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Stable-Isotope Probing of Microorganisms Thriving at Thermodynamic Limits: Syntrophic Propionate Oxidation in Flooded Soil

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Propionate is an important intermediate of the degradation of organic matter in many anoxic environments. In methanogenic environments, due to thermodynamic constraints, the oxidation of propionate requires syntrophic cooperation of propionate-fermenting proton-reducing bacteria and H_2-consuming methanogens. We have identified here microorganisms that were active in syntrophic propionate oxidation in anoxic paddy soil by rRNA-based stable-isotope probing (SIP). After 7 weeks of incubation with [^{13}C]propionate (<10 mM) and the oxidation of ~30 μmol of ^{13}C-labeled substrate per g dry weight of soil, we found that archaeal nucleic acids were ^{13}C labeled to a larger extent than those of the bacterial partners. Nevertheless, both terminal restriction fragment length polymorphism and cloning analyses revealed *Syntrophobacter* spp., *Smithella* spp., and the novel *Pelotomaculum* spp. to predominate in "heavy" ^{13}C-labeled bacterial rRNA, clearly showing that these were active in situ in syntrophic propionate oxidation. Among the *Archaea*, mostly *Methanobacterium* and *Methanosarcina* spp. and also members of the yet-uncultured "rice cluster I" lineage had incorporated substantial amounts of ^{13}C label, suggesting that these methanogens were directly involved in syntrophic associations and/or thriving on the [^{13}C]acetate released by the syntrophs. With this first application of SIP in an anoxic soil environment, we were able to clearly demonstrate that even guilds of microorganisms growing under thermodynamic constraints, as well as phylogenetically diverse syntrophic associations, can be identified by using SIP. This approach holds great promise for determining the structure and function relationships of further syntrophic or other nutritional associations in natural environments and for defining metabolic functions of yet-uncultivated microorganisms.

In many anoxic environments, which are low in electron acceptors such as oxygen, nitrate, sulfate, and iron or manganese oxides, complex organic matter is degraded to methane and CO_2 by the cooperation of anaerobic microorganisms of several metabolic guilds (47). An important intermediate of organic matter conversion under methanogenic conditions is propionate, which may account for up to 35% of methanogenesis in anaerobic digestors (35) and up to 30% in paddy soil (15, 26). The degradation of propionate to acetate, CO_2, and 3 H_2 is highly endergonic under standard conditions ($\Delta G^{\circ\prime}$ = +76.1 kJ/mol), but it can be accomplished by syntrophic cooperation of propionate-oxidizing hydrogen-producing bacteria and hydrogen (or formate)-scavenging partner microorganisms (methanogens), which maintain a low hydrogen partial pressure (for a review, see reference 47). Only in such syntrophic associations does propionate degradation become feasible under methanogenic conditions.

The process has been studied extensively in flooded rice field soil (26, 27, 58), in upflow anaerobic sludge blanket reactors (52), and sediments (46, 49). In all environments studied, the methyl-malonyl-coenzyme A ("randomizing") pathway of propionate degradation appears to be predominantly operative (see reference 26 and references therein). Propionate oxidation in rice field soil is under tight thermodynamic constraint, since the observed Gibbs free energies available to both syntrophic propionate-oxidizing microorganisms and their methanogenic partners range only between −3 and −25 kJ per mol of propionate (2, 15, 26, 58). Thus, less than one-quarter ATP may be available to both organisms if a minimum of −70 kJ per mol of ATP is assumed for irreversible ATP synthesis (47).

Our current knowledge of the diversity and ecology of syntrophically propionate-oxidizing bacteria is based on only few pure cultures such as the δ-proteobacterial *Syntrophobacter wolinii* (1, 55), *Syntrophobacter fumaroxidans* (17), *Syntrophobacter pfennigii* (56), and *Smithella propionica* (28). Recently, two thermophilic gram-positive spore-forming strains, *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* (39) and *Pelotomaculum thermopropionicum* (22), have also been described. Cultivation-independent studies have focused on the distribution and dynamics of *Syntrophobacter* spp. in anaerobic granular sludge (9, 12, 16, 18, 21, 50). Because of many difficulties in cultivating microorganisms of this highly fastidious guild and the lack of a suitable molecular marker for targeting syntrophic propionate-oxidizing acetogens, the natural diversity and ecology of syntrophic propionate-oxidizing bacteria remains largely unexplored.

A direct way to identify microbial populations active in a defined metabolic process is stable-isotope probing (SIP). SIP is based on the incorporation of ^{13}C-labeled substrate into cellular biomarkers such as nucleic acids, separation of labeled from unlabeled nucleic acids by density gradient centrifugation, and molecular identification of active populations carrying labeled nucleic acid (40). DNA-SIP has been successfully applied to directly link structure and function of microbial communities (14, 20, 38, 41). Recently, SIP has been extended

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to the analysis of rRNA (33, 34, 36) and has also been directly applied in the field (24).

In the present study, we have used SIP of rRNA for the first time in an anoxic soil system to study populations involved in propionate degradation under methanogenic conditions. In principle, propionate should be an excellent substrate for SIP, since it can only be degraded syntrophically under methanogenic conditions, i.e., by a narrowly defined guild of microorganisms. Nevertheless, the intermediates of syntrophic propionate oxidation acetate and bicarbonate (or formate) may be assimilated by other microorganisms nonspecifically, which is an inherent limitation of SIP. But when electron acceptors other than CO_2 are not available in the system, acetate, for instance, can only be catabolized by methanogens and not fermented for thermodynamic reasons (47). However, the diversity of species involved as anticipated from the currently known range of syntrophic propionate oxidizers is limited, and thus targeting syntrophs should be most suitable to test whether SIP can also be successfully applied to detect specifically active microorganism under anoxic conditions. Although cell yield and specific substrate degradation rates of syntrophic microorganism are low compared to microorganisms that thrive on thermodynamically more favorable reactions, we have been able to detect bacteria known to be involved in syntrophic propionate oxidation by RNA-SIP.

MATERIALS AND METHODS

Incubation setup. Rice field soil was collected from an Italian rice field soil near Vercelli in 2001, air dried, stored, and sieved as described previously (2). Soil from the same sampling site has been described previously to contain ~1.5% organic carbon (59). In 150-ml serum vials, 20 g of dry soil was mixed with 20 ml of sterile, anoxic water. Vials were sealed with sterile rubber septa after the headspace was flushed with N_2. The soil was preincubated statically at 25°C in the dark for 14 days to allow for the activation and growth of a methanogenic rice field soil microbial community and the reduction of alternative electron acceptors such as iron(III) and sulfate (32). Also, a 10-ml glass tube containing 2 ml of 5 M NaOH was fitted vertically into vials as a CO_2 trap. Isotope labeling of microbial populations was initiated by adding $^{13}C_3$-labeled propionate (99 atom%; Isotec, Miamisburg, Ohio) to two slurries at a final concentration of ~10 mM in pore water (200 μmol in all), whereas controls received unlabeled propionate. At intervals of 3 to 4 days, the total CH_4 formed in slurries was monitored by gas chromatography (GC) (45), and $^{13}C/^{12}C$ isotope ratios of CH_4 and CO_2 were determined by GC-isotope ratio mass spectrometry (5). At intervals of ~10 days, 0.2 ml of slurry water was sampled for quantification of fatty acids by high-pressure liquid chromatography (25). After 2 days, 3 weeks, and 7 weeks of incubation, microcosms were opened, and soil samples stored frozen (−20°C) for subsequent nucleic acid extraction.

Nucleic acid extraction and gradient centrifugation. Ribosomal nucleic acids were extracted from ~2 g of slurry samples according to a cell lysis protocol involving bead beating in the presence of the denaturant sodium dodecyl sulfate, phenol-chloroform-isoamyl alcohol extraction, and polyethylene glycol precipitation as previously described (33). After purification and quantification, 500 ng of rRNA extracts were loaded into cesium trifluoroacetate (CsTFA) centrifugation medium and resolved by equilibrium density gradient centrifugation (33). Gradients were then fractionated, and nucleic acids were precipitated for subsequent quantitative and qualitative community analyses.

Domain-specific PCR quantification of density-resolved nucleic acids. rRNA from gradient fractions was quantified by real-time reverse transcription-PCR with primers Ba519f/Ba907r and Ar109f/Ar912rt targeting all *Bacteria* and *Archaea*, respectively (33). Copy numbers were standardized by using dilution series (10^7 to 10^2 molecules μl^{-1}) of 16S rRNA in vitro transcripts from cloned almost full-length 16S rRNA genes of a *Methylophilus* spp. (accession number AY360527) and a *Methanosarcina barkeri* strain (accession number AY641448) by using a RiboProbe in vitro transcription kit according to the manufacturer's instructions (Promega, Mannheim, Germany).

Community analyses. Terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of density-resolved bacterial and archaeal communities from gradient fractions was done with primers Ba27f-FAM/Ba907r and Ar109f/Ar912rt-FAM (32, 34), and amplicons were digested by using MspI and *Taq* for *Bacteria* and *Archaea*, respectively. Digested amplicons (100 ng in 10 μl) were desalted by using AutoSeq G-50 columns (Amersham Biosciences, Freiburg, Germany). Desalted digests (1 μl) were mixed with 12 μl of Hi-Di formamide (Applied Biosystems) and 0.2 μl of ROX-labeled MapMarker 1000 ladder (BioVentures, Murfreesboro, Tenn.), denatured (3 min at 95°C), cooled on ice, and size separated on an ABI 310 genetic analyzer (Applied Biosystems, Weiterstadt, Germany). Electrophoresis was performed with POP-4 polymer in 47-cm-by-50-μm capillaries (Applied Biosystems) under the following conditions: a 2-s injection time, a 15-kV injection voltage, a 15-kV run voltage, a 60°C run temperature, and a 45-min analysis time.

Amplicons for sequence analysis were generated from rRNA gradients with the primers Ba27f/Ba907r and Ar109f/Ar912rt and PCR conditions as described earlier (32, 34). Fragments were cloned by using the pGEM-T Vector System II (Promega, Mannheim, Germany), and randomly selected clones were sequenced on an ABI Prism 377 sequencer (Applied Biosystems) by using BigDye terminator cycle sequencing chemistry as specified by the manufacturer. Phylogenetic analyses were conducted by using the ARB software package (http://www.arb-home.de), as described previously (13, 30), and sequence data were deposited with GenBank under accession numbers AY607105 to AY607271.

RESULTS

Consumption of [^{13}C]propionate. Before administering the ^{13}C-labeled substrate, we preincubated microcosms for 14 days to allow for the activation and development of an anoxic rice field soil microbiota. Electron acceptors such as nitrate, sulfate, and iron(III) are typically reduced after 8 to 12 days in Vercelli rice field soil (23, 26, 32), which is an important prerequisite for the selective labeling of propionate-oxidizing syntrophs intended in this experiment. After 2 weeks of preincubation, methane in the headspace had accumulated to ~3.5 kPa, which is indicative of the reduction of sulfate and iron(III) in rice field soils (57). In incubations of homogenized and sieved rice field soil slurries, i.e., rice straw and root pieces were removed, propionate may transiently accumulate up to a concentration of 1.5 mM within the first 2 weeks after flooding (2, 32, 43, 58), which corroborates the transition from iron(III)-dependent to syntrophic propionate oxidation (32, 58). In the present experiment, we supplemented [^{13}C]propionate at a concentration of 10 mM, which is about 1 order of magnitude higher than the substrate concentrations transiently encountered in incubations with sieved and homogenized rice field soil. Locally in anoxic rice field soil, however, where high concentrations of organic matter (i.e., decomposing rice straw fibers or roots) prevail, propionate may accumulate up to 8 mM, as shown in rice field soil incubations amended with rice straw (15).

Syntrophic propionate oxidation was evidently operative by the time of ^{13}C-labeled-substrate addition in our experiment: the ^{13}C atom% of formed CH_4 increased to ~3% after 4 days. Values constantly increased to more than 40% after 4 weeks of incubation and approached 50% near the end of the experiment, after 7 weeks. The partial pressure of methane (pCH_4) in the headspace was ~30 kPa after this time, whereas pCO_2 was kept low (~0.1 kPa) throughout the incubation by alkaline trapping. Because of this constant removal, the ^{13}C atom% of formed CO_2 was a much more sensitive indicator of [^{13}C]propionate depletion in the slurry than that of accumulated CH_4. Twice during the experiment, after 19 and 33 days of incubation, when decreasing ^{13}C atom% values of CO_2 were indicative of the depletion of the labeled substrate, [^{13}C]propionate

(10 mM final concentration each) was replenished to the microcosm. At these time points, propionate was below the detection level. Thus, in total, 600 μmol of [^{13}C]propionate were supplemented to the soil. The amount of carbon added (23 mg in total) thus represented only a small fraction of the ~1.5% organic carbon (i.e., ~300 mg in 20 g of soil) naturally present in Italian rice field soils from Vercelli (43, 59). Since [^{13}C]propionate was almost completely consumed after 7 weeks (0.77 mM final concentration or 15 μmol in total remained in the system), the average rates of propionate consumption were ~0.6 μmol per g (dry weight) and day, or ~30 μmol per g (dry weight) in all. A total of ~670 μmol of $^{13}CH_4$ was formed during incubation corresponding to ~64% of the expected amount of $^{13}CH_4$, assuming that 1 mol of propionate is completely converted to 1.75 mol of CH_4 and 1.25 mol of CO_2 (47).

Nucleic acid distribution in centrifugation gradients. rRNA was extracted from slurries after 2 days of incubation with [^{12}C]propionate (as unlabeled control) and after 3 and 7 weeks of incubation with [^{13}C]propionate. This time series was used to trace and identify microorganisms by RNA-SIP, which became specifically active under methanogenic conditions and oxidized propionate syntrophically. The day 2 control gradient clearly showed peaks of bacterial and archaeal rRNA at a BD, which is characteristic of unlabeled nucleic acids, i.e., <1.79 g/ml in CsTFA (33, 34) (Fig. 1). Maximum archaeal rRNA amounts equaled ~12% of the maximum bacterial rRNA quantities detectable.

After 3 weeks of incubation, the rRNA gradient profile of [^{13}C]propionate-oxidizing rice field soil had not changed and was virtually identical to the control gradient of day 2 (data not shown). Hence, even though propionate had been oxidized syntrophically for 3 weeks and ~10 μmol of [^{13}C]propionate had been consumed per g (dry weight) of soil, label incorporation into any of the involved microbial populations was still too weak to be detected by RNA-SIP.

After 7 weeks of incubation, however, gradient profiles had changed (Fig. 1). Although the bulk of bacterial rRNA still remained in the "light" fractions and only a minute tailing of "heavy" rRNA was detected, the entire archaeal rRNA had completed a substantial shift toward "heavier" BDs and now banded between 1.79 and 1.81 g/ml in CsTFA. Also, a quantitative stimulation of the archaeal population was apparent, and maximum amounts of detectable archaeal nucleic acids now equaled ~19% of bacterial rRNA quantities.

Fingerprinting of density-resolved nucleic acids. Bacterial and archaeal rRNA templates resolved within the centrifugation gradients of day 2 and day 50 were analyzed by T-RFLP fingerprinting to trace specific members of the rice field soil microbial community that had incorporated ^{13}C label into their rRNA and therefore had potentially been active in syntrophic [^{13}C]propionate oxidation. For bacterial templates, the gradient profile displayed only small amounts of "heavy" rRNA (Fig. 1), indicating a low rate of label assimilation. Control fingerprints generated for different gradient fractions of the unlabeled day 2 control rRNA (1.772 to 1.798 g ml^{-1} in CsTFA) showed highly similar microbial communities dominated by the 146-, 154-, 162-, 273-, and 513-bp terminal restriction fragments (T-RFs) (Fig. 2). However, after 50 days of incubation with [^{13}C]propionate, the anoxic rice field soil bacterial community detectable in the "light" gradient fractions (between 1.769 and 1.802 g ml^{-1}) had apparently shifted significantly in relative composition. Although the 146-, 154-, 273-, and 513-bp T-RFs were still detectable (with reduced abundance), the 217-, 298-, and 519-bp T-RFs had increased their prominence, and a T-RF of 207 bp now clearly dominated the electropherograms. However, most strikingly, in the "heaviest" fraction (1.812 g ml^{-1}) containing considerable amounts of rRNA, the bacterial community composition completely changed. A 151-bp T-RF became dominant, and T-RFs of 70, 167, 302, and 510 bp also appeared. However, the 207- and 298-bp T-RFs also remained detectable. These data show clearly that, despite a low rate of ^{13}C label assimilation, RNA-SIP effectively resolved a small fraction of the bacterial community that had incorporated substantial amounts of ^{13}C label (fully ^{13}C-labeled bacterial rRNA bands between 1.81 and 1.82 g ml^{-1} in CsTFA [33]).

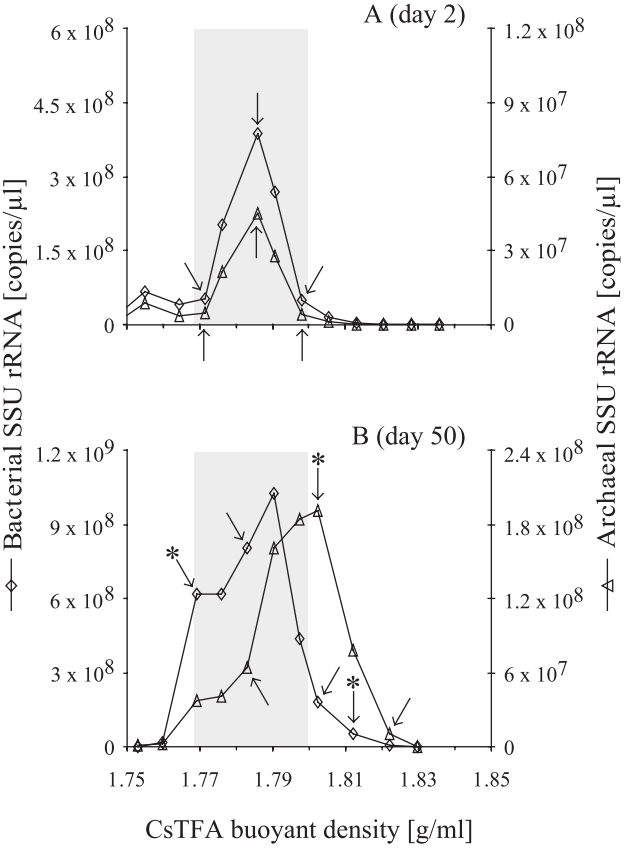

FIG. 1. CsTFA density gradient centrifugation of rRNA extracted from anoxic rice field soil after 2 (A) and 50 (B) days of syntrophic propionate oxidation. Domain-specific template distribution within gradient fractions was quantified with real-time reverse transcription-PCR. The density-range characteristic for the light rRNA is shaded in gray. Fractions from which T-RFLP fingerprints (arrows) or clone libraries (asterisks) of selected templates were generated subsequently are indicated.

In contrast, although ^{13}C labeling appeared to be much more efficient for *Archaea* than for *Bacteria* (see Fig. 1), fingerprints of density-resolved archaeal rRNA showed no pronounced dynamics, either with time or with BD (Fig. 3). Thus, the day 2 and day 50 T-RFLP electropherograms, although generated over different density ranges (1.772 to 1.798 g ml^{-1}

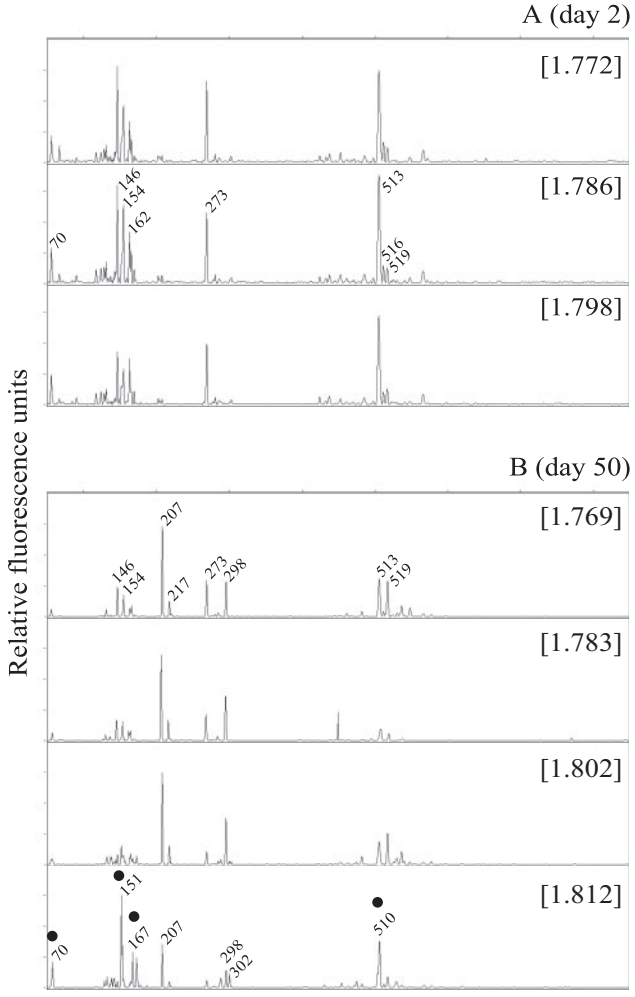

FIG. 2. T-RFLP fingerprints of density-resolved bacterial communities generated from selected rRNA gradient fractions as indicated in Fig. 1. CsTFA BDs (in grams per milliliter) of fractions are given in brackets. The specific fragment lengths (in base pairs) of important T-RFs (as mentioned in the text) are given. T-RFs representing identified lineages of syntrophic propionate oxidizers are also marked (●).

and 1.783 to 1.822 g ml^{-1}, respectively), showed very similar archaeal communities. Both bulks of archaeal rRNA were dominated by the 86-, 94-, 188-, 286-, 395-, and ~800-bp T-RFs, and only the 94-bp T-RF was of clearly increased abundance after 50 days of incubation.

Sequence analysis of density-resolved populations. Having shown, with the help of T-RFLP fingerprinting, that bacterial populations detected in the "heaviest" rRNA fraction after 50 days of [^{13}C]propionate oxidation were distinct, clone libraries were generated to identify and distinguish microbes represented in the resolved rRNA fractions. Randomly selected clones of two libraries were sequenced that were generated from "light" (X9Ba clones [$n = 74$] at 1.769 g ml^{-1}) and "heavy" (X3Ba clones [$n = 48$] at 1.812 g ml^{-1}) bacterial templates. Phylogenetic analysis of the "light" clones revealed a community clearly dominated by highly diverse populations of clostridia (68% of all clones; Table 1), but bacilli (14%), *Geobacteriaceae* (8%), and sequences related to the "T78 group" (51) of uncultured bacteria (5%) were also detected. In contrast, the clone library of "heavy" templates resolved within the same gradient was of a completely different composition (Table 1). Here, 60% of all clones were related closely to syntrophic propionate oxidizers (29% to *Syntrophobacter* spp., 21% to the novel *Pelotomaculum* spp., and 10% to *Smithella* spp.), and a further 6% were related to butyrate oxidizers of the genus *Syntrophomonas*. However, sequences related to microorganisms not known as syntrophs were also detected in this library, and the remaining one-third of the clones was related to diverse lineages of the anoxic rice field soil community (19).

The phylogenetic placement of selected clones, mostly related to syntrophic propionate oxidizers, is depicted in Fig. 4 and 5. Sequence data also showed that the composition of the clone libraries was consistent with generated T-RFLP fingerprints of the resolved gradient fractions. Thus, the diverse *Clostridia* detected were mostly reflected in the 207-bp T-RF but also the 217-, 273-, 298-, 513-, and 519-bp T-RFs (Table 1). Another abundant T-RF in "light" fractions (146 bp) was affiliated with the detected bacilli. On the other hand, most of the prominent T-RFs detected in the "heavy" rRNA clearly represented syntrophic propionate oxidizers, e.g., *Syntrophobacter* spp. (70 and 167 bp) and *Smithella* spp. (510 bp)

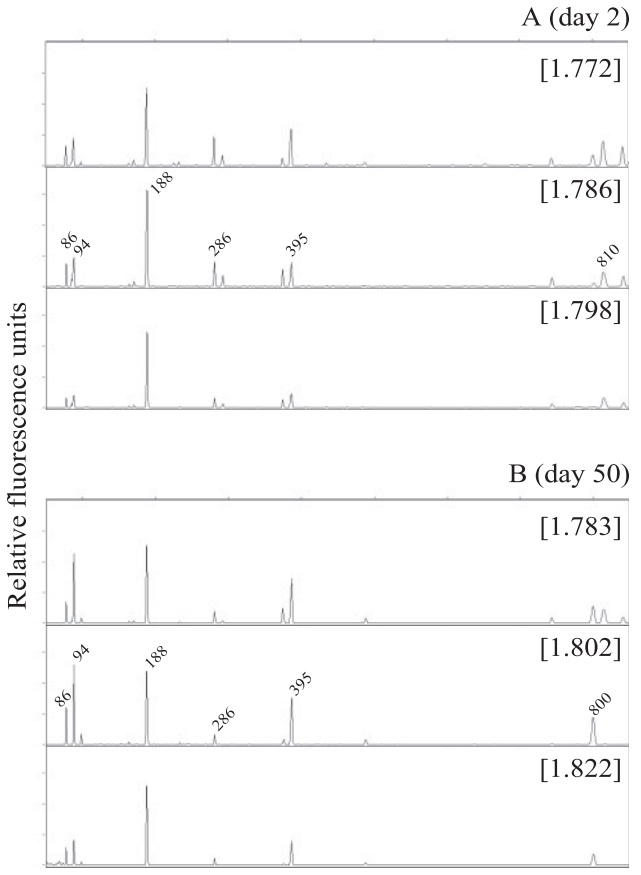

FIG. 3. T-RFLP fingerprints of density-resolved archaeal communities generated from selected rRNA gradient fractions as indicated in Fig. 1. CsTFA BDs (in grams per milliliter) of fractions are given in brackets. The specific fragment lengths (in base pairs) of important T-RFs (as mentioned in the text) are given.

TABLE 1. Phylogenetic affiliations and numbers of bacterial 16S rRNA clones retrieved in libraries generated from density-resolved nucleic acids[a]

Phylogenetic group	No. of clones		Characteristic[b] T-RFs (bp)
	X3Ba ("heavy")[c]	X9Ba ("light")[c]	
δ-*Proteobacteria*			
Syntrophobacter spp.	14		**69, 70, 167**
Smithella spp.	5		**510**
Geobacter spp.	1	6	**131**
WCH1B-27 group	1		163
Firmicutes			
Bacilli	1	10	**146**
Clostridia	5	50	**196, 207, 217, 273, 298, 513, 519**
Pelotomaculum spp.	10		**151**
Desulfosporosinus spp.		1	142
Selenomonas spp.		1	300
Syntrophomonas spp.	3		**302**
Actinobacteria	1		136
Acidobacteria	1		?
Chlorobi		1	219
Bacteroidetes	2		140
Clone T78 group	1	4	**516**
Candidate division BRC-1	1		494
Candidate division OP-10	1	1	165, 305
Unidentified	1		136

[a] Characteristic T-RFs for different clone groups are given.
[b] T-RFs were detected for a major group of clones within a lineage. T-RFs detected for more than one clone within a group are indicated in boldface.
[c] ^{13}C enrichment of rRNA.

(Fig. 4), and the novel gram-positive *Pelotomaculum* spp. (151-bp T-RF) (Fig. 5). Furthermore, the minor 302-bp T-RF, found only in the "heavy" fraction, was assigned to *Syntrophomonas* spp.

An archaeal clone library from intermediately ^{13}C-labeled rRNA templates (X4Ar clones [$n = 45$] at 1.802 g ml^{-1}) was also generated, and randomly selected clones were sequenced. Rice field soil archaeal communities are well studied by T-RFLP fingerprinting, and most detectable T-RFs have already been assigned to defined archaeal lineages for this habitat (4, 31, 44). As indicated already by the fingerprints of archaeal rRNA resolved within the different gradient fractions (Fig. 3), mostly well-known methanogens were identified in the archaeal clone library, e.g., *Methanosarcina* spp. (56% of all clones, 188-bp T-RF), *Methanobacterium* spp. (24%, 94 bp), *Methanomicrobiaceae* (11%, 86 bp), and the yet-uncultured methanogens of rice cluster I (7%, 395 bp).

DISCUSSION

To date, nucleic acid-based SIP has been successfully used to identify microbes, which thrive on thermodynamically favorable reactions, e.g., the aerobic oxidation of methanol (34, 40) or methane (20, 38), nitrate-dependent methanol oxidation (14), and the aerobic oxidation of aromatic compounds (24, 36). Here, we present the first identification of active anaerobic microorganisms by SIP, which are involved in the thermodynamically unfavorable syntrophic oxidation of propionate under methanogenic conditions. More importantly, we have been able to identify syntrophic propionate oxidizers for the first time as a whole guild by a molecular approach in their natural habitat. Since known syntrophic microorganisms are not a phylogenetically coherent group but instead are scattered over several lineages of descent, previous molecular studies were bound to utilize group-specific probes for a rather incomplete assessment of this specialized guild of microorganisms (9, 11, 16, 21, 50).

Thus far, the sensitivity of SIP has not been explored by targeting microorganisms with low substrate conversion rates. In a previous SIP study investigating aerobic methanol oxidation in rice field soil, methanol oxidation rates were ~16 μmol per g (dry weight) and day, and ^{13}C-labeled rRNA was detectable already after 6 days of incubation. Despite the low rate of [^{13}C]propionate consumption (~0.6 μmol per g [dry weight] and day) measured in our experiment, sufficient amounts of ^{13}C label for detection were incorporated into rRNA, albeit after 7 weeks of incubation. This shows clearly that even slowly growing bacteria that utilize small amounts of energy for growth such as syntrophic acetogens can in fact be detected by SIP.

Although the prolonged incubation clearly caused a shift within the entire bacterial community, the bulk of bacterial rRNA was still unlabeled, probably also due to the constant removal of formed $^{13}CO_2$ via an alkaline trap, which minimized possible cross-feeding of label into community members not involved in syntrophic propionate oxidation. After 50 days, the "light" bacterial rRNA was clearly dominated by the 207- and 298-bp T-RFs, representing defined groups of clones related to *Clostridium cellulolyticum* and *Clostridium aminovalericum*. Clostridia represent numerically dominant members of the anoxic bulk soil community in rice field soil, which are involved in the degradation of polymeric rice plant residues (3).

Although most of the bacterial community was not active in [^{13}C]propionate consumption, as indicated by the massive bulk of "light" rRNA detected after 7 weeks of incubation, we were indeed able to detect a small amount of bacterial rRNA present in the gradient fraction where "heavy" rRNA is typically found (33). More important, by T-RFLP fingerprinting we could prove that the rRNA templates present in this fraction were distinct from the "light" community, which is an important prerequisite for the correct interpretation of environmental SIP data (34, 36). And finally, cloning of these "heavy" templates clearly showed that three different lineages of syntrophic propionate oxidizers were active at the same time, belonging to the δ-*Proteobacteria* (*Syntrophobacter* and *Smithella* spp.) and to the novel genus *Pelotomaculum*, which is closely related to the gram-positive sulfate-reducing members of the genus *Desulfotomaculum* (22). *Smithella* spp. have not been detected thus far in environments other than methanogenic bioreactors (28). It is unlikely (although we did not explicitly measure this) that sulfate was available as electron acceptor after the 2 weeks of preincubation to predominant bacterial species detected, i.e., *Syntrophobacter*. This would have facilitated nonsyntrophic propionate oxidation by sulfate reducers and, in fact, *Syntrophobacter wolinii* and *Syntrophobacter pfennigii* but not *Syntrophobacter fumaroxidans* are capable of dissimilatory sulfate reduction. Numerous studies analyzing sequential reduction processes directly after flooding of rice field soil have demonstrated that pore water sulfate concentrations are typically low (up to 2 mM for a nonacid sulfur soil) and that sulfate is typically reduced within 8 to 10

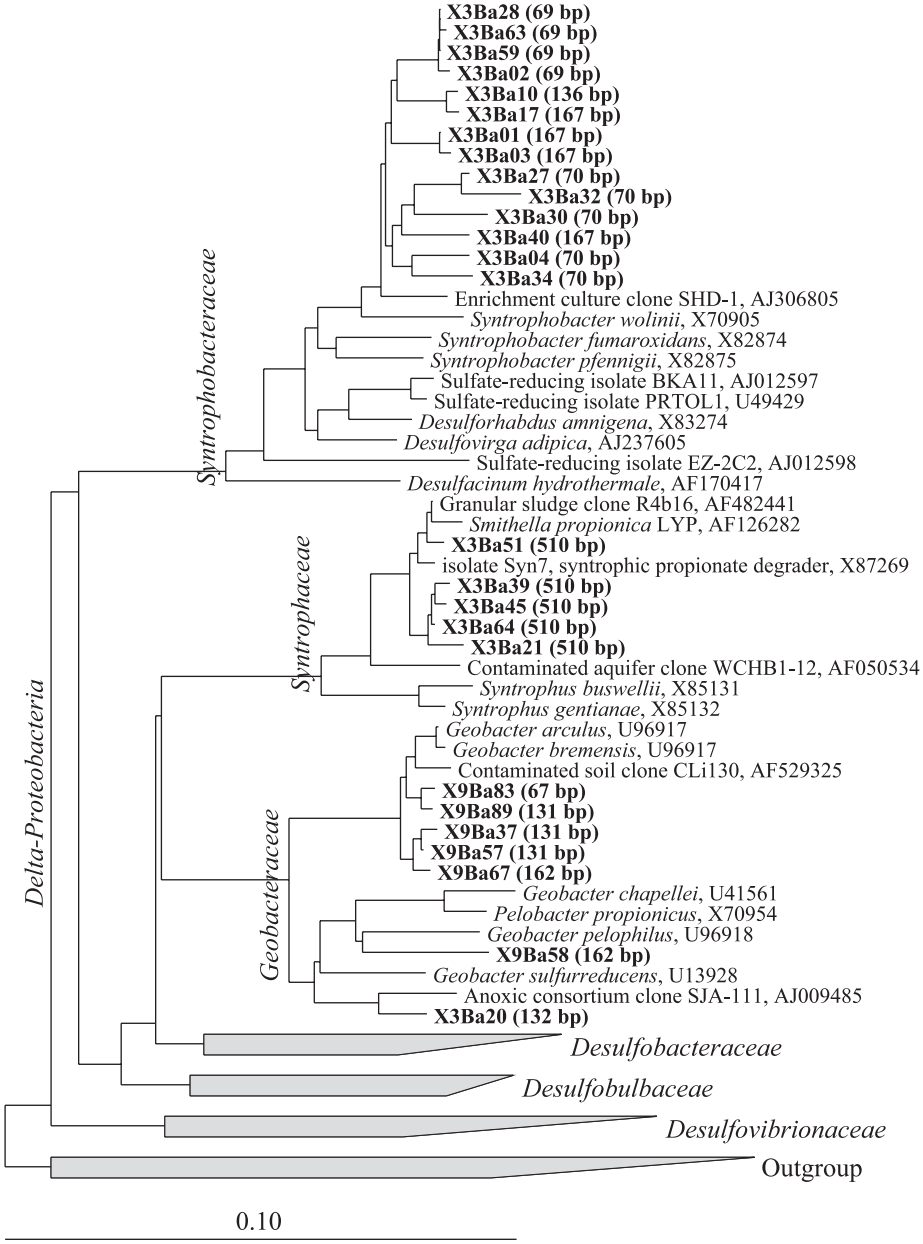

FIG. 4. Phylogenetic affiliation of representative δ-proteobacterial clones from density-resolved rRNA extracted from [^{13}C]propionate-consuming anoxic rice field soil relative to members of the δ-*Proteobacteria*. X3Ba clones, "heavy"; X9Ba clones, "light." Numbers in parentheses (base pairs) indicate expected T-RFs of clones. The scale bar represents 10% sequence divergence. GenBank accession numbers of reference sequences are given.

days in rice field soils from Vercelli (31, 32, 57, 59). More importantly, the onset of the phase of vigorous methane production is generally indicative of the complete reduction of sulfate and iron(III) as electron acceptors (57). The type species of the genus *Pelotomaculum*, *Pelotomaculum thermopropionicum*, which has been described recently as a novel thermophilic syntrophic propionate oxidizer, is also unable to reduce sulfate in a dissimilatory fashion (22). Furthermore, a strain proposed as "*Pelotomaculum schinkii*" has been isolated from mesophilic sludge, which may represent the first true obligately syntrophic anaerobic bacterium isolated (7), i.e., a syntroph that cannot be cultivated in pure culture with another substrate and/or alternative electron acceptors (7). In fact, we show here for the first time, with the help of SIP, that bacteria clearly affiliated with the genus *Pelotomaculum* were not only present in anoxic rice field soil but were also active as propionate-oxidizing syntrophs. Also, according to the T-RFLP fingerprint of the "heavy" rRNA, *Pelotomaculum* spp. may even form a prominent part of the syntrophic population in anoxic rice field soil, at least under the given experimental conditions. Similar sequences related to the novel genus *Pelotomaculum* were detected in anoxic rice field soil previously but assigned to *Desulfotomaculum* lineage I (53), since a role of *Pelotomaculum* as syntrophic propionate oxidizers was not evident at that time point. These *Pelotomaculum*-like se-

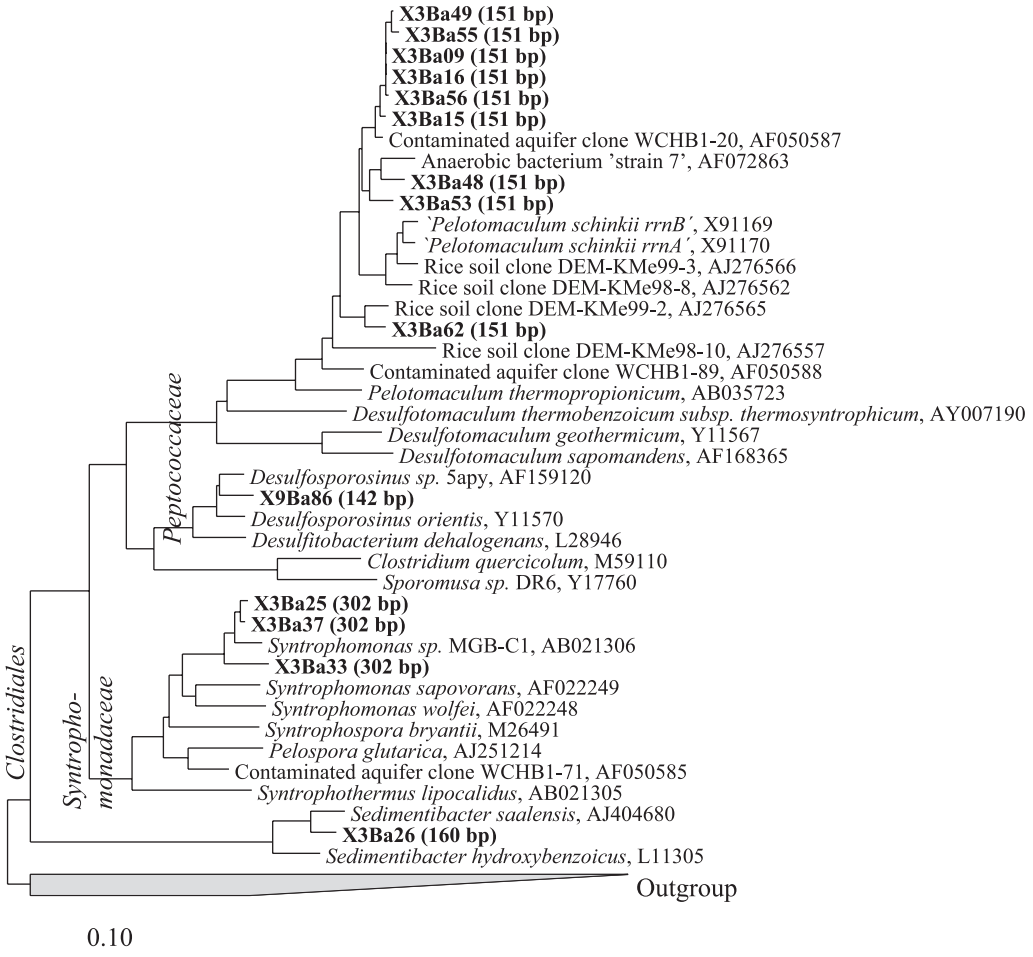

FIG. 5. Phylogenetic affiliation of representative clones related to gram-positive syntrophs generated from density-resolved rRNA of [^{13}C]propionate-consuming anoxic rice field soil to members of the *Clostridiales*. X3Ba clones, "heavy"; X9Ba clones, "light". Numbers in parentheses (base pairs) indicate expected T-RFs of clones. The scale bar represents 10% sequence divergence. GenBank accession numbers of reference sequences are given.

quences were shown to make up 1% of all bacterial templates (54).

Furthermore, we detected rRNA molecules related to *Syntrophomonas* spp. with low frequency in both the "heavy" fingerprint and the clone library. Thus far, *Syntrophomonas* spp. are known as syntrophic oxidizers of butyrate but not of propionate (29, 37, 47). Presently, we cannot rule out that novel *Syntrophomonas* spp. detected in our SIP experiment are capable of syntrophic propionate oxidation. However, it is more likely that *Syntrophomonas* spp. became labeled via a food chain that involves the intermediate formation of [^{13}C]butyrate from [^{13}C]propionate by the δ-proteobacterial *Smithella* spp., which were detected in addition to *Syntrophobacter* spp. in ^{13}C-labeled rRNA fractions. *Smithella propionica* dismutates propionate to acetate and butyrate, which is further metabolized syntrophically by β-oxidation to acetate (8). However, in rice field soil thus far only the randomizing pathway of propionate degradation was found to be operative, and butyrate as a characteristic intermediate of propionate degradation by *Smithella propionica* was not detected (26). However, the soil samples of Vercelli rice field soil used in the study of Krylova et al. (26) were different from the samples that we used, which may explain differences in the presence of *Smithella* spp. Apparently, more research is necessary to elucidate the role of *Smithella* spp. in flooded soils.

Growth of microorganisms that utilize reactions with a low Gibbs free-energy yield, such as propionate oxidation, requires energy sharing among the syntrophic partners (47), i.e., all partners have to share the energy available from the complete degradation of propionate to methane and CO_2. Energy sharing in syntrophic propionate oxidation has been measured by determining growth yields of all partners in defined batch and continuous cultures (48). SIP does not allow measurement of growth yields of the syntrophic partners involved, since it targets only the nucleic acids, but it can provide insight into carbon assimilation efficiencies of the microorganisms detected in "heavy" nucleic acid fractions. We found that the absolute numbers of archaeal rRNA molecules detected in the "heavy" 1.812-g ml^{-1} fraction (7.8×10^7 copies μl^{-1}) was slightly larger than the number of bacterial templates (5.2×10^7 copies μl^{-1}) in this fraction. It has to be considered, however, that roughly two-thirds of the ribotypes detected could be affiliated with syntrophic propionate oxidizers, suggesting that the other bacterial ribotypes detected were not involved in propionate

oxidation and thus likely to originate from unspecific carryover of unlabeled "background" rRNA in centrifugation gradients (33). However, the entire methanogen community clearly shifted to intermediate gradient fractions and therefore had apparently incorporated substantial amounts of ^{13}C label. Both observations suggest that the labeling efficiency for the different functional guilds in syntrophic associations was apparently higher for the methanogenic partners than for the syntrophic propionate oxidizers. Obviously, these data cannot reveal differences in energy sharing among the syntrophic partners involved (47). The high degree of labeling of methanogens suggests that syntrophic associations were closely juxtaposed (6). This may have helped to assimilate ^{13}C-labeled intermediates (i.e., acetate and CO_2) from [^{13}C]propionate more efficiently and probably prevented dilution into the pore water pool of dissolved carbon or alkaline trapping of CO_2 prior to assimilation. Apart from that, we cannot rule out direct assimilation of [^{13}C]propionate by methanogens; however, thus far it has been shown only that some strains of methanogens assimilate propionate as a precursor for isoleucine and not as a general carbon source (10).

Our study shows that to a large extent ^{13}C-labeled rRNA of species known to be involved in the syntrophic degradation were found. This corroborates results from other SIP studies, which targeted other nutritionally highly defined guilds. Collectively, in all cases labeling of active populations was achieved by stimulating degradation of a substrate, i.e., by dissimilation and assimilation. However, substrates such as propionate or the intermediately formed acetate may—in principle—be coassimilated by other bacteria that are present and active in the same habitat but that utilize a different type of substrate for dissimilatory purposes. Factors controlling the predominant labeling of the dissimilatorily active populations over other populations could be linked to the overall higher abundance of labeled anabolic precursors originating from a substrate that flows through the catabolite pool. Future studies may show whether active populations stimulated with a nonlabeled catabolic substrate can be detected in the presence of ^{13}C-labeled anabolic precursors. Possibly, the methanogenic conditions prevailing during the present SIP incubation, with only CO_2 available as a terminal electron acceptor, may have aided in predominantly labeling the syntrophic propionate oxidizers and methanogens. Hence, intermediately formed [^{13}C]acetate cannot be fermented for thermodynamic reasons (47), and even if nonspecific assimilation by other community members is to be expected, a strong dilution of label by continuously formed [^{12}C]acetate would clearly mitigate possible cross-feeding effects.

In conclusion, we provide here the first evidence that SIP can also be used under thermodynamic constraints to successfully trace and identify microorganisms involved in processes with only low energy yield. We identified three distinct lineages of syntrophic propionate oxidizers (*Syntrophobacter*, *Smithella*, and *Pelotomaculum* spp.) to operate simultaneously in anoxic rice field soil slurries. Also, we have detected their hydrogenotrophic and acetotrophic methanogenic partner organisms (mostly *Methanobacterium* and *Methanosarcina* spp. but also members of the *Methanomicrobiaceae* and rice cluster I) by assimilation of labeled intermediates, i.e., $^{13}CO_2$ and [^{13}C]acetate. Of course, we cannot rule out that the composition of active species detected may have been affected by the incubation in the presence of propionate concentrations (i.e., 10 mM) 1 order of magnitude higher than normally found in rice field soil slurries over an extended period of time (i.e., 7 weeks). However, SIP essentially requires for the addition of label in amounts that allow to obtain ^{13}C-labeled rRNA. Therefore, we have to clearly limit our conclusions on the ecological relevance of the community structure of syntrophic propionate oxidizers detected under the applied conditions. It remains to be shown what microbes are active in syntrophic propionate oxidation under much lower substrate concentrations (in the range of only a few micromoles) in natural rice field soil environments. Nevertheless, RNA-SIP (36, 42) was superior to DNA-SIP, since parallel analyses by DNA-SIP, although allowing the retrieval of labeled archaeal DNA, resulted in only minor labeling of syntrophs (data not shown) compared to interfering GC effects (33). Similar to an earlier study (34), we used RNA-SIP to demonstrate the activity of defined functional groups of microorganisms in the conversion of an added ^{13}C-labeled substrate and also their nutritional interactions and engagement in food chains. For the first time, however, this has now been accomplished in an anaerobic soil ecosystem, which holds great promise for future applications of SIP in general and for RNA-SIP specifically. However, more importantly, this strategy will allow researchers to unravel structure and function relationships of further syntrophic or other nutritional associations in natural environments and to define metabolic functions of yet-uncultivated microorganisms.

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REFERENCES

1. **Boone, D. R., and M. P. Bryant.** 1980. Propionate-degrading bacterium, *Syntrophobacter wolinii* sp. nov. gen. nov. from methanogenic ecosystems. Appl. Environ. Microbiol. **40:**626–632.
2. **Chin, K. J., and R. Conrad.** 1995. Intermediary metabolism in methanogenic paddy soil and the influence of temperature. FEMS Microbiol. Ecol. **18:**85–102.
3. **Chin, K. J., D. Hahn, U. Hengstmann, W. Liesack, and P. H. Janssen.** 1999. Characterization and identification of numerically abundant culturable bacteria from the anoxic bulk soil of rice paddy microcosms. Appl. Environ. Microbiol. **65:**5042–5049.
4. **Chin, K. J., T. Lukow, and R. Conrad.** 1999. Effect of temperature on structure and function of the methanogenic archaeal community in an anoxic rice field soil. Appl. Environ. Microbiol. **65:**2341–2349.
5. **Conrad, R., M. Klose, and P. Claus.** 2000. Phosphate inhibits acetotrophic methanogenesis on rice roots. Appl. Environ. Microbiol. **66:**828–831.
6. **Conrad, R., B. Schink, and T. J. Phelps.** 1986. Thermodynamics of H_2-consuming and H_2-producing metabolic reactions in diverse methanogenic environments under in situ conditions. FEMS Microbiol. Ecol. **38:**353–360.
7. **De Bok, F. A. M.** 2002. Ph.D. thesis. Wageningen University, Wageningen, The Netherlands.
8. **De Bok, F. A. M., A. J. M. Stams, C. Dijkema, and D. R. Boone.** 2001. Pathway of propionate oxidation by a syntrophic culture of *Smithella propionica* and *Methanospirillum hungatei.* Appl. Environ. Microbiol. **67:**1800–1804.
9. **Diaz, E., R. Amils, and J. L. Sanz.** 2003. Molecular ecology of anaerobic granular sludge grown at different conditions. Water Sci. Technol. **48:**57–64.
10. **Eikmanns, B., R. Jaenchen, and R. K. Thauer.** 1983. Propionate assimilation by methanogenic bacteria. Arch. Microbiol. **136:**106–110.
11. **Elferink, S. J. W.H.O., H. T. S. Boschker, and A. J. M. Stams.** 1998. Identification of sulfate reducers and *Syntrophobacter* sp. in anaerobic granular sludge by fatty acid biomarkers and 16S rRNA probing. Geomicrobiol. J. **15:**3–17.

12. **Elferink, S. J. W.H.O., W. J. C. Vorstman, A. Sopjes, and A. J. M. Stams.** 1998. Characterization of the sulfate-reducing and syntrophic population in granular sludge from a full-scale anaerobic reactor treating papermill wastewater. FEMS Microbiol. Ecol. **27:**185–194.
13. **Friedrich, M. W.** 2002. Phylogenetic analysis reveals multiple lateral transfers of adenosine-5′-phosphosulfate reductase genes among sulfate-reducing microorganisms. J. Bacteriol. **184:**278–289.
14. **Ginige, M. P., P. Hugenholtz, H. Daims, M. Wagner, J. Keller, and L. L. Blackall.** 2004. Use of stable-isotope probing, full-cycle rRNA analysis, and fluorescence in situ hybridization-microautoradiography to study a methanol-fed denitrifying microbial community. Appl. Environ. Microbiol. **70:**588–596.
15. **Glissmann, K., and R. Conrad.** 2000. Fermentation pattern of methanogenic degradation of rice straw in anoxic paddy soil. FEMS Microbiol. Ecol. **31:** 117–126.
16. **Harmsen, H. J., A. D. Akkermans, A. J. Stams, and W. M. De Vos.** 1996. Population dynamics of propionate-oxidizing bacteria under methanogenic and sulfidogenic conditions in anaerobic granular sludge. Appl. Environ. Microbiol. **62:**2163–2168.
17. **Harmsen, H. J., B. L. M. Van Kuijk, C. M. Plugge, A. D. L. Akkermans, W. M. De Vos, and A. J. M. Stams.** 1998. *Syntrophobacter fumaroxidans* sp. nov., a syntrophic propionate-degrading sulfate-reducing bacterium. Int. J. Syst. Bacteriol. **48:**1383–1387.
18. **Harmsen, H. J. M., H. M. P. Kengen, A. D. L. Akkermans, A. J. M. Stams, and V. De.** 1996. Detection and localization of syntrophic propionate-oxidizing bacteria in granular sludge by in situ hybridization using 16S rRNA-based oligonucleotide probes. Appl. Environ. Microbiol. **62:**1656–1663.
19. **Hengstmann, U., K. J. Chin, P. H. Janssen, and W. Liesack.** 1999. Comparative phylogenetic assignment of environmental sequences of genes encoding 16S rRNA and numerically abundant culturable bacteria from an anoxic rice paddy soil. Appl. Environ. Microbiol. **65:**5050–5058.
20. **Hutchens, E., S. Radajewski, M. G. Dumont, I. R. McDonald, and J. C. Murrell.** 2004. Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. Environ. Microbiol. **6:**111–120.
21. **Imachi, H., Y. Sekiguchi, Y. Kamagata, A. Ohashi, and H. Harada.** 2000. Cultivation and in situ detection of a thermophilic bacterium capable of oxidizing propionate in syntrophic association with hydrogenotrophic methanogens in a thermophilic methanogenic granular sludge. Appl. Environ. Microbiol. **66:**3608–3615.
22. **Imachi, H., Y. Sekiguchi, Y. Kamagata, S. Hanada, A. Ohashi, and H. Harada.** 2002. *Pelotomaculum thermopropionicum* gen. nov., sp. nov., an anaerobic, thermophilic, syntrophic propionate-oxidizing bacterium. Int. J. Syst. Evol. Microbiol. **52:**1729–1735.
23. **Jäckel, U., and S. Schnell.** 2000. Suppression of methane emission from rice paddies by ferric iron fertilization. Soil Biol. Biochem. **32:**1811–1814.
24. **Jeon, C. O., W. Park, P. Padmanabhan, C. DeRito, J. R. Snape, and E. L. Madsen.** 2003. Discovery of a bacterium, with distinctive dioxygenase, that is responsible for in situ biodegradation in contaminated sediment. Proc. Natl. Acad. Sci. USA **100:**13591–13596.
25. **Krumböck, M., and R. Conrad.** 1991. Metabolism of position-labeled glucose in anoxic methanogenic paddy soil and lake sediment. FEMS Microbiol. Ecol. **85:**247–256.
26. **Krylova, N., P. H. Janssen, and R. Conrad.** 1997. Turnover of propionate in methanogenic paddy soil. FEMS Microbiol. Ecol. **23:**107–117.
27. **Krylova, N. I., and R. Conrad.** 1998. Thermodynamics of propionate degradation in methanogenic paddy soil. FEMS Microbiol. Ecol. **26:**281–288.
28. **Liu, Y. T., D. L. Balkwill, H. C. Aldrich, G. R. Drake, and D. R. Boone.** 1999. Characterization of the anaerobic propionate-degrading syntrophs *Smithella propionica* gen. nov., sp. nov. and *Syntrophobacter wolinii.* Int. J. Syst. Bacteriol. **49:**545–556.
29. **Lorowitz, W. H., H. X. Zhao, and M. P. Bryant.** 1989. *Syntrophomonas wolfei* subsp. *saponavida* subsp. nov., a long-chain fatty-acid-degrading, anaerobic, syntrophic bacterium—*Syntrophomonas wolfei* subsp. *wolfei* subsp. nov.—and emended descriptions of the genus and species. Int. J. Syst. Bacteriol. **39:**122–126.
30. **Lueders, T., K. J. Chin, R. Conrad, and M. Friedrich.** 2001. Molecular analyses of methyl-coenzyme M reductase-subunit (*mcrA*) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. Environ. Microbiol. **3:**194–204.
31. **Lueders, T., and M. Friedrich.** 2000. Archaeal population dynamics during sequential reduction processes in rice field soil. Appl. Environ. Microbiol. **66:**2732–2742.
32. **Lueders, T., and M. W. Friedrich.** 2002. Effects of amendment with ferrihydrite and gypsum on the structure and activity of methanogenic populations in rice field soil. Appl. Environ. Microbiol. **68:**2484–2494.
33. **Lueders, T., M. Manefield, and M. W. Friedrich.** 2004. Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. Environ. Microbiol. **6:**73–78.
34. **Lueders, T., B. Wagner, P. Claus, and M. W. Friedrich.** 2004. Stable isotope probing of rRNA and DNA reveals a dynamic methylotroph community and trophic interactions with fungi and protozoa in oxic rice field soil. Environ. Microbiol. **6:**60–72.
35. **Mah, R. A., L.-Y. Xun, D. R. Boone, B. Ahring, P. H. Smith, and A. Wilkie.** 1991. Methanogenesis from propionate in sludge and enrichment systems, p. 99–119. Microbiology and biochemistry of strict anaerobes involved in interspecies hydrogen transfer. Plenum Press, Inc., New York, N.Y.
36. **Manefield, M., A. S. Whiteley, R. I. Griffiths, and M. J. Bailey.** 2002. RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. Appl. Environ. Microbiol. **68:**5367–5373.
37. **McInerney, M. J., M. P. Bryant, R. B. Hespell, and J. W. Costerton.** 1981. *Syntrophomonas wolfei* gen. nov. sp. nov, an anaerobic, syntrophic, fatty-acid oxidizing bacterium. Appl. Environ. Microbiol. **41:**1029–1039.
38. **Morris, S. A., S. Radajewski, T. W. Willison, and J. C. Murrell.** 2002. Identification of the functionally active methanotroph population in a peat soil microcosm by stable-isotope probing. Appl. Environ. Microbiol. **68:**1446–1453.
39. **Plugge, C. M., M. Balk, and A. J. M. Stams.** 2002. *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* subsp. nov., a thermophilic, syntrophic, propionate-oxidizing, spore-forming bacterium. Int. J. Syst. Evol. Microbiol. **52:**391–399.
40. **Radajewski, S., P. Ineson, N. R. Parekh, and J. C. Murrell.** 2000. Stable-isotope probing as a tool in microbial ecology. Nature **403:**646–649.
41. **Radajewski, S., G. Webster, D. S. Reay, S. A. Morris, P. Ineson, D. B. Nedwell, J. I. Prosser, and J. C. Murrell.** 2002. Identification of active methylotroph populations in an acidic forest soil by stable-isotope probing. Microbiology **148:**2331–2342.
42. **Radajewski, S., I. R. McDonald, and J. C. Murrell.** 2003. Stable-isotope probing of nucleic acids: a window to the function of uncultured microorganisms. Curr. Opin. Biotechnol. **14:**296–302.
43. **Ramakrishnan, B., T. Lueders, R. Conrad, and M. Friedrich.** 2000. Effect of soil aggregate size on methanogenesis and archaeal community structure in anoxic rice field soil. FEMS Microbiol. Ecol. **32:**261–270.
44. **Ramakrishnan, B., T. Lueders, P. F. Dunfield, R. Conrad, and M. W. Friedrich.** 2001. Archaeal community structures in rice soils from different geographical regions before and after initiation of methane production. FEMS Microbiol. Ecol. **37:**175–186.
45. **Roy, R., H. D. Klüber, and R. Conrad.** 1997. Early initiation of methane production in anoxic rice soil despite the presence of oxidants. FEMS Microbiol. Ecol. **24:**311–320.
46. **Schink, B.** 1985. Mechanisms and kinetics of succinate and propionate degradation in anoxic freshwater sediments and sewage sludge. J. Gen. Microbiol. **131:**643–650.
47. **Schink, B., and A. J. M. Stams.** 2002. Syntrophism among prokaryotes. *In* M. Dworkin (ed.), The prokaryotes. [Online.] Springer, New York, N.Y. http://link.springer-ny.com/link/service/books/10125/
48. **Scholten, J. C. M., and R. Conrad.** 2000. Energetics of syntrophic propionate oxidation in defined batch and chemostat cocultures. Appl. Environ. Microbiol. **66:**2934–2942.
49. **Schulz, S., H. Matsuyama, and R. Conrad.** 1997. Temperature dependence of methane production from different precursors in a profundal sediment (Lake Constance). FEMS Microbiol. Ecol. **22:**207–213.
50. **Sekiguchi, Y., Y. Kamagata, K. Nakamura, A. Ohashi, and H. Harada.** 1999. Fluorescence in situ hybridization using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. Appl. Environ. Microbiol. **65:** 1280–1288.
51. **Snaidr, J., R. Amann, I. Huber, W. Ludwig, and K. H. Schleifer.** 1997. Phylogenetic analysis and in situ identification of bacteria in activated sludge. Appl. Environ. Microbiol. **63:**2884–2896.
52. **Stams, A. J. M., C. Dijkema, C. M. Plugge, and P. Lens.** 1998. Contribution of C-13-NMR spectroscopy to the elucidation of pathways of propionate formation and degradation in methanogenic environments. Biodegradation **9:**463–473.
53. **Stubner, S., and K. Meuser.** 2000. Detection of *Desulfotomaculum* in an Italian rice paddy soil by 16S ribosomal nucleic acid analyses. FEMS Microbiol. Ecol. **34:**73–80.
54. **Stubner, S.** 2002. Enumeration of 16S rDNA of *Desulfotomaculum* lineage 1 in rice field soil by real-time PCR with SybrGreen[trade] detection. J. Microbiol. Methods **50:**155–164.
55. **Wallrabenstein, C., E. Hauschild, and B. Schink.** 1994. Pure culture and cytological properties of '*Syntrophobacter wolinii*.' FEMS Microbiol. Lett. **123:**249–254.
56. **Wallrabenstein, C., E. Hauschild, and B. Schink.** 1995. *Syntrophobacter pfennigii* sp. nov., new syntrophically propionate-oxidizing anaerobe growing in pure culture with propionate and sulfate. Arch. Microbiol. **164:**346–352.
57. **Yao, H., and R. Conrad.** 1999. Thermodynamics of methane production in different rice paddy soils from China, the Philippines, and Italy. Soil Biol. Biochem. **31:**463–473.
58. **Yao, H., and R. Conrad.** 2001. Thermodynamics of propionate degradation in anoxic paddy soils from different rice-growing regions. Soil Biol. Biochem. **33:**359–364.
59. **Yao, H., R. Conrad, R. Wassmann, and H. U. Neue.** 1999. Effect of soil characteristics on sequential reduction and methane production in sixteen rice paddy soils from China, the Philippines, and Italy. Biogeochemistry **47:**269–295.