Visualization of Candidate Division OP3 Cocci in Limonene-Degrading Methanogenic Cultures

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Members of candidate division OP3 were detected in 16S rRNA gene clone libraries from methanogenic enrichment cultures that utilized limonene as a carbon and energy source. We developed probes for the visualization of OP3 cells. *In situ* hybridization experiments with newly designed OP3-specific probes [OP3-565 and Eub-338(VI)] revealed abundant small OP3 cocci attached to larger cells. Syntrophic *Deltaproteobacteria*, OP3 cells, and methanogens affiliating with *Methanoculleus* and *Methanoseta* formed the limonene-degrading community.

Keywords: Candidate division OP3, monoterpene, limonene, hydrocarbon degradation, methanogenesis, syntrophy

The bacterial candidate division OP3 was first discovered in 16S rRNA gene libraries generated from sediment of the Obsidian Pool, a hot spring in the Yellowstone National Park [11]. Phylogenetic analysis placed this lineage together with anaerobic ammonium oxidizers (anammox bacteria) and *Planctomycetales* [22, 31]. Candidate division OP3 is ubiquitous in anoxic environments, such as wastewater treatment plants, and was recently found in methanogenic reactors [1, 4, 26, 29]. Metagenomic studies of OP3 identified the catalytic subunit of the anaerobic carbon monoxide dehydrogenase/acetyl-CoA synthase, the obligate anaerobic key enzyme of the Wood–Ljungdahl pathway [6]. The metabolism of OP3 is yet unknown and cultivation of members of this candidate division will assist in revealing their physiology and function in syntrophic communities. In this note, we report the observation of OP3 cells by catalyzed reporter deposition-fluorescence *in situ* hybridization experiments (CARD-FISH) in methanogenic enrichment

cultures initiated on monoterpenes [7]. Limonene, the monoterpene substrate used in this study, is produced by the plastids of plants [12, 15] and is, alongside toluene, a major indoor volatile organic compound. Sediment-free enrichments were transferred once a year with 2–5% limonene in 30 ml of 2,2,4,6,8,8-heptamethylnonane (HMN), in 300 ml of methanogenic medium [7]. Cultures produced methane for up to two years (Fig. 1). Limonene consumption and methane formation were demonstrated by headspace gas chromatography on a Shimadzu 14B gas chromatograph with flame ionization detector and an isothermal separation

Fig. 1. Methane production (diamonds) and growth (circles) of limonene-utilizing enrichment cultures in the presence or absence of acetate (2 mM, filled and open symbols, respectively).

Lower panel: transient acetate formation in the culture without 2 mM acetate coincided with a longer lag phase of the culture. Observations represent average values of two replicates.

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at 200°C and 110°C, respectively. Over 250 days of incubation, cell-free controls did not show limonene loss. After 250 days of incubation, analyses of limonene

consumption and methane production revealed a recovery of nearly 60% of the carbon from limonene as methane gas. This suggested the stoichiometry: $C_{10}H_{16} + 6 H_2O \rightarrow$

Fig. 2. Maximum parsimony tree of *Bacteria* 16S rRNA gene sequences.

Crenarchaeota were used as the outgroup. The phylotypes obtained in this study are emphasized in bold, and in parenthesis is the number of full 16S rRNA gene sequences with identity above 98.5%. Related sequences of representative microorganisms can be accessed at the NCBI using the tags given in parenthesis. The scale bar represents 10 substitutions per 100 nucleotides.

7 CH₄ + 3 CO₂ ($\Delta G_0' = -348 \text{ kJ mol}^{-1}$ limonene). Transient formation of fatty acids in the medium (Fig. 1) was observed by HPLC [25]. Acetate was routinely added in small amounts in the medium (2 mM). It facilitated maintenance of the methanogenic community after transfer. In the absence of acetate, solely with limonene, a longer lag phase of 3 months was observed before methane production started (Fig. 1). These cultures transiently produced acetate, up to 0.2 mM (Fig. 1). Thus, acetate is involved as an intermediate in the syntrophic limonene degradation, likely being synthesized by syntrophic *Bacteria* and consumed by acetoclastic, methanogenic *Archaea*.

We selected the 16S rRNA gene approach to identify the community diversity and CARD-FISH studies to reveal the community structure. Genomic DNA of a 5 ml cell culture was extracted with the Qiagen genomic tip 100/G according to the manufacturer's instruction (Qiagen, Hilden, Germany). A 25 ng amount of high-molecular weight DNA was used to amplify the 16S rRNA gene with bacterial primers 8F and 1492R [9, 14] and archaeal primer pairs 21F-958R and 21F-1492R [5, 28]. The reaction mix consisted of 2 μM of each primer, 0.2 mM dNTPs, 0.04 U Taq polymerase, and 1 $\times$ PCR buffer in 50 μl . After an initial denaturing step of 4 min at 94°C, polymerase was added at 80°C. The 32 cycles involved a denaturing step for 1 min at 94°C, 1 min primer annealing at 42°C for *Bacteria* and 58°C for *Archaea*, and 3 min elongation at 72°C. The A-overhang for cloning was introduced by a final elongation at 60°C for 60 min. The bacterial and archaeal amplicons were purified and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and the pCR4-TOPO vector (Invitrogen, Grand Island, NY, USA), respectively. The recombinant plasmids were introduced into *E. coli* DH5 α (Promega, Madison, WI, USA) and *E. coli* TOP10 (Invitrogen, Grand Island, NY, USA), respectively. Amplified rDNA restriction analysis (ARDRA) with the restriction endonucleases *Bsu*RI and *Rsa*I on 327 *Bacteria* clones and 141 *Archaea* clones and partial sequencing of 100 randomly selected clones directed the full sequence analyses of representative 16S rRNA genes (accession numbers FN646432 to FN646495). Maximum parsimony phylogenetic trees were calculated excluding the influence of highly variable positions in ARB [18, 23]. 16S rRNA gene sequences with more than 98.5% identity were regarded as one operational taxonomic unit (OTU) [27]. Phylogenetic affiliations of the 35 representative 16S *Bacteria* sequences and 28 representative 16S *Archaea* sequences are shown in Fig. 2 and 3, respectively.

The characterization of the community structure required the design of novel probes targeting OP3 sequences, Eub-338(VI) (5'-GCAGCCTCCCGTAGGAGT-3') and OP3-565 (5'-TACCTGCCCTTTACACCC-3'). Probes were developed *in silico* with the software ARB [http://www.arb-home.de, 18, 23]. OP3-565 had 1.2 weighted mismatches to

Fig. 3. Maximum parsimony tree of *Archaea* 16S rRNA gene sequences.

Crenarchaeota were used as the outgroup. The phylotypes obtained in this study are emphasized in bold, and in parenthesis is the number of full 16S rRNA gene sequences with identity above 98.5%. Related sequences of representative microorganisms can be accessed at the NCBI using the tags given in parenthesis. The scale bar represents 10 substitutions per 100 nucleotides.

Thialkalivibrio halophylus (DSM 15791) and *Marinitoga piezophila* (DSM 14283), which served as the control for hybridization experiments with horseradish peroxidase (HRP) 5'-modified probes [21]. Newly designed and previously tested probes [17] were applied: Eub-338(I) [2] in equimolar mix with probe Eub-338(VI) designed in this study, Arch-915 [28], CF-319a [19], Delta-495a in equimolar mix with its competitor [16], and the newly designed OP3-565. As the control, we used the nonsense probe Non-338, which gave one signal for 300 DAPI-stained cells.

Microscopic observations showed the presence of numerous morphotypes in the enrichment, from small cocci and vibrios to long filaments (Fig. 4a). *Bacteria* represented 40% of the microbial community, whereas *Archaea* represented 33%, as detected by CARD-FISH with phyla-specific probes (Eub338 I+VI, Arch915). The general *Archaea* probe Arch-915 targeted *Methanosaeta*-like filaments and numerous rod-shaped cells. *Archaea* 16S rRNA gene sequences were related to *Methanosaeta* (>99% identity) and far related to *Methanoculleus palmolei* (91.6% identity) and *Methanospirillum hungatei*

Fig. 4. Microscopic images of samples from methanogenic enrichment cultures thriving on limonene, as visualized by (a) phase contrast microscopy and (b) epifluorescence microscopy with a newly designed OP3-specific probe, OP3-565, and (c) the same microscopic field visualized by a general DNA dye, DAPI.

(Fig. 3). *Methanosaeta* are slowly growing acetoclastic methanogens with high affinity to acetate [13]. The genera *Methanospirillum* and *Methanoculleus* represent hydrogen- and formate-utilizing methanogens [32]. This suggests that acetate as well as formate and hydrogen are involved in the electron transfer from limonene-degrading microorganisms to methanogens.

Most of the bacterial clones retrieved from the enrichment cultures showed low 16S similarity with cultured microorganisms of the following *Bacteria* lineages: candidate division OP3, *Deltaproteobacteria*, *Bacteroidetes*, and *Firmicutes* (Fig. 2). The first three groups were dominant in the clone library. However, specific probes for *Deltaproteobacteria* (Delta-495a) and OP3 (OP3-565) accounted collectively for almost the entire bacterial population (30% of all cells), whereas *Bacteroidetes* accounted for less than 1% of all DAPI-stained cells.

Bacteroidetes were related to *Prolixibacter bellariivorans* (81% to 89% identity) and *Alistipes putredinis* (84.1%). Genus *Prolixibacter* is represented by facultative anaerobes [10], whereas genus *Alistipes* includes strictly anaerobic microorganisms [24]. The low numbers of *Bacteroidetes* suggests no or a minor function for these cells in the syntrophic degradation of limonene.

Firmicutes were represented by only one phylotype with 91.2% identity to *Syntrophomonas* sp. and only 95% to the closest uncultured relative, a sequence retrieved from a reactor degrading organic waste. *Syntrophomonas* and *Syntrophobacter* are the ideal candidates for syntrophic transformation of fatty acids and alcohols into acetate, hydrogen, and carbon dioxide [20]. The *Deltaproteobacteria* curved rods were found abundant (12%) in limonene-degrading enrichments during mid-exponential growth, as identified by CARD-FISH with a specific probe (Delta-495a). *Deltaproteobacteria* phylotypes were related to *Syntrophobacter* (Fig. 2), having as closest relative (90–96% identity) *Syntrophobacter fumaroxidans* strain MBOP [8].

The abundance of candidate division OP3 cells attached to rod-shaped microorganisms indicates a more complex

degradation pathway. They made up for 18% of all cells in the limonene-consuming methanogenic community, as determined by a specific designed probe (OP3-565). Cells of candidate division OP3 were small and round-shaped (Fig. 4b), and they were free-living or found attached to larger cells of distinct phylogeny (Fig. 4b and 4c). The abundance of candidate division OP3 members during exponential growth on limonene suggests an important role in the degradation of this monoterpene. Because syntrophic cultures are often limited by the flux of electron-transferring molecules between the partners, we consider based on the attachment to larger cells that candidate division OP3 cells could actually contribute to the pool of electron-transferring intermediates in limonene syntrophic degradation. One possibility is the production of formate, described as a superior alternative to hydrogen as a transfer intermediate under mesophilic conditions [3, 30].

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