Quantitative Analysis of Three Hydrogenotrophic Microbial Groups, Methanogenic Archaea, Sulfate-Reducing Bacteria, and Acetogenic Bacteria, within Plaque Biofilms Associated with Human Periodontal Disease

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Received 27 November 2007/Accepted 1 March 2008

Human subgingival plaque biofilms are highly complex microbial ecosystems that may depend on H₂-metabolizing processes. Here we investigated the ubiquity and proportions of methanogenic archaea, sulfate reducers, and acetogens in plaque samples from 102 periodontitis patients. In contrast to the case for 65 healthy control subjects, hydrogenotrophic groups were almost consistently detected in periodontal pockets, with the proportions of methanogens and sulfate reducers being significantly elevated in severe cases. In addition, antagonistic interactions among the three microbial groups indicated that they may function as alternative syntrophic partners of secondary fermenting periodontal pathogens.

Periodontal disease is a polymicrobial anaerobic infection that, besides possibly leading to loss of the involved teeth (if untreated), is considered to constitute a risk factor contributing to the development of life-threatening systemic diseases, such as endocarditis, atherosclerosis, and stroke, as well as being causative for preterm birth (6, 7, 33). The list of oral microorganisms involved in periodontal disease is large, consisting of several hundred species, of which approximately 50% represent as-yet-uncultivable phylotypes (4, 16, 31, 37). No single species has been identified so far as the ultimate causative agent. Instead, the disease is likely to be a result of the activities of different and varying microbial complexes (36).

The recent implication of methanogenic archaea (methanogens) and dissimilatory sulfate-reducing bacteria (SRB) in periodontal pockets (18, 23) prompted us to perform a cross-sectional examination of the ubiquity of hydrogen (H₂)-utilizing organisms (hydrogenotrophs) in subgingival plaque biofilms from periodontitis patients. Such knowledge could provide basic information on the role of H₂ consumption in the regulation of the periodontal biofilm ecosystem (i.e., “inter-species hydrogen transfer” [35] as a possible driving force to promote proliferation of fermenting pathogens). Because of their polyphyletic nature, hydrogenotrophs are not amenable for study as functional microbial entities with 16S rRNA gene-based approaches. We therefore surveyed the major functional microbial guild methanogens, SRB, and acetogenic bacteria (acetogens) by recovering and analyzing three unifying group-specific genes (i.e., *mcrA*, *dsrAB*, and *fts* encoded key enzymes involved in H₂ consumption, namely, methyl-coenzyme M reductase, dissimilatory sulfite reductase, and formyltetrahydrofolate synthetase, respectively.

Our analysis encompassed 102 plaque samples from patients at different stages of severity of chronic periodontal disease (the most common form of periodontitis) along with 65 samples from age-matched healthy control individuals. Patient inclusion (42 males and 60 females attending the Clinic of Operative and Preventive Dentistry and Periodontology, RWTH Aachen; mean age, 50.7 [standard deviation, 11.23] was in accordance with the guidelines of the Ethics Committee of the RWTH University Hospital, Aachen. Subgingival plaque samples were 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. For the healthy subjects, plaque was collected and pooled from vestibular sulcus of first molars from all quadrants with sterile paper points. Periodontitis sites with periodontal probing depths of ≥6 mm (clinical attachment loss, ≥4 mm) were defined as “severe” cases and those with periodontal probing depths of <6 mm (clinical attachment loss, <4 mm) as “moderate” cases (30). Healthy sites were defined as those with periodontal probing depths of <3 mm and no bleeding on probing.

Whole genomic community DNA was extracted as described previously (14). The abundance of target microorganisms was determined by real-time quantitative PCR using primer pairs with validated target specificity as published earlier (15, 21, 27, 29, 42) and thermal profiles modified from the original protocols for adaptation to a LightCycler-based amplification (LightCycler 2.0) according to the recommendations of the manufacturer (Roche Molecular Biochemicals technical note 2/99; Roche Applied Science, Penzberg, Germany) (Table 1). Reactions were performed using LightCycler FastStart DNA Master **SYBR green I** in a total volume of 20 μL. Final reaction mixtures contained 500 nM of each primer and 3 μL of template DNA (approximately 75 ng). Quantification (i.e., de-
termination of crossing points and conversion to initial gene target molecule numbers based on calibration standards) as well as melting curve analysis followed the protocol described by Vianna et al. (41) except for preparations of DNA standards. For this purpose, DNA from *Methanobrevibacter oralis* DSM 7256T was amplified with primers ME1 (11) and LuR, DNA from *Desulfovibrio piger* DSM 749T was amplified with primers DSR1F (42) and DSR4R, and DNA from *Eubacterium limosum* DSM 20543T was amplified with primers FTHFS-F and FTHFS-R. Purified amplicons (Qiagen purification kit; Qiagen, Hilden, Germany) were quantified with the PicoGreen double-stranded DNA quantification kit (Molecular Probes, Leiden, The Netherlands). Knowing the exact size of the amplicons (in base pairs) and using the average molecular weight of a single DNA base pair (i.e., 650), the target molecule numbers for each PCR product could be determined and appropriate dilutions series of the PCR products could be used as standards. The linear scope of detection ranged from 10^2 to 10^8 target molecule numbers, with amplification efficiencies of 1.695 (error, 0.025) for *mcrA*, 1.755 (error, 0.01) for *dsrAB*, and 1.830 (error, 0.007) for *fhs*. For quantifying sulfate reducers, a preamplification with the external primer DSR1F and the assay primer DSR4R was performed with the following temperature profile: 96°C for 10 min and then 16 cycles of 96°C for 10 s, 56°C for 10 s, and 72°C for 42 s; fluorescence read at 85°C. The efficiency of this reaction measured on the basis of dilution series of genomic DNA from *D. piger* 749T was found to be 1.57 (error, 0.06). Thus, *dsrAB* gene levels were assumed to be enriched by the preamplification step by a factor of 1.57^{16} (=1,363). Total bacteria were quantified exactly as described previously (41). All samples were run in triplicate, with the mean value used for analysis. The coefficient of variation among replicates was below 1%.

A representative set of real-time quantitative PCR products from randomly chosen patient samples was directly sequenced as described previously (41) and phylogenetic analysis performed using the ARB software package (26). Differences in the microbial population size between disease states and between patients grouped by presence/absence of hydrogenotrophic groups were assessed by the Mann-Whitney test (SPSS 10.0.1; SPSS, Chicago, IL). All errors are reported as standard errors.

### Nucleotide sequence accession numbers.

The partial *mcrA*, *dsrAB*, and *fhs* gene sequences determined in this study have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. EU294497 to EU294506.

### Prevalence, proportions, and interactions of hydrogenotrophic groups.

While methanogens and SRB were detected in samples from periodontitis patients only (43.1 and 41.2%, respectively) acetogens were found in 84.3% of patient samples but also in 64.4% of healthy control subjects (Fig. 1). Only 4.9% of patient samples did not contain any of three functional groups. Single presence occurred in 3.9% (methanogens), 2.9% (SRB), and 30.4% (acetogens) of cases, respectively. Cooccurrence of acetogens with one or both groups was found with roughly equal frequency (between 15 and 19% of cases), while cooccurrence of methanogens and SRB was a rarity (3.9%). The absolute abundance of total bacteria and acetogens was significantly lower in healthy control subjects than in periodontitis patients (*P* < 0.0001) (Fig. 2). Among the periodontitis patients, roughly equal amounts of methanogens and acetogens were found, but the level of SRB was about one

### Table 1. Primer description and thermal profiles for PCR

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Sequence (5’→3’)</th>
<th>Target DNA (approximate size, bp)</th>
<th>Temp profile</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LuF/LuR</td>
<td>GGTGGTGTGGATTTCACACARTAYGCWACAGC/TTTATTGCTAGTTGGRTAGTT</td>
<td><em>mcrA</em> (464)</td>
<td>95°C for 10 min; 40 cycles of 95°C for 10 s, 56°C for 7 s, and 72°C for 25 s; fluorescence read at 78°C</td>
<td>27</td>
</tr>
<tr>
<td>DSR2F/DSR4R</td>
<td>CTGGAAGGAYGACATCAA/GTGTAGCAGTACCAGCA</td>
<td><em>dsrAB</em> (1,400)</td>
<td>96°C for 10 min; 40 cycles of 96°C for 10 s, 56°C for 10 s, and 72°C for 120 s; fluorescence read at 90°C</td>
<td>42</td>
</tr>
<tr>
<td>FTHFS-F/FTHFS-R</td>
<td>TTTACAGGTGACTTCCATGC/GTATTGDGTYTRGCCATACA</td>
<td><em>fhs</em> (1,100)</td>
<td>95°C for 10 min; 40 cycles of 95°C for 10 s, 58°C for 20 s, and 72°C for 42 s; fluorescence read at 85°C</td>
<td>21 (modified)</td>
</tr>
<tr>
<td>EuF/EuR</td>
<td>TCCTACGGGAGGCAAGCAGT/GGACTACCAGGGTATCATAATCCTGTT</td>
<td>16S rRNA gene (466)</td>
<td>95°C for 10 min; 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 25 s; fluorescence read at 80°C</td>
<td>29</td>
</tr>
</tbody>
</table>
order of magnitude lower (Fig. 2). In order to normalize for variations in microbial biomass among samples, absolute abundance data were referred to the total bacterial load. Methanogens made up the largest proportion, with 0.26%, followed by acetogens (0.11%) and SRB (0.01%). The mean proportion of acetogens in the healthy control subjects was 1.67%. Significant antagonistic interactions among the three hydrogenotrophic groups were such that the mean proportion of acetogens was twice as high in SRB-negative samples \((n = 51)\) as in SRB-positive samples \((n = 42)\) \((P = 0.001)\) and 2.4 times higher in methanogen-negative samples \((n = 50)\) than in methanogen-positive samples \((n = 44)\) \((P = 0.028)\). Likewise, the mean proportion of SRB was 2.7 times higher in methanogen-negative samples \((n = 22)\) than in methanogen-positive samples \((n = 44)\) \((P = 0.002)\), but cooccurrence with acetogens did not alter the population size of SRB. Lastly, the mean proportion of methanogens was 3.3 times higher in SRB-negative samples \((n = 24)\) than in SRB-positive samples \((n = 42)\) \((P = 0.001)\) and 9.0 times higher in samples devoid of acetogens \((n = 8)\) than in acetogen-positive samples \((n = 86)\) \((P = 0.001)\).

While the total bacterial loads did not significantly differ in the moderate and severe cases of periodontitis (Fig. 3A), the mean proportions of the hydrogenotrophs were significantly different, although with opposite amplitudes (Fig. 3B to D). The mean proportion of acetogens was reduced by a factor of 3 in the severe cases compared to the moderate cases \((P = 0.088)\). Conversely, the mean proportion of methanogens was elevated by a factor of 4 \((P = 0.038)\) and that of sulfate reducers by a factor of 10 \((P = 0.032)\) in the severe cases compared to the moderate cases.

**Identity of hydrogenotrophic groups.** Phylogenetic analysis of sequenced PCR products confirmed the identity of the target genes. All \(mcrA\) gene sequences except one were highly related to that of *Methanobrevibacter oralis*, with 100% identity at the protein level (Fig. 4) while the remaining sequence was approximately 96% identical at the protein level. Most \(dsrAB\) gene sequences determined in this study formed a distinct clade related to *Desulfovibrio piger* and *Desulfovibrio desulfuricans*, with a sequence identity of approximately 93% at the protein level (Fig. 5). Sequence identities within this clade would be necessary to confirm the identity of the target genes.
were greater than 98%. Its position within the overall tree topology prompted us to test for a possible close relationship to the recently reported periodontal isolate "Desulfovibrio strain NY682" (DSM12803 [19]). A 1,320-bp stretch of the dsrAB sequence of this strain was therefore determined and incorporated into the ARB database. In fact, the dsrAB clade determined in our study shared ancestry with strain NY682 to the exclusion of *D. piger* and *D. desulfuricans*, with a sequence identity of approximately 96% at the protein level. The fhs sequence types determined in our study were highly related to each other, with a protein sequence identity of at least 95%. They formed a distinct lineage with a moderate relationship to the fhs gene from an uncultured microorganism previously identified in the gut systems of ruminants (i.e., clone FPH06; H. Matsui, K. Tajima, and K. Ogata, unpublished data), with a sequence identity of approximately 80% (Fig. 6).

**Hydrogenotrophy and periodontal disease.** This epidemiological survey provides evidence for the ubiquity of hydrogenotrophs in periodontal pockets with niche exclusion (single presence of one hydrogenotrophic group) in approximately 40% of patients. The cooccurrence of two (or all three) groups in the remaining 60% indicates H₂ levels to be sufficiently high.

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**FIG. 3.** Total bacterial amount (rRNA gene copy number) (A) and relative abundances of acetogenic bacteria (B), SRB (C), and methanogenic archaea (D) in subgingival plaque samples from periodontal pockets with a pocket depth of less than 6 mm or of 6 mm and greater. For this analysis, only cases in which a minimum of three periodontal sites belonged clearly to one group were considered. Error bars represent standard errors.

**FIG. 4.** Phylogenetic tree showing the positions of the mcrA gene types identified in subgingival plaque of periodontitis patients relative to those of representative members of methanogenic archaea. Sequences determined in this study are shown in boldface; numbers in parentheses indicate the number of sequenced plaque samples. The tree is based on a distance matrix of deduced amino acid sequences using 150 unambiguously aligned positions and the neighbor-joining approach (with the Kimura correction), via the ARB program package. The scale bar corresponds to 0.1 substitution per amino acid.
in periodontal plaque to allow partitioning of H₂ consumption or alternative metabolic strategies of SRB and/or acetogens. This could include the use of electron donors others than H₂ or the fermentation of short-chain fatty acids with possible production of H₂ (8, 12). In the latter case, a mutual relationship with methanogens rather than competition could be possible (3, 13, 22, 40). The actual in situ activity may depend on the availability of nutrients and the redox potential of the terminal electron acceptors (5). This in turn may vary from host to host and also within plaque biofilms of one patient during the course of disease. Irrespective of such site-specific and temporary varying interactions and of the intersubject variability of the subgingival microflora, our data suggest that from a global point of view, antagonistic interactions, and hence competition among H₂ utilizers, seem to prevail. Although SRB should outcompete methanogens for the substrate H₂ if sulfate is not a limiting factor (10, 25), a recent study provided evidence for the possibility of an opposite order of competitivity (i.e., methanogens outcompeting SRB) in the human colon (38). The close phylogenetic affinity of gut and oral methanogens as well as their proportional dominance over SRB as determined in our study could be a reflection of an analogous situation in subgingival plaque.

The proportions of the hydrogenotrophs were in most cases below 1% of the total microflora. This agrees with a study by Kumar et al. (17), in which 93% of 274 phylotypes identified in subgingival plaque and healthy sites had a proportion of below 1% and approximately 50% of phylotypes were even below 0.1%. Similar magnitudes have also been reported in another study, which found that 99% of phylotypes in soil samples each made up less than 1% of the community (1). This means not only that complex microbial communities may be dominated by “rare” species but that the population size of any given species is not an appropriate measure for assessing its ecological impact. This should in particular be true for organisms for which a high degree of functional redundancy does not exist and which are considered to parallel keystone species in certain environments (e.g., hydrogenotrophs [2]).

The mcrA and dsrAB sequence types determined in our study are in line with previous findings, including those of culture-based studies. For instance, M. oralis and closely related phylotypes are the dominant oral methanogens (9, 23, 23).

![FIG. 5. Phylogenetic tree showing the positions of the dsrAB gene types identified in subgingival plaque of periodontitis patients relative to those of representative members of dissimilatory sulfate reducers. Sequences determined in this study are shown in boldface; numbers in parentheses indicate the number of sequenced plaque samples. The tree is based on a distance matrix of deduced amino acid sequences using 150 unambiguously aligned positions and the neighbor-joining approach (with the Kimura correction), via the ARB program package. The scale bar corresponds to 0.1 substitution per amino acid.

![FIG. 6. Phylogenetic tree showing the positions of the fhs gene types identified in subgingival plaque of periodontitis patients relative to those of representative members of acetogenic bacteria. Sequences determined in this study are shown in boldface; numbers in parentheses indicate the number of sequenced plaque samples. The tree is based on a distance matrix of deduced amino acid sequences using 150 unambiguously aligned positions and the neighbor-joining approach (with the Kimura correction), via the ARB program package. The tree was rooted with the fhs gene sequence of the nonacetogenic Proteus vulgaris. The scale bar corresponds to 0.1 substitution per amino acid.]
41), yet strikingly, the overall archaeal diversity in the human oral cavity seems to be restricted to few members of the genus *Methanobrevibacter*. In addition, most *dsrAB* gene types determined in our study were closely related to the oral isolate *Desulfovibrio* strain NY682* (19), which in turn matches *Desulfovibrio fairfieldensis* based on 16S rRNA tree analysis (19). *D. fairfieldensis*, a resident of the human gastrointestinal tract, has frequently been isolated not only from periodontal pockets (24) but also from various other sites of infection, such as from a pyogenic liver abscess (39), from blood (28), and in association with choleodocholithiasis (34), making this species a potential human pathogen. Its 10-fold increase in severe periodontitis cases as observed in the present study underscores this hypothesis. It should be noted, though, that the diversity of SRB in subgingival plaque is not restricted to *Desulfovibrio* species (19).

In contrast to the sequences discussed above, it remains unclear to which organisms the *fls* sequence types correspond; however, their position within the phylogenetic radiation of known acetogenic organisms (Fig. 6) and the antagonistic interactions with methanogens and SRB, as observed in this study, suggest that they reflect authentic acetogens. It could be speculated that these *fls* sequences correspond to *Treponema* spp. since Lepp et al. (23) found larger proportions of *Treponema* populations at periodontal sites lacking methanogenic archaea, hypothesizing a possible competition for *H2* between these two groups of organisms. However, as opposed to *Treponema* colonizing the guts of termites (20, 32), human-associated *Treponema* spp. have not yet been demonstrated to be capable of (hydrogen-consuming) acetogenesis. Clearly, more work is needed to assess the identity and function of acetogenic bacteria in human plaque biofilms.

**Final consideration.** Unlike the case for acetogens, detectable levels of methanogens and sulfate reducers were only found at diseased sites, with proportions of methanogens and sulfate reducers being significantly elevated in the more severe cases. Although this finding demonstrates association with, rather than causation of, disease, the increased importance of both groups for the progression of periodontitis is evident and principally designates the genes *mcrA* and *dsrAB* as potential biomarkers for progressive periodontal disease with high positive predictive values.

With respect to species diversity and species function, we are only at the beginning of understanding the polymicrobial periodontal disease. An ultimate etiological agent of the various forms of periodontitis may not exist. Instead, the concerted activity of the microbial community as a whole may be one cause of the disease. Attempts to identify fundamental principles driving the infectious process regardless of site- or host-specific community structure might strongly advance our understanding of the disease. The patterns of hydrogenotrophic colonization as described in this study show that interactions among *H2* consumers and *H2* producers in plaque biofilms may be as important as those in other anaerobic environments for the overall functioning of this disease-associated microbial ecosystem.

This work was supported by the START program of the Faculty of Medicine, RWTH Aachen University Hospital, Germany.


