Syntrophic acetate oxidation under thermophilic methanogenic condition in Chinese paddy field soil

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Abstract
The aim of the present work was to determine and compare the degradation of acetate in a Chinese rice field soil at 25°C and 50°C, respectively, and to identify specifically the active organisms involved in syntrophic acetate oxidation. Soil was preincubated anaerobically for 30 days to reduce alternative electron acceptors other than CO₂. The [2-13C] acetate (99% 13C) was added twice: 0 day and 19 days after preincubation. Addition of [2-13C] acetate resulted in an immediate increase of 13C labeled CH₄ but non-labeling of CO₂ at 25°C. The methanogen community was dominated by Methanosarcinaceae and Methanocellales at 25°C. In contrast, the addition of [2-13C] acetate at 50°C resulted in a rapid increase of 13CO₂. The 13C labeling of CH₄ gradually increased and reached a similar value to CO₂ (13% 13C) at the end of incubation (40 days). Nearly all archaeal 16S rRNA genes detected at 50°C belonged to hydrogenotrophic Methanocellales. DNA-based stable isotope probing analysis revealed that the organisms related to Thermacetogenium lineage and the unclassified Thermoanaerobacteraceae group were intensively labeled with 13C in the incubations at 50°C. Thus, acetate was converted to CH₄ and CO₂ through aceticlastic methanogenesis at 25°C, while syntrophic acetate oxidation occurred at 50°C.

Introduction
Methane (CH₄) is produced and emitted into the atmosphere from paddy field soils. Acetate and H₂/CO₂ roughly account for 67% and 33% of CH₄ precursors, respectively, in the anoxic soil (Conrad, 1999). Aceticlastic methanogenesis, however, could be substantially suppressed or was even absent under certain circumstances. For instance, hydrogenotrophic methanogenesis was found exclusively dominant when soil temperature increased to 45°C and higher (Fey & Conrad, 2000; Peng et al., 2008; Noll et al., 2010). CH₄ production from excised rice root material when incubated anaerobically resulted primarily from CO₂ reduction (Conrad & Klose, 1999; Lehmann-Richter et al., 1999; Conrad et al., 2002; Chin et al., 2004) and addition of ≥20 mM phosphate (a common fertilizer in rice soil) could substantially suppress aceticlastic methanogenesis without significant effect on the H₂-dependent pathway (Conrad et al., 2000). However, under these conditions, the rate of organic material degradation was not greatly affected, and acetate accumulated only transiently (Glissmann & Conrad, 2000; Peng et al., 2008; Noll et al., 2010).

Theoretically, conversion of acetate to CH₄ and CO₂ can follow two pathways under methanogenic conditions – aceticlastic methanogenesis and syntrophic acetate oxidation. The latter mechanism was first envisaged by Barker (1936) but was not appreciated until Zinder & Koch (1984) reported a co-culture involving a thermophilic syntrophic acetate oxidizer and a hydrogenotrophic methanogen and isolated the first pure culture (strain AOR) (Lee & Zinder, 1988b). Thereafter, several syntrophic acetate oxidizers were obtained from bioreactors including thermophilic Thermacetogenium phaeum (Hattoni et al., 2000) and Thermotoga lettingae (Balk et al., 2002), and mesophilic Clostridium ultunense (Schnürer et al., 1994, 1996) and Syntrophaceticus schinkii (Westerholm et al., 2010). These organisms, particularly for the strain T. phaeum PB, possibly utilize the reversal Wood/Ljungdahl pathway for the syntrophic acetate oxidation.
The syntrophic acetate oxidizers in environments, however, are probably less competitive for acetate than the aceticlastic methanogens, which convert acetate directly into CH₄ and CO₂. In anaerobic biogas reactors, it was found that CH₄ production from syntrophic acetate oxidation occurred only under conditions of high ammonia concentration (Schnürrer et al., 1999; Karakashev et al., 2006), which probably suppressed the aceticlastic methanogens (Karakashev et al., 2006). In the profound lake sediment, CH₄ production was found primarily from syntrophic acetate oxidation when hydrogenotrophic members were dominant (Nüsslein et al., 2001) but shifted to aceticlastic methanogenesis when aceticlastic methanogens were present (Schwarz et al., 2007).

So far knowledge of syntrophic acetate oxidation and the responsible organisms is limited to only a few pure cultures isolated from bioreactors. The mechanisms in natural environments remain largely unknown. Liu & Conrad (2010) reported the first case of syntrophic acetate oxidation coupled to CH₄ production in an Italian rice field soil at 50 °C. It is unknown whether such a process also occurs in other soils. The soil temperature of 50 °C is uncommon in the rice cultivation area in Asia. However, in summer, the temperature in flooding water and the surface soil of paddy fields in the southeastern China can reach temperatures of over 45 °C. The objectives of the present study, therefore, were to determine and compare the pathway of acetate conversion in a Chinese rice soil at 25 and 50 °C and to identify the potential organisms involved in syntrophic acetate oxidation. The DNA-based stable isotope probing (DNA-SIP) approach was employed in the experiment.

**Materials and methods**

**Anaerobic incubation**

Rice field soil was collected from the experimental station of the China National Rice Research Institute in Hangzhou, China (30°04′37″N, 119°54′37″E). The soil properties have been described previously (Peng et al., 2008; Wu et al., 2009). For anaerobic incubation, 3 g dry soil was mixed with 4.5 mL autoclaved and degassed water in 15-mL glass vials, and 15 mg of rice straw residues (dry weight) was added. The vials were sealed with butyl stoppers and flushed with N₂ for 5 min. Soil slurries were then anaerobically incubated for 30 days at 25 and 50 °C before the addition of acetate. The addition of rice straw in this preincubation activated the soil microorganisms and eliminated available oxidants such as nitrate, sulfate and ferric iron before the formal experiment (Lueders & Friedrich, 2000; Yuan et al., 2009).

Three treatments were prepared: (1) addition of nonlabeled acetate at a final concentration of 25 mM in soil slurry, (2) addition of the same amount of [2-13C] acetate (99% 13C, Sigma) and (3) addition of the same volume of distilled water. Addition of labeled and nonlabeled acetate was performed twice at day 0 and day 19 after the preincubation. At 25 °C, phosphate (20, 40 and 80 mM KH₂PO₄) or ammonium chloride (1 M NH₄Cl) was added to specifically inhibit the aceticlastic methanogens, creating conditions mesophilic syntrophic acetate oxidation. Each treatment was carried out in triplicate and statically incubated for 42 days.

**Measurement of gases and volatile fatty acids**

Gas samples (0.2 mL) were taken from headspace with a syringe. Liquid samples were collected and centrifuged as described previously (Rui et al., 2009). The supernatants were passed through 0.45-μm pore-size filters (Sangon, Shanghai) and stored at ~20 °C. The concentrations of CH₄, CO₂ and H₂ were determined with HPLC (Shimadzu LC-10A) using Shim-Pack RP-C18 ODS column (150 × 6.0 mm) under conditions described previously (Shen, 1998).

**DNA extraction and terminal restriction fragment length polymorphism (T-RFLP) analyses**

Genomic DNA was extracted as described previously (Peng et al., 2008). PCR amplification of bacterial and archaeal DNA used primer pairs of Ba27f/Ba907r and Ar109f/Ar934r, respectively (Lueders et al., 2004; Lu et al., 2006). The 5′ end of Ba27f and Ar934r was labeled with 6-carboxyfluorescein (FAM). The 50-μL PCR reaction mixture contained the following components: 1 μL of DNA template (in 1:10 dilution of original extracts), 5 μL of 10 × buffers (TaKaRa), 3 μL of 25 mM MgCl₂ (TaKaRa), 4 μL of 2.5 mM dNTP (TaKaRa), 0.5 μL of each 50 μM primer (Sangon) and 2.5 U Taq DNA polymerase (TaKaRa). The thermal profile for bacterial amplification was: 2 min at 94 °C; and 28 cycles of 30 s at 94 °C, 45 s at 52.5 °C and 80 s at 72 °C; and finally 10 min at 72 °C. The thermal profile for archael amplification was: 3 min at 94 °C; and 32 cycles of 60 s at 94 °C, 45 s at 52 °C and 90 s at 72 °C; and finally 5 min at 72 °C. The FAM-labeled PCR product was purified using an agarose gel DNA extraction kit (TaKaRa). The PCR product of bacterial DNA was digested at 37 °C for 5 h by MspI (TaKaRa), and that of archaeal DNA at 65 °C for 5 h by TaqI (TaKaRa). The digestion products were purified with SigmaSpin™.
Post-Reaction Clean-Up Columns (Sigma) and were then size-separated using the 3130xl Genetic Analyzer as described previously (Peng et al., 2008).

DNA-SIP
Two $^{13}$C-labeled DNA samples and one control ($^{12}$C-DNA) sample were selected to perform DNA-SIP analyses. The $^{13}$C-labeled samples were obtained from soil under [2-$^{13}$C] acetate incubation at day 6 and day 23, respectively. PicoGreen (Invitrogen) was used to quantify the DNA content in the extracts. DNA was density resolved by equilibrium density gradient centrifugation in cesium trifluoroacetate (CsTFA) (Amersham Biosciences) following a similar protocol described previously (Lu et al., 2005; Qiu et al., 2009). After centrifugation, the density of the collected gradient fractions was determined refractometrically, and DNA was precipitated and resuspended in 30 μL nuclease-free water for subsequent community analyses. PCR and T-RFLP analyses for DNA fractions of different buoyant density were performed as described above.

Cloning, sequencing and phylogenetic analyses
The PCR amplification of bacterial and archaeal 16S rRNA genes used the same primers as indicated above. PCR products were purified and ligated into the pMD19-T vector (TaKaRa) according to the manufacturer’s instructions. Plasmids were transformed into Escherichia coli cells and clones randomly selected were sequenced using an ABI 3730xl sequencer as described previously (Peng et al., 2008; Rui et al., 2009).

The sequence data were analyzed using the ARB software package (http://www.arb-home.de) (Ludwig et al., 2004). Phylogenetic trees were constructed using the neighbor-joining algorithm as described in Peng et al. (2008) and Rui et al. (2009). The sequence data obtained from this study have been deposited in the EMBL nucleotide sequence database under the accession numbers from FN643421 to FN643571.

Ordination analyses of T-RFLP fingerprints
The relative abundance of terminal restriction fragments (T-RFs) was calculated as the percentage of an individual T-RF in sum of all concerned peak heights in a given T-RFLP profile. Ordination analyses of T-RFLP profiles were performed using CANOCO 4.5 software (Microcomputer Power, Ithaca, NY) (Ter Braak & Smilauer, 1998). The linear model of redundancy analysis was chosen for analysis. The temporal change of bacterial dynamics was evaluated as described previously (Rui et al., 2009). The correlations between different species could be estimated by perpendicularly projecting the arrow tip of a species of interest onto the arrow of another species (Leps & Smilauer, 2003).

The correlation between a species and the explanatory variable (time) can be estimated similarly.

Results and discussion

Dynamics of acetate and CH₄
Over 90% of acetate was consumed within 12 days after the first addition (Fig. 1). The depletion rate of acetate was very similar between 25 and 50 °C. When the second dose of acetate was applied, a similar pattern of acetate depletion was observed at 50 °C. For the 25 °C incubation, NH₄Cl (1 M) was added in some vials at the time of second acetate addition. Methanogenesis of both aceticlastic and hydrogenotrophic appeared completely inhibited and hence acetate concentration did not decrease in the second phase of incubation at 25 °C. In contrast, the addition of phosphate (20, 40 and 80 mM) had no detectable effect on either acetate degradation or CH₄ production (data not shown).

In correspondence with acetate consumption, CH₄ concentration in the headspace increased almost linearly with time (Fig. 2). The rate of CH₄ production was initially faster at 50 °C (Fig. 2a) than at 25 °C (Fig. 2b). After a transient slowdown, CH₄ production increased again with the addition of second acetate dosage. For the control without acetate addition, CH₄ in the headspace from 50 °C incubations accumulated to a concentration of almost twice that at 25 °C. CH₄ concentration did not increase in the presence of NH₄Cl at 25 °C (Fig. 2b).

![Fig. 1. Dynamics of acetate during anoxic incubation in rice field soil.](https://academic.oup.com/femsec/article-abstract/77/2/264/498620)
The analysis of isotopic carbon ratios revealed that the \(^{13}C\) atom% of CO\(_2\) sharply increased after addition of [\(^2\text{-}^{13}C\) acetate in incubation at 50 °C and reached the first maximum (24%) at day 4 and then gradually decreased until day 18 (Fig. 3a). The \(^{13}C\) atom% of CH\(_4\) increased and decreased again after the addition of the second acetate dosage, reaching a second maximum at day 25. The \(^{13}C\) atom% of CH\(_4\) increased rapidly after addition of [\(^2\text{-}^{13}C\) acetate, reaching 22% at day 8. Following a brief decrease, \(^{13}C\)CH\(_4\) increased again with the addition of a second dose of [\(^2\text{-}^{13}C\) acetate, reaching a final maximum of 26% at the end of experiment.

Carbon isotopic labeling is the most straightforward approach for distinguishing CH\(_4\) production of acetoclastic methanogenesis and syntrophic oxidation of acetate (Zinder & Koch, 1984; Nüsslein et al., 2001; Karakashev et al., 2006). The rapid increase of \(^{13}C\)CH\(_4\) but nonlabeling of CO\(_2\) at 25 °C indicated that CH\(_4\) production was primarily from acetoclastic methanogenesis. In contrast, the rapid increase of \(^{13}C\)CO\(_2\) followed by a gradual increase of \(^{13}C\)CH\(_4\) at 50 °C (Fig. 3b) indicated that the methyl group of acetate was first oxidized to CO\(_2\), and CH\(_4\) was then produced from CO\(_2\) reduction. Theoretically, 50% of CH\(_4\) and 50% of CO\(_2\) should be labeled with \(^{13}C\) if [\(^2\text{-}^{13}C\) acetate (99% \(^{13}C\) was syntrophically oxidized and CH\(_4\) was produced completely from the H\(_2\)/CO\(_2\) produced. However, only 24% of CO\(_2\) was labeled with \(^{13}C\) at the maximum, indicating that isotopic dilution from an indigenous carbon source occurred. This
indigenous carbon source might result from organic residues added before the preincubation. Nevertheless, the $^{13}$C-labeling pattern of CH$_4$ and CO$_2$ obtained suggested that syntrophic oxidation of acetate was the main pathway for CH$_4$ and CO$_2$ production in the soil at 50°C. This pattern differed from that in the Italian rice field soil (Liu & Conrad, 2010), where the isotopic signature of CH$_4$ increased more quickly and reached a higher value than that of CO$_2$ after [2-$^{13}$C] acetate addition. This suggested that CH$_4$ should also be produced actively from aceticlastic methanogenesis in Italian rice soil at 50°C.

**Archaeal and bacterial communities**

T-RFLP fingerprinting was used to follow the dynamics of archaeal community in the soil over a 42-day incubation. Four major T-RFs (93, 158, 258 and 393 bp) were detected in T-RFLP profiles retrieved from soil samples at 50°C (Fig. 4a). The 393-bp T-RF was exclusively dominant, with the relative abundance being higher than 85% over the entire incubation period. The 93-bp T-RF was detected occasionally at a low frequency (relative abundance < 3.2%). The 158-bp T-RF showed an increase in the later period of incubation, with the relative abundance reaching 8.2% at the end of experiment. The 258-bp T-RF was also detected at a low frequency in the later period. A clone library of archaeal 16S rRNA genes constructed from a soil sample at 50°C indicated that all clone sequences (n = 37) were affiliated with *Methanocellales* and showed a characteristic T-RF of 393 bp (Supporting Information, Fig. S1). We referred to several previous studies for the affiliation of other three T-RFs detected (Wu et al., 2006; Peng et al., 2008; Yuan & Lu, 2009). Thus, the 93-bp T-RF could be linked to *Methanobacteriaceae*, and the 158- and 258-bp T-RFs were related to different members of *Methanocellales*. Combination of both clone library and T-RFLP profiles revealed that hydrogenotrophic methanogens were exclusively dominant, whereas aceticlastic methanogens were absent in soil slurries at 50°C. On the other hand, T-RFLP analysis revealed that the archaeal community was more diverse at 25°C and both *Methanosarcinaceae* (186 bp) and *Methanocellales* (393 bp) were dominant over the 42-day incubation (Fig. 4b). The addition of NH$_4$Cl at day 19 probably increased the relative abundance of *Methanocellales* compared with *Methanosarcinaceae*.

For the bacterial community, the $^{13}$C-labeled DNA retrieved at day 6 and day 23 after [2-$^{13}$C] acetate incubation were fractionated and analyzed across a buoyant density gradient from 1.518 to 1.613 g mL$^{-1}$. For comparison, DNA from unlabeled samples at day 23 was also analyzed in parallel. T-RFLP fingerprints of the unlabeled control (added with nonlabeled acetate) did not change across DNA buoyant density gradients, with the exception of the 518-bp T-RF, which showed a tendency to decrease with increase of buoyant density (Fig. 5a). For the labeled sample at day 6 (Fig. 5b), T-RFLP patterns similar to those of the control soil were obtained. However, there was already evidence of a tendency of the 143- and 148-bp T-RFs in ‘heavy’ DNA fractions to increase, based on relative abundance calculation. For the labeled sample at day 23 (Fig. 5c), T-RFLP patterns changed markedly among DNA fractions of different buoyant densities. In the ‘light’ DNA fractions (BD < 1.574 g mL$^{-1}$), T-RFLP patterns still remained similar to that of control soil. However, in the ‘heavy’ fractions (BD > 1.584 g mL$^{-1}$), there was a reduction in the diversity of T-RFLP fingerprints, and the 143- and 148-bp T-RFs became exclusively dominant. The 518-bp T-RF almost disappeared in these ‘heavy’ fractions.
The temporal patterns of T-RFLP profiles for the bulk DNA samples (without isopycnic centrifugation) revealed that the relative abundance of 143-, 148-, 297- and 81-bp T-RFs increased with time, whereas those of 170, 518, 522 and 568 bp decreased relatively (Fig. 6). No significant change over the incubation period was seen for other T-RFs, for example 95, 150, 172 and 160 bp.

A 'heavy' (BD of 1.584 g mL\(^{-1}\)) and a 'light' (BD of 1.563 g mL\(^{-1}\)) DNA fraction were selected for construction of the bacterial clone libraries (Table 1). Phylogenetic analysis revealed that most of sequences were affiliated with class Clostridia (Fig. 7). The dominant sequences belonged to members of Clostridiaceae and Thermanaerobacteraceae. Clostridiaceae consisted mainly of clostridial clusters I and III groups and Thermanaerobacteraceae encompassed organisms related to Thermacetogenium and the unclassified Thermoaerobacteraceae groups. The clusters I and III clostridia accounted for 27% and 19% in the 'light' clone library but only 6% and 11% in 'heavy' library, respectively (Table 1). By contrast, organisms related to Thermacetogenium and unclassified Thermoaerobacteraceae groups accounted for 19% and 26% in the 'heavy' library but only 6% and 16% in the 'light' clone library.

In silico analysis of clone sequences revealed that the clostridial clusters I and III groups were characterized by 170-, 518-, 522- and 568-bp T-RFs and 85-, 143- and 273-bp T-RFs, respectively (Table 1, Fig. 7). Three clones of cluster III clostridia characterized by T-RF 143 bp were detected only in the 'light' library. Organisms related to Thermacetogenium were characterized by T-RFs of 140, 143 and 158 bp.

The subgroup characterized by the 158-bp T-RF showed only a minor abundance. The subgroup represented by 143-bp T-RF had 16S rRNA gene similarity of 98% to T. phaeum PB strain (Hattori et al., 2000) and showed the highest abundance in the 'heavy' fraction. The subgroup characterized by 140-bp T-RF was also related to Thermacetogenium but...
showed a similarity of only 94% to *T. phaeum*. These two T-RFs were indistinguishable in the real T-RFLP fingerprints and hence presented as a single peak in the electropherograms (Fig. 5). Members of the unclassified *Thermoanaerobacteraeae* group were characterized by a single T-RF of 148 bp. Thus, both T-RFLP fingerprint and clone library analyses revealed that organisms related to *Thermacetogenium* and the unclassified *Thermoanaerobacteraeae* groups actively assimilated [2-13C] acetate during the incubation at 50 °C.

The first strain of *T. phaeum* was isolated from a thermophilic anaerobic bioreactor (Hattori et al., 2000). This organism was affiliated with acetogens and utilized the Wood–Ljungdahl acetyl-CoA pathway reversibly for acetate synthesis and oxidation (Hattori et al., 2005). Recently, a new syntrophic, acetate-oxidizing bacterium, namely *S. schikii*, was isolated from a mesophilic methanogenic bioreactor (Westerholm et al., 2010). This organism showed the 16S rRNA gene similarity of 92% to *T. phaeum* and hence was proposed as a new genus (Westerholm et al., 2010). The organisms actively assimilating 13C in our soil can be separated into three subgroups (Fig. 7). Cluster I (represented by T-RF of 143 bp) was closely related to *T. phaeum* (16S rRNA gene similarity of 98%). Cluster II (T-RF 140 bp) showed a similarity of 94% to *T. phaeum*, thus possibly representing a new species of *Thermacetogenium*. Cluster III (T-RF 148 bp) represented the unclassified *Thermoanaerobacteraeae* group and was only distantly related to *Thermacetogenium*. Similar bacterial sequences were detected in the syntrophic acetate oxidation in Italian rice soil (Liu & Conrad, 2010). Therefore, it appears that the organisms with their 16S rRNA gene sequences scattered within the radiation of *Thermacetogenium* are widespread in anoxic rice soils and probably share a common physiology of syntrophic acetate oxidation in the soil.

Clusters I and III clostridia characterized by T-RFs of 170, 273 and 518 bp were dominant but apparently did not assimilate 13C in our soil at 50 °C. We have shown earlier that clusters I and III clostridia are abundant and the cluster III in particular are adapted to high temperatures during anaerobic degradation of organic residues in rice soil (Rui et al., 2009). Active CH4 production was detected in the control soil without acetate addition (Fig. 2), indicating the degradation of indigenous organic matter. Given that thermophilic syntrophic oxidation took place upon acetate addition without delay (Fig. 3a), it could be assumed that the syntrophic interactions were already established during the preincubation. Clusters I and III clostridia, therefore, were possibly involved in fermentation of degradable organic residues and generated acetate for the syntrophic oxidation and CH4 production (Rui et al., 2009). The isotopic dilution of CO2 and CH4 after addition of [2-13C] acetate indicated that these fermentors were active even during the acetate incubation.

The dominance of *Methanocellales* and the absence of acetoclastic methanogens indicated that members of *Methanocellales* served as a partner in the syntrophic interaction at 50 °C. Previous studies showed that these hydrogenotrophic methanogens in rice soil were favored under low H2 conditions and high temperatures compared with other methanogens (Fey & Conrad, 2000; Lu et al., 2005; Peng et al., 2008). A mesophilic and a thermophilic strain have been obtained recently (Sakai et al., 2008, 2010). The thermophilic and low H2 preferences offer these methanogens a specific advantage to serve as H2 remover in the thermophilic syntrophic oxidation of acetate. Some rare T-RFs belonging to *Methanocellales* (T-RFs of 158 and 258 bp) appeared in the later stage of syntrophic acetate oxidation (Fig. 4a), indicating that these members of *Methanocellales* might be favored under conditions of syntrophic acetate oxidation.

Both 13C labeling and methanogen composition revealed that CH4 production was primarily from acetoclastic methanogenesis at 25 °C. We tried to establish conditions for the mesophilic syntrophic acetate oxidation. Addition of phosphate at 20, 40 and 80 mM failed to inhibit the acetoclastic CH4 production, but addition of NH4Cl at 1 M appeared to inhibit not only acetoclastic but also hydrogenotrophic methanogenesis completely. Therefore, no further attempt

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**Table 1.** Phylogenetic affiliations and clone number of 16S rRNA genes retrieved from ‘heavy’ (D23L6, BD of 1.584 g mL–1) and ‘light’ (D23L8, BD of 1.563 g mL–1) DNA fractions of anoxic rice soil at day 23 and 50 °C.

<table>
<thead>
<tr>
<th>Phylogenetic group</th>
<th>Heavy</th>
<th>Light</th>
<th>T-RF (bp)*</th>
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<tr>
<td>Clostridiales</td>
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<td>81, 138</td>
</tr>
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</table>

* T-RF lengths (in silico) are shown in base pairs (bp).
* T-RFs detected in more than one phylogenetic group.
* T-RFs with a relative abundance of more than 4% in total (114 clones) are indicated in boldface.

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Fig. 7. Phylogenetic relationship of representative bacterial 16S rRNA gene clone sequences generated from ‘heavy’ and ‘light’ gradient fractions of anoxic rice soil at day 23 and 50 °C. Sequences of D23L6 library represented the ‘heavy’ DNA fraction; those of D23L8 library represented the ‘light’ DNA fraction. Reference sequences are shown in roman type and the sequences generated in this study are in boldface. The scale bar represents 10% sequence divergence; GenBank accession numbers of sequences are indicated; the in silico T-RF sizes are given after the sequence names. ‘I–III’ represent three subgroups of bacteria which actively assimilated $^{13}$C.
was made to determine syntrophic acetate oxidation at 25 °C. Bacterial information at this temperature is given in our previous report (Rui et al., 2009).

In conclusion, acetate was syntrophically oxidized and CH4 was produced from H2/CO2 in rice soil at 50 °C. The organisms related to Thermacetogenium and the unclassified Thermoanaerobacteriaceae groups are responsible for syntrophic oxidation of acetate with the hydrogenotrophic Methanocellales serving as partner for H2 removing and CH4 production. Given that this type of syntrophic acetate oxidizers was also active in Italian rice soil (Liu & Conrad, 2010), we assume that the thermophilic syntrophic oxidation of acetate is widespread in flooded rice soils of different geographical origins.

Acknowledgements

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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Phylogenetic relationship of representative archaeal 16S rRNA gene clone sequences generated from anoxic rice soil at day 23 and 50 °C.

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