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Identification of oral *Actinomyces* species using DNA probes

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Ximénez-Fyvie LA, Haffajee AD, Martin L, Tanner A, Macuch P, Socransky SS. Identification of oral Actinomyces *species using DNA probes.*
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Oral *Actinomyces* comprise a major segment of both the supra- and subgingival microbiota; however, little is known about the distribution of individual species in different sites or clinical conditions. The purpose of the present investigation was to develop DNA probes for suggested species and genotypes of oral *Actinomyces*. Whole genomic DNA probes to 12 human oral species and/or serotypes were labeled with digoxigenin and used to seek cross-reactions among the taxa using the checkerboard DNA-DNA hybridization assay. The *Actinomyces* formed three distinct groups: 1) *Actinomyces georgiae*, *Actinomyces meyeri* and *Actinomyces odontolyticus* serotypes I and II; 2) *Actinomyces viscosus* and *Actinomyces naeslundii* serotypes I, II, III and WVA 963; and 3) *Actinomyces gerencseriae* and *Actinomyces israelii*. Cross-reactions among taxa were detected and minimized by increasing the temperature of the post-hybridization high-stringency wash to 80°C. Despite the elevation in high stringency wash temperature, cross-reactions among strains of the *A. naeslundii*/*A. viscosus* group persisted. Probes for two of the three currently recognized genospecies in this group were prepared by removing the DNA in common between cross-reacting species using subtraction hybridization and polymerase chain reaction. Nine species and genospecies could be clearly separated by a combination of whole genomic and subtraction hybridization probes and by increasing the high-stringency wash temperature. A total of 195 fresh isolates of *Actinomyces* were grouped in a blind study using DNA probes and separately by SDS-PAGE protein profiles. Concordance between the two methods was 97.3%. The probes and hybridization conditions were tested for their ability to detect the *Actinomyces* species and genospecies in samples of supragingival and subgingival plaque from periodontitis subjects using checkerboard DNA-DNA hybridization. The probes detected the species in samples of supragingival and subgingival plaque. We concluded that whole genomic and subtraction hybridization DNA probes facilitate the detection and enumeration of species and genospecies of *Actinomyces* in plaque samples.

Key words: *Actinomyces*; DNA probes; subtraction hybridization; periodontal microorganism

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Actinomyces comprise a major segment of the indigenous human oral microbiota and are among the most predominant microorganisms in both supragingival and subgingival plaque (3, 12, 36, 43, 45). Little is known, however, about the distribution of individual species in different oral sites and of their role in distinct clinical conditions. Animal model studies in the late 1960s and early 1970s showed that *Actinomyces viscosus* and *Actinomyces naeslundii* were capable of causing destructive periodontal disease in hamsters and germ-free rats (23, 24, 44), suggesting that such organisms could play an important role in the pathogenesis of periodontal disease. Findings of the late 1980s, however, contradicted this hypothesis. Dzink et al. (10) observed a decreased proportion of *Actinomyces* in sites showing periodontal disease progression as compared to non-progressing sites. Haffajee et al. (19) showed an increase in *Actinomyces* species in successfully treated individuals after surgery and systemically administered tetracycline. Furthermore, *Actinomyces* were isolated more frequently from healthy compared with periodontally diseased pockets (32). Thus, the concepts of the role of *Actinomyces* in periodontal processes changed radically from suspected pathogens to possible beneficial species.

More recent studies renewed interest in the possible role of specific *Actinomyces* species in gingivitis and other periodontal conditions. Tanner et al. (50, 51) observed an association of *A. naeslundii* with putative active and buccal active periodontal subjects, while *A. naeslundii* serotype III was associated with gingivitis (32). Moore & Moore (33) found significantly elevated levels

Table 1. Reference strains employed for the development of DNA probe

Species or serotype	Strain[a]
Actinomyces georgiae	49285
Actinomyces meyeri	35568
Actinomyces odontolyticus serotype I	17929
Actinomyces odontolyticus serotype II	29323
Actinomyces viscosus serotype II	19246
Actinomyces viscosus	43146
Actinomyces naeslundii serotype I	12104
Actinomyces naeslundii serotype II	49339
Actinomyces naeslundii serotype III	49340
Actinomyces naeslundii serotype WVA963	49338
Actinomyces gerencseriae	23860
Actinomyces israelii	12102

[a] Source of strains: ATCC, American Type Culture Collection.

of *Actinomyces meyeri* in active destructive periodontitis as compared with adult gingivitis. Thus, available data suggest that specific *Actinomyces* species might play quite different roles in oral health or disease and suggest the need for their rapid and precise differentiation in plaque samples.

Much effort has been directed in recent years towards the taxonomy of *Actinomyces*. *Bergey's manual of systemic bacteriology* (38) listed 12 *Actinomyces* species. With improvements in the taxonomic methods used for characterizing such organisms, at least 20 new *Actinomyces* species from human sources have been described (26, 35). Unfortunately, discrimination of some taxa within the genus *Actinomyces* still presents difficulties. It is generally accepted that *Actinomyces israelii*, *Actinomyces odontolyticus* serotypes I and II and *A. meyeri* are distinct. Johnson et al. (22) recently described two other distinct species, *Actinomyces gerencseriae* (formerly recognized as *A. israelii* serotype II) and *Actinomyces georgiae*. However, a clear distinction between *A. naeslundii* and human isolates of *A. viscosus* has not been reported. Gerencser (17) noted that *A. naeslundii* and *A. viscosus* might be varieties of a single species. Using cluster analysis of polyacrylamide gel electrophoresis whole cell protein patterns, McCormick et al. (31) observed that one of the two major clusters found contained strains of *A. naeslundii* serotypes I, II and III and *A. viscosus* serotypes I and II, while strains of *A. naeslundii* serotype WVA 963 (previously known as *A. naeslundii* serotype IV) were distinct from this group. Coykendall & Munzenmaier (8) reported 71% to 107% DNA relatedness between *A. naeslundii* and human strains of *A. viscosus* using DNA hybridization. A recent classification of oral *Actinomyces* recognized 3 genospecies within the *A. naeslundii*/*A. viscosus* group (22). Genospecies 1 consisted of strains of *A. naeslundii* serotype I; genospecies 2, strains of *A. naeslundii* serotypes II & III and *A. viscosus* serotype II; and genospecies 3, strains of *A. naeslundii* serotype WVA 963 (serotype IV). However, due to the inability to differentiate between such genospecies, in most studies the different groups are frequently classified simply as *A. naeslundii*.

While the taxonomy of *Actinomyces* continues to improve, precise identification and enumeration of these taxa in large numbers of samples remains a challenge. The high degree of phenotypic and serological similarity presents difficulty in distinguishing among species, particularly taxa within *A. naeslundii*. Thus, studies attempting to relate specific species or genospecies to oral sites or to pathological processes, such as caries and periodontal disease, have been difficult. The purpose of the present investigation was to develop DNA probes and hybridization conditions to allow precise differentiation of pure cultures of oral *Actinomyces* species or genospecies and to facilitate their enumeration in large numbers of plaque samples.

Material and methods

Bacterial strains and growth conditions

Twelve reference *Actinomyces* strains were used in this study for the preparation of DNA probes (Table 1). All strains were obtained as lyophilized stocks from the American Type Culture Collection (ATCC, Rockville, MD). Bacterial stocks were rehydrated in Mycoplasma broth (Difco Laboratories, Detroit, MI) and grown on Trypticase soy agar with 5% defibrinated sheep blood (BBL, Baltimore Biological Laboratories, Cockeysville, MD) at 35°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2).

DNA isolation and preparation of whole-genomic DNA probes

Bacterial strains were grown anaerobically for 3 days on 2 blood agar plates. The growth was harvested and placed in 1.5-ml microcentrifuge tubes containing 1 ml of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6). Cells were washed twice by centrifugation in TE buffer at 3500 rpm for 10 min. The cells were resuspended and lysed in 150 µl of an enzyme mixture containing 15 mg/ml lysozyme (Sigma Chemical Co., St. Louis, MO) and 5 mg/ml achromopeptidase (Sigma Chemical Co.) in TE buffer (pH 8.0). The pelleted cells were resuspended by 15 s of sonication and incubated at 37°C for 1 h. DNA was isolated and purified using the method described by Smith et al. (42). The concentration of the purified DNA was determined by spectrophotometric measurement of the absorbance at 260 nm. The purity of the preparations was assessed by the ratio of DNA to protein as measured by the ratio of the absorbances at 260 nm and 280 nm. Whole-genomic DNA probes were prepared from each of the 12 test strains by labeling 1 µg of DNA with digoxigenin using a random primer technique (14).

Hybridization conditions

The checkerboard DNA-DNA hybridization technique (46) was used to evaluate cross-reactions among strains. Purified DNA from each reference strain (100 ng, equivalent to approximately 10^7 cells) was diluted in a total volume of 100 µl of TE buffer in 1.5 ml microcentrifuge tubes. One hundred µl of 0.5 M NaOH was added to each tube. The samples were boiled in a water bath for 10 min and neutralized with 0.8 ml of 5 M ammonium acetate. The DNA was placed into the extended channels of a MiniSlot (Immunetics, Cambridge, MA), concentrated onto four separate 15×15 cm positively charged nylon membranes (Boehringer Mannheim, Indianapolis, IN) and fixed to the membranes by exposure to ultraviolet light followed by baking at 120°C for 20 min. The membranes were prehybridized at 42°C for 1 h in 50% formamide, 5× SSC (1× SSC=150 mM NaCl plus 15 mM sodium citrate, pH 7.0), 1% casein (Sigma Chemical Co.), 5× Denhardt's solution, 25 mM sodium phosphate (pH 6.5) and 0.5 mg/ml yeast RNA (Boehringer Mannheim). The membranes with fixed DNA were placed in Miniblotter 45 (Immunetics) devices with the DNA-lanes rotated 90° to the channels of each apparatus. The probes were diluted to 20 ng/ml in hybridization solution (45% formamide, 5× SSC, 1× Denhardt's solution, 20 mM Na

Table 2. Strains employed for the preparation of subtraction hybridization DNA probes

Probe strain	Subtracter strains	
A. viscosus serotype II (ATCC 19246)	*A. naeslundii* serotype I	(ATCC 12104)
	A. naeslundii serotype II	(ATCC 49339)
	A. naeslundii serotype III	(ATCC 49340)
	A. naeslundii serotype WVA963	(ATCC 49338)
A. naeslundii serotype I (ATCC 12104)	*A. viscosus* serotype II	(ATCC 19246)
	A. naeslundii serotype II	(ATCC 49339)
	A. naeslundii serotype III	(ATCC 49340)
	A. naeslundii serotype WVA963	(ATCC 49338)

phosphate (pH 6.5), 0.2 mg/ml yeast RNA, 10% dextran sulfate, 1% casein), placed in individual lanes of the Miniblotters and hybridized overnight at 42°C with the devices sealed inside a plastic bag.

The membranes were washed twice at high stringency for 20 min each time in phosphate buffer (0.1× SSC, 0.1% sodium dodecyl sulfate) using a Disk Wisk apparatus (Schleicher and Schuell, Keene, NH). Four high stringency wash temperatures were tested (65, 70, 75 and 80°C). The membranes were blocked by incubating for 1 h in a blocking buffer containing 1% casein in maleate buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5). Hybrids were detected by incubating the membranes in a 1:15,000 dilution of anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) using the modification described by Engler-Blum et al. (13). Signals were detected by chemiluminescence after incubating the membranes in Lumiphos 530 (Lumigen, Southfield, MI) for 45 min at 35°C and placing them in autoradiographic film cassettes with Reflection NEF film (DuPont, Boston, MA) for 1 h.

Subtraction hybridization DNA probes

Probes to *A. viscosus* serotype II (ATCC 19246) and *A. naeslundii* serotype I (ATCC 12104) were prepared using a modified subtraction hybridization technique (2).

Preparation of probe-strain DNA. Three µg of DNA from each probe strain was digested with *Sau*3AI restriction endonuclease. The restriction fragments were purified using the High Pure polymerase chain reaction Product kit (Boehringer Mannheim) according to the manufacturer's instructions. Double-stranded L-P linkers containing 5′-phosphorylated *Sau*3AI-compatible overhangs at one end were prepared by mixing 5 µg of each oligonucleotide TB7006 and TB7008 (Gibco BRL, Life Technologies, Grand Island, NY) (AGC GGA TAA CAA TTT CAC ACA GGA and ATC TCC TGT GTG AAA TTG TTA TCC GCT, respectively). Mixtures were heated to 65°C for 25 min and cooled slowly to room temperature (2). Linkers were ligated to the digested probe-strain DNA by incubating 200 ng of *Sau*3AI-digested DNA, 600 ng of the L-P linker, 1 µl of T4 DNA ligase (Promega Corporation, Madison, WI) and 10× ligation buffer containing 300 mM Tris-HCL (pH 7.8), 100 mM $MgCl_2$, 100 mM DTT and 5 mM ATP to a final volume of 20 µl at 16°C for 1 h. Excess linkers were removed using the High Pure polymerase chain reaction Product Kit. One ng of the ligated restriction fragments was amplified by polymerase chain reaction for 45 cycles using oligonucleotide TB7006 as primer. Each cycle consisted of denaturation at 94°C for 80 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Reactions were performed in sterile 0.2 ml microcentrifuge tubes with a final reaction volume of 100 µl containing the following concentrations: 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µM each deoxynucleoside triphosphates, 1 µM primer, and 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA). Mixtures were overlaid with 100 µl of mineral oil to avoid evaporation. The efficiency of amplification and the size of the fragments were examined by gel electrophoresis of 10-µl aliquots of each reaction mixture in a composite agarose gel (3% NuSieve agarose, 1% SeaKem agarose; FMC BioProducts, Rockland, ME).

Preparation of subtracter-strain DNA. Equal amounts of DNA from all of the cross-reacting strains were pooled to a final volume of 10 µl (1 µg/µl of pooled DNA) (Table 2). An equal volume of PhotoActivable Biotin acetate (Clontech Laboratories, Palo Alto, CA) was added and conjugated to the pooled subtracter DNA by placing the tubes in ice and irradiating them for 30 min with a 500-W mercury vapor reflector sunlamp (Clontech Laboratories) placed 8 to 10 cm above the open tubes. The reaction volume was brought to 100 µl with TE buffer and extracted twice with equal volumes of 2-butanol (Sigma Chemical Co.) The DNA was precipitated with 3 volumes of 95% ethanol and 0.5 volumes of 7.5 M ammonium acetate, collected by centrifugation for 15 min at 12,000 rpm at 4°C and redissolved for 1 h at 55°C in 10 µl of TE buffer.

Table 3. Distribution of fresh isolates identified by DNA probes and by phenotypic traits. Strains of *A. meyeri* were not detected by either method

	Phenotypic characterization						
DNA Probes	*A. georgiae*	*A. odontolyticus*	*A. naeslundii/ A. viscosus*	*A. naeslundii*	*A. gerencseriae*	*A. israelii*	Unidentified
A. georgiae	15						3
A. odontolyticus		2					3
A. naeslundii genospecies 1*				26			
A. viscosus serotype II*			19	69			1
A. naeslundii genospecies 3				2			
A. gerencseriae				2	38		
A. israelii					3	12	

* DNA probes prepared by subtraction hybridization and polymerase chain reaction.

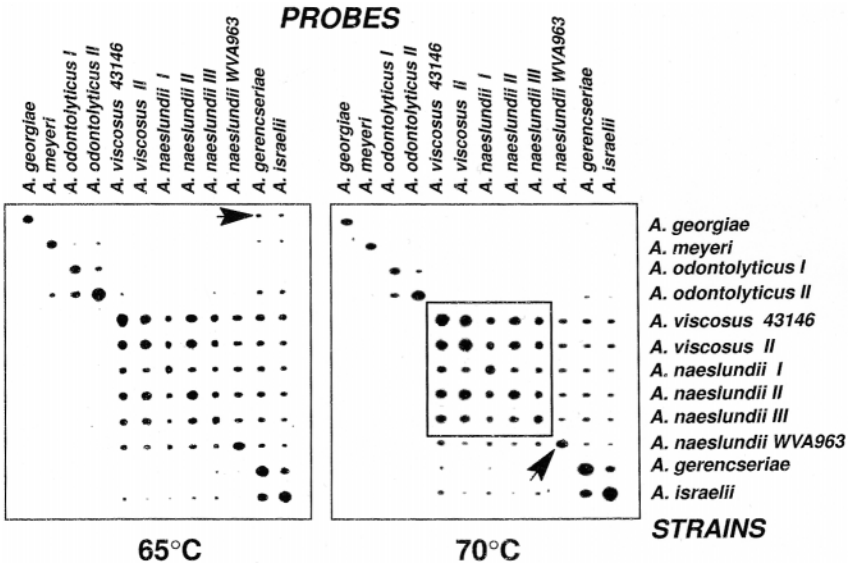

Fig. 1. Checkerboard DNA-DNA hybridization of whole genomic DNA probes to 12 *Actinomyces* taxa. The horizontal lanes contained 100 ng of DNA from each test organism. The vertical lanes contained the DNA probes at a concentration of 20 ng/ml in hybridization buffer. A signal at the intersection of the vertical and horizontal lanes indicated detection of complementary DNA. The intensity of the signal was related to the proportion of complementary DNA sequences in the target DNA. The left and right panels represent the results of stringency washing at either 65°C or 70°C respectively. The arrow in the left panel points to an example of cross-reactions between distantly related taxa such as the probes to *A. gerencseriae* and *A. israelii* reacting with DNA from *A. georgiae* and *A. meyeri*. The arrow in the right panel indicates that the *A. naeslundii* serotype WVA 963 DNA probe signal to DNA from the same strain was more intense than signals to other taxa in the *A. naeslundii* group. The box in the right panel focuses on cross-reactions within the *A. naeslundii* genospecies 1 and 2 group.

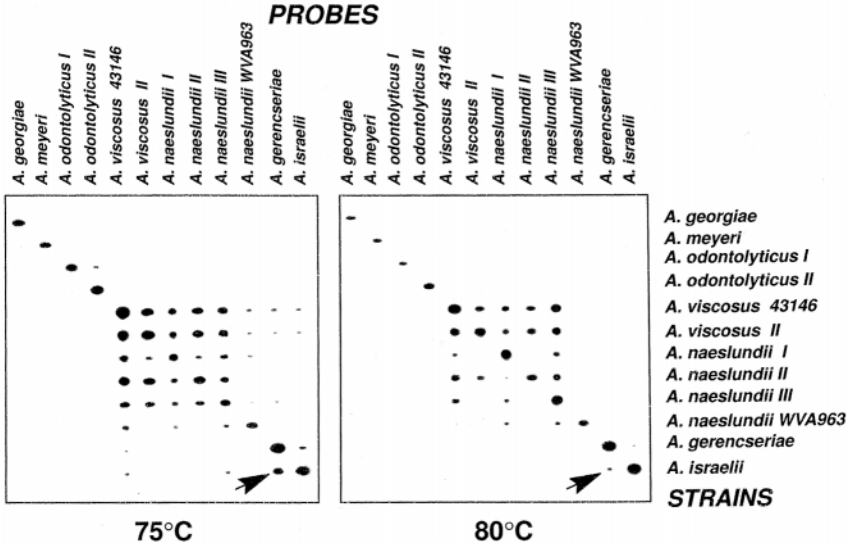

Fig. 2. Checkerboard DNA-DNA hybridization of whole genomic probes to 12 *Actinomyces* taxa after stringency washing at 75 and 80°C. The membrane format is as described in Fig. 1. The arrow in the left (75°C) panel indicates a cross-reaction between *A. gerencseriae* and *A. israelii*. The majority of cross-reactions in this panel occurred within *A. naeslundii* genospecies 1, 2. The arrow in the right panel demonstrates that the cross-reaction between *A. gerencseriae* and *A. israelii* was diminished after stringency washing at 80°C.

Subtraction hybridization. The probe-strain DNA and biotinylated subtracter DNA were hybridized at a 1:20,000 ratio (200 pg of probe-strain DNA and 4 μg of subtracter DNA) in a hybridization solution containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5), 500 mM NaCl, 1 mM EDTA and 0.1% sodium dodecyl sulfate (SDS) (final concentrations) by denaturing the DNA at 99°C for 10 min, rapidly cooling the reaction on ice and incubating at 64°C for 48 h. The total volume of the hybridization reactions was 20 μl, and the ratio of total DNA solution volume to hybridization solution used was 1:5 (v/v).

Removal of cross-reacting DNA. A total of 30 μg of streptavidin (Promega) was added to the hybridization mixture and thoroughly mixed. The biotin containing hybrids were removed by 4 consecutive extractions with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1, v/v). The DNA was precipitated with 99% ethanol and recovered by 5 min of centrifugation at 12,000 rpm. The pellet was washed once with 70% ethanol and redissolved in 10 μl of TE buffer at 50°C for 30 min. Two additional rounds of subtraction hybridization were carried out with 5-μl aliquots of the probe DNA remaining from each previous subtraction. For each round of subtraction, 4 μg of subtracter DNA was added to the hybridization mixture.

Amplification and labeling of subtracted probe-DNA fragments. Two μl of the DNA fragments remaining after subtraction hybridization were amplified by polymerase chain reaction using 5 μl of primer TB7006 (at 200 μM) and the conditions previously described for amplification of the probe-strain DNA. Polymerase chain reaction products were detected by gel electrophoresis of 10-μl aliquots of each reaction in composite agarose gels as described above. Ten μl of the amplified fragments was labeled with digoxigenin-11-dUTP (Boehringer Mannheim) using polymerase chain reaction (ratio of dTTP:DIG-11-dUTP=1:4) with 30 cycles of labeling. Each cycle consisted of denaturation at 94°C for 2 min, annealing at 55°C for 2 min and extension at 72°C for 3 min. The subtracted probes were then tested for specificity using the same hybridization conditions

described above for the whole genomic DNA probes.

Fresh isolates and plaque samples

Whole-genomic and subtraction-hybridization probes were used in a double-blind study to identify 195 freshly isolated strains of *Actinomyces* that were also characterized by selected phenotypic tests and SDS-PAGE protein profiles as described by Maiden et al. (30). One investigator identified the isolates using phenotypic traits while a second investigator used the DNA probes. The results were compared after completion of all isolates.

Samples of plaque were collected from the mesial-buccal supragingival and subgingival surfaces of two teeth in each of five subjects with adult periodontitis. Plaque samples were placed in individual microcentrifuge tubes, and the DNA was released from the microorganisms by adding 100 µl of 0.5 M NaOH and boiling for 10 min. After neutralization, the released DNA was transferred to the surface of a positively charged nylon membrane (Boehringer Mannheim) using the 30 channels of a Minislot (Immunetics). The DNA was fixed to the membrane by UV light and baking. The membrane was then placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at right angles to the channels of the Miniblotter device. Whole genomic and subtraction hybridization DNA probes were labeled with digoxigenin and placed in hybridization buffer into individual lanes and hybridized overnight. After stringency washing at 79°C, the signals were detected using phosphatase-conjugated antibody to digoxigenin and a chemiluminescence substrate. Probes were used to seek the test species and genospecies of *Actinomyces* in samples of supragingival and subgingival plaque using the checkerboard DNA-DNA hybridization method as described above.

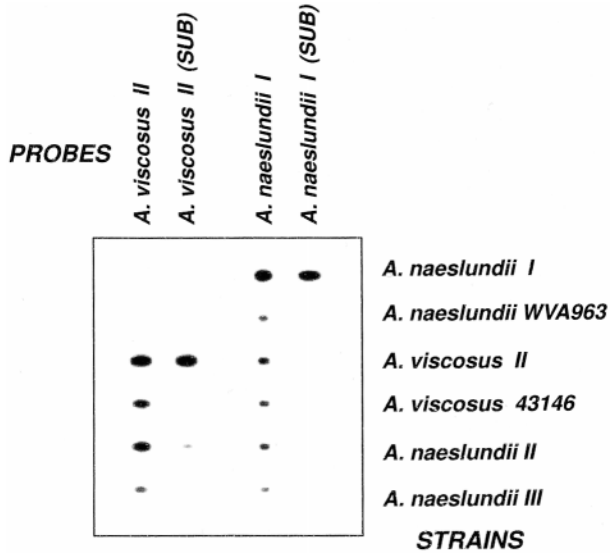

Fig. 3. Comparison between whole genomic DNA probes and probes prepared using subtraction hybridization and polymerase chain reaction to strains of *Actinomyces* within genospecies 1, 2. The subtraction hybridization probes are indicated by (SUB). The stringency wash temperature was 70°C.

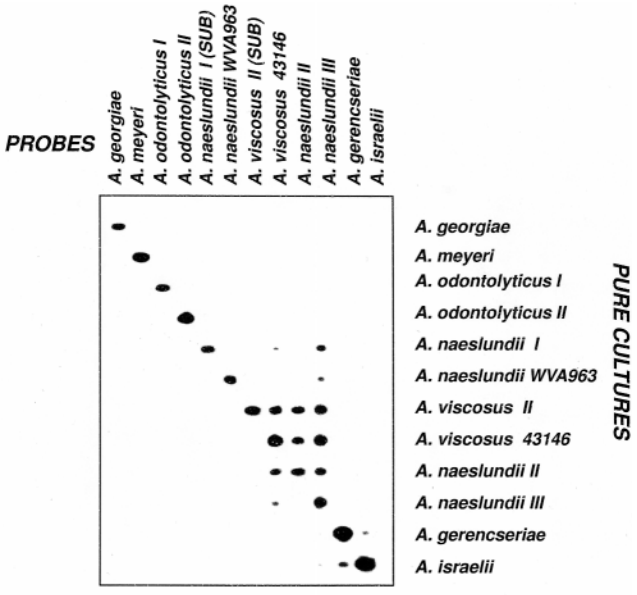

Fig. 4. Checkerboard DNA-DNA hybridization using both whole genomic DNA probes and probes prepared using subtraction hybridization and polymerase chain reaction (indicated by SUB). The probes are in the vertical lanes and the target DNA in the horizontal lanes. The high-stringency wash temperature was 79°C.

Results

Effect of high-stringency wash temperature on DNA probe specificity

Figs. 1 and 2 show the effect of different high-stringency wash temperatures on the specificity of whole genomic DNA probes tested against pure cultures of 12 *Actinomyces* taxa. When the membranes were washed at 65°C, a high degree of cross-reactivity was observed for 8 of the 12 DNA probes tested. Probes to *A. viscosus* serotype II, *A. viscosus* (ATCC 43146), *A. naeslundii* serotype I, *A. naeslundii* serotype II, *A. naeslundii* serotype III, *A. naeslundii* serotype WVA963, *A. gerencseriae* and *A. israelii* presented cross-reactions to six or more taxa. Only the probe to *A. georgiae* was considered species specific. A reduction in the number of cross-reactions was observed at 70°C, particularly among species that had lower percentage of DNA homology. Such probes included those to *A. meyeri, A. odontolyticus* serotypes I and II, *A. naeslundii* serotype WVA963, *A. gerencseriae* and *A. israelii*.

The highest specificity was obtained by raising the high-stringency wash temperature above 70°C. At 75°C only the five probes belonging to the *A. naes-*

lundii/A. viscosus group exhibited cross-reactions to four or more taxa within this particular group. *A. naeslundii* serotype WVA963 presented only weak cross-reactions with *A. viscosus* serotype II, *A. viscosus* (ATCC 43146) and *A. naeslundii* serotype I, confirming that this serotype of *A. naeslundii* was different from the rest of the taxa in the group. The highest stringency wash temperature tested (80°C) resulted in a significant reduction of most of the cross-reactions initially observed. Six probes were now considered species specific. The remaining cross-reactions were confined to taxa belonging to *A. naeslundii* genospecies 1 (*A. naeslundii* serotype I) and *A. naeslundii* genospecies 2 (*A. viscosus* serotype II, *A. viscosus* ATCC 43146, *A. naeslundii* serotype II and *A. naeslundii* serotype III) or between *A. gerencseriae* and *A. israelii* (Fig. 2). While the specificity of the DNA probes was greatly improved by increasing the high-stringency wash temperature, the sensitivity of the probes diminished at 80°C and decreased markedly by raising the temperature to 85°C (data not shown).

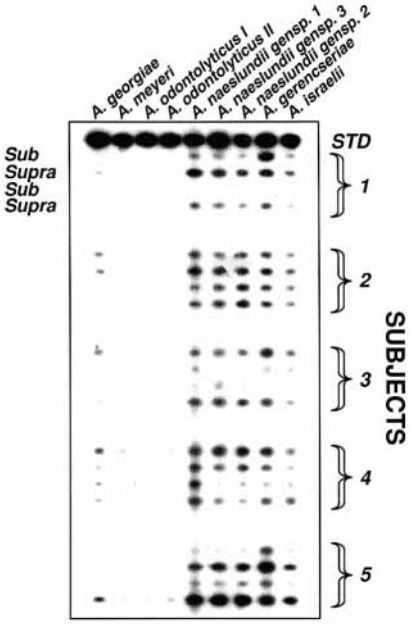

Fig. 5. Checkerboard DNA-DNA hybridization detection of *Actinomyces* in supragingival and subgingival plaque samples from two teeth in each of 5 periodontitis subjects. The vertical lanes are the whole genomic DNA probes or the probe prepared using subtraction hybridization and polymerase chain reaction (*A. naeslundii* genospecies 1). The whole genomic probe to *A. viscosus* serotype II was used to identify *A. naeslundii* genospecies 2. The supra- and subgingival plaque samples from the five subjects are in the horizontal lanes. The top horizontal lane (STD) contained standards at a concentration of 10^7 cells of each taxon.

Differentiation within the *A. naeslundii/A. viscosus* group using probes prepared by subtraction hybridization and polymerase chain reaction

Fig. 3 illustrates the specificity of subtraction hybridization probes compared with whole genomic probes for the same organisms. Whole genomic probes exhibited cross-reactions with most of the other test species within the *A. naeslundii/A. viscosus* group, while the probes prepared using subtraction hybridization and polymerase chain reaction were able to precisely differentiate both *A. naeslundii* genospecies 1 and *A. viscosus* serotype II. Fig. 4 demonstrates that together, the whole genomic and subtraction hybridization probes, could be used on the same membrane to distinguish nine taxa of *Actinomyces*.

Identification of fresh isolates of *Actinomyces*

The results obtained in the identification of fresh isolates by both DNA probes and phenotypic tests are summarized in Table 3. Seven of the 195 isolates were not definitively identified by phenotypic traits. The two methods agreed for 183 of 188 isolates (97.3%). None of the fresh isolates were identified as *A. meyeri* by either method. The most predominant taxa detected among the isolates was *A. viscosus* serotype II (termed *A. naeslundii/A. viscosus* or *A. naeslundii* using phenotypic characterization).

Detection of *Actinomyces* in plaque samples

The probes were tested for their ability to detect the *Actinomyces* species and genospecies in plaque samples taken from individual supra and subgingival sites using the conditions of hybridization and the whole genomic and subtraction hybridization probes previously described. All taxa were detected in the plaque samples except for *A. meyeri* (Fig. 5). There were suggestions of differences in the relative proportions of the taxa in supra- and subgingival plaque. However, the data presented in the present study are too limited to draw conclusions.

Discussion

Accurate identification and enumeration of *Actinomyces* species is required to determine their role in oral ecology and dental disease. Several investigators (4, 7, 8, 15, 17, 18, 20–22, 39, 40) have observed a high degree of similarity in phenotypic and other traits between *Actinomyces* species, genospecies and serotypes. This poses difficulties for accurate differentiation of oral isolates, particularly those belonging to the *A. naeslundii* genospecies. Although species such as *A. georgiae* and *A. meyeri* can be easily separated from other members of this genus, it is the separation of strains within the *A. naeslundii* genospecies that presents problems. Because of the limitations of phenotypic traits, additional methods have been tested for their ability to identify the members of this genus.

Gerencser (17) demonstrated that serotyping could be used to distinguish *Actinomyces* species. Putnins & Bowden (34) concurred that differentiation between the *Actinomyces* genospecies was possible by agglutination and that serotypes could be identified with fluorescent-labeled antibody. Bowden et al. (4) and Johnson et al. (22), however, observed that serology suffered from the unavailability of standard antisera and from the known variation in antisera from different animals. Using a series of 65 phenotypic and serological tests, Fillery et al. (15) grouped 43 strains of *A. naeslundii* and *A. viscosus* from human and animal sources into 7 clusters using monoclonal antibodies. Using DNA fingerprinting and ribotype patterns, Bowden et al. (4) found that some strains within a given cluster had very similar fingerprints and ribotypes, while strains in other clusters were very different. By identification of the antigenic determinants of strains representative of the seven clusters, Firtel & Fillery (16) concluded that the surface of strains of *A. naeslundii* and *A. viscosus* presented a complicated mosaic of epitopes responsible for the cross-reactions between isolates. DNA fingerprinting has recently been used to distinguish between different strains of a number of bacterial species, including *Streptococcus mutans* (5), *Haemophilus influenzae* (28), *Eikenella corrodens* (6), *Porphyromonas gingivalis* (28–29), *Fusobacterium nucleatum* (9) and *Actinobacillus actinomycetemcomitans* (52). DNA fingerprinting can provide im-

portant new information on the relatedness of oral bacterial strains. Nonetheless, Bowden et al. (4) observed that, while the large number of bands found in agarose gels rendered the technique useful for successful identification of identical patterns, it was less well suited for building a strain identification scheme. Other genetic approaches, such as the 16S rDNA polymerase chain reaction–restriction fragment-length polymorphism, have also been suggested for the differentiation of oral *Actinomyces* species. In a recent report by Sato et al. (37), the use of 16S rDNA polymerase chain reaction–restriction fragment-length polymorphism was proposed for the separation of oral *Actinomyces* species. It was suggested that the technique could be a rapid method for the identification of seven species of oral *Actinomyces*, however, it is not a practical approach when processing large numbers of plaque samples.

Johnson et al. (22) observed that *A. viscosus* serotype II and *A. naeslundii* serotypes II and III (currently recognized as *A. naeslundii* genospecies 2) had a DNA relatedness of 35 to 62%. *A. naeslundii* serotype I (*A. naeslundii* genospecies 1) was somewhat less related to the members of genospecies 2 with a DNA relatedness of 22 to 45%, while *A. naeslundii* serotype WVA 963 (*A. naeslundii* genospecies 3) was the least related with percentages of DNA homology ranging from 29% to 38%. Similarly, Schofield & Schaal (41) used phenotypic traits and the Jaccard coefficient to define clusters based on 50 strains of *A. naeslundii*, *A. viscosus* and related organisms. They found that *A. viscosus* serotype II clustered with the type strain of *A. naeslundii* as well as with strains of *A. naeslundii* serotypes II and III at a similarity level of about 60%. The results derived from the use of whole genomic DNA probes in the present investigation agreed with these findings. Strains of *A. viscosus* and *A. naeslundii* serotypes I, II and III could not be unequivocally distinguished from each other by whole genomic DNA probes, even when the temperature of the high-stringency wash was elevated to 80°C. However, *A. naeslundii* serotype WVA 963 as well as the six other test species were clearly separated from each other and from the cross-reacting strains of the *A. naeslundii*/*A. viscosus* group using whole genomic DNA probes. These results are in accord with the findings of Johnson et al. (22), Schofield & Schaal (41) and McCormick et al. (31) in terms of the genomic relatedness of these organisms.

In the present investigation, whole genomic DNA probes in combination with an elevated high-stringency wash temperature provided an effective method for accurately identifying pure cultures of all the tested taxa of oral *Actinomyces*, with the exception of *A. naeslundii* genospecies 1 and 2 and to a lesser extent *A. gerencseriae* and *A. israelii*. Raising the high-stringency wash temperature above 70°C increased the specificity of the DNA probes, but the sensitivity decreased, particularly over 75°C. The decreased sensitivity did not present a problem when identifying pure cultures but could present a limitation for the simultaneous detection of *Actinomyces* and other genera in small plaque samples using checkerboard DNA-DNA hybridization. The high moles % guanine+cytosine (G+C) DNA probes to *Actinomyces* can be employed using elevated high-stringency wash temperatures without excessive loss of sensitivity and with significant gain in specificity. Unfortunately, the low moles % G+C content of DNA probes to other genera exhibit decreased sensitivity after using elevated stringency wash temperatures. In practice, this limitation may be overcome by employing a high-stringency wash temperature of 68–70°C for low moles % G+C taxa prior to signal detection. The membrane can then be rewashed at 75–80°C followed by signal detection to improve specificity for the high moles % G+C genera.

The inability to distinguish between the members of *A. naeslundii* genospecies 1 and 2 by elevating the high-stringency wash temperature led to the development of more specific probes prepared using subtraction hybridization and polymerase chain reaction. *A. naeslundii* genospecies 1 (*A. naeslundii* serotype I) and one member of *A. naeslundii* genospecies 2 (*A. viscosus* serotype II) were selected for preparation of subtraction hybridization probes because they demonstrated the strongest cross-reactions among the organisms tested. Previous investigations (2, 47) showed that subtraction hybridization is a powerful technique for obtaining highly specific probes. Our results were in agreement with such findings. Subtraction hybridization probes prepared for the two selected taxa were highly specific and presented a sensitivity comparable to that of whole genomic probes. Furthermore, subtraction hybridization probes could be used to differentiate the 2 taxa in plaque samples using a stringency wash temperature of 68°C, the same temperature employed for members of other genera. Probes prepared using subtraction hybridization and polymerase chain reaction are currently being developed for *A. gerencseriae* and *A. israelii*.

The development of DNA probes by subtraction hybridization techniques requires considerable effort both in their preparation and validation. Probes prepared in different laboratories using this technique may exhibit different sensitivity and specificity. Thus, it is possible that inter-laboratory differences could occur.

The species and genospecies of *Actinomyces* play an important role in the ecology of dental plaque. Several taxa have been implicated as initial colonizers on the tooth surfaces (1, 25), and some reports suggest a possible involvement of specific species in oral disease processes such as root caries (11, 23, 48) and gingivitis (27, 49). However, accurate discrimination of the species and genospecies of oral *Actinomyces* is necessary to fully appreciate their role in oral ecology and disease. The present investigation indicated that DNA probes are useful for the identification of *Actinomyces* species and genospecies in pure cultures as well as in samples of supragingival and subgingival plaque.

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