ORAL MICROBIOLOGY AND IMMUNOLOGY

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T-RFLP-based *mcrA* gene analysis of methanogenic archaea in association with oral infections and evidence of a novel *Methanobrevibacter* phylotype

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Introduction: Increasing evidence suggests a role for methanogenic archaea (methanogens) in human health and disease via syntrophic interactions with bacteria. Here we assessed the prevalence and distribution of methanogens and possible associations with bacteria in oral biofilms.
Methods: Forty-four periodontal and 32 endodontic samples from necrotic teeth with radiographic evidence of apical periodontitis were analysed. Terminal restriction fragment length polymorphism analysis based on the *mcrA* gene, specific to methanogens, was applied. The prevalence and amounts of methanogens in endodontic samples were compared with those of *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Treponema* spp. and *Synergistes* spp. based on real-time quantitative polymerase chain reactions.
Results: Besides dominance of the *mcrA* gene corresponding to *Methanobrevibacter oralis*, one *mcrA* gene type, for which no cultivated member has been reported previously, was identified in five periodontal samples and one endodontic sample. Rates of non-synonymous vs. synonymous nucleotide substitutions suggest that this *mcrA* gene type codes for a functionally active methyl-coenzyme M reductase. *Methanobrevibacter smithii*, the prominent methanogen in the human gut system, was not detected. Mean proportions of methanogens were comparable to *Synergistes* spp. ranging from 0.5 to 1.0% of the total microbial community. *Treponema* spp. dominated with a mean proportion of 10%, while the mean proportions of the other endodontic pathogens were below 0.1%. A positive association between methanogens and *Synergistes* spp. was found.
Conclusion: Our data provide evidence of a novel, as yet uncultured methanogenic phylotype in association with oral infections, and indicate possible interactions between methanogens and *Synergistes* spp., the nature of which deserves further investigation.

Key words: *Archaea;* endodontic infections; *mcrA; Methanobrevibacter oralis;* methyl-coenzyme M reductase, periodontitis; terminal restriction fragment length polymorphism

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The two species *Methanobrevibacter smithii* and *Methanobrevibacter oralis* are the only representatives of the distinct prokaryote domain *Archaea* that have been regularly found within the human microbial ecosystem. While *M. smithii* predominates in the gastrointestinal tract (19), *M. oralis* predominates in the oral cavity (14, 15) indicating adaptation of these two methane-producing archaea (methanogens) to defined ecological niches in humans. Recently, *M. oralis* has been identified in infected root canals with necrotic pulp tissue, in both asymptomatic and symptomatic clinical cases by two independent studies (23, 26). This finding hints at the intriguing possibility that some archaea may have the potential to invade primary sterile anatomical sites and to contribute to the pathogenesis of microbial diseases. Although no definite virulence genes or factors as commonly defined (e.g. the means for enabling tissue invasion and release of toxins) have been described in archaea there is an emerging view of an 'indirect pathogenicity' of human methanogens (5). With the unique metabolic capacity of methanogens [i.e. consumption of molecular hydrogen (H_2) with formation of methane] it has been suggested that they have the potential to indirectly promote anaerobic infectious diseases through syntrophic interactions with true pathogenic, fermenting bacteria [i.e. 'interspecies H_2 transfer', a well-known mechanism in natural environments (4, 6)]. This hypothesis is borne out by the established link between methanogens and the severity of the major oral disease periodontitis (15). If human methanogens actually use H_2 as substrate in anoxic compartments in the oral cavity they may on the one hand be interacting with distinct syntrophic partners but on the other hand also be competing with H_2-consuming functional guilds, others than methanogens. The first evidence for such antagonistic interactions in subgingival plaque biofilms (with dissimilatory sulfate-reducers and acetogens, both of which can consume H_2) was provided recently by our group (25). That study was based on the surveillance of functional genes encoding for key enzymes involved in H_2 consumption, including the *mcrA* gene of methanogens. The gene *mcrA* encodes for the alpha subunit of the methyl-coenzyme M reductase, which catalyses the terminal step in methane formation. Two distinct *mcrA* gene types were found in this study, one corresponding to *M. oralis*, the other – referred to as 'novel *mcrA* gene type' – grouping within the phylogenetic radiation of the genus *Methanobrevibacter* but with no cultured representative counterpart (25).

Detection of this novel *mcrA* gene type raises the question whether it actually corresponds to an authentic methanogenic species or rather represents an additional gene copy or even a pseudo-gene of the known methanogens *M. oralis* or *M. smithii*. If corresponding to an authentic methanogenic species, the additional question regarding its overall prevalence compared with *M. oralis* in subgingival plaque arises and of particular interest would be whether it can also be identified within infected dental root canals. To address these questions we performed terminal restriction fragment length polymorphism (T-RFLP) analysis based on the *mcrA* gene complemented by generation of clone libraries and sequencing of *mcrA* and 16S ribosomal RNA (rRNA) gene types from subgingival plaque and endodontic samples. As a first attempt to identify possible syntrophic partners we compared the prevalence, coexistence and relative proportions of methanogens with a selection of recognized and putative pathogens, including *Tannerella forsythia*, *Prevotella intermedia*, *Porphyromonas gingivalis*, *Treponema* spp. and *Synergistes* spp. While the latter analysis was focused on endodontic samples, the assessment of possible associations between methanogens and bacteria in subgingival plaque samples from patients with periodontitis is the subject of an ongoing study and will not be addressed in this article.

Materials and methods

Sample collection and DNA extraction

We selected subgingival plaque samples from 44 human periodontitis sites that had tested positive for the presence of methanogens in a previous study, which also provides in depth details regarding inclusion criteria for patients and sample collection (25). Briefly, subgingival plaque was collected and pooled from the four deepest periodontal pockets of each patient with sterile paper points (ISO 45; Alfred Becht GmbH, Offenburg, Germany), after isolation and supragingival plaque removal. Patient inclusion for subgingival plaque samples was in accordance with the guidelines of the Ethics Committee of the Medical Faculty, RWTH Aachen University, Germany.

In addition, 32 endodontic samples from patients with chronic primary endodontic infections were selected from another study, for which details regarding patient inclusion and sample collection are provided elsewhere (24). Briefly, after isolation of the teeth with a rubber dam, and disinfection of the crown and the surrounding area, an access cavity was prepared with a sterile high-speed diamond bur under irrigation with sterile saline. Samples were collected with five sterile paper points (Malleifer-Dentsply, Bailagues, Switzerland) and pooled in a sterile tube containing 1 ml reduced transport fluid. Patient inclusion for the endodontic samples was in accordance with the Human Volunteers Research and Ethics Committee of the Dental School of Piracicaba, Brazil.

For both subgingival plaque and endodontic samples whole genomic community DNA was extracted with the Qiamp DNA minikit (Qiagen, Hilden, Germany) as described previously (12).

Detection and quantification of methanogenic archaea and oral pathogens in endodontic samples using real-time quantitative polymerase chain reaction

Detection and quantification of *P. gingivalis*, *P. intermedia*, *Treponema* spp., *Synergistes* spp. and total bacteria from the 32 endodontic samples and of methanogenic archaea from a subset of 20 endodontic samples were performed previously by real-time quantitative polymerase chain reaction (RTQ-PCR) (23, 24). In the current study we enlarged the dataset by quantifying methanogens in the remaining 12 samples and *T. forsythia* in all 32 samples using the same experimental design for RTQ-PCR as described in detail previously (23, 24). Specific to the quantification of *T. forsythia* was the use of the *T. forsythia* type strain ATCC 43037 for establishing the calibration standards and the temperature profile for RTQ-PCR, which was 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 56°C for 10 s and 72°C for 35 s. The fluorescence was read at 84°C. The primers used for quantifying *T. forsythia* were PF1 5′-AGAGTTTGATCCTGGCTCAG-3′ (7) and Bf 5′-TGCTTCAGTGTCAGTTA TACCT-3′, (20). The linear scope of detection ranged from 10^2 to 10^8 target molecule numbers, with an amplification efficiency of 1.93 (error 0.03). The relative proportions were calculated through referring the amount of *T. forsythia* to the total bacterial load in the same sample.

Conventional PCR amplification for T-RFLP analysis

For T-RFLP analysis of methanogens the *mcrA* gene was preamplified with the

forward primer ME1: 5′-GCMATGCAR ATHGGWATGTC-3′ (11) and the reverse primer LuR: 5′-TTCATTGCRTAGTTW GGRTAGTT-3′ (17) in a total volume of 50 μl using an Eppendorf thermal cycler (Eppendorf AG, Hamburg, Germany). The reaction mixture contained 500 nM of each primer and 1 μl of template DNA (approximately 25 ng of template DNA). The temperature profile was 95°C for 2 min; followed by 18 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1.5 min, with a final extension of 72°C for 10 min. Aliquots of the products of this PCR (1 μl) were used as the template in a second round of PCR using the forward primer LuF: 5′-GGTGGTGTMGGATTCACACARTA YGCWACAGC-3′ (17) and LuR. In the second PCR the reverse primer was labeled with the fluorescence dye carboxytetramethylrhodamine (TAMRA). PCR conditions here were 95°C, 2 min; followed by 32 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 1.0 min, with a final extension of 72°C for 20 min. Aliquots of the amplicons (5 μl) were analysed by electrophoresis on a 1.5% agarose gel.

Conventional PCR amplification for clonal analysis

PCR amplification of the *mcrA* gene for creation of clone libraries was identical to that described above except that no TAMRA-labeled primer was used. PCR amplification of archaeal 16S rRNA genes was performed using the primers A109F 5′-ACKGCTCAGTAACACGT-3′ (10) and A934R 5′-GTGCTCCCCCGCCAATTCC T-3′ (21). PCR were performed in a total volume of 50 μl using an Eppendorf thermal cycler (Eppendorf AG). The reaction mixture contained 500 nM of each primer, and 1 μl template DNA (approximately 25 ng template DNA). The temperature profile was 95°C for 2 min; followed by 35 cycles of 95°C for 30 s, 53°C for 30 s and 72°C for 1.5 min, with a final extension of 72°C for 20 min.

T-RFLP analysis

After purification with QIAquick spin columns (Qiagen, Alameda, CA), approximately 100 ng of the amplicons was digested separately with 20 U of the restriction endonucleases *Alu*I, *Msp*I and *Hpy*F31 (all New England BioLabs, Beverly, MA). The digestions were carried out in a total volume of 10 μl for 4 h at 37°C according to the instructions of the manufacturer. Enzyme inactivation was carried out by incubation at 80°C for 20 min.

A volume of 1 μl of the restriction reaction was mixed with 18.7 HiDi formamide and with the internal lane standards 0.3 ROX 500 (both reagents from Applied Biosystems, Foster City, CA). Subsequent electrophoresis was performed on an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems). Based on the internal Rox-standard the terminal restriction fragments (T-RFs) were sized using GeneMapper software (Applied Biosystems). Fragments with a peak height below 1% (regarded as background noise) as well as those with a size less than 50 base pairs (bp) were excluded from the analysis.

Cloning and sequencing

PCR amplicons (*mcrA* gene and the 16S rRNA gene) were cloned using the TOPO TA Cloning® Kit for Sequencing with the pCR®4-Topo-Vector (Invitrogen, Carlsbad, CA), according to the instructions of the manufacturer. Clones were randomly selected for further analysis. For preparation of clone DNA, colonies grown on agar plates were picked, and the material was resuspended in 20 μl sterile, DNA-free water. Cells were lysed by boiling, and the resulting debris was pelleted by centrifugation. The supernatant was transferred to a new tube, and 1-μl aliquots were directly used for PCR. Cloned inserts were amplified using the primers M13F/M13R (provided with the cloning kit) that targeted vector sequences using PCR conditions as described in the manual for the TOPO TA Cloning kit. Sequence analysis of the resulting PCR amplicons was performed using the Big Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems) on an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems). The quality of the sequences was visually inspected using Vector NTI Advance 9.0 (Invitrogen Life Science Software, Frederick, MD). The identities of the gene sequences were confirmed by searching the international sequence databases using the Blast program (URL: http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree reconstruction was performed using the Arb software package (16). *McrA* gene and 16S rRNA gene sequences of the novel methanogenic phylotype have been submitted to GenBank.

Statistical analysis

Differences in prevalence in the root canal samples were tested with a Cochrane test for nominal non-parametric data (present vs. not present). The Fisher's Exact test was applied to test the null hypothesis that there was no relationship between any of the species ($P > 0.05$). When there was a significant difference, indicating the presence of a relationship between species, the odds ratio was calculated. Positive associations were those with an odds ratio >2 and negative associations were those with an odds ratio <0.5.

Results

McrA-based T-RFLP analysis

For T-RFLP analysis we selected three different restriction enzymes that were able to distinguish the *mcrA* genes from *M. oralis*, the novel *mcrA* gene type and those from *M. smithii*. Table 1 shows the three selected enzymes (*Alu*I, *Msp*I and *Hpy*F31I) along with the theoretical cutting sites predicted from the sequence information. *McrA* amplicons generated from strains of *M. oralis* (DSM 7256^T) and *M. smithii* (DSM 861^T), as well as from cloned DNA obtained from the novel *mcrA* gene type previously (25), were subjected to restriction digestion with all three enzymes separately. Each sample produced a distinct T-RF that could be assigned to the theoretical T-RFs deduced from the sequence information (data not shown).

PCR amplification of the *mcrA* genes from the methanogen-positive clinical samples (i.e. 44 subgingival plaque samples and eight endodontic samples) was performed using conventional PCR and a TAMRA-labeled reverse primer for T-RFLP analysis. PCR amplicons for the

Table 1. Restriction enzymes (recognition site) and resulting terminal restriction fragments (T-RFs) to distinguish oral *mcrA* gene types[1]

	T-RFs length (base pairs)		
Restriction enzymes (recognition site)	*Alu*I (AG′CT)	*Msp*I (C′CGG)	*Hpy*F31 (C′TNAG)
Methanobrevibacter oralis	**217**	470	470
Methanobrevibacter smithii	79	470	**158**
Novel *mcrA* gene type	79	**276**	470

[1]Numbers in bold indicate the size of T-RFs unique for one *mcrA* gene type. A fragment size of 470 base pairs represents the entire amplicon, as no recognition site exists for the respective enzyme.

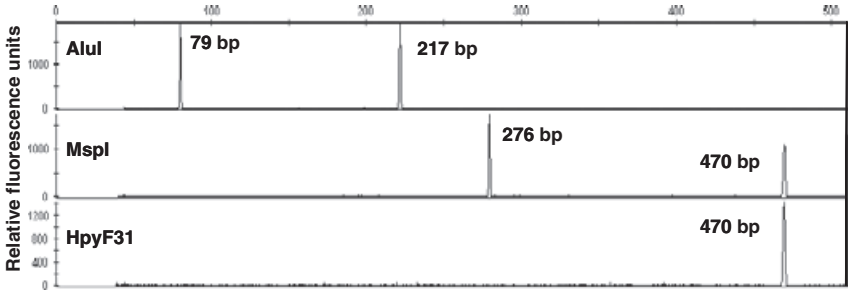

Fig. 1. Representative *mcrA*-based terminal restriction fragment length polymorphism profiles from one subgingival plaque sample generated after digestion with the restriction enzymes *Alu*I, *Msp*I and *Hpy*F31. Note that the fragment size of 470 base pairs (bp) represents the entire amplicon, as no recognition site for the restriction enzyme exists, compare with Table 1.

mcrA gene were obtained for all samples. Subsequently performed T-RFLP analysis led to the recovery of single T-RFs in the majority of samples. *M. oralis* was the sole methanogenic organism in 39 periodontal and seven endodontic samples, whereas the novel *mcrA* gene type was found in three periodontal samples but also in one endodontic sample. Two T-RFs, indicative of a coexistence of both, *M. oralis* and the novel *mcrA* gene type, were detected in two periodontal samples (as representatively shown in Fig. 1). To verify the identity of the T-RFs, PCR amplicons from two periodontal and two endodontic samples with single T-RFs (either indicative of *M. oralis* or the novel *mcrA* gene type) were cloned and from each clone library 10 clones were selected for sequence analysis. A BLAST search of the GenBank database confirmed 100% sequence identity to *M. oralis* (accession number DQ251045) in the case of samples with a unique *Alu*I-based T-RF of 217 bp. Likewise, sequences obtained from samples with a unique *Alu*I-based T-RF of 79 bp were 100% identical to the novel *mcrA* gene type (accession number EU294498).

Characterization of the novel *mcrA* gene type

One periodontal sample and one endodontic sample with single occurrence of the novel *mcrA* gene type were subjected to PCR amplification using broad-range 16S rRNA gene primers specific to the domain *Archaea*. PCR products of the correct amplicon size were obtained for both samples, cloned and again 10 clones each were selected for sequence analysis. According to the GenBank search all clone sequences were 100% identical to 'phylotype 3', (accession number AJ001711) and 99.5% identical to 'SBGA-2', (accession number AY374554), both of which are archaeal 16S rRNA gene sequences previously identified in subgingival plaque (14, 15). Nucleotide differences with 'SBGA-2' occurred at three positions over a region of 565 bp. Reconstruction of evolutionary dendrograms based on the *mcrA* gene and of the 16S rRNA gene (using our endodontic sample 'E24' as representative sequence for treeing analysis) showed good agreement in the overall tree topology but with the *mcrA*-gene-based tree allowing for a higher phylogenetic resolution (Fig. 2). While the sequence identity of the 16S rRNA gene from 'phylotype 3' with *M. oralis* and *M. smithii* ranged between 98 and 99%, the sequence identity of the corresponding *mcrA* gene with *M. oralis* and *M. smithii* ranged between 88 and 89% at the nucleotide level and was approximately 96% at the amino acid level. There were 41 synonymous nucleotide changes out of a total of 53 nucleotide changes along the branch to *M. oralis* and 35 synonymous nucleotide changes out of a total of 48 nucleotide changes along the branch leading to *M. smithii*.

Comparison of prevalence and relative proportions of methanogens with endodontic pathogens

The order of prevalence found in the 32 endodontic samples tested were *Treponema*

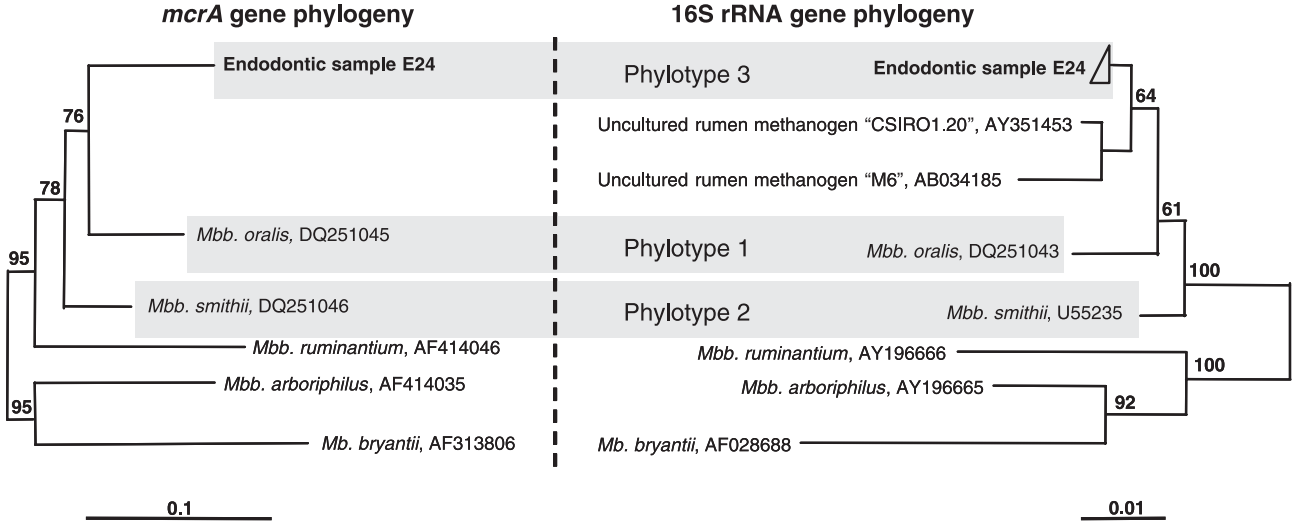

Fig. 2. Phylogenetic tree showing the position of the *mcrA* gene type sequences and 16S ribosomal RNA (rRNA) gene sequences of three methanogenic phylotypes identified in humans relative to other members of methanogenic archaea. Sequences determined in this study are shown in bold type. The scale bar corresponds to 0.1 substitutions per nucleotide (*mcrA* tree) and 0.01 substitutions per nucleotide (16S rRNA tree), respectively. The tree was calculated using 470 *mcrA* nucleotide positions and 798 16S rRNA gene nucleotide positions and the neighbor joining approach (with the Felsenstein correction), via the ARB program package (16). The triangle indicates representation of Phylotype 3 by two further 16S rRNA gene sequences (i.e 'phylotype 3', AJ001711) and 'SBGA-2', (AY374554). The statistical significance levels of interior nodes shown as percentages were determined by performing bootstrap analyses (1000 replications, only values over 50% are shown). Mb, *Methanobacterium*; Mbb, *Methanobrevibacter.*

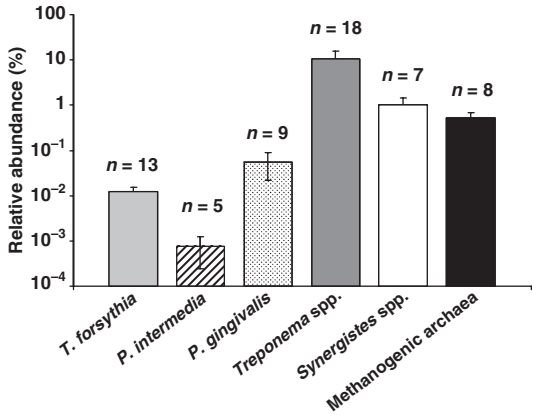

Fig. 3. Prevalence and relative abundance of the target microorganisms in 32 analysed endodontic samples.

spp., (18 positive cases), *T. forsythia* (13 positive cases), *P. gingivalis* (nine positive cases), methanogenic archaea (eight positive cases), *Synergistes* spp. (seven positive cases) and *P. intermedia* (five positive cases). In total, 24 samples were positive for at least one of the five target microorganisms. With a mean proportion of approximately 10%, *Treponema* spp. made up the largest proportion followed by *Synergistes* spp. and *Methanobrevibacter* spp., both of which made up proportions between 0.5 and 1% of the total endodontic microbial community (Fig. 3). Mean proportions of *P. gingivalis*, *T. forsythia* and *P. intermedia* were much lower, ranging from 0.001 to 0.08% (Fig. 3). Species combination varied from single species to the combined presence of all target microorganisms. A positive association between methanogenic archaea and *Synergistes* spp. was found with an odds ratio of 7 (95% confidence interval ranging from 1.11 to 44.06, $P = 0.047$).

Discussion

According to T-RFLP analysis the *mcrA* gene corresponding to *M. oralis* was found much more often than the novel *mcrA* gene type. Despite its 'rarity', the novel *mcrA* gene type was also identified (for the first time) in a sample from dental root canal infections, stressing the importance of methanogens in clinical microbiology. It could be speculated that *M. oralis* has the potential to outcompete other methanogens in the oral ecosystem; however, co-occurrence of both *mcrA* gene types as found in some periodontal samples indicated no strict niche exclusion between *M. oralis* and the putative new methanogenic organism. *M. smithii* was not detected in any of the samples confirming the study of Lepp et al. (15), but contrasting with the results of Kulik et al. (14). The latter authors amplified archaeal DNA from subgingival plaque samples using up to 65 PCR cycles in a two-step PCR, while in our study and in that of Lepp et al. (15) far fewer PCR cycles were used. It is therefore possible that *M. smithii* exists only in very low numbers and probably only as a transient member of the oral microflora.

The recovery of the novel *mcrA* gene type and of one distinct archaeal 16S rRNA gene sequence (i.e. 'phylotype 3') from the same sample along with the congruency of the treeing analysis provides strong evidence for the true existence of a novel, as yet uncultivated, methanogenic phylotype represented by the two different genes. Consequently, 'phylotype 3' is unlikely to represent an artificial (chimeric) 16S rDNA molecule as had been speculated by Kulik et al. (14). Further conclusions can be drawn from the *mcrA* gene sequence. The strong predominance of synonymous over non-synonymous changes indicates an evolutionary pressure that retains the function of the encoded enzyme (namely the methyl-coenzyme M reductase), a mechanism generally referred to as purifying selection (27). Purifying selection in turn means that the *mcrA* gene of this novel phylotype is biologically active and that methane may be actually produced in the oral cavity. The mechanism of purifying selection also indicates that the oral microenvironment (i.e. subgingival plaque and infected root canals) does not allow for a large functional variability of the methyl-coenzyme M reductase. This restrictive effect may explain in part the low diversity of oral methanogens and possibly also the entire absence of other, non-methanogenic archaea in humans, although more verification is needed here (18).

Comparison with endodontic pathogens

There is an increasing trend toward recognition that most anaerobic infections are polymicrobial with two or more microorganisms acting synergistically or in succession to drive complex disease processes (3). Although each polymicrobial disease has unique aspects, interactions among microorganisms involved in the disease may follow distinct ecological principles (e.g. niche generation of one microbe for subsequent colonization of other microorganisms, or syntrophic growth on substrates). In this context, the possible role of methanogenic archaea as keystone species to support the growth of pathogenic bacteria through reducing inhibitory levels of H_2 may be considered as a novel paradigm of microbial pathogenesis (5). Assuming the existence of such interactions the identity of the syntrophic partner(s) of methanogens could be pivotal for disease progression. In the current study no association between methanogens and *T. forsythia*, *P. gingivalis*, *P. intermedia* and *Treponema* spp. in endodontic samples were found, either because no associations exist, or because the number of samples analysed ($n = 32$) was too low to ascertain such relationships. However, the positive association that we observed between methanogens and *Synergistes* spp., both of which occurred with roughly similar prevalence and proportions in endodontic samples (Fig. 3), warrants further investigation for three reasons. First, *Synergistes* spp. are known for their ability to degrade a variety of amino acids anaerobically with the production of H_2 and CO_2, which are the substrates required for methanogenesis (8, 9). Second, syntrophic interactions between methanogens and *Synergistes* spp. actually exist, as has been demonstrated for the first time based on a full-scale anaerobic sludge digester (2). Lastly, *Synergistes* spp. have been found at multiple sites of human infections including oral infections, soft tissue infections and peritonitis, and so are considered candidate human pathogens. (12, 13, 22, 24).

Although more work is needed for definite conclusions, these observations underscore the possibility that methanogens may actually support distinct pathogenic bacteria.

Final remarks

Like bacteria, archaea occur in a wide variety of environments, and in close association with eukaryotes including metazoa. Although the *Archaea* seem

inherently less diverse than bacteria in the same environment (1), their diversity in humans is strikingly low. While on the one hand fundamental features shared by most archaea might exist that preclude them from colonizing the human body, one might on the other hand ask what features *M. oralis*, *M. smithii* and 'phylotype 3' share that enable them to do so. Besides successful evasion of the host immune system, these features may include tolerance to temporary oxygen stress and adaptation to the levels of H_2 provided by their syntrophic partners. Whole genome analysis has recently demonstrated that *M. smithii* is in fact highly adapted to the human intestinal tract system (19). Analogous genomic approaches applied to *M. oralis* and 'phylotype 3' in future may reveal key information about their adaptive strategy in the oral cavity, including the participation in – and the support of – pathogen-associated plaque biofilms.

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