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Short communication

Prevalence and molecular diversity of *Archaea* in subgingival pockets of periodontitis patients

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Introduction: The aim of this study was to investigate the prevalence and molecular diversity of *Archaea* in the subgingival crevices of patients with chronic periodontitis.
Methods: Subgingival plaque was collected from 41 patients with chronic periodontitis and 15 healthy subjects. The prevalence of *Archaea* in those plaque samples was tested by polymerase chain reaction with two broad-range archaeal primer sets. Amplicons from eight *Archaea*-positive plaque samples were cloned and sequenced for molecular diversity analysis using one of these two primer sets and a novel third primer set.
Results: *Archaea* were detected in the subgingival plaque of patients with chronic periodontitis at a prevalence of 70.7–73.2%, but were not detected in healthy subjects. Using one primer set, all sequences of the archaeal amplicons were identified as *Methanobrevibacter oralis*-like species. With another primer set, the amplicons were also found to be identical to the uncultured *M. oralis*-like species except one phylotype was found to belong to the class *Thermoplasmata*.
Conclusion: *Archaea* might be correlated with periodontal diseases. The diversity of *Archaea* associated with periodontitis was limited. Almost all sequenced amplicons fell into the genus *Methanobrevibacter* of the *Euryarcheota* phylum. *M. oralis*-like species was the predominant but non-exclusive archaeon in the subgingival dental plaque of patients with periodontitis.

Key words: *Archaea*; molecular diversity; periodontitis

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Archaea are a group of single-celled microorganisms and were first classified as a separate group of prokaryotes in phylogenetic trees based on the sequences of ribosomal RNA genes in 1977 (22). They can be found in most ecosystems and are often prevalent in supposedly uninhabitable environments. Despite their abundant and ubiquitous association with humans, animals, and plants, no pathogenic *Archaea* have been described (19). In addition, some *Archaea* could be isolated from human dental plaque and infected root canals (4, 20), as well as from the human gut system and vagina (3, 9, 14, 17). Moreover, methanogenic bacteria have also been found in some foodstuffs (5); however, no archaeon has been established as a cause of human disease.

Chronic periodontitis is a chronic inflammatory disease associated with polymicrobial infection in adults (13). This disease has been linked to a complex microflora comprising several hundred mainly strictly anaerobic microorganisms (15). The periodontal subgingival pocket is a unique environment and serves as home to billions of microbes. These microbes form a complex community of organisms that interact with each other and play an essential role in the etiology of periodontal disease. In 1986 Brusa et al. (4) first isolated a methanobacterium as a member of *Archaea* from human subgingival plaque. In that study, subgingival plaque samples were obtained from adult subjects after 24 h refraining from oral hygiene and were incubated in an anaerobic cabinet for 10 and 20 days. Methanobacteria were detected in three of the 10 samples by means of gas chromatography and fluorescence microscopy. Since then, several studies have shown that *Archaea* can be isolated from the deep periodontal pockets in different types of periodontitis, but not

from a healthy periodontal sulcus (7, 10). In addition, the presence of archaeal cells at subgingival sites with periodontal disease was confirmed by polymerase chain reaction (PCR) and by fluorescent detection with *in situ* hybridization (12). Furthermore, the progress of periodontitis showed a corresponding change in the diversity of microorganisms and the relative abundance of each member in the subgingival niche (12). However, in most studies, the number of the patients examined was not large enough to determine the true distribution of *Archaea*. In this study, we investigate the prevalence of *Archaea* in subgingival crevices of patients with chronic periodontitis and analyse the molecular diversity of *Archaea* detected in periodontal pockets.

The subjects of this study comprised 41 Chinese adults (25 women, 16 men) from 36 to 65 years old (mean age 54 years) who sought treatment for periodontitis at the Shanghai Ninth People's Hospital. They were otherwise healthy, and had not received antibiotic treatment during the previous 3 months. The study was performed in accordance with the Helsinki Declaration of 1975, as revised in 2000, and was approved by the ethics committee of the Shanghai Jiao Tong University School of Medicine. All patients received a detailed description of the proposed treatment and gave informed consent. They were diagnosed as having generalized chronic periodontitis based on radiographic evidence, with an absence of periapical periodontitis and other oral soft tissue diseases. Subgingival samples were collected from periodontal pockets with a probing depth >4 mm without site selection using sterile curettes. Before sampling, the supragingival plaque was removed from tooth surfaces. Clinical assessments at each site included the presence or absence of bleeding on probing, probing depth, and clinical attachment loss. The mean probing depth of sampling sites in patients with chronic periodontitis was 6.1 mm (range, 5–8 mm). Fifteen periodontally healthy subjects without bleeding on probing and clinical attachment loss for all sites were selected as the control (nine women and six men from 27 to 43 years old with a mean age of 37 years). The mean probing depth of control subjects was 1.7 mm (range, 1–2 mm). The plaque samples from the control subjects were collected from the gingival crevices. A detailed medical and dental history was obtained from each subject. Clinical assessments and sample collections were performed by the same researcher. Samples from one subject were pooled in a sterile tube containing 0.3 ml reduced transport medium and were stored at −20°C until molecular analysis.

The frozen plaque samples were thawed and dispersed by vortexing for 15 s, then centrifuged for 5 min at 13,000 ***g***. The supernatant was discarded and the microbial DNA was extracted by using a Bacterial Genomic DNA Extraction Kit (Tiangen, Beijing, China), according to the manufacturer's protocol. 16S ribosomal DNA fragments of oral *Archaea* were amplified using two broad-range archaeal primers for which the specificity and sensitivity had been determined previously (10, 12). The oligonucleotide sequences of the primers were as follows: primer set 1, 300fEyAr (5′-AGCRRGAGCCCGGAGATGG-3′) and 954rEyAr (5′-CGGCGTTGARTCCAATTAAAC-3′), and primer set 2: SDArch0333aS15 (5′-TCCAGGCCCTACGGG-3′) and SDArch0958aA19 (5′-YCCGGCGTTGAMTCCAATT-3′). The PCR amplifications were performed by using Ex *Taq* DNA polymerase (Takara, Dalian, China) and a negative control was performed without DNA templates. The following PCR protocol was used: after predenaturation at 95°C for 3 min, the reaction comprised 35 cycles of denaturation at 94°C for 30 s, annealing at a 58°C (60°C for primer set 2) for 30 s, and extension at 72°C for 30 s (40 s for primer set 2), followed by a final extension at 72°C for 7 min. The PCR products were separated by electrophoresis in 1.5% agarose gels in 1 × tris-acetate-ethylenediaminetetraacetic acid buffer and visualized under ultraviolet light, following ethidium bromide staining. The positive samples were recorded, and the prevalence of *Archaea* in plaque samples was calculated.

The prevalence of *Archaea* phylotypes in subgingival plaque samples from patients with chronic periodontitis and the healthy subjects is shown in Table 1. Thirty of the 41 samples (73.2%) from patients with periodontitis showed positive amplicons with primer set 1 and 29 of the 41 samples (70.7%) showed positive amplicons with primer set 2. *Archaea* were not detected in the group of healthy subjects. No statistically significant differences were observed with respect to clinical parameters between patients with chronic periodontitis carrying plaque containing or lacking *Archaea* (data not shown).

Next, we used primer set 2 and another broad-range archaeal primer set, set 3: Arch516F (5′-TGYCAGCCGCCGCGGTAAHACCVGC-3′) and SDArch1378-aA20 (5′-TGTGTGCAAGGAGCAGGGAC-3′) (12, 18), to clone the fragment of *Archaea* 16S ribsomal DNA and analyse the molecular diversity of *Archaea* detected in periodontal pockets. The PCR protocol for primer set 3 was as follows: denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 55 s, and a final extension at 72°C for 7 min. Eight *Archaea*-positive samples were selected and the PCR products with primer set 2 and set 3 were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA), according to the manufacturer's instructions. About 10 colonies of each sample were screened by colony PCR for the appropriately sized inserts. Samples were sent to Invitrogen (Shanghai, China) for sequence determination using their publication grade sequencing service. The DNA sequences were submitted to the Ribosomal Database Project II (21) for phylogenetic analysis. BLAST service was used to search for close evolutionary relatives in the GenBank (1) and those DNA sequences were aligned to related sequences with CLUSTALW (8). Phylogenetic trees were constructed by 1000-fold bootstrap analysis using neighbor-joining methods with MEGA 3.1. Sequences with 99% similarity were designated as being from the same species. Parts of the 16S ribosomal DNA (rDNA) archaeal sequences detected in the periodontal pockets were submitted to GenBank and were assigned the following accession numbers: FJ468179–FJ468289 and FJ458322–FJ458402.

Of the 111 *Archaea* 16S rDNA sequences obtained from eight samples by primer set 2, all were phylogenetically most similar to *Methanobrevibacter oralis* (data not shown). The results from another 81 sequences acquired by primer set 3 were almost the same as those from primer set 2 except that one sequence was different. The topologies of the phylogenetic trees were constructed using these data.

Table 1. Prevalence of *Archaea* in periodontal pockets and healthy sulcus

Group	Primer set 1[1]	Primer set 2[2]
Periodontitis (*n*)	41	41
Archaea-positive (*n*)	30	29
Prevalence (%)	73.2[3]	70.7[3]
Healthy (*n*)	15	15
Archaea-positive (*n*)	0	0
Prevalence (%)	0	0

[1]Primer 300fAr and 954rAr.
[2]Primer SDArch0333aS15 and SDArch0958-aA19.
[3]$P < 0.001$ periodontitis group vs. healthy group.

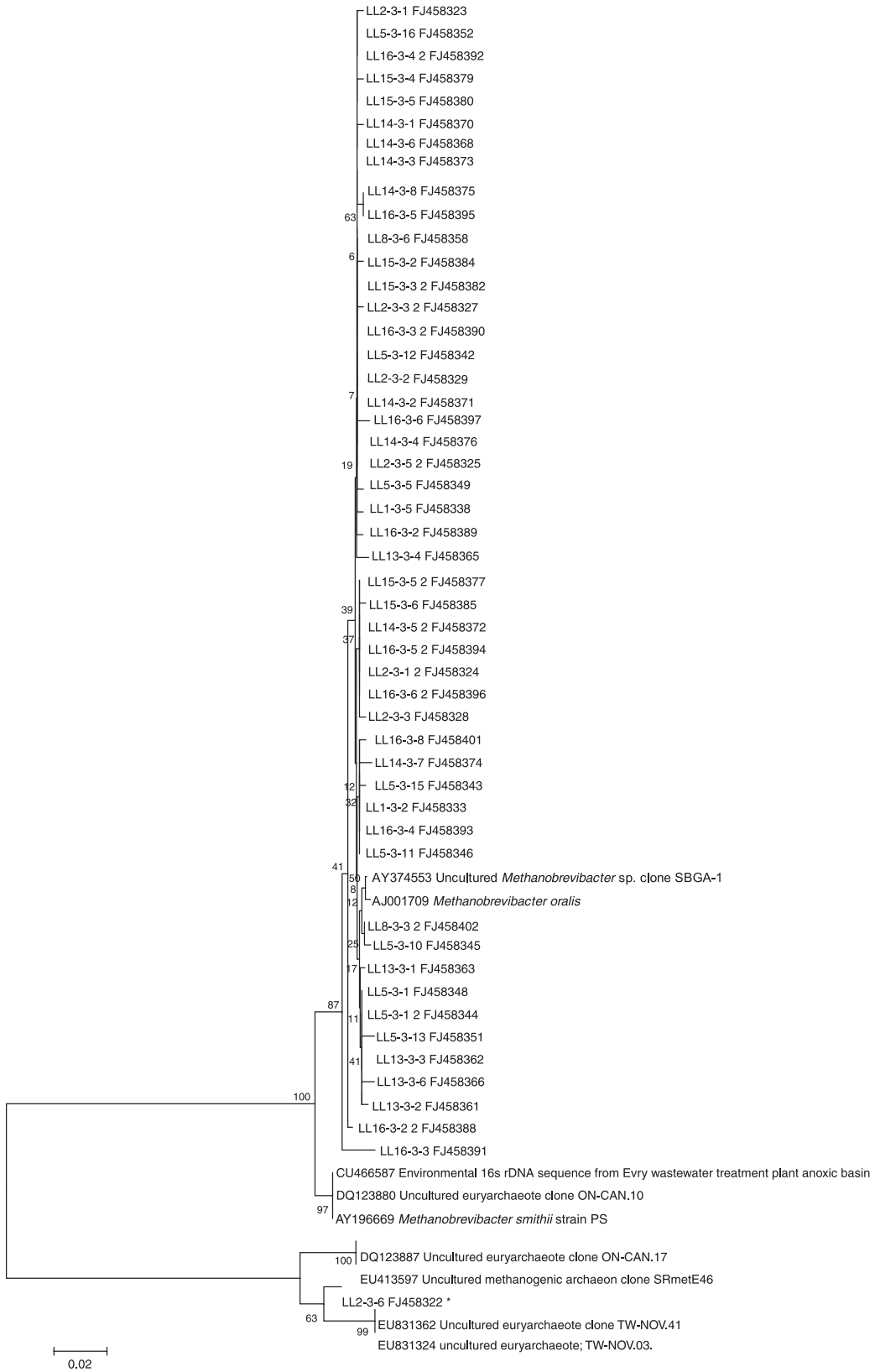

Fig. 1. Unrooted phylogenetic tree of *Archaea* inferred from 50 partial 16S ribosomal DNA sequence (excluding 31 repetitions from 81 sequences) generated with *Archaea* specific primer Arch516F and SDArch1378aA20 (primer set 3). Clones from this study are shown in front of their GenBank accession numbers. The evolutionary history was inferred by 1000-fold bootstrap analysis using a neighbor-joining approach via program MEGA 3.1. The tree was calculated using 894 nucleotide positions. The scale bar corresponds to 0.02 substitutions per nucleotide. *A new archaeal phylotype (clone LL2-3-6, GenBank accession number FJ458322, belonging to class *Thermoplasmata*) that was different from *Methanobrevibacter oralis* (belong to class Methanobacteria) was found. The other phylotype sequences were retrieved from GenBank (accession numbers indicated with their names).

Figure 1 shows the dendrogram for the archaeal partial rDNA sequences obtained with primer set 3. Almost all of the sequences belonged to one single phylotype. Only one new phylotype (clone LL2-3-6, GenBank accession number FJ458322), belonging to class *Thermoplasmata*, was found by primer set 3 from the sample of a 54-year-old woman (with probing depth 7 mm). More sequences obtained from this sample were determined and four of the 36 additional 16S rDNA sequences showed phylogenetic homology with *Thermoplasmata*, similar to the previous determination (1 of the 11 sequences).

Recent molecular studies have revealed that *Archaea* not only exist in supposedly uninhabitable environments, but also inhabit eukaryotic hosts, including humans (7). However, no member of the *Archaea* has been described as a human pathogen (16). In this research, *Archaea* 16S rDNA were detected in 29 (70.7%) or 30 (73.2%) of 41 subgingival plaque samples from patients with chronic periodontitis, depending on the primer set used, while none was detected in the 15 healthy subjects. These data obtained from Chinese patients are similar to previous reports that showed the detection of *Archaea* in moderate or advanced periodontitis, but not in healthy subjects, suggesting that *Archaea* might play a role in the progress of chronic periodontitis (2, 10). There were some differences between patients and healthy controls for age and sex in our study. These differences were the result of a random selection of subjects from the clinical patients. The relationship between oral microbial composition and age or sex demands further investigation.

M. oralis has been cultivated from human plaque and feces. This is an coccobacillary non-motile, gram-positive, methane-producing organism. Both hydrogen and carbon dioxide were required for its growth. In this study, the partial sequences of the 16S rDNA were aligned to those of *M. oralis* and analysed together with other sequences from *Methanobrevibacter* of human and animal intestinal tract origin by phylogenetic inference. Among the 192 cloned 16S rDNA fragments obtained using two pairs of *Archaea*-specific primers from eight samples, almost all clones displayed the sequence similarity with the *M. oralis* (>99% identical). Interestingly, another archaeal phylotype that belongs to the class *Thermoplasmata* was detected in the subgingival pocket of one woman. The class *Thermoplasmata*, which is also widely distributed in different oceanic regions, contains moderately thermophilic acidophilic organisms known since the 1970s (11). However, it has not been detected in dental plaque samples. Our results suggest that apart from the predominant colonizers of *M. oralis*, other *Archaea* also have opportunities to colonize the periodontal sulcus.

The genetic diversity of oral *Archaea* is low, and low diversity is not untypical for *Archaea* species. Studies of the human intestinal canal have shown that all 1524 archaeal sequences from 21 fecal samples belonged to a single phylotype *Methanobrevibacter smithii* (6). The low diversity showed the specificity of *Archaea* in the ecosystem of the human oral cavity and intestinal tract. *M. oralis* was found in the current research to be the predominant archaeon in the subgingival dental plaque. Reasons for the low diversity of oral *Archaea* were still unknown. With our data indicating that the *Thermoplasmata* may exist in subgingival dental plaque, an investigation of the non-predominant *Archaea* should be conducted to establish the microbial community structure of human subgingival plaque.

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