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Genotypic diversity of oral *Actinomyces naeslundii* genospecies 1 and 2 in caries-active preschool children

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A total of 991 isolates of *Actinomyces naeslundii* were obtained from sound approximal tooth sites in either caries-active (n = 35) or caries-free (n = 20) preschool children. From this group of isolates, 101 strains were chosen to study the genotypic diversity of *A. naeslundii* genospecies 1 (n = 30), catalase-positive (n = 30), and catalase-negative genospecies 2 (n = 41). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), with a pair of primers targeting the 16S ribosome RNA gene (16S rDNA), and *Mnl*I digestion together with randomly amplified polymorphic DNA (RAPD) with eight arbitrary, single 10-mer primers were performed to generate genetic profiles of selected *Actinomyces* isolates. The hierarchic relationships of genetic profiles were finally analyzed using computerized dendrograms. There was no significant difference in the prevalence rates and proportions of either genospecies 1 or 2 between the caries-free and caries-active groups, although a higher prevalence of genospecies 2 was noted in the total population. Dendrogram analyses of the 16S rDNA PCR-RFLP profiles revealed that all strains belonging to *A. naeslundii* genospecies 1 could be subgrouped into three genotypes (T7, T18, and T19), with a single predominant genotype, T18 (27/30). Catalase-positive strains for genospecies 2 fell into three subtypes (T4, T7, and T17), whereas the catalase-negative counterparts were distributed amongst 16 subtypes. No specific genotype was significantly associated with caries activity. We conclude that heterogeneous subgroups of *A. naeslundii* genospecies 1 and 2, particularly the latter, are the constituent flora of dental plaque in children and may contribute to the pathogenesis of childhood caries.

Key words: *actinomyces;* caries; genetic diversity; primary dentition

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The human oral cavity harbors a diverse array of bacteria comprising more than 500 different species (22). However, only a limited number of species, including streptococci and *Actinomyces* spp., predominate. *Actinomyces* comprises a group of either facultative or obligate anaerobic, nonspore-forming, nonmotile and gram-positive pleomorphic robs. These primary colonizers initiate a biofilm substrate for the coadhesion of other bacteria, including streptococci and gram-negative bacteria (21), resulting in the development of a plaque community with ever-increasing bacterial diversity and leading to disease states such as caries (3, 8, 18) and periodontal diseases (26, 34). *Actinomyces* spp. may also invade the tissue via damaged mucosa, leading to bacteremia and systemic infections (9) in both healthy individuals (11, 13) and immunocompromised patients (1). Of the common oral *Actinomyces* spp., genetically heterogeneous *Actinomyces naeslundii,* with cell-surface fimbriae, has attracted much attention. It is currently subgrouped into genospecies 1 and 2, according to their genetic relatedness (20), and differentiated either using molecular methods (3) or genospecies-specific antisera (29).

Actinomyces spp. colonize the human oral cavity in early childhood, and the prevalence rates of total *Actinomycetes* flora increase from 31% to 97% within the first 2 years of life (31). Among oral *Actinomyces* spp., *A. naeslundii* is the second most common early colonizer, after the predominant *Actinomyces odontolyticus* (31). However, *A. naeslundii* does not colonize the mouth until after the eruption of teeth (10, 31). This suggests that tooth surface presents an ideal environment for the initial adhesion of this flora. This property may be related to type 1 fimbriae (fimP) of *A. naeslundii*, which mediate their adherence to teeth through binding with salivary acid proline-rich proteins, covering enamel surfaces (27).

Previous studies have associated the presence of *A. naeslundii* with root caries (7), particularly its initiation (16, 33). However, its role in the cariogenicity of children's teeth has not been studied (3, 25, 36). Marchant et al. (25) and Becker et al. (3) have found a higher proportion of *A. naeslundii* in the plaque samples from children than from caries lesions. The genotypic diversity of *A. naeslundii*, particularly genospecies 2, suggests that recognizing the role of *A. naeslundii* at a species level is only the first step to further clarify different genotypes and their contribution to host colonization, plaque development, and disease initiation in varying cohorts (5, 38).

Dental caries in primary teeth is one of the most common chronic diseases in children (14). But only sparse data are available on the genetic characteristics of *A. naeslundii* genospecies 1 and 2 from children and their association with cariogenicity. We therefore undertook the present study

- to investigate the distribution of *A. naeslundii* genospecies 1 and 2 in supragingival plaque of caries-free and caries-active children;
- to compare the genotypic profiles of genospecies 1 (previously *A. naeslundii* serotype I), catalase-negative (previously *A. naeslundii* serotypes I and II) and catalase-positive genospecies 2 (previously *Actinomyces viscosus* serotype II) from these two groups of children;
- to correlate genetic characteristics of *A. naeslundii* with caries activity.

Material and methods

Subjects and sampling

The study recruited 55 ethnic Chinese children aged 3–4 years. These children were in good general health and divided into two groups:

- caries-free group (n = 20): children with no detectable caries on naked-eye examination;
- caries-active group (n = 35): children with at least three primary teeth with active cavitated caries.

All plaque samples were obtained from intact interproximal sites using dental floss; in preferential order the sampled sites were 52/53, 62/63, and other interproximal sites in the maxilla. Plaque samples were obtained by a trained and calibrated examiner by passing sterile dental floss through the interproximal site. Immediately after sampling, the section of the dental floss with plaque was cut with sterile scissors and put into a sterile vial with 1 ml of PBSTC (1.58 g $K_2HPO_4 \cdot 3H_2O$, 0.34 g KH_2PO_4, 8 g NaCl, 0.1 ml CTAB, 1 g sodium thioglycollate in 1 liter of water (36).

Microbiological processing

In the laboratory, each plaque sample was well dispersed by whirl mixing for 10 s and spiral-plated on fastidious anaerobic agar (FAA, LAB M, Bury, Lancashire, UK) supplemented with 5% defibrinated horse blood, using Autoplate 4000 (Spiral Biotech, Bethesda, MD). After a 7-day growth period in an anaerobic environment (85% N_2, 10% H_2, 5% CO_2), the number of colony-forming units (CFUs) per sample was calculated. From the same plate, colonies with different morphology were grouped, calculated and subcultured, and up to 50 colonies, initially classified as gram-positive pleomorphic rods, were randomly picked up for subculture under a microscope for each sample.

Identification and DNA purification

The identification of *A. naeslundii* genospecies 1, and catalase-negative and catalase-positive genospecies 2 was carried out using genospecies-specific rabbit antisera (29) and the catalase-test. During identification, seven American Type Culture Collection (ATCC) reference strains (Manassas, VA) were used as controls, namely, *Actinomyces bovis* (ATCC 13683), *Actinomyces gerencseriae* (ATCC 23860), *Actinomyces israelii* (ATCC 10048), *Actinomyces meyeri* (ATCC 35568), *A. naeslundii* genospecies 1 (ATCC 12104), *A. odontolyticus* (ATCC 17929) and *A. viscosus* (ATCC 15987).

A total of 101 strains of *A. naeslundii*, including genospecies 1 (n = 30), catalase-negative (n = 41) and catalase-positive genospecies 2 (n = 30) were randomly selected for further genotypic analyses. All purified isolates were recovered by incubation anaerobically (85% N_2, 10% H_2, 5% CO_2) at 37°C for 5 days on Columbia agar (Oxoid, Basingstoke, England) supplemented with defibrinated horse blood (5%), hemin (0.0005%), and menadione (0.00005%). The identity of the bacteria was then reconfirmed with antisera and the catalase-test. Afterwards, bacteria from a 3-day anaerobic growth period were harvested and resuspended in 480 μl of 50 mM EDTA for DNA isolation. After cell lysis with 0.6 mg lysozyme, bacterial DNA was isolated and purified, using the Wizard Genomic DNA Purification Kit, according to the manufacturer's instructions (Promega, Madison, WI).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

The DNA obtained was then amplified with a pair of universal primers (Forward primer: 5′-GGGTGAGTAACACGTGAGTAA-3′, AF 543282: position 103–123; Reverse Primer: 5′-CGAGCTGACGACAACCAT-3′, AF 543282: position 1090–1107) targeting 16S ribosome RNA gene (16S rDNA, 35), using GeneAmp PCR system 9700 (Perkin Elmer, Foster City, CA). The final volume of each PCR reaction was 50 μl, and contained 1.5 mM $MgCl_2$, 1 μl of 10 mM dNTP (containing 200 μM dATP, dTTP, dCTP, dGTP each, Roche Molecular Biochemicals, Mannheim, Germany), 0.5 μM each of the primers (Invitrogen Life Technologies, Tokyo, Japan), 1 unit of polymerase (Invitrogen, Roche Molecular System Inc., Alameda, CA), and 1–10 ng bacterial DNA template. The PCR reaction was carried out as follows: predenaturation at 94°C for 5 min; denaturation at 94°C for 1 min; annealing at 57°C for 1 min; elongation at 72°C for 1 min; and a run of 35 cycles. Final elongation was at 72°C for 10 min (35). The generated 988-bp amplicons were digested with *Mnl*I (New England BioLabs Inc, Beverly, MA) and the obtained fragments were separated in 3% agarose (Sigma, St. Louis, MO) by electrophoresis at 80 V for 4 h. Afterwards, the fragments were stained with ethidium bromide (0.5 μg/ml) for 30 min, gently washed with deionized water for other 30 min, and visualized with the Gel Doc system (BIO-RAD, Laboratory-Segrate, Milan, Italy).

Randomly amplified polymorphic DNA (RAPD)

The RAPD profiles were obtained with eight arbitrary, single, 10-mer primers in a 50-μl PCR reaction (Table 1). The final volume of each reaction contained 1 μl each of the primers, and other reagents were identical to those used in the PCR-RFLP analysis section. The single primers included OPA 01, OPA 02, OPA 04, OPA 10, OPA 12, OPA 18, OPE 04, and OPE 18 (Invitrogen Life Technologies) (2, 17, 24, 30). All these primers were used for genospecies 1 and catalase-positive genospecies 2, and OPA 02, OPA 10, OPE 04, and OPE 18 were used for catalase-negative genospecies 2, as defined by our previously conducted pilot study. The PCR was performed as follows: the initial five cycles consisted of denaturation at 94°C for 30 s, annealing at 31°C for 2 min, and extension at 72°C for 2 min. The subsequent 45 cycles included denaturation at 94°C for 30 s, annealing at 36°C for 2 min, and extension at 72°C for 2 min, with final elongation at 72°C for 10 min. The control tube without DNA template was included in each run (12). All the reactions were duplicated and the generated PCR amplicons were separated in 1% agarose by electrophoresis at 100 V for 2 h. The visualization procedure was the same as described in the PCR-RFLP analysis.

Analyses of genetic profiles with Dendrongram

The PCR-RFLP profiles and RAPD fingerprint patterns were analyzed using a band-position-based method with a computerized Dendrogram (Version 3.0, Solltech Inc., Oakdale, IA). According to the manual, the data for two banding patterns (lanes A and B) were classified by the binary values 0 and 1, where 0 indicates no band at a position and 1 indicates a band at that position. The similarity coefficient (S_{AB}) for each pair of strains A and B was calculated with the following formula:

$$S_{AB} = 1.0 - \frac{\sqrt{b + c}}{2a + b + c}$$

where 'a' is the number of bands common for both lanes A and B (coded as 1, 1), 'b' the number of bands in lane A without counterparts in lane B (coded as 1, 0), and 'c' the number of bands in lane B without counterparts in lane A (coded as 0, 1). The aforementioned band-position-based method of similarity analysis (mean square difference/total bands) and the unweighted pair-group method using arithmetic average (UPGMA) clustering analysis (4) were used to generate dendrograms in our study.

Statistical analyses

The Mann–Whitney test, Wilcoxon matched-pairs signed-ranks test and Fisher's exact test were used to analyze the association between *A. naeslundii* and caries activity in children. Statistical analysis was performed with GRAPHPAD INSTAT software (GraphPad InStat version 3.00 for WINDOWS, GraphPad Software, San Diego, CA). The level of significance was set at $P < 0.05$.

Table 1. Eight arbitrary, single, 10-mer primers used for RAPD

Primers	Sequences	Previous reports in genotyping (ref.)
OPA 01	5′-CAGGCCCTTA-3′	*Actinomyces* spp. (30)
OPA 02	5′-TGCCGAGCTG-3′	*Actinomyces* spp. (30), *Streptococcus* spp. (24)
OPA 04	5′-AATCGGGCTG-3′	*Actinomyces* spp. (30)
OPA 10	5′-GTGATCGCAG-3′	*Actinomyces* spp. (30)
OPA 12	5′-TCGGCGATAG-3′	*Actinomyces* spp. (30)
OPA 18	5′-AGGTGACCGT-3′	*Actinomyces* spp. (30), *Streptococcus* spp. (24)
OPE 04	5′-GTGACATGCC-3′	*Streptococcus* spp. (17), *Candida* spp. (2)
OPE 18	5′-GGACTGCAGA-3′	*Candida* spp. (2)

Results

Distribution of *A. naeslundii* in caries-free vs. caries-active children

A total of 911 isolates of *A. naeslundii* were recovered from all sample sites. These included genospecies 1 (n = 115), catalase-positive (n = 420) and catalase-negative genospecies 2 (n = 339). There was no significant difference in the prevalence rate of either genospecies 1 between the caries-free and caries-active group, or genospecies 2 between the above two groups (Table 2). The prevalence rate of genospecies 2 was significantly higher than genospecies 1 in the caries-free group ($P = 0.01$), but not in the caries-active group ($P = 0.05$). In the total population studied, the prevalence rate of genospecies 2 was also significantly higher than the genospecies 1 ($P = 0.001$). On further investigation, similar proportions of genospecies 1 and 2 were isolated from both caries-free and caries-active subjects ($P > 0.05$), and higher proportions of genospecies 2 from the caries-free and the caries-active groups, compared with genospecies 1 ($P < 0.05$, Table 3).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) *with* MnlI digestion profiles

The 101 strains of *A. naeslundii* were grouped into 19 different subtypes, T1–T19, according to the PCR-RFLP with *Mnl*I digestion profiles (Fig. 1A). The 19 patterns of *A. naeslundii* generated were different from those generated from other six *Actinomyces* spp., including *A. bovis* (ATCC 13683), *A. gerencseriae* (ATCC 23860), *A. israelii* (ATCC 10048), *A. meyeri* (ATCC 35568), *A. odontolyticus* (ATCC 17929), and *A. viscosus* (ATCC 15987). The predominant T18 pattern of genospecies 1 was identical to that of *A. naeslundii* genospecies 1 (ATCC 12104, Fig. 1B). Further investigation showed that

Table 2. Prevalence of *A. naeslundii* genospecies 1 and 2 in supragingival plaque of caries-free and caries-active children (percentages in parentheses)

	A. naeslundii	*A. naeslundii* Genospecies 2			*A. naeslundii*
	Genospecies 1	Cat (+/–)	Cat (–)	Cat (+)	genospecies 1 and 2
Caries-free (n = 20)	6 (30%)	15 (75%)	11 (55%)	10 (50%)	16 (80%)
Caries-active (n = 35)	11 (31%)	20 (57%)	15 (43%)	11 (31%)	24 (69%)
Total (n = 55)	17 (31%)	35 (64%)	26 (47%)	21 (38%)	40 (73%)

Fisher's exact test: caries-free vs. caries-active groups ($P > 0.05$).
Cat (+): catalase-positive; Cat (–): catalase-negative; Cat (+/–): catalase-positive and -negative.

Table 3. Total cell number and proportions of *A. naeslundii* genospecies 1 and 2 in supragingival plaque of caries-free and caries-active children (percentages in parentheses)

	Total cells/ml per sample	*A. naeslundii* (cells/ml)	*A. naeslundii* genospecies 1 (cells/ml)	*A. naeslundii* genospecies 2 (cells/ml)		
				Cat (+/–)	Cat (–)	Cat (+)
Caries-free		(16%)	(3%)	(14%)	(8%)	(6%)
Mean ± SE*	1.35×10^7 ± 5.97×10^6	7.57×10^5 ± 2.51×10^5	1.98×10^5 ± 8.50×10^4	2.19×10^6 ± 1.56×10^6	4.60×10^5 ± 1.95×10^5	1.50×10^5 ± 7.96×10^4
Median	2.60×10^6	1.20×10^5	0.00	1.20×10^5	1.50×10^4	1.58
Caries-active		(8%)	(2%)	(6%)	(3%)	(3%)
Mean ± SE*	9.34×10^6 ± 2.31×10^6	4.05×10^5 ± 8.60×10^4	1.02×10^5 ± 3.13×10^4	3.03×10^5 ± 7.93×10^4	1.96×10^5 ± 7.26×10^4	1.07×10^5 ± 3.57×10^4
Median	4.52×10^6	2.00×10^5	0.00	8.00×10^4	0.00	0.00

*SE: Standard error.
Mann–Whitney Test: caries-free vs. caries-active groups ($P > 0.05$).
Wilcoxon matched-pairs signed-ranks test: genospecies 1 vs. 2 ($P < 0.05$).

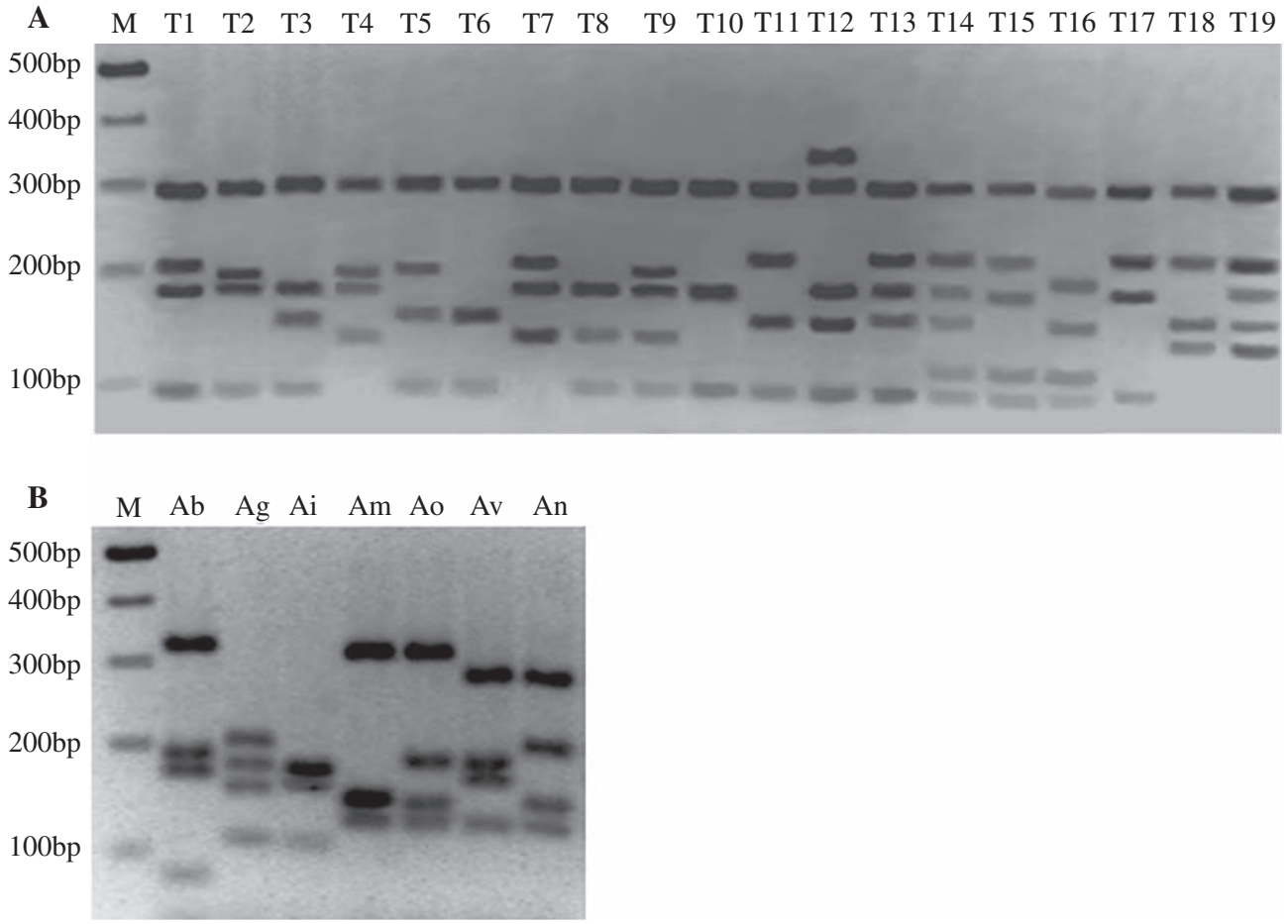

Fig. 1. The PCR-RFLP patterns of *Actinomyces* spp. Of 19 patterns generated from *A. naeslundii*, the T4 pattern was found both in catalase-negative and catalase-positive genospecies 2 and the T7 pattern in genospecies 1 and 2 (1 A). The patterns generated from seven ATCC strains, including Ab (*A. bovis* ATCC 13683), Ag (*A. gerencseriae* ATCC 23860), Ai (*A. israelii* ATCC 10048), Am (*A. meyeri* ATCC 35568), Ao (*A. odontolyticus* ATCC 17929), Avenue (*A. viscosus* ATCC 15987) and An (*A. naeslundii* ATCC 12104) were different from each other. The pattern generated from ATCC 12104 was identical with the predominant T18 pattern of genospecies 1 (1B).

all isolates belonging to *A. naeslundii* genospecies 1 (n = 30) fell into three genotypes (T7, T18, and T19), with a single predominant genotype, T18 (27/30). With regard to genospecies 2, catalase-positive isolates (n = 30) fell into three subtypes (T4, T7, and T17), and the catalase-negative counterparts (n = 41) were distributed among 16 subtypes (Table 4).

Among 19 patterns generated by PCR-RFLP, the T4 pattern was found both in catalase-negative and catalase-positive genospecies 2; the T7 pattern was found in both genospecies 1 and 2. When the similarity coefficient (S_{AB}) values of the 19 profiles were compared using dendrogram analyses, the relationship between genospecies 1 (T7, T18, and T19) and

Table 4. PCR-RFLP with MnlI digestion generated patterns of *A. naeslundii*

Pattern	Genospecies 1 (n = 30)	Genospecies 2		Caries-free (n = 46)	Caries-active (n = 55)	Total (n = 101)
		Cat (–) (n = 41)	Cat (+) (n = 30)			
T1	0	10	0	5	5	10
T2	0	3	0	1	2	3
T3	0	3	0	1	2	3
T4	0	3	16	9[a]	10[b]	19
T5	0	1	0	1	0	1
T6	0	1	0	1	0	1
T7	**2**	**4**	**11**	**10**[c]	**7**[d]	**17**
T8	0	1	0	0	1	1
T9	0	2	0	0	2	2
T10	0	2	0	0	2	1
T11	0	1	0	0	1	1
T12	0	1	0	0	1	3
T13	0	3	0	0	3	4
T14	0	4	0	1	3	1
T15	0	1	0	1	0	1
T16	0	1	0	1	0	3
T17	0	0	3	1	2	27
T18[e]	27	0	0	14	13	1
T19	1	0	0	0	1	

[a]Catalase-positive (n = 6) and catalase-negative (n = 3) genospecies 2.
[b]All belonging to catalase-positive genospecies 2.
[c]Genospecies 1 (n = 1), catalase-positive (n = 7) and catalase-negative genospecies 2 (n = 2).
[d]Genospecies 1 (n = 1), catalase-positive (n = 4) and catalase-negative genospecies 2 (n = 2).
[e]*A. naeslundii* genospecies 1 (ATCC 12104) belonging to T18 group.

genospecies 2 (the other 16 patterns) was found to be 0.76 (i.e. distantly related) compared with T13 and T14 profiles with a S_{AB} value of 0.91 (i.e. closely related, Fig. 2). Further analyses of the relationship between each of the seven examined species demonstrated S_{AB} values ranging from 0.65 to 0.85 (Fig. 2). A S_{AB} of 0.85 was noted for the following pairs: *A. odontolyticus* (ATCC 17929) and *A. meyeri* (ATCC 35568); and *A. gerencseriae* (ATCC 23860) and *A. israelii* (ATCC 10048).

According to the profiles generated by PCR-RFLP with *Mnl*I digestion, no significant association was found between caries activity and any of the 19 subtypes of *A. naeslundii* ($P > 0.05$) (Table 4).

Randomly amplified polymorphic DNA (RAPD) profiles

The relationship between different isolates within the same subgroup based on their RAPD profiles was also analyzed according to the dendrogram evaluations. The S_{AB} values of *A. naeslundii* genospecies 1 (n = 30) with eight different primers ranged from 0.48–1.00 for the primer OPA 12 to 0.67–1.00 for the primer OPA 02. Those of catalase-positive genospecies 2 (n = 30) ranged from 0.36–1.00 for the primer OPE 18 to 0.63–1.00 for the primer OPA 10. The genotypic profiles of catalase-negative genospecies 2 generated by four primers showed less diversity (Table 5). When a S_{AB} value of 0.8 was set as the threshold cut-off value for clustering similar strains, genospecies 1 as well as 2 isolated from both caries-free and caries-active children showed a similar degree of genetic diversity (Table 6). No specific cluster in either genospecies 1 or 2 was found to be significantly related to caries activity of children, based on the RAPD generated through the arbitrary primers we used.

Discussion

The oral cavity is a complex environment in which divergent groups of microorganisms interact with other plaque bacteria and host tissues both in health and disease. The emphasis of most previous studies has been on bacteria, such as mutans streptococci and periodontopathic flora, that are thought to be the more important oral pathogens. However, it is well known that the remaining vast numbers of oral commensal inhabitants play a critical role in the maintenance of a healthy oral cavity while contributing to the initiation or progression of oral diseases (21, 22, 38). *Actinomyces* appears to be one such group that is present in the oral cavity from infancy to adulthood. Among various *Actinomyces* spp., strains belonging to *A. naeslundii* remain the most heterogeneous (38). Previous workers have used different methodology to study the genetic diversity of this species, including genomic DNA fingerprinting (6, 30), ribotyping (6, 19), PCR-RFLP with different endonuclease (26, 34), and arbitrarily primed-polymerase chain reaction (AP-PCR) (30).

In this study, we used two methods to genotype 101 *Actinomyces* isolates from the primary dentition of 3–4-year-old children. The methods included PCR-RFLP with a pair of universal primers targeting the variable region of 16S rDNA and *Mnl*I digestion, and RAPD with eight arbitrary, single 10-mer primers. PCR-RFLP with *Mnl*I digestion has been recommended as a reliable method for the differentiation of *Actinomyces* spp. (30, 32). We can confirm that *Mnl*I is an excellent endonuclease for differentiating *Actinomyces* species for use with PCR-RFLP. The primers used previously (30, 32) are different from ours, but all have targeted the variable regions of 16S rDNA. Furthermore, our study suggests that PCR-RFLP with *Mnl*I digestion could be also utilized for further subgrouping heterogeneous *A. naeslundii* (Figs 1 and 2). Of 19 patterns generated from PCR-RFLP, the T4 pattern was shared both by catalase-positive (n = 16) and catalase-negative genospecies 2 (n = 3). The T7 pattern (in bold, Table 4) was shared by genospecies 1 (n = 2), catalase-positive (n = 11) and catalase-negative genospecies 2 (n = 4). However, very interestingly, the dendrograms suggest that the T7 pattern is closely related to two other clusters of genospecies 1 (T18 and T19, $S_{AB} = 0.78$), but not to the 16 patterns derived from genospecies 2 ($S_{AB} = 0.76$, Fig. 2). Although the present data are limited, it is tempting to suggest that the heterogeneity of *A. naeslundii* may be more complex than generally understood and its taxonomy as yet unclear.

Overall, of 19 genetic profiles generated from the PCR-RFLP with *Mnl*I, 12 were derived from the caries-free group and 15 from the caries-active group. Four patterns were only observed in the caries-free group, seven only in the caries-active group and the remaining eight patterns shared by both groups (Table 4). Further analyses of this data did not show any significant association between any of the 19 profiles and caries activity, a finding consistent with a previous investigation where diverse ribotypes of *A. naeslundii* were associated with root caries (7).

RAPD or AP-PCR is one of the popular PCR-based techniques used for studying the genomic polymorphism of bacteria (24, 30), fungi (12), and mammalian cells (15). In the RAPD reaction performed here, the first five cycles were performed at

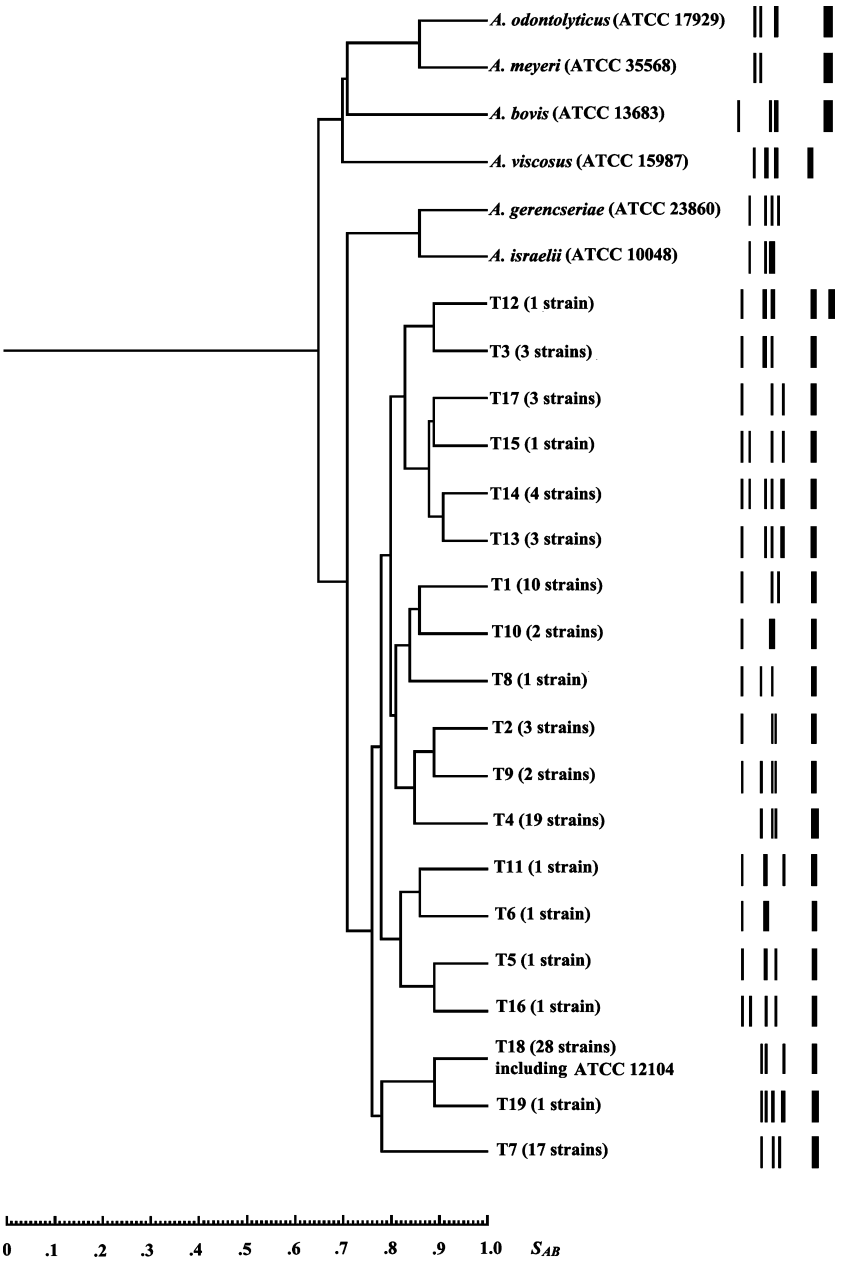

Fig. 2. Dendrograms of PCR-RFLP patterns of *Actinomyces* spp. The S_{AB} ranged from 0.76 for the relationship between *A. naeslundii* genospecies 1 (T7, T18, and T19) and genospecies 2 (other 16 patterns) to 0.91 for the relationship between patterns T13 and T14.

Table 5. The range of S_{AB} values according to RAPD profiles generated with eight arbitrary, single 10-mer primers

Primers	S_{AB} for genospecies 1 (n = 30)	S_{AB} for catalase-positive genospecies 2 (n = 30)	S_{AB} for catalase-negative genospecies 2 (n = 41)
OPA 01	0.56–1.00	0.41–1.00	
OPA 02	0.67–1.00	0.53–1.00	0.54–1.00
OPA 04	0.61–1.00	0.52–1.00	
OPA 10	0.51–1.00	0.63–1.00	0.42–1.00
OPA 12	0.48–1.00	0.43–1.00	
OPA 18	0.65–1.00	0.52–1.00	
OPE 04	0.62–1.00	0.58–1.00	0.41–1.00
OPE 18	0.50–1.00	0.36–1.00	0.56–1.00

low stringency (31°C) to generate initial products by priming with mismatches between the primer and template. The subsequent 45 cycles were performed at higher stringency (36°C), based on those initial products, to generate final amplicons that have ends complementary to the primer (12, 37). The RAPD profiles obtained in this manner clearly demonstrated the multiplicity of anonymous sites that represent the genomic characteristics of *A. naeslundii*. With respect to eight arbitrary, single, 10-mer primers used for RAPD (Table 1), six OPA primers have been used in previous studies for genotyping both *Actinomyces* spp. (30) and *Streptococci* spp. (24). The other two OPE primers have also been utilized in subtyping *Streptococci* spp. (17) and *Candida* spp. (2). The present study suggests that OPE 04 and OPE 18 are also suitable primers for studying genotypic diversity of *A. naeslundii* through RAPD.

We did not find significant differences in the prevalence rates and proportions of *A. naeslundii* in the caries-free and caries-active groups. Thus, the profiles generated from either PCR-RFLP with *Mnl*I digestion (Table 4) or RAPD with eight single primers, showed diverse genetic subtypes of *A. naeslundii* in supragingival plaque of caries-free or caries-active children (Tables 5 and 6), consistent with similar studies conducted in adults (7, 28). Moreover, this study showed that *A. naeslundii* predominated in children's supragingival plaque both in its prevalence (Table 2) and proportion (Table 3). Therefore, *A. naeslundii* plays an important role in the supragingival dental plaque of children. Our data, together with previous findings (7, 10, 28, 31), tend to suggest that various subtypes of *A. naeslundii* may, to a greater or less extent, play a role in the oral microenvironment of humans from infancy (10, 31) and childhood (this study) to adulthood (7, 28).

There was a higher prevalence and proportion of *A. naeslundii* genospecies 2, compared with genospecies 1, recovered from the total population of our study. This is consistent with finding in previous studies (28). It is tempting to speculate that this phenomenon may be related to certain special surface features of these organisms. *A. naeslundii* genospecies 2 has both type 1 (fimP) and 2 (fimA) fimbriae, but typical genospecies 1 only possesses type 2 fimbriae (23, 38). Type 1 fimbriae mediate their attachment to salivary acidic proline-rich protein-coated tooth surfaces (27), while type 2 fimbriae help *A. naeslundii* bind to mammalian cells and

Table 6. The RAPD-generated clusters of *A. naeslundii* from caries-free and caries-active children*

	Genospecies 1 (30 strains)			Genospecies 2 (71 strains)								
	Catalase-positive (30 strains)			Catalase-negative (41 strains)			Genospecies 2 (71 strains)					
Primers	Total	Caries-active (15 strains)	Caries-free (15 strains)	Total	Caries-active (16 strains)	Caries-free (14 strains)	Total	Caries-active (24 strains)	Caries-free (17 strains)	Total	Caries-active (40 strains)	Caries-free (31 strains)
OPA 01	27	14	13	24	13	13						
OPA 02	8	6	2	9	8	4	21	13	13	30	21	17
OPA 04	11	8	9	23	14	11						
OPA 10	21	11	12	22	14	11	27	15	15	46	27	26
OPA 12	23	13	12	26	13	13						
OPA 18	13	9	7	23	14	10						
OPE 04	14	9	8	21	13	9	28	16	14	49	29	23
OPE 18	20	12	11	22	13	12	24	15	15	44	27	26

*$S_{AB} = 0.8$ was set as the threshold for clustering similar strains.

other bacterial surfaces (23, 38). Another reason for the high prevalence of *A. naeslundii* genospecies 2 in dental plaque may be associated with the genotypic diversity of this subspecies. In contrast to genospecies 1, *A. naeslundii* genospecies 2 represents an antigenically heterogeneous group, with 33–79% antigenic similarity among strains (29). The highly diverse genotypic spectrum of *A. naeslundii* genospecies 2 was clearly demonstrated in our study from both the PCR-RFLP and RAPD data. Such inherent genetic plasticity of a species is thought to be closely associated with their ability to survive and persist in the complex oral microenvironment with fluctuating environmental stresses (28).

In conclusion, our data imply that the heterogeneous *A. naeslundii* genospecies 1 and 2, particularly the latter, contribute to the development of dental plaque in children with primary dentition. Their predominance in supragingival plaque and diverse genetic profiles implies that these organisms may also contribute to the pathogenesis of dental caries in this cohort, together with other putative pathogens sharing the same ecologic niche.

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