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Asaccharolytic anaerobic gram-negative coccobacilli (AAGNC) isolated from infected root canals and periodontal pockets

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Background/aims: Culture-difficult bacteria, including asaccharolytic anaerobic gram-negative coccobacilli (AAGNC), may constitute a predominant group of organisms in oral sites. This study aimed to characterize phylogenetically 10 AAGNC isolated from endodontic lesions and periodontal pockets.
Methods: 16S rDNA sequence and G + C content were determined. Strains sharing more than 98% sequence similarities and similar G + C content were considered the same bacterial species.
Results: One isolate resembled *Dialister pneumosintes* (the type species of the genus *Dialister*) with 35 mol% G+C content and 97% sequence similarity. Of eight isolates having 45–47 mol% G+C content, seven were identified as *D. invisus* and one resembled *Dialister invisus* with 97% sequence similarity. However the 16S rDNA sequence similarities with *D. pneumosintes* were relatively low, indicating the strains may belong to a new genus. The last isolate revealed 35 mol% G+C content, but had higher 16S rDNA sequence similarity with *D. invisus* than with *D. pneumosintes*.
Conclusion: The group of oral AAGNC isolates need to be reclassified.

Key words: asaccharolytic anaerobic gram-negative anaerobic coccobacilli, AAGNC; *Dialister invisus*; *Dialister pneumosintes*; gram-negative anaerobic coccobacilli; phylogenetic characterization

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Bacterial 16S rDNA fragments have been frequently detected in oral samples, including those taken from dental plaque (1, 2, 12, 13, 22, 24), periodontal pockets (3, 17, 27), and endodontic lesions (7, 16, 26). Comparison of gene sequence data indicates that some fragments are derived from established bacterial species, but the taxon of some has remained unclear. The sequences of unidentified 16S rDNA fragments have often resembled those of *Dialister pneumosintes* (5), one asaccharolytic anaerobic gram-negative short rod, although with low similarity (around 90%). Asaccharolytic anaerobic gram-negative coccobacilli (AAGNC) have rarely been isolated from oral bacterial florae (26), indicating that some AAGNC may be culture-difficult or may remain uncultured, and thus unclassified. There are also few reports on the isolation and the culture of AAGNC from oral lesions. The aim of the present study was to phylogenetically classify 10 AAGNC isolated from infected root canals and periodontal pockets, by analyses of 16S rDNA gene sequences and G+C contents.

Material and methods

Bacterial strains

D. pneumosintes ATCC 33048^T was obtained from the American Type Culture Collection (ATCC). Ten clinical strains of AAGNC, isolated from infected root canals and periodontal pockets (Table 1), were cultured on Trypticase Soy Broth (TSB) blood (sheep) agar plates (modified BHI blood agar plates) (9). Tiny colonies on the plates were collected and the microorganisms were resuspended in TSB, then inoculated onto multiple agar plates

Table 1. Phylogenetic groups

Bacterial strain	Group	G+C content (mol%)	Percentage similarity with *D. invisus*	Percentage similarity with *D. pneumosintes*	Sources
D. invisus					
CCUG 47026^T	I	45	100	93.0	Endodontic lesion*
9–74	I	45	99.4	91.3	Periodontal pocket
C11b-g	I	46	99.3	90.8	Root canal
7–92	I	47	98.6	91.6	Periodontal pocket
UJB13e-1	I	47	98.6	91.2	Root canal
BN11a-k	I	46	98.6	90.5	Root canal
1–18	I	45	98.6	91.3	Periodontal pocket
9–67	I	46	98.1	90.2	Periodontal pocket
D. invisus-like					
P11a-i	II	47	97.1	89.2	Root canal
D. pneumosintes					
ATCC 33048^T	III	35	90.3	99.5	Nasal abscess
D. pneumosintes-like					
9–73	IV	35	89.7	97.3	Periodontal pocket
Unestablished bacteria					
AB13a-h	V	35	97.9	89.6	Root canal

*Data from Downes et al. (5).

to obtain enough organisms for DNA extraction. Succinate was added as a metabolic substrate to strains producing propionate.

G+C contents

Bacterial DNA was extracted using Insta-Gene Matrix (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The DNA concentration was determined at 260 nm (A_{260}), and the purity was estimated by measuring the ratio A_{260}/A_{280} (10, 20). High performance liquid chromatography (HPLC) was used to determine the G+C content (11, 14, 15, 19, 20). A DNA preparation (1 mg/ml distilled water) was denatured at 100°C for 5 min. After rapid cooling in an ice bath, the denatured DNA was decomposed with 0.05 U nuclease P1 (Yamasa Shoyu Co., Chiba, Japan) in 40 mM sodium acetate buffer containing 0.2 mM $ZnCl_2$ at 50°C for 1 h. The hydrolysates and a standard solution of deoxyribonucleotide monophosphates (dAMP, dCMP, dGMP and dTMP) were each subjected to HPLC (L-6200, Hitachi, Japan) with a 3013-N column (4 × 150 mm), eluted with a mobile phase containing 30 mM KCl, 10 mM KH_2PO_4 and 10% CH_3CN (pH 3.5), and detected with a Hitachi L-4200 apparatus at 267 nm (20).

16S rDNA polymerase chain reaction (PCR) amplification

16S rDNA was amplified by PCR with a nucleotide primer set [5′-AGA GTT TGA TCM TGG CTC AG-3′, located at position 8–27 (*Escherichia coli* numbering) and 3′-TTC AGC ATT GTT CCA TYG GCAT-5′, located at position 1492–1513 (*E. coli* numbering)] (19), using Premix Tag (Takara, Tokyo, Japan) according to the manufacturer's instructions in a thermal controller PTC-100 (MJ Research, Watertown, MA). PCR thermal cycles were programmed as follows: 4 min at 94°C for initial heat activation and 35 cycles of 1 min at 94°C for denaturation, 1 min at 54°C for annealing, and 2 min at 72°C for extension (25). The amplification products were subjected to electrophoresis on 2.0% agarose gel and visualized by ethidium bromide staining (24).

Sequencing of 16S rDNA and phylogenetic analysis

The PCR products were used for template DNA in the sequence analysis. Sequencing was performed using a Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) with the 10 universal primer sets labeled with Cy-5, following the manufacturer's protocol, and analyzed with a DNA sequencer (ALFexpress, Amersham Pharmacia Biotech). The segmented nucleotide sequences of 16S rDNA were connected using Seqman in the LASERGENE computer program (DNA Star, Madison, WI). 16S rDNA sequences were compared with those available at the GenBank using BLAST algorithms software. Sequence similarity was analyzed with the CLUSTAL method, which was programmed by Megalign in the LASERGENE computer program. A 16S rDNA sequence similarity of 98% was used as the cut-off point for positive identification of taxa (6, 16, 23, 28).

Results

G+C content

The DNA G+C contents of 10 clinical isolates and a reference strain ranged from 35 to 47 mol%, as determined by HPLC (Table 1). Of those, eight clinical isolates (9–74, C11b-g, 7–92, UJB13e-1, BN11a-k, 1–18, 9–67, P11a-i) revealed 45–47 mol% G+C. These strains were distinct from the other three strains (*D. pneumosintes* ATCC 33048, 9–73, AB13a-h), which showed 35 mol% G+C.

Sequence analyses of 16S rDNA

16S rDNA sequences of seven clinical isolates (9–74, C11b-g, 7–92, UJB13e-1, BN11a-k, 1–18, 9–67) highly resembled each other, and the similarity with the reported sequences of *Dialister invisus* (4) was more than 98.1% (98.1–99.4%, Table 1). As these seven strains revealed a similar G+C content (45–47 mol%), they were designated as *D. invisus*. Another clinical strain (P11a-i) resembled *D. invisus*; it belonged to the same G+C content group (47%) and had a rather high 16S rDNA similarity to that of *D. invisus*, but showed less than 98.0% sequence similarity (Table 1). This strain was tentatively designated as a *D. invisus*-like strain. Strain 9–73 had 16S rDNA strongly resembling that of the reference strain of *D. pneumosintes* ATCC 33048, but the similarity with ATCC 33048 was less than 98% (Table 1), although both revealed identical G+C content (35 mol%). This strain was tentatively designated as a *D. pneumosintes*-like strain.

The other strain (AB13a-h) had 35 mol% G+C content, similar to that *D. pneumosintes* ATCC 33048, but the similarity of 16S rDNA with *D. invisus* and *D. pneumosintes* ATCC 33048 was low (Table 1). This strain could not be assigned to any established bacterial species and may need new classification.

It should be noted that the similarities of 16S rDNA of the nine clinical isolates were lower than that of *D. pneumosintes* ATCC 33048, which is the type species of the genus *Dialister*. This indicates that the strains may in fact not belong to the *Dialister* genus and may need to be reclassified. In addition, the 16S rDNA similarity of *D. invisus* type strain (CCUG 47026) with that of *D. pneumosintes* ATCC 33048 was low (93%) (Table 1).

Phylogenetic tree

Isolates in the present study were phylogenetically located close to *Megasphaera*

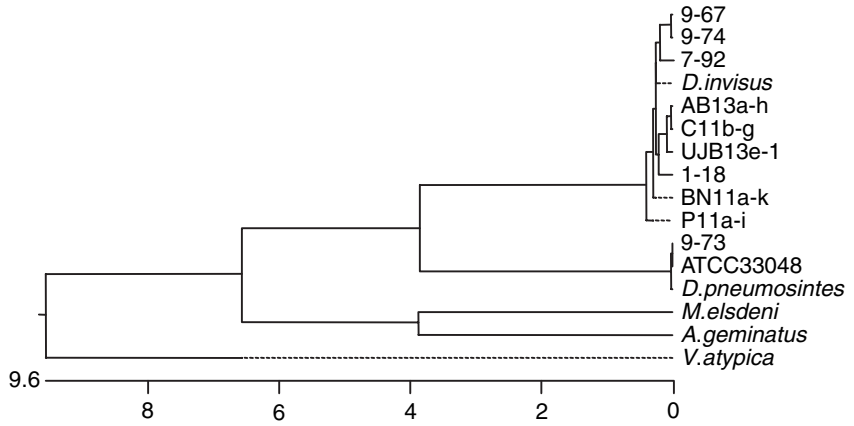

Fig. 1. Phylogenetic relationship between AAGNC, *D. pneumosintes*, *D. invisus,* and related bacteria on the basis of 16S rRNA gene sequences. The sequence data for species *D. pneumosintes*, *D. invisus*, *M. elsdenii*, *A. geminatus,* and *V. atypica* were obtained from GenBank database. The dendogram was created by using the neighbor-joining method.

elsdenii, *Anaeroglobus geminatus* and *Veillonella atypica*, members of the family Acidaminococcaceae (14, 31), but were distinct from these relatives (Fig. 1).

Discussion

Bacteria in oral florae have not been fully identified. Some have remained uncultured and have therefore often been ignored. Obligate anaerobic and asaccharolytic bacteria may be among such bacteria. We stock large numbers of unclassified bacteria that have been isolated from various oral sites. Among them, asaccharolytic anaerobic gram-positive rods were assigned new classifications such as *Eubacterium minutum* (21), *Eubacterium saphenum* (29), *Mogibacterium timidum* (19), *Mogibacterium diversum* (18), *Mogibacterium neglectum* (18), *Mogibacterium vescum* (19), *Mogibacterium pumilum* (19), *Cryptobacterium curtum* (17), and *Eubacterium exiguum* (reclassified to *Slackia exigua*). Most of these bacteria were culture-difficult. In the present study, a group of isolates, asaccharolytic anaerobic gram-negative coccobacilli (AAGNC), that are culture-difficult and thus have remained unclassified, were characterized phylogenetically. AAGNC are not well described and may include heterogeneous bacterial groups. Since *Veillonella* is a diplococcus and *Johnsonella* is a rod, both of which should be excluded from the AAGNC group.

Asaccharolytic bacteria do not utilize carbohydrates as their growth nutrients, but they do most likely utilize amino acids, peptides, and other noncarbohydrate nutrients. They might not, therefore, have any advantage over saccharolytic bacteria at sites where enough sugars are provided, such as in supragingival dental plaque. But they could favorably reside near inflamed areas, such as in periodontal pockets and infected root canals, where amino acids and peptides are present as inflammatory decomposed products of tissue proteins.

Unknown 16S rDNA fragments have often been detected from oral samples (3, 7, 16, 27, 30), especially from periodontal pockets and endodontic lesions. Some have been suggested to resemble fragments of *D. pneumosintes*, although the similarities are not very high (around 93%) (5), indicating they may not be derived from *D. pneumosintes* but from relatives of the species. There are also no accepted guidelines for computer-aided comparison of sequence similarity for 16S rDNA-based bacterial identification. We set a 98% similarity as a suitable cut-off point for species identification (28) because the 97% cut-off has been questioned (8).

In the present study, the 16S rDNA sequences of eight of the 10 isolates that were tested showed a rather low similarity to the sequences of *D. pneumosintes* ATCC 33048, which is the type strain of the genus *Dialister*, indicating the isolates may not belong to the *Dialister* genus. Although seven of the eight isolates were identical to *D. invisus*, which has recently established within the genus *Dialister*, they may need to be re-classified under a different genus name. One isolate was unique because its 16S rDNA sequence resembled that of *D. pneumosintes* but its G+C content was different. This may indicate there are limitations in bacterial classification using partial phylogenetic determination.

In a previous study, *D. invisus* species were isolated from infected root canals and periodontal pockets. They produced only tiny colonies, and thus required a stereoscope at a magnification at 30× for visualization (5). The necessity of such specialized conditions may have led to an underestimation of the bacterial populations in periodontal pockets and infected root canals. In addition to the genomic analyses in the present paper, biological multifactorial analyses would be needed to understand the biological characteristics of these bacteria. Studies are now in progress to culture this group of bacteria.

In conclusion, the present study demonstrated five phylogenetic groups of oral AAGNC strains. Seven isolates were identified as *D. invisus*. The remaining three isolates could not be classified into any established bacterial species. In addition, there were large differences in G+C content and 16S rDNA sequence between *D. invisus* and *D. pneumosintes*. This finding confirms a need for reclassification of this group of microorganisms.

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