ORIGINAL ARTICLE

Chemolithotrophic acetogenic H_2/CO_2 utilization in Italian rice field soil

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Acetate oxidation in Italian rice field at 50 °C is achieved by uncultured syntrophic acetate oxidizers. As these bacteria are closely related to acetogens, they may potentially also be able to synthesize acetate chemolithoautotrophically. Labeling studies using exogenous H_2 (80%) and $^{13}CO_2$ (20%), indeed demonstrated production of acetate as almost exclusive primary product not only at 50 °C but also at 15 °C. Small amounts of formate, propionate and butyrate were also produced from $^{13}CO_2$. At 50 °C, acetate was first produced but later on consumed with formation of CH_4 . Acetate was also produced in the absence of exogenous H_2 albeit to lower concentrations. The acetogenic bacteria and methanogenic archaea were targeted by stable isotope probing of ribosomal RNA (rRNA). Using quantitative PCR, ^{13}C -labeled bacterial rRNA was detected after 20 days of incubation with $^{13}CO_2$. In the heavy fractions at 15 °C, terminal restriction fragment length polymorphism, cloning and sequencing of 16S rRNA showed that *Clostridium* cluster I and uncultured *Peptococcaceae* assimilated $^{13}CO_2$ in the presence and absence of exogenous H_2 , respectively. A similar experiment showed that *Thermoanaerobacteriaceae* and *Acidobacteriaceae* were dominant in the ^{13}C treatment at 50 °C. Assimilation of $^{13}CO_2$ into archaeal rRNA was detected at 15 °C and 50 °C, mostly into *Methanocellales*, *Methanobacteriales* and rice cluster III. Acetoclastic methanogenic archaea were not detected. The above results showed the potential for acetogenesis in the presence and absence of exogenous H_2 at both 15 °C and 50 °C. However, syntrophic acetate oxidizers seemed to be only active at 50 °C, while other bacterial groups were active at 15 °C.

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Introduction

Acetogens contribute about 10% to the annual output of acetic acid in terrestrial habitats, such as soils and sediments, which is over 10^{13} kg (100 billion US tons) globally (Wood and Ljungdahl, 1991). Although most acetogens like *Moorella thermoacetica* are members of the phylum *Firmicutes*, acetogens also include *Spirochaetes*, δ -*Proteobacteria* and *Acidobacteria* (Drake *et al.*, 2006). Acetogens have been isolated from diverse environments, including the gastro intestinal tracts of animals and termites (Breznak, 1994; Mackie and Bryant, 1994), rice paddy soils (Rosencrantz *et al.*, 1999), hypersaline waters (Ollivier *et al.*, 1994), surface soils (Peters and Conrad, 1995; Kusel *et al.*, 1999) and deep subsurface sediments (Liu and Suflita, 1993). In these ecosystems, chemolithoautotrophic acetogenic bacteria can compete directly with hydrogenotrophic methanogenic archaea, or

interact syntrophically with acetoclastic methanogens to produce methane (Chassard and Bernalier-Donadille, 2006). Among the above environments, rice field soil may be considered as a rather simple model system for vegetated wetland ecosystems. H_2 – CO_2 -dependent methanogenesis ($\Delta G'^{\circ} = -130$ kJ per mole reaction) is more exergonic than H_2 – CO_2 -dependent acetogenesis ($\Delta G'^{\circ} = -95$ kJ per mole reaction). Both processes are more exergonic at low versus high temperatures. At the *in situ* concentrations of H_2 found in many anoxic environments, methanogenesis is usually energetically more favorable than acetogenesis, and hydrogenotrophic methanogens outcompete chemolithoautotrophic acetogens (Drake *et al.*, 2006). Nevertheless, it was found that acetogens can outcompete methanogens at low temperature (Conrad *et al.*, 1989; Kotsyurbenko *et al.*, 1993) as many acetogens seem to have a higher growth rate at low temperature than most methanogens (Kotsyurbenko *et al.*, 1996, 2001). Therefore, acetogenesis might become a quantitatively important process in anaerobic environments when H_2 partial pressures are high and/or temperatures are low.

Syntrophic acetate oxidation is the reversal of chemolithoautotrophic acetogenesis and becomes

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more exergonic as temperature increases (Lee and Zinder, 1988). Our previous study showed that members of *Thermacetogenium*, *Thermoanaerobacteriaceae* and *Symbiobacterium* were involved in syntrophic acetate oxidation in methanogenic Italian rice field soil at 50 °C (Liu and Conrad, 2010). The *Thermacetogenium* genus contains the thermophilic syntrophic acetate oxidizer *Thermacetogenium phaeum*, which can operate the reductive acetyl-CoA pathway reversibly both for acetate oxidation and reductive acetogenesis (Hattori *et al.*, 2005). Correspondingly, one may suspect that the same bacterial groups can also operate in the reverse mode, that is, chemolithoautotrophic acetogenesis.

The purpose of this study was to identify the acetogens that could potentially become active in rice field soil at 50 °C and determine whether these acetogens are identical (or similar) to those identified as putative syntrophic acetate oxidizers in the same soil. However, the soil microbial community probably rarely, if at all, experiences the temperature (50 °C) that is used for this study. Nevertheless, it is noteworthy that Italian rice field soil in which the temperatures range typically from 15 °C to 30 °C (Schütz *et al.*, 1990) contains a methanogenic microbial community with acetogens and acetate oxidizers functioning under thermal conditions. Rice field soils from other geographical regions apparently also contain a thermophilic methanogenic community (Wu *et al.*, 2006; Rui *et al.*, 2009). Moreover, we were interested whether these bacteria could also become active as acetogens, when the temperature was decreased to 15 °C, which is at the low end of the temperature range in Italian rice fields *in situ*. The investigation was motivated by our lack of knowledge of the temperature range of chemolithoautotrophic acetogens present and potentially active in a natural environment and by our desire to learn more about the ecophysiological potential of non-phototrophic CO₂-fixing microbes.

Materials and methods

Soil incubation

Soil from rice fields at the Italian Rice Research Institute in Vercelli, Italy, was a sandy loam (Holzapfel-Pschorn *et al.*, 1986). The soil and the experimental procedures were the same as that used by Liu and Conrad (2010). Briefly, soil slurries were prepared in 2-l bottles using 700 g dry soil, 0.35 g dry ground rice straw and 700-ml anoxic sterile water. The bottles were closed with red butyl rubber stoppers, flushed with N₂, pressurized to 0.5 bar overpressure, and incubated at 50 °C for 81 days. Then, the slurry was dispensed into 26-ml pressure tubes using about 10 g for each tube.

All incubation experiments were done with rice field soil pre-incubated at 50 °C. The tubes with soil slurry were prepared in numerous parallels.

Three sets of slurries each were incubated at 15 °C with a headspace of (1) 20% ¹³CO₂ (99 atom%; Sigma, Munich, Germany) plus 80% H₂; (2) 20% ¹²CO₂ plus 80% H₂ (unlabeled control); and (3) 20% ¹³CO₂ plus 80% N₂ (control without exogenous H₂). Three further sets of slurries were incubated at 50 °C with a headspace of 20% ¹³CO₂ plus 80% H₂. Triplicates were sacrificed for analysis. Aliquot samples from the gas headspace were analyzed, and the soil slurries were stored frozen (−75 °C) for nucleic acid extraction.

Methane and CO₂ were quantified in a gas chromatograph (GC) equipped with methanizer and flame ionization detector (Conrad and Klose, 1999). Hydrogen was analyzed in a GC equipped with a HgO-to-Hg conversion detector (Schuler and Conrad, 1990). Whenever most of the added CO₂ was consumed, as analyzed by GC, it was added again (two times during incubation, about 375 μmol in total). The ¹³C content of CH₄ and CO₂ was analyzed in a GC combustion isotope ratio mass spectrometer system (GC-C-IRMS) (Thermoquest, Bremen, Germany) as described before (Conrad *et al.*, 2007). In the incubations with unlabeled ¹²CO₂, the ¹³C content of CH₄ and CO₂ was in the range of the natural abundance and therefore, is given as δ¹³C. The apparent isotopic fractionation factor for conversion of CO₂ to CH₄ was determined by $\alpha_{app} = (\delta^{13}C_{CO_2} + 10^3) / (\delta^{13}C_{CH_4} + 10^3)$.

Liquid samples for analysis of organic acids dissolved in the pore water were recovered as described by Hori *et al.* (2007). The isotopic composition of fatty acids was determined in a high pressure liquid chromatograph combustion isotope ratio mass spectrometer system (HPLC-C-IRMS) (Thermoquest) as described before (Conrad *et al.*, 2007).

The concentrations of gases and dissolved compounds in the slurry incubations were used to calculate the Gibbs free energy (ΔG) of hydrogenotrophic methanogenesis, chemolithotrophic acetogenesis and other processes for the incubation conditions at either 15 °C or 50 °C (Conrad and Wetter, 1990).

RNA extraction and gradient centrifugation

Extraction of RNA was conducted using ~0.5 g (wet weight) soil samples as previously described (Liu and Conrad, 2010). RNA was obtained by removal of co-extracted DNA with RNase-Free DNase Set (Qiagen, Hilden, Germany) at room temperature for 30 min. RNA was further recovered using the Qiagen RNeasy Mini Kit (Qiagen) and stored at −80 °C until used as template for complementary DNA synthesis. RNA was confirmed to be DNA-free by the absence of PCR products after amplification of 16S ribosomal RNA (rRNA) genes with universal primers 27F/1492R (Weisburg *et al.*, 1991) for bacteria and Ar7f/Ar1384r (Lueders *et al.*, 2004) for archaea.

Isopycnic centrifugation of soil rRNA was done, as previously described (Lueders *et al.*, 2004) with minor modifications of the number and volume of the gradient fractions, using cesium trifluoroacetate centrifugation gradients. Twelve equal volume fractions ($\sim 450 \mu\text{l}$) of the density-separated rRNA were obtained, and the cesium trifluoroacetate buoyant density of each fraction was determined, and RNA was precipitated for subsequent quantitative and qualitative community analyses.

Quantification of rRNA in density gradient fractions

Bacterial and archaeal 16S rRNA from gradient fractions was quantified by quantitative reverse-transcription RT-PCR using primer pair Ba519f/Ba907r (Stubner, 2002) and A364aF/A934b (Kemnitz *et al.*, 2005), respectively. Copy numbers were standardized by using dilution series (10^2 – 10^7 molecules μl^{-1}) of almost full-length 16S rRNA genes of *Escherichia coli* strain JM109 or a *Methanosarcina barkeri* strain (accession number AY641448) as described by Lueders *et al.* (2004).

Community analyses by T-RFLP analysis

Terminal restriction fragment length polymorphism (T-RFLP) analysis of density-resolved bacterial and archaeal communities from gradient fractions was performed by RT-PCR using primer pairs Ba27f-FAM/Ba907r and Ar109f/Ar912rt-FAM, respectively. Reverse transcription was described previously by Liu and Conrad (2010). After inactivation of the reverse transcriptase by heating at 70°C for 15 min, the reaction product was subjected to PCR to amplify the DNA. The thermal profile of the PCR included 30 cycles of primer annealing at 52°C for 45 s, primer extension at 72°C for 1.5 min and denaturing at 94°C for 45 s. The final elongation step was 5 min. Amplicons were digested by *MspI* (*Bacteria*) and *TaqI* (*Archaea*, Chin *et al.*, 1999) and size-separated on an ABI Prism 373 DNA sequencer (Applied Biosystems, Darmstadt, Germany).

Sequencing and phylogenetic analysis

Selected density fractions of bacterial and archaeal rRNA were amplified for cloning using the primer set Ba27f/Ba907r and A364aF/A934b, respectively, under the thermal conditions mentioned above. The RT-PCR product was ligated into the plasmid vector pGEM-T Easy (Promega, Mannheim, Germany), and the ligation mixture was used to transform *E. coli* TOP10 competent cells (Invitrogen) according to the manufacturer's instructions. Clones were randomly selected and sequenced at GATC Biotech AG (Konstanz, Germany). Raw sequence data were assembled and checked with the Lasergene software package DNASTAR (Madison, WI, USA). Chimeric structures were detected by Bellerophon programme on the Greengenes website (DeSantis *et al.*, 2006). Phylogenetic analyses were conducted using the ARB software package (<http://www.arb-home.de>) as

described previously (Liu *et al.*, 2009). Bootstrapped phylogenetic trees with reference 16S rRNA sequences (>1400 nucleotides) from SILVA (Pruesse *et al.*, 2007) using 'aligner' tool were calculated using the neighborjoining and maximum likelihood methods with 100 resamplings. Sequence data have been submitted to the GenBank database under accession numbers HQ266779 to HQ266953.

Results

H_2/CO_2 utilization

All experiments were done with rice field soil pre-incubated at 50°C . Thus pre-incubated soil contains an active community of thermophilic syntrophic acetate oxidizers (Liu and Conrad, 2010). In the presence of exogenous H_2 , CO_2 was fixed for incubations both at 15°C and 50°C (Figure 1a). After the added CO_2 was depleted, CO_2 was replaced another time giving a total of $375 \mu\text{mol}$ exogenous CO_2 .

The H_2 partial pressures decreased in parallel to CO_2 (Figure 1b). H_2 partial pressures were lower in the incubation at 50°C than that at 15°C , in particular after the second addition of $^{13}\text{CO}_2$ at day 13. Acetate accumulated linearly after a lag phase of 3 days in the incubations at 15°C except when exogenous H_2 was absent (Figure 1e). Coincidentally, the pH value decreased linearly from 6.9 at the beginning to 5.3 at day 27 (Figure 1d). A total of $350 \mu\text{mol}$ carbon, that is, 93% of the $^{13}\text{CO}_2$ added, was finally recovered as acetate at day 27. In contrast, acetate was produced without lag phase in the incubation at 50°C . However, it was consumed gradually after reaching the highest concentration of 16.4 mM at day 8. Accordingly, the pH value decreased to 6.3 at day 6 and recovered to 6.8 at day 13, dropped down again to 6.1 at day 17 and then came up to 7.0 at day 27. A total of $222 \mu\text{mol}$ carbon, that is, 71% of the $^{13}\text{CO}_2$ added, was finally recovered as gaseous CH_4 and acetate at day 27 (Figure 1e).

When soil was incubated with $^{13}\text{CO}_2$, the ^{13}C content of acetate in the incubation at 50°C (82 atom%) was initially higher than that at 15°C (21 atom%). Nevertheless, significant amounts of ^{13}C -labeled acetate accumulated at 15°C and eventually reached a similar high atom percentage (70–80 atom% ^{13}C) after 8 days (Figure 1f), demonstrating that most of the carbon atoms in acetate were produced from $^{13}\text{CO}_2$. The ^{13}C content of acetate in the incubation with N_2 at 15°C increased slowly from 7 atom% at day 3 to 27 atom% at day 27 although the highest acetate concentration was only 0.8 mM (Figure 1f), indicating that a small amount of $^{13}\text{CO}_2$ was also fixed without exogenous H_2 or isotopically exchanged with the carboxyl group of acetate.

The concentrations of formate, propionate and butyrate were measured in the four sets of rice soil slurry incubations. Peaks of formate accumulation

Figure 1 Concentrations of (a) CO₂, (b) H₂, (c) CH₄ and (e) acetate in soil slurries after addition of CO₂ (unlabeled and ¹³CO₂) with H₂ or N₂ (20:80 (vol/vol)) during incubation at 15 °C or 50 °C over 27 days; (d) pH values; (f) relative abundance of ¹³C (atom%) in acetate; means ± s.e. of triplicates. CO₂ addition is indicated by black closed triangle. Samples selected for rRNA-SIP analysis (Figure 3) are indicated by arrows in (e).

were observed after the addition of CO₂ in the incubations with CO₂ and H₂ both at 15 °C and 50 °C (Figure 2a). Propionate was produced in the incubation at 50 °C with a maximum of 0.4 mM at day 17 and then decreasing below the detection limit (Figure 2b). Butyrate was also detected in the incubations with CO₂ and H₂ both at 15 °C and 50 °C (Figure 2c). At day 17 and 15 °C, the ¹³C in formate, propionate and butyrate from the incubation with ¹³CO₂ and H₂ was 62, 57 and 28 atom%, respectively. At 50 °C, the ¹³C content was 27, 60 and 72 atom%, respectively (Figure 2e).

Hydrogenotrophic methanogenesis was generally exergonic ($\Delta G = -160$ to -20 kJ mol⁻¹). The same was true for the conversion of H₂ and CO₂ to formate ($\Delta G < -5$ kJ mol⁻¹) or acetate ($\Delta G < -50$ kJ mol⁻¹), for the conversion of H₂, acetate and CO₂ to propionate ($\Delta G < -50$ kJ mol⁻¹), and for the conversion of H₂ and acetate to butyrate ($\Delta G < -40$ kJ mol⁻¹), when the soil was incubated in the presence of exogenous H₂. At 50 °C, however, ΔG values generally increased during the second half of incubation (after day 14), when H₂ partial pressures decreased to very low values (Figure 1b). At this time, production of formate, acetate, propionate and butyrate eventually became endergonic.

Methane was produced in all incubations. However, in the incubation at 50 °C, accumulation

was faster and reached much higher amounts (266 μmol) till the end of incubation (27 days). In contrast, only 0.2–2 μmol CH₄ was produced in the incubation at 15 °C (Figure 1c). The ¹³C content of CH₄ and CO₂ was only analyzed in the soil slurries incubated with unlabeled CO₂ (natural abundance of ¹³C). The resulting $\delta^{13}\text{C}$ values of CH₄ and CO₂ were used to calculate the apparent fractionation factor α_{app} for the conversion of CO₂ to CH₄ (Figure 2d). After the addition of CO₂, α_{app} increased gradually to a relatively high value of $\alpha_{\text{app}} \approx 1.08$ – 1.09 , which is characteristic for CH₄ production by hydrogenotrophic methanogenesis only (Fey *et al.*, 2004).

Stable isotope probing (SIP) targeting bacterial 16S rRNA

After 20 days of incubation with ¹³CO₂ and H₂ at 15 °C, the bulk of bacterial 16S rRNA was found in two 'heavy' peaks at about 1.79 and 1.82 g ml⁻¹ of cesium trifluoroacetate gradients (Figure 3a). Bacterial rRNA from the incubation with ¹³CO₂ and H₂ at 50 °C was only found in the 'light' fractions of a cesium trifluoroacetate gradient after 8 days of incubation, but after 13 and 20 days of incubation, a substantial peak of 'heavy' density at about 1.80 g ml⁻¹ was obtained (Figure 3c). For the

Figure 2 Concentrations of (a) formate, (b) propionate and (c) butyrate in soil slurries after addition of CO_2 (unlabeled and $^{13}\text{CO}_2$) with or without H_2 during incubation at 15 °C or 50 °C over 27 days; and (d) apparent isotope fractionation factor (α_{app}) after addition of unlabeled CO_2 ; (e) relative abundance of ^{13}C (atom%) in formate, propionate and butyrate after labeling with $^{13}\text{CO}_2$; means $\pm$ s.e. of triplicates.

incubation with $^{13}\text{CO}_2$ and N_2 (15 °C), only a slight shift to 'heavy' fractions was observed at the end of incubation (27 days, Figure 3b). However, at day 20,

'heavy' peaks were obtained only in the incubations with $^{13}\text{CO}_2$ and H_2 both at 15 and 50 °C (Figure 3d).

Analysis of the relative abundance of the nine most abundant bacterial terminal restriction fragments (T-RFs) across the rRNA density gradient after different days of incubation with either $^{12}\text{CO}_2$ or $^{13}\text{CO}_2$ is shown in Figure 4a. For the incubation with $^{13}\text{CO}_2$ and H_2 at 15 °C, the T-RF of 520-bp length was predominant and became exclusive at high buoyant density. For the incubation with N_2 at 15 °C, the T-RF of 289bp in addition to that of 520-bp length increased in abundance at higher density, in particular at day 20, while the T-RF of 150-bp length decreased strongly. By contrast, for the incubation at 50 °C the T-RFs of 140- and 171-bp length increased dramatically at higher density, in particular after 13 days, while the T-RF of 150-bp length decreased sharply (Figure 4a).

In order to affiliate the detected T-RFs to phylogenetic bacterial groups, five clone libraries were generated using samples of different buoyant densities from three sets of rice soil slurry incubations as indicated by asterisks in Figures 3a–c. One clone library used 'light' templates of rRNA as a control, and four clone libraries used 'heavy' templates. Some samples were selected at the shoulders of the peaks because they located in the heavy fractions characterized in our previous SIP study (Liu and Conrad, 2010). The phylogenetic affiliation of all bacterial clones analyzed is summarized in Table 1. The phylogenetic placement of selected representative clones from 'heavy' templates is shown in Figure 5.

Phylogenetic analysis of the 'heavy' clones from the incubation with $^{13}\text{CO}_2$ and H_2 (15 °C) at day 20 revealed a community (coverage 78%) that was exclusively dominated by populations of *Clostridium* cluster I (88% of all clones; Table 1). From the incubation with N_2 (15 °C) at day 20, the 'light' community (coverage 72%) was dominated by populations of *Acidobacteriaceae* (27%). However, sequences related to *Clostridium* cluster I (19%), the genus *Symbiobacterium* (16%) and the *Bacillales* (15%) were also detected. To a minor extent (<8%) also members of *Chloroflexi*, *Clostridium* cluster III and XIVa were detected in light-density rRNA. By contrast, the clones derived from 'heavy' rRNA at day 20 (N_2 , 15 °C, coverage 75%) showed a clear predominance of sequences related to members of the uncultured *Peptococcaceae* (34%, Table 1, Figure 5). Further clones formed a distinct cluster related to *Clostridium* cluster I (28%), III and the *Bacillales* (Table 1, Figure 5). Furthermore, we detected two clones and one clone related to the *Clostridium* cluster XIVa and *Acidobacteriaceae*, respectively (Table 1).

For the incubation with $^{13}\text{CO}_2$ plus H_2 at 50 °C, populations of *Acidobacteriaceae* (42%) were predominant in the 'heavy' bacterial community (coverage 70%) after 8 days (acetate accumulated). Sequences related to *Thermoanaerobacteriaceae*

Figure 3 Quantification of (a–d) bacterial and (e–h) archaeal templates across gradient fractions by quantitative reverse transcription-PCR. The gradient fractions, characterized by their buoyant densities, were generated using cesium trifluoroacetate (CsTFA) density gradient centrifugation of rRNA extracted from anoxic rice field soil after addition of CO₂ (unlabeled and ¹³CO₂) with H₂ at 15 °C (day 20) (a and e); addition of ¹³CO₂ with N₂ at 15 °C (day 1, 20 and 27) (b and f); addition of ¹³CO₂ with H₂ at 50 °C (day 8, 13 and 20) (c and g) and all treatments at day 20 (d and h). Fractions from which clone libraries (asterisks or with the same symbol as corresponding treatment) were generated are indicated.

(17%), the genus *Desulfosporosinus* (17%) and *Symbiobacterium* (8%) were also detected. In contrast, members of the *Thermoanaerobacteriaceae* (35%) dominated the ‘heavy’ rRNA after 20 days (acetate was consumed, coverage 73%). Sequences related to *Acidobacteriaceae* (31%), the genus *Symbiobacterium* (13%) and *Thermacetogenium* (9%) were also detected.

We used the sequence data to tentatively assign major T-RFs observed in the different bacterial fingerprints to defined phylogenetic lineages. Thus, the predominant 512 and 520 bp T-RFs represented members of *Clostridium* cluster I, which dominated the ‘heavy’ rRNA clone library from the incubation

with ¹³CO₂ and H₂ (15 °C) at day 20 (Figure 4a). The predominant 289-bp T-RF represented members of the uncultured *Peptococcaceae*, which dominated the ‘heavy’ rRNA clone library from the incubation with N₂ (15 °C) at day 20. The predominant 150- and 152-bp T-RFs represented members of the *Acidobacteriaceae*, which also dominated the ‘light’ rRNA clone library from the incubation with N₂ (15 °C) at day 20 (Table 1). In contrast, the T-RFs dominating the ‘heavy’ fractions from the incubation with ¹³CO₂ and H₂ at 50 °C clearly represented members of the *Thermoanaerobacteriaceae* (140 and 171 bp) and *Thermacetogenium* (171 bp) (Figure 4a). Clones clustering with *Symbiobacterium* exhibited various

Figure 4 Relative abundance of different (a) bacterial and (b) archaeal T-RFs across cesium trifluoroacetate (CsTFA) buoyant densities of isolated ^{13}C -labeled rRNA. Mb, *Methanobacteriales*; Msr, *Methanosarcinaceae*; Msa, *Methanosaetaceae*; RC III, rice cluster III; RC I, rice cluster I (*Methanocellales*); Cren, *Crenarchaeota*.

T-RF lengths, including T-RFs of 124- and 132-bp lengths (Table 1).

SIP targeting archaeal 16S rRNA

SIP targeting rRNA was also used to identify the archaea that assimilated $^{13}\text{CO}_2$. No obvious 'heavy' peak was found in archaeal 16S rRNA from the incubation with $^{13}\text{CO}_2$ and H_2 at 15°C after 20 days (Figure 3e). However, 'heavy' peaks of 1.79 and 1.80 g ml^{-1} were observed in the incubations with N_2 (day 20; Figure 3f) and also in the incubations with $^{13}\text{CO}_2$ and H_2 at 50°C (day 13; Figure 3g). Comparison of the results at day 20 showed that heavily labeled archaeal 16S rRNA was only obtained in the incubation at 50°C (Figure 3h).

The T-RFLP of the archaeal 16S rRNA templates showed four major T-RFs of 92, 381, 393 and 738 bp. The relative abundance of these T-RFs changed with

the buoyant density of the gradient centrifugation and with the time of different incubations (Figure 4b). Although the 393-bp T-RF was the dominant one, the 381- and 738-bp T-RFs became increasingly more abundant, in particular in the heavy fractions from incubations at 15°C , indicating that archaea representing this T-RF reacted on CO_2 addition by synthesizing ribosomes at low temperature. For the incubation with $^{13}\text{CO}_2$ plus H_2 at 50°C , the 92-bp T-RF specifically increased in the heavy fractions, showing its response to this incubation conditions. Nevertheless, the archaea represented by the 393-bp T-RF usually dominated the rRNA (Figure 4b).

Three clone libraries were generated using samples of 'heavy' buoyant densities from three sets of rice soil slurry incubations as indicated by asterisks in Figures 3e–g. The phylogenetic placement of selected representative clones is shown in Figure 6. Phylogenetic analysis of the 'heavy' clones from the incubation with $^{13}\text{CO}_2$ and H_2 (15°C) at day 20 revealed an archaeal community that was dominated by populations of *Methanocellales* (rice cluster I) (5 of 10 clones) and rice cluster III (5 of 10 clones). From the incubation with N_2 (15°C) at day 20, the 'heavy' community was also dominated by populations of *Methanocellales* (8 of 18 clones) and rice cluster III (8 of 18 clones). However, sequences related to *Crenarchaeota* (2 of 18 clones) were also detected. For the incubation with $^{13}\text{CO}_2$ plus H_2 at 50°C , populations of *Methanobacteriaceae* (5 of 8 clones) were predominant in the 'heavy' archaeal community after 8 days. Sequences related to *Methanocellales* (2 of 8 clones) and rice cluster III (1 of 8 clones) were also detected.

We used the sequence data to assign major T-RFs observed in the different archaeal fingerprints to defined phylogenetic lineages. Thus, the predominant 393-bp T-RF represented members of *Methanocellales*, which dominated the 'heavy' rRNA clone library from all the incubations (Figure 4b). The predominant 92-bp T-RF represented members of the *Methanobacteriales*, which dominated the 'heavy' rRNA clone library from the incubation with $^{13}\text{CO}_2$ and H_2 (50°C) at day 8 (Figure 6). By contrast, the 381-bp T-RF increasing in the 'heavy' fractions from the incubation at 15°C with either H_2 or N_2 represented members of rice cluster III (Figures 4b and 6). The 738-bp T-RF represented members of the *Crenarchaeota*.

Discussion

Our study showed that the syntrophic acetate oxidizers, that is, *Thermoanaerobacteriaceae*, which had been identified in Italian rice field soil incubated at 50°C (Liu and Conrad, 2010) can also operate as chemolithoautotrophic acetogens by assimilating CO_2 with exogenous H_2 . At 15°C , however, different acetogens were active. Results of

The data are compared with previously published data (RC21-C9, R21-C4) on syntrophic acetate oxidizers^c.

Figure 5 Neighbor-joining tree of representative bacterial 16S rRNA clone sequences generated from density-resolved nucleic acids extracted from ¹³CO₂ consuming anoxic rice field soil. Bootstrap values of ≥75% and 50–75% are shown as closed circles and open circles, respectively. R20-¹³C-A4 clones: ‘heavy’ rRNA fraction (1.801 g ml⁻¹ cesium trifluoroacetate (CsTFA)) extracted from rice field soil after addition of ¹³CO₂ and H₂ (20:80 (vol/vol), 15 °C) at day 20; R20-N₂-B9 clones: ‘light’ rRNA fraction (1.764 g ml⁻¹ CsTFA) extracted from that with ¹³CO₂ and N₂ (20:80 (vol/vol), 15 °C) at day 20; R20-N₂-B3 clones: ‘heavy’ rRNA fraction (1.811 g ml⁻¹ CsTFA) extracted from that with ¹³CO₂ and N₂ (20:80 (vol/vol), 15 °C) at day 20; R8-50 °C-C4 clones: ‘heavy’ rRNA fraction (1.801 g ml⁻¹ CsTFA) extracted from that with ¹³CO₂ and H₂ (20:80 (vol/vol), 50 °C) at day 8; R20-50 °C-C4 clones: ‘heavy’ rRNA fraction (1.801 g ml⁻¹ CsTFA) extracted from that with ¹³CO₂ and H₂ (20:80 (vol/vol), 50 °C) at day 20. Numbers in parentheses (base pairs) indicate the lengths of T-RFs determined *in silico*. The scale bar represents 10% sequence divergence; GenBank accession numbers of reference sequences as indicated.

Figure 6 Neighbor-joining tree of representative archaeal 16S rRNA clone sequences generated from density-resolved nucleic acids extracted from ¹³CO₂ consuming anoxic rice field soil. Bootstrap values of ≥75 and 50–75% are shown as closed circles and open circles, respectively. R20-¹³C-E3 clones: ‘heavy’ rRNA fraction (1.811 g ml⁻¹ cesium trifluoroacetate (CsTFA)) extracted from rice field soil after addition of ¹³CO₂ and H₂ (20:80 (vol/vol), 15 °C) at day 20; R20-N₂-F3 clones: ‘heavy’ rRNA fraction (1.811 g ml⁻¹ CsTFA) extracted from that with ¹³CO₂ and N₂ (20:80 (vol/vol), 15 °C) at day 20; R8-50°C-G3 clones: ‘heavy’ rRNA fraction (1.811 g ml⁻¹ CsTFA) extracted from that with ¹³CO₂ and H₂ (20:80 (vol/vol), 50 °C) at day 8. Numbers in parentheses (base pairs) indicate the lengths of T-RFs determined *in silico*. The scale bar represents 10% sequence divergence; GenBank accession numbers of reference sequences as indicated.

rRNA–SIP experiments identified populations of *Clostridium* cluster I being responsible for chemolithoautotrophic acetogenesis at 15 °C. Without exogenous H₂ at 15 °C, *Peptococcaceae* eventually became the dominant bacteria assimilating CO₂. The results show a remarkable potential for acetogenesis in Italian rice field soil. This potential could be initiated at 15 °C as well as at 50 °C, although the soil

was pre-incubated at 50 °C for 81 days indicating that the resident acetogens were quite heat-resistant.

Acetate accumulated in the incubation with CO₂ plus H₂ at 50 °C without lag phase. In the first 8 days, when acetate increased steadily the ¹³C-labeled bacterial community was dominated by *Acidobacteriaceae* and *Thermoanaerobacteriaceae*, demonstrating that they were responsible for acetate

production. Later on, at day 20, when acetate concentrations decreased gradually, the bacterial community was dominated by other members of *Thermoanaerobacteriaceae* and *Acidobacteriaceae*. Bacteria belonging to these phylogenetic groups had also been identified as active syntrophic acetate oxidizers in rice field soil incubated at 50 °C (Liu and Conrad, 2010). However, it remains unclear whether these were identical species that performed both syntrophic acetate oxidation and chemolithoautotrophic acetogenesis. Nevertheless, Italian rice field can apparently not only convert acetate to CO₂ and H₂, but can also convert CO₂ with H₂ to acetate. Our data show that the latter reaction is not only possible at 50 °C but also at 15 °C. At 15 °C, as much as 33.4 mM acetate accumulated after 27 days of incubation.

In the presence of exogenous H₂, the acetogens in the rice field soil almost completely outcompeted H₂-utilizing methanogens at low temperature (15 °C). Only little CH₄ was formed under these conditions, although thermodynamic conditions were favorable for both H₂-CO₂-dependent methanogenesis and acetogenesis. It is likely due to their complex and diverse heterotrophic (rather than autotrophic) capabilities, and higher growth rates at low temperature than the methanogens (Drake *et al.*, 2006). This result is consistent with earlier studies using rice field soil, lake sediments and other anoxic environments (Conrad *et al.*, 1989; Kotsyurbenko *et al.*, 2001). It is also consistent with the observation, that only bacterial (acetogenic) but not archaeal (methanogenic) rRNA became labeled with ¹³CO₂.

Already after 20 days of incubation with ¹³CO₂ and H₂ at 15 °C, the bacterial community with ¹³C-labeled rRNA was dominated by members of *Clostridium* cluster I. This cluster has been found being responsible for acetate production from CO₂ in rice root preparations incubated at 25 °C (Lu *et al.*, personal communication). In addition, members of *Clostridium* cluster I have been found being responsible for the decomposition of easily degradable residue components and for the fast accumulation of fatty acids in a Chinese rice field soil during the early phase of incubation at 15 and 30 °C (Rui *et al.*, 2009). Noll *et al.* (2010) reported that *Clostridium* cluster I and III developed in parallel to the thermal (45 °C) turnover of acetate and propionate. Therefore, *Clostridium* cluster I seems to have a rather large temperature range.

Clostridium cluster I contains the acetogenic species *Clostridium magnum* and *Clostridium drakei*, which exhibit 98% and 96% sequence similarity, respectively, to sequences retrieved in this study (Figure 5). *Clostridium magnum* was isolated from pasteurized freshwater sediment (Schink, 1984). H₂-CO₂, formate, methanol and a few other compounds serve as substrates, and acetate is the sole reduced end product. *C. magnum* can assimilate N₂ (Bomar *et al.*, 1991) and tolerate and consume small

amounts of O₂ (Karnholz *et al.*, 2002). Growth is possible at 15–45 °C with the optimum at 30–32 °C. The pH optimum is around pH 7.0 and no growth occurs at pH >7.8 or pH <6.0. *Clostridium drakei* was isolated from sediment collected from an acidic coal-mine pond in East-Central Germany. It is an obligate anaerobe with an optimum growth temperature of 30–37 °C and an optimum pH of 5.5–7.5. It grows chemolithoautotrophically with H₂-CO₂ or CO and chemoorganotrophically with glucose, cellulose, and so on. Acetic acid, ethanol, butyrate and butanol are the end-products of its catabolism (Liou *et al.*, 2005). One of the sequences (HQ266872) detected in our clone libraries has 90% similarity with acetogenic *Sporomusa acidovorans* retrieved from a distillation wastewater fermenter (Ollivier *et al.*, 1985). *S. acidovorans* is a Gram-negative, motile, curved, spore-forming rod and obligate anaerobic. The temperature range for growth is 20–40 °C with an optimum at 35 °C. Growth occurs within a pH range of 5.4–7.5, with an optimum at pH 6.5. Growth substrates include methanol, H₂-CO₂, formate, fructose, ribose, fumarate, succinate and glycerol. The only fermentation product is acetate; CO₂ is the only electron acceptor used.

Assimilation of CO₂ was greatly reduced when H₂ was replaced with N₂ indicating that chemolithoautotrophic acetogenesis was limited by H₂. Under these conditions at 15 °C, *Acidobacteriaceae* were the most abundant bacteria, but members of *Clostridium* cluster I and *Bacillales* were also abundant. However, members of uncultured *Peptococcaceae* were probably the most dominant CO₂ fixers as they were in particular prominent in the 'heavy' rRNA fractions. In the absence of exogenous H₂, methanogenic archaea were also prominent CO₂ fixers, as their rRNA became labeled from ¹³CO₂. Under these conditions, both acetogens and methanogens apparently reduced CO₂ to their respective products. Similar observations for acetogens and methanogens in peatland soil were recently reported (Drake *et al.*, 2009; Wüst *et al.*, 2009).

It is worth mentioning that all the microorganisms detected by SIP could be activated, although they had undergone a pre-incubation at 50 °C for 81 days. Nevertheless, the bacterial and archaeal groups detected were still able to assimilate CO₂ at 15 °C. A possible interpretation is that they can endure high temperature (50 °C) by forming spores and recover activity when the temperature is decreased. However, the microorganisms with potential recalcitrance against 50 °C included not only spore-forming *Clostridium* species and *Bacillales* but also *Acidobacteriaceae*, *Peptococcaceae* and various archaea (for example, *Methanobacteriales*). Although their mode of survival is unknown, such survival is not without precedence, as recovery of various anaerobic processes and microbial populations have been observed before in preheated Italian rice field soil (Conrad *et al.*, 2009; Noll *et al.*, 2010).

Beside acetate, formate, propionate and butyrate were also formed in the incubations, in particular in those at 50 °C, but maximum concentrations were much lower than for acetate, generally <0.4 mM. The H₂ partial pressures were high enough for exergonic production from H₂, CO₂ and acetate. Formate was reported as an interspecies electron carrier, similarly as H₂, in the syntrophic acetate oxidizer *Thermacetogenium phaeum* cocultured with methanogens (Hattori *et al.*, 2001). All these four fatty acids contained an excess of ¹³C ranging from 27 to 72 atom%, indicating that they were formed to a large part from CO₂ assimilation. Possibly acetogens were responsible for production, as many of them are able to form small amounts of fatty acids other than acetate (Drake *et al.*, 2006).

Acetate, an abundant potential substrate for acetoclastic methanogens, was accumulated at both 50 °C and 15 °C, however, was only consumed at 50 °C but not at 15 °C. Nevertheless, acetoclastic methanogens were not detected in the present experiments and also had only occasionally been detected in previous experiments when the soil was pre-incubated at 50 °C (Liu and Conrad, 2010). A possible explanation is that acetoclastic methanogens do not thrive after the long pre-incubation at 50 °C. Similar results have been obtained in previous studies (Fey *et al.*, 2001; Conrad *et al.*, 2009; Noll *et al.*, 2010). These results show that syntrophic acetate oxidizers were only active at 50 °C but not at 15 °C.

By contrast, hydrogenotrophic methanogens were detected in all the samples whether incubated at 15 °C or 50 °C, with or without H₂. Stable isotope fractionation ($\alpha_{\text{app}} \approx 1.08\text{--}1.09$) in the absence of exogenous H₂ indicated that CH₄ was mainly formed by hydrogenotrophic methanogenesis. Initially, *Methanocellales* (formerly rice cluster I, Sakai *et al.*, 2008) (T-RF = 393 bp) were dominant. However, after 20 days of incubation at 50 °C there was a gradual increase in the relative abundance of *Methanobacteriales* (T-RF = 92 bp) eventually becoming the main methanogenic populations.

Interestingly, the T-RF of 381-bp length (rice cluster III) also dramatically increased in abundance at higher buoyant density in the incubations with ¹³CO₂ at 15 °C both with and without H₂. Members of rice cluster III have not yet been isolated in pure culture. An enrichment culture containing about 2% rice cluster III archaea grew anaerobically on peptides and produced H₂ and acetate as intermediates that were eventually converted to CH₄ (Kemnitz *et al.*, 2005). The present experiments indicate that rice cluster III archaea are able to assimilate CO₂ into biomass even when H₂ is limiting.

In conclusion, acetogenic acetate production and CO₂ assimilation were found in Italian rice field soil, and syntrophic acetate oxidizers were identified to act also as acetogens at 50 °C when exogenous H₂ is supplied. At 15 °C, however, the syntrophic acetate oxidizers were not active and instead, other bacteria

were active in CO₂ fixation with and without exogenous H₂. These bacteria were apparently able to survive the 81 days of pre-incubation at high temperature and became active when the temperature was decreased to 15 °C. Hence, there was a considerable diversity of acetogens that could potentially be active as dark CO₂ fixers. For optimizing the process of dark CO₂ fixation it remains to be shown what are the maximum CO₂ amounts to be fixed. For environmental considerations it remains to be shown to what extent these bacteria can be active under *in situ* conditions.

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