization among various strains of A. viscosus and A. naeslundii. Hybrids were formed on membrane filters. Symbols: ▽, A. viscosus strains from humans; ●, A. viscosus strains from animals; ○, A. viscosus strains from hamsters; ■, A. naeslundii strains. The index strains were: experiment 1, WVU845; experiment 2, WVU398A; experiment 3, WVU745; experiment 4, A1353. They are always at 100% homology.*

content of the two hamster strains is notable in view of the lack of DNA homology between them and any other strains.

DISCUSSION

Currently the differentiation between *A. naeslundii* and *A. viscosus* is based on serological differences, the catalase reaction, and cell wall composition. The numerical taxonomy study of Fillery et al. (6) showed that typical *A. viscosus* serotype 2 strains could be separated from typical *A. naeslundii* strains when the overall results of 65 tests were compared (6), although their similarity was high (88%) on a numerical scale. Thus, these two species are similar but

TABLE 4. *Extent of DNA hybridization of Actinomyces strains as determined by the S₁ nuclease method*

Source of unlabeled DNA	% of homologous reaction attained with the following reference strains:		
	WVU627 ^a	B74 ^b	WVU398A ^c
Cluster 1 (typical <i>A. viscosus</i> strains)			
WVU627	100	38	35
AN6	90	53	32
W752	98	46	33
WVU626	85	78	30
A1353	87	53	35
Be66	87	48	38
H103	92	48	37
Cluster 3 (atypical <i>A. naeslundii</i> strains)			
B74	56	100	45
B120	65	89	46
H272	66	82	50
Cluster 5 (typical <i>A. naeslundii</i> strains)			
WVU398A	46	21	100
WVU45	43	32	43
4A05	37	30	84
W1048	37	32	73
X600	42	38	61
W1096	28	27	52
Cluster 6			
W1053	70	30	36
Unclustered strains			
T14V	76	37	23
T14AV	55	30	46
<i>E. coli</i>	1	2	— ^d

^a Experiment 5.

^b Experiment 6.

^c Experiment 7.

^d —, Not done.

FIG. 2. Extent of DNA hybridization among *A. viscosus* and *A. naeslundii* strains which have been placed in clusters by numerical taxonomy. Hybrids were formed in solution. Cluster 1 strains are typical *A. viscosus* strains from humans (▽); cluster 3 strains are atypical *A. naeslundii* strains (○); cluster 5 strains are typical of *A. naeslundii* strains (■); cluster 6 strain is an atypical *A. viscosus* strain (●); and T14V and T14AV are *A. viscosus* strains (▽). The index strains were: experiment 5, WVU627; experiment 6, B74; experiment 7, WVU398A. They are always at 100% homology.

TABLE 5. DNA base contents of some *A. viscosus* and *A. naeslundii* strains

Strain	DNA base content ^a
<i>A. viscosus</i> (serotype 1)	
WVU745	68.5 ± 0.21
WVU440	68.6 ± 0.45
A1231	67.9 ± 0.58
A1351	67.2 ± 0.96
A1353	67.5 ± 0.98
<i>A. viscosus</i> (serotype 2)	
WVU845	66.5 ± 0.51
P1	67.8 ± 1.24
P2	67.4 ± 0.21
34a ₁	66.7 ± 0.52
M100	67.2 ± 0.33
UCHC-BIB	68.0 ± 0.29
UCHC-HAs	68.2 ± 0.11
UCHC-R3A	67.2 ± 0.10
UCHC-TO4	67.5 ± 0.29
T14AV	67.5 ± 0.92
T14V	66.7 ± 0.51
<i>A. naeslundii</i>	
WVU398A	66.4 ± 1.09
WVU631	68.1 ± 0.69
WVU820	66.9 ± 0.52
WVU45	67.4 ± 0.47
C2	67.5 ± 0.39
N9	68.1 ± 1.11
43e ₁	67.5 ± 0.61
<i>E. coli</i>	50.5 ± 0.91

^a Values indicate mol% GC ± standard deviation.

not identical. The atypical strains of *A. naeslundii* (cluster 3) also formed a discrete cluster.

When strains of the two species were compared by DNA hybridization, the results were in

general agreement with taxonomic divisions based on serological and numerical taxonomy methods. The filter method of hybridization was capable of separating *A. viscosus* serotype 1 (hamster strains) from *A. viscosus* serotype 2 (human strains) and also showed the similarity of serotype 2 and veterinary strains. On the other hand, *A. naeslundii* and *A. viscosus* serotype 2 were barely separable by the filter hybridization method. Use of the more discriminating S₁ hybridization technique revealed clear distinctions among typical *A. viscosus* strains from humans and typical *A. naeslundii* strains, as well as atypical *A. naeslundii* strains (Fillery's clusters 1, 5, and 3, respectively). Thus, the phenotypic differences observed by Fillery et al. (6) and compared and contrasted by their computer methods, are manifestations of molecular differences whose underlying DNA base sequences are large enough to be detected by a lack of complete DNA hybridization, especially with the S₁ nuclease method.

The ability to separate these various groups on the basis of their genetic material as well as by traditional biochemical and serological methods has some taxonomic significance. We believe that *A. viscosus* serotype 2 and *A. naeslundii* should be separate species. In addition to their demonstrated serological, biochemical, and genetic differences, they have different ecologies (4), which may be important in the etiology of oral diseases. Their separation at species level seems logical and useful.

A. viscosus serotype 1 (hamster strains) appears to be quite different from *A. viscosus* serotype 2. It may be necessary to regard hamster strains as a species separate from the organism of humans. Since the type strain of *A. viscosus* is a member of serotype 1, the name *A. viscosus* must be reserved for serotype 1. Thus, the serotype 2 strains would require a new species name if they were segregated from the serotype 1 (hamster) strains.

The strains of cluster 3 were also distinct from the other strains in this study, both by DNA hybridization and numerical taxonomy. They are catalase negative but share an agglutinable antigen with cluster 1 (*A. viscosus*) (6). They lack the precipitable antigens common to cluster 1 and 5 strains and have an α-6-phosphogluconate dehydrogenase that is different from the enzyme of cluster 1 and 5 strains (6). These organisms also may deserve taxonomic recognition; they certainly deserve more study.

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REPRINT REQUESTS

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