Competing Formate- and Carbon Dioxide-Utilizing Prokaryotes in an Anoxic Methane-emitting Fen Soil

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Running title: Competing Prokaryotes in a Methane-emitting Fen Soil

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Methanogenesis in wetlands is dependent on intermediary substrates derived from the degradation of biopolymers. Formate is one such substrate and is stimulatory to methanogenesis and acetogenesis in anoxic microcosms of soil from the fen Schlöppnerbrunnen. Formate dissimilation also yields CO\(_2\) as a potential secondary substrate. The objective of this study was to resolve potential differences between anaerobic formate- and CO\(_2\)-utilizing prokaryotes of this fen by stable isotope probing. Anoxic soil microcosms were pulsed daily with low concentrations of \([^{13}\text{C}]\)-formate or \(^{13}\text{CO}_2\) (i.e., \([^{13}\text{C}]\)-bicarbonate). Taxa were evaluated by assessment of 16S rRNA genes, \(mcrA\) (encodes for the alpha-subunit of methyl-CoM reductase), and \(fhs\) (encodes for formyltetrahydrofolate synthetase). Methanogens, acetogens, and formate-hydrogen lyase-containing taxa appeared to compete for formate. Genes affiliated with \(\text{Methanocellaceae, Methanobacteriaceae, Acetobacteraceae, and Rhodospirillaceae}\) were \([^{13}\text{C}]\)-enriched (i.e., labeled) in \([^{13}\text{C}]\)-formate treatments, whereas genes affiliated with \(\text{Methanosarcinaceae, Conexibacteraceae, and Solirubrobacteraceae}\) were labeled in \(^{13}\text{CO}_2\) treatments. \([^{13}\text{C}]\)-acetate was enriched in \([^{13}\text{C}]\)-formate treatments, but labeling of known acetogenic taxa was not detected. However, several phylotypes were affiliated with acetogen-containing taxa (e.g., \(\text{Sporomusa}\)). \(\text{Methanosetaeae}\)-affiliated methanogens appeared to participate in the consumption of acetate. Twelve and 58 family-level archaeal and bacterial 16S rRNA phylotypes, respectively, were detected, approximately half of which had no isolated representatives. \(\text{Crenarchaeota}\) constituted half of the detected archaeal 16S rRNA phylotypes. The results (a) highlight the unresolved microbial diversity of the fen Schlöppnerbrunnen, (b) suggest that differing taxa competed for the same substrate, (c) indicate that
Methanocellaceae, Methanobacteriaceae, Methanosarcinaceae, and Methanosaetaceae were linked to the production of methane, but (d) did not clearly resolve the taxa responsible for the apparent conversion of formate to acetate.

**INTRODUCTION**

Methane is the second most important greenhouse gas (76), and its atmospheric concentration has increased to approximately 1775 ppb (39, evaluation from 2005). Wetlands contribute 27-53% (i.e., 160 to 314 Tg methane) to the global emission of methane (39), underscoring the importance of understanding microbial-mediated processes that are linked to methanogenesis in wetlands. Methanogens have a very limited substrate range (34, 95), and their in situ activities are linked to ‘intermediary ecosystem metabolism’, i.e., a complex food-web of interconnected microorganisms that catalyze essential intermediary processes that ultimately drive methanogenesis (19, 64, 94). Thus, methane production in many ecosystems including wetlands is dependent on intermediary substrates formed during the degradation of plant-derived polymers. Fifty to 80 percent of plant-derived organic matter consists of lignocelluloses (2), polymers that can be degraded by fungi and bacteria to glucose, xylose, and aromatic compounds (82). Chitin, a biopolymer of \( N \)-acetylglucosamine, is another potentially important source of organic carbon in various ecosystems (75). Primary fermenters (e.g., *Aeromonadaceae, Clostridiaceae*) in wetland soils can produce organic acids, alcohols, molecular hydrogen (\( H_2 \)), and carbon dioxide (\( CO_2 \)) from potential breakdown products of biopolymers (i.e., glucose, xylose, and \( N \)-acetylglucosamine) (32, 90). Organic acids
and alcohols are further metabolized to H\textsubscript{2} and CO\textsubscript{2} by secondary fermenters (e.g., Synctrophobacteraceae [6]). The terminal stage of the methanogenic food-web is catalyzed by methanogens (e.g., Methanomicrobiaceae, Methanosarcinaceae) that collectively convert formate, acetate, methanol, and H\textsubscript{2}-CO\textsubscript{2} to methane (1, 19, 34, 64, 95).

Several studies have demonstrated that wetland soils contain complex prokaryotic communities (e.g., 10, 15, 40, 43), a finding consistent with the aforementioned network of trophically linked processes that yield methane. The methanogenic community of the fen Schöllpnerbrunnen in southeast Germany is composed of Methanobacteriaceae, Methanomicrobiaceae, Methanosaetaceae, and Methanosarcinaceae (32, 90). Formate is a significant driver of methanogenesis under experimental conditions and (a) might be derived from the fermentation of monosaccharides such as glucose and N-acetylglucosamine and (b) also stimulates acetogenesis (i.e., the reductive synthesis of acetate from CO\textsubscript{2} via the acetyl-CoA 'Wood-Ljungdahl' pathway [20]) (32, 90). The periodic occurrence of up to 0.65 mM formate in fen porewater (47) reinforces the likelihood that formate is a relevant in situ substrate for fen methanogens and other competing prokaryotic taxa. Dissimilation of formate also yields CO\textsubscript{2}, which could subsequently be utilized as a secondary source of carbon. The main objective of the present study was to resolve potential differences between anaerobic formate- and CO\textsubscript{2}-utilizing prokaryotic taxa in soil from the fen Schöllpnerbrunnen by stable isotope probing.
MATERIAL AND METHODS

Sampling site. Fens are specialized mires (29), and the moderately acidic, methane-emitting fen Schlöppnerbrunnen is located 700 m above sea level (50° 07' 53" N, 11° 52' 51" E) in the Lehstenbach catchment of the Fichtelgebirge (translates as Spruce Mountains) in southeast Germany (for site description, see 32, 71). The pH of fen pore water approximates 4.5, and formate in fen pore water can range from 0 to 0.65 mM (47). Three 0-20 cm depth soil cores were taken in July 2008 (4-5 m apart) with a soil corer, transported in airtight sterile plastic bags, and stored on ice until processed within 6 hours of sampling.

Anoxic microcosms. The three soil cores were homogenized together; the homogenized fen soil had a 83.5% water content. Thirty-five grams of fresh weight homogenized soil were placed in sterile 500 ml-infusion flasks (Merck ABS, Dietikon, Switzerland) and diluted with 125 ml anoxic mineral solution (pH 4.8) containing mineral salts, trace metals, and vitamins (90). The infusion flasks were closed with rubber stoppers and crimp seals, and flushed with sterile N\textsubscript{2} (100%). Anoxic solutions were prepared by using modified Hungate techniques (14).

A 15 day anoxic pre-incubation of soil slurry microcosms was used to ensure that endogenous nitrate, sulfate, and iron(III) were reduced (19, 32, 90). Formate treated soil slurry microcosms were then pulsed daily with approximately 64 µmol formate per microcosm, yielding approximately 0.3 to 0.6 mM formate in the aqueous phase (i.e., 9.7 to 17.4 µmol formate g\textsubscript{soil\textsubscript{dw}}\textsuperscript{-1}) during the incubation, a variation due to the daily sampling that yielded a changing volume. The formate that was pulsed was from a filter sterilized solution of either sodium \textsuperscript{[13]C}-formate (99 atom% \textsuperscript{13}C) or sodium \textsuperscript{[12]C}-formate (i.e., unlabeled formate with a natural abundance of \textsuperscript{13}C).
Additional microcosms were pulsed daily with approximately 160 µmol CO₂ (from a filter sterilized solution of sodium [¹³C]-bicarbonate or sodium [¹²C]-bicarbonate) per microcosm, yielding approximately 1.0 to 3.1 mM CO₂ in the combined aqueous and the gas phases (i.e., 28.9 to 87.1 µmol CO₂ g[soil dw]⁻¹) during the incubation. The CO₂ that was pulsed was from a filter sterilized solution of either sodium [¹³C]-bicarbonate (99 atom% [¹³C]) or sodium [¹²C]-bicarbonate (i.e., unlabeled bicarbonate with a natural abundance of [¹³C]). Control microcosms lacked supplemental formate or CO₂. The purpose of the [¹³C]CO₂ treatment was two-fold: (a) to control for potential cross feeding (i.e., labeling of microorganisms by assimilation of [¹³C]-formate-derived [¹²C]CO₂), and (b) to assess taxa capable of utilizing CO₂ (i.e., assimilating CO₂ at the expense of endogenous reductant). Two additional safeguards against CO₂ cross-feeding were taken: (a) formate treatments were pulsed daily with 192 µmol [¹²C]CO₂ (i.e., sodium [¹²C]-bicarbonate, yielding 1.9 to 4.4 mM CO₂ in the combined aqueous and the gas phases, equivalent to 53.7 to 122.0 µmol CO₂ g[soil dw]⁻¹) per microcosm, and (b) the gas phases of microcosms were exchanged with sterile N₂ (100%) before substrate pulsing was initiated and every subsequent fourth day. For exchanging the gas phase with N₂, microcosms were evacuated under sterile conditions for 30 minutes at approximately -800 mbar, after which the gas phase was replaced with sterile N₂ (100%). This procedure was repeated after 15 min. Microcosms were then flushed with sterile N₂ (100%) for 20 min. The pH was adjusted every fourth day to approximately pH 4.5 with anoxic sterile 5M HCl. Soil slurries were incubated horizontally in the dark at 15°C and were exposed to light only during analyses. The gas and liquid phases of soil slurries were sampled with sterile syringes. Liquid samples were stored at -20°C for chemical analyses or at -80°C for molecular analyses.
Nucleic acid extraction. Nucleic acids were extracted by bead-beating lysis, organic solvent extraction, and precipitation (30). DNA was purified and separated from RNA with Qiagen RNA/DNA mini-kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol.

Density gradient centrifugation. DNA stable isotope probing was performed by published protocol (68). DNA was added to a gradient solution (buoyant density 1.725 g ml\(^{-1}\)) containing a cesium chloride solution (buoyant density 1.881 g ml\(^{-1}\); 80.8% of total) and gradient buffer (pH 8; 100 mM Tris; 100 mM KCl; 1 mM EDTA; 19.2% of total) and filled into OptiSeal Tubes (Beckmann, Fullerton, CA, USA). Differences within the gradient density could cause differences in the gene libraries prepared from gradient fractions, thus resulting in inconsistencies in determining which microorganisms are labeled. This problem was minimized by preparing all gradients with the same gradient solution. DNA was subjected to isopycnic centrifugation (177,200 g [44,100 rpm] at 20°C for 40 hours; Vti 65.2 vertical rotor, Beckman Coulter, Fullton, USA) and fractionated. The buoyant density of the gradient solution and fractions (Supplemental Fig. S1A) were determined by weighing gradient solutions and fractions at 20°C. DNA was precipitated with glycogen and polyethylene glycol 6000, and DNA concentrations in gradients (Supplemental Fig. S1A) were measured with Quanti-iT PicoGreen Assay Kit (Invitrogen, Karlsruhe, Germany).

PCR conditions and cloning. Complementary DNA was amplified with the following primer sets: (a) mcrAf (5’-TAYGAYCARATHTGGYT-3’) and mcrAr (5’-ACRTTCATNGCRTARTT-3’) for mcrA (83), (b) FTHFSf (5’-TTYACWGGHGAYTTCCATGC-3’) and FTHFSr (5’-GTATTGDGTYTTRGCCATACA-3’) for fhs (53), (c) 27f (5’-AGAGTTTGATCMTGGCTC-3’) and 907r (5’-
CCGTCAATTCMTTTRAGT-3') for bacterial 16S rRNA genes (52), and (d) Arc21Fa
(5'-TCCGGTTGATCCYGSCRG-3') (38) and Arc915 (5'-
GTGCTCCCCCGCAATTCT-3') (74) for archaeal 16S rRNA genes. Amplification
of mcrA was as described (59) with the following modifications: (a) initial denaturation
at 94°C for 5 min, (b) 35 cycles of denaturation at 94°C for 45 s with annealing at
50°C for 45 s and elongation at 72°C for 45 s, and (c) terminal elongation at 72°C for
5 min. Fhs was amplified as follows: (a) initial denaturation at 94°C for 5 min, (b) 35
cycles of denaturation at 94°C for 30 s with annealing at 58°C for 45 s and elongation
at 72°C for 70 s, and (c) terminal elongation at 72°C for 5 min. Bacterial 16S rRNA
genes were amplified as follows: (a) initial denaturation at 95°C for 5 min, (b) 5 pre-
cycles of denaturation at 95°C at 60 s with annealing at 40°C for 60 s and elongation
at 72°C for 60 s, (c) 30 subsequent cycles of denaturation at 95°C for 30 s with
annealing at 43°C for 30 s and elongation at 72°C for 5 s, and (d) terminal elongation
at 72°C for 5 min. Archaeal 16S rRNA genes were amplified as described (32) with
the following modifications: (a) initial denaturation at 95°C for 5 min, (b) 33 cycles of
denaturation at 95°C for 50 s with annealing at 55°C for 50 s and elongation at 72°C
for 110 s, and (c) terminal elongation at 72°C for 5 min. Each PCR assay was
facilitated with 5 Prime mastermix (5 Prime, Hamburg, Germany). Final
concentrations of PCR reagents were: 0.4 mg bovine serum albumin ml⁻¹, 4 µM
(mcrA, fhs) or 0.6 µM (16S rRNA genes) of each primer, 0.6 U Taq DNA polymerase,
0.2 mM of each dNTP, 2.6 mM (mcrA) or 3.6 mM (fhs, 16S rRNA genes) MgCl₂.

PCR products for cloning were ligated into pGEM-T vector plasmids (Promega,
Mannheim, Germany). Competent cells of *Escherichia coli* JM109 (Promega;
protocol as per manufacturer’s instructions) were transformed with ligated pGEM-T
vector plasmid. Clones were randomly picked, and the correct insert was determined
by M13 PCR (primer set M13f/M13r) according to published protocol (65) and selected for sequencing at Macrogen (Seoul South Korea).

**Analysis of DNA.** The DNA in fractions 3 to 9 from density gradients prepared from $[^{13}]C$-formate supplemented fen microcosms was evaluated with terminal restriction fragment length polymorphism (TRFLP) analysis. PCR was performed with fluorescently labeled primers mcrAf-DY681 and mcrAr (Biomers GmbH, Ulm, Germany) (83). Gel-purified DNA (Montage™ DNA Gel Extraction Kit, Millipore Corporation, Bedford, USA) was digested with mung bean endonuclease (New England Biolabs, Frankfurt/Main, Germany) according to the manufacturer’s protocol to minimize the occurrence of pseudo-terminal restriction fragments (21). DNA was then cleaved at specific restriction sites with two units of each restriction enzyme MspI and RsaI (New England Biolabs, Frankfurt/Main, Germany). Remaining DNA was quantified with Quanti-iT PicoGreen Assay Kit, and TRFLP analysis was performed as described (32).

The 210 bp terminal restriction fragments of ‘heavy’ fractions increased in relative intensity (Supplemental Fig. S1B), indicating a greater abundance of mcrA genes in the heavier fractions. ‘Heavy’ fraction 4 contained enough DNA to obtain a clear PCR signal. DNA of ‘heavy’ fraction 4 was used for establishing gene libraries of mcrA and 16S rRNA genes from $[^{13}]C$-formate-, $[^{12}]C$-formate-, $^{13}CO_2$-, and $^{12}CO_2$-supplemented microcosms to identify active consumers of formate and CO$_2$. Adequate PCR signals for fhs were not detected in fraction 4, but were detected in fraction 5. Fraction 7 also yielded an adequate fhs PCR signal and was therefore analyzed for fhs to increase the detection of the overall diversity of this gene. Thus, fhs was analyzed from DNA of fraction 5 and fraction 7 of $[^{13}]C$-formate-
supplemented microcosms and fraction 5 of $^{12}$C-formate-supplemented microcosms after 23 days of incubation with formate.

**Sequence analyses and identification of phylotypes.** All sequences were analysed with Mega (85) and ARB software (58). MegaBLAST was used to compare sequences to those in public databases (67). Chimeric sequences of 16S rRNA gene sequences were identified by the greengenes tool Bellerophon (16) and excluded from further analyses. Phylotypes of 16S rRNA genes were determined with RDP Classifier at a confidence threshold of 80% (88), aligned with SINA webaligner, and merged with the latest 16S rRNA gene database from the SILVA homepage (www.arb-silva.de) (72). Sequences of 16S rRNA genes were assigned to novel family-level phylotypes if they had a <87.5% similarity to the next cultured taxa (93). Sequences of *mcrA* and *fhs* were translated in silico and aligned with reference sequences obtained from MegaBLAST using ClustalW algorithm implemented in ARB software. Coverages were calculated by published protocol (81).

Phylogenetic correlation plots (70, 73) of 16S rRNA gene sequence similarities and amino acid sequence similarities of *mcrA* or *fhs* were prepared with the following filters: for *mcrA*, 100% similarity filter and 131 valid amino acids between positions 98 and 227 of *mcrA* of *Methanocella paludicola* SANAE; for *fhs*, 100% similarity filter and 351 valid amino acids between positions 134 and 486 of *fhs* of *Clostridium difficile* 630. Assignment of *mcrA* and *fhs* sequences to taxonomic hierarchic phylotypes was based on correlations between amino acid sequences of the translated structural gene to the 16S rRNA gene sequence of cultured organisms (Fig. 1). 16S rRNA gene sequence similarities of 97.0% and 87.5% are conservative threshold values for determining species- and family-level differences, respectively,
between organisms (93). These values yielded species- and family-level thresholds for (a) mcrA-encoded amino acid sequences of 85.7% and 75.4%, respectively, and (b) bacterial fhs-encoded amino acid sequences of 76.4% and 50.0%, respectively (Fig. 1).

Phylogenic trees of mcrA and fhs were calculated with neighbor-joining (Dayhoff correction) (77), maximum likelihood (Jukes-Cantor or Dayhoff correction), and maximum parsimony methods. McrA trees used a 100% similarity filter and 131 valid amino acid positions between 98 and 227 of mcrA of Methanocella pauldicola SANAE. Fhs trees used either a 100% similarity filter and 197 valid amino acids between positions 138 and 335 of fhs of Clostridium difficile 630 or a 100% similarity filter and 175 valid amino acids between positions 292 and 468 of fhs of Clostridium difficile 630. Phylogenetic trees of 16S rRNA genes were calculated with neighbor-joining (Felsenstein correction) (77), AxML, and maximum parsimony methods. Archaeal 16S rRNA gene sequence trees used a 100% similarity filter and 759 valid nucleotide positions between 103 and 894 of 16S rRNA sequence of E. coli ATCC11775. Bacterial 16S rRNA gene sequence trees used a 100% similarity filter and 412 valid nucleotide positions between 311 and 745 of 16S rRNA sequence of E. coli ATCC11775. Calculated trees contained a bootstrap test with 100-10,000 replicates (24).

A species or family was considered labeled when its relative abundance in the gene library from ‘heavy’ fraction 4 of the $^{13}$C treatment was higher than its relative abundance in the gene library from ‘heavy’ fraction 4 of the $^{12}$C treatment (79).

**Sequencing of mcrA sequence of Methanoplanus limicola DSM 2279.** M. limicola was obtained from the ‘Deutsche Sammlung von Mikroorganismen und
Zellkulturen GmbH' (DSMZ) (Braunschweig, Germany). McrA of *M. limicola* was amplified, sequenced, and used as a reference sequence for tree calculations.

**Calculations of Gibbs free energy.** The calculated Gibbs free energies (ΔG) are based on the concentrations of reactants and products in the liquid phase of soil microcosms at 15 °C and the pH of the liquid phase (87). ΔG° values were calculated from the standard Gibbs energies of formation (ΔGf°) at the pH of the liquid phase (that varied from pH 4.2 to 6.3) and utilized to calculate ΔG values for the following reactions: 4 HCOO− + 4 H+ → CH4 + 3 CO2 + 2 H2O (ΔG° varied from -161 to -208 kJ mol⁻¹ CH₄); CH₃COO− + H+ → CH₃CO + CO₂ (ΔG° varied from -44 to -50 kJ mol⁻¹ CH₄); 4 HCOO− + 3 H+ → CH₃COO− + 2 CO₂ + 2 H₂O (ΔG° varied from -121 to -157 kJ mol⁻¹ acetate). Estimated concentrations of H⁺ and estimated ΔGf° for H⁺ are based on the pH of the liquid phase (i.e., the ΔGf° for H⁺ at pH 4.2 to 6.3 was -23.9 to -35.8 kJ mol⁻¹, respectively [61]).

**Analytical techniques.** Dry weight of soil was determined by weighing soil before and after drying at 60 °C for 72 hours. Iron(II) was determined photometrically (86). Nitrate and sulfate were analyzed with a Dx500 ion chromatograph equipped with an ED 40 detector and AS 4A-SC column (Dionex Corporation, Sunnyvale, USA) at the Center for Analytical Chemistry (Bayreuth Centre of Ecological and Environmental Research, University of Bayreuth, Bayreuth, Germany). The mobile phase was 1.8 mM sodium carbonate and 1.7 mM sodium bicarbonate at a flow rate of 2 ml min⁻¹. The column temperature was 35 °C. pH was measured with a InLab R422 pH electrode (Mettler Toledo, InLab Semi-Micro, Gießen, Germany). Organic acids were determined by high performance liquid chromatography (1090 series II with UV detector, Hewlett Packard, Palo, USA) (91). H₂ was measured with a gas chromatograph (5890 series II with a thermal conductivity detector, Hewlett-Packard,
CO₂ and methane were separated with a hayesep-D column (2m x 1/8 in, SRI Instruments, Torrance, USA) and analyzed with a flame ionization detector (SRI Instruments, Torrance, USA). The carrier gas was helium at a flow rate of 40 ml min⁻¹, injector and column temperatures were 60°C, and detector temperature was 380°C. Units of concentration are per gram soil dry weight [g soil dw]. Concentrations of gases are combined concentrations from gas and liquid phases and were calculated from the ideal gas law taking into consideration the actual pressure, temperature, pH, and volume of gas and liquid phases in incubation flasks (5, 44). The ¹³C content of acetate was determined by liquid chromatography coupled to isotope ratio mass spectrometry (Finnigan™ LC IsoLink, Thermo Fisher Scientific Inc., Waltham, USA) (46). Acetate was separated from other organic compounds by high performance liquid chromatography, oxidation and acid/catalyst reagents (ammonium peroxodisulfate, phosphoric acid, silver nitrate) were added, and organic compounds were oxidized to CO₂ in an oxidation reactor at 100°C (46). CO₂ of the liquid phase were degassed by a helium counter flow, which was then dried in an on-line gas-drying unit and injected into the mass spectrometer (46). Sodium [¹³C]-formate and sodium [¹³C]-bicarbonate were obtained from Campro Scientific (Berlin, Germany). In this study, no distinction is made between CO₂ and its carbonate forms.

**Nucleotide sequence accession numbers.** The sequences obtained in this study are available from EMBL nucleotide sequence database under accession numbers FR725451-FR725861 (mcrA), FR725862-FR725930 (fhs), FR732102-FR732501 (bacterial 16S rRNA genes), FR744942-FR745247 (archaeal 16S rRNA genes), and FR745248 (mcrA from *M. limicola* DSM 2279).
Effect of supplemental formate and CO$_2$ on product profiles. Alternative electron acceptors (i.e., approximately 103 µmol iron(III) and 15 µmol sulfate g$^{-1}$ soil$_{dw}$) in fen microcosms were reduced during 15 days of anoxic pre-incubation prior to supplementation of substrates. Sulfate was not detected and iron(II) reached a stable end concentration at the end of the pre-incubation period. Nitrate was not detected during the pre-incubation period (detection limit was 0.13 µmol nitrate g$^{-1}$ soil$_{dw}$). Concentrations of methane, acetate, and CO$_2$ approximated 2.5, 4, and 85 µmol g$^{-1}$ soil$_{dw}$, respectively, at the end of the pre-incubation period. A total of approximately 25 µmol methane g$^{-1}$ soil$_{dw}$, 5 µmol acetate g$^{-1}$ soil$_{dw}$, and 2 µmol propionate g$^{-1}$ soil$_{dw}$ were produced in the subsequent 23 days in unsupplemented controls (Fig. 2). Formate and H$_2$ remained below 1 µmol g$^{-1}$ soil$_{dw}$ in unsupplemented controls. Traces of butyrate, iso-butyrate, and methyl-butyrate were detected after 15 days in all treatments and controls (data not shown).

Each formate pulse was essentially consumed within 24 hours (Fig. 2A). In total, an additional 63 µmol H$_2$ g$^{-1}$ soil$_{dw}$, 43 µmol methane g$^{-1}$ soil$_{dw}$, 29 µmol acetate g$^{-1}$ soil$_{dw}$, and 8 µmol propionate g$^{-1}$ soil$_{dw}$ were detected in formate treatments compared to unsupplemented controls, indicating that formate stimulated the production of these compounds. The apparent formate-dependent stimulation of the production of H$_2$ suggested that formate-hydrogen lyase-containing taxa were active in formate treatments. Approximately 17 atom% and 1 atom% of acetate-derived carbon was enriched with $^{13}$C in the $[^{13}$C]-formate and $[^{12}$C]-formate treatments, respectively, reinforcing the likelihood that acetogens participated in the synthesis of acetate in formate treatments. Formate-derived carbon is incorporated
preferentially into the methyl carbon of acetate during acetogenesis (i.e., formate preferentially enters the methyl branch of the acetyl-CoA pathway [54, 63]), and it is therefore possible that the $^{13}$C enrichment of $[^{13}C]$-formate-derived acetate was mostly in the methyl carbon.

An additional 20 µmol methane g[$\text{soil}_{dw}$]$^{-1}$ and 1.7 µmol propionate g[$\text{soil}_{dw}$]$^{-1}$ were detected in CO$_2$ treatments compared to unsupplemented controls (Fig. 3), indicating that CO$_2$ stimulated the production of these compounds. Supplemental CO$_2$ also appeared to stimulate the consumption of endogenously produced acetate (i.e., acetate produced before addition of CO$_2$), and the production of methane increased during the disappearance of acetate (Fig. 3B and 3C).

Product profiles of $^{13}$C and $^{12}$C treatments (Fig. 2 and 3) were very similar, indicating that similar microbial activities occurred in these treatments. For example, at 23 days post supplementation and on a per gram soil dry weight basis (values in parentheses are the percent of reductant theoretically recovered from formate-derived reductant), approximately 39 µmol methane (54%), 33 µmol acetate (46%), 70 µmol H$_2$ (24%), and 9 µmol propionate (22%) were produced from 287 µmol $[^{13}C]$-formate, whereas approximately 47 µmol methane (70%), 25 µmol acetate (38%), 57 µmol H$_2$ (21%), and 7 µmol propionate (18%) were produced from 267 µmol $[^{12}C]$-formate (values have been corrected by values from unsupplemented controls). These values indicated that most reducing equivalents from supplemental formate were recovered in methane and acetate. As shown above, recovery of supplemental formate-derived reductant exceeded 100% in both $^{13}$C and $^{12}$C treatments. A recovery of greater than 100% suggested that supplemental substrate enhanced the use of endogenous substrates, a priming effect observed in other studies (25, 31, 79).
**Bioenergetics.** The estimated Gibb’s free energies of the apparent formate-dependent methanogenesis and apparent formate-dependent acetogenesis in $[^{13}\text{C}]-$formate-supplemented microcosms averaged -104 kJ mol$^{-1}$ CH$_4$ and -42 kJ mol$^{-1}$ acetate, respectively (Fig. 4A). The estimated Gibb’s free energy of the apparent acetoclastic methanogenesis in $^{13}\text{CO}_2$-supplemented microcosms averaged -64 kJ mol$^{-1}$ CH$_4$ (Fig. 4B).

**Formate- and CO$_2$-consuming methanogens.** A total of 365 $mcrA$ sequences and 306 archaeal 16S rRNA gene sequences were analyzed from $[^{13}\text{C}]-$formate-, $[^{12}\text{C}]-$formate-, $^{13}\text{CO}_2$-, and $^{12}\text{CO}_2$-supplemented microcosms. The percentage of methanogens and non-methanogens in the 16S rRNA gene library approximated 67% and 33%, respectively. Family-level coverage of $mcrA$ and archaeal 16S rRNA genes were >99%. $McrA$ sequences were affiliated with the families Methanocellaceae (48% of total), Methanosarcinaceae (17% of total), Methanomicrobiaceae (15% of total), Methanosaetaceae (4% of total), Methanobacteriaceae (3% of total), and an unclassified family (13% of total, all of which were affiliated with Methanoregula boonei) (Table 1), whereas archaeal 16S rRNA gene sequences were affiliated with Methanocellaceae (27% of total), Methanosarcinaceae (22% of total), Methanobacteriaceae (16% of total), 2 unclassified families (11% and 1% of total were affiliated with “Candidatus Nitrosopumilus maritimus” and Methanoregula boonei, respectively), and 7 novel family-level phylotypes (23% of total) (Table 2). At 9 days post supplementation, higher relative abundances of Methanobacterium formicicum-affiliated $mcrA$ sequences and novel species-level $mcrA$ phylotype 1 (most closely related to Methanocella paludicola) were obtained from ‘heavy’ fractions of $[^{13}\text{C}]-$formate supplemented microcosms than those of $[^{12}\text{C}]-$formate supplemented microcosms.
(Table 1), indicating that organisms of these phylotypes were early assimilators of formate. In contrast, at this same time interval, higher relative abundances of Methanosarcina mazei-affiliated mcrA sequences and novel phylotype 1 were obtained from 'heavy' fractions of $^{13}$CO$_2$-supplemented microcosms than from those of $^{12}$CO$_2$-supplemented microcosms (Table 1), indicating that organisms of these phylotype were early assimilators of CO$_2$.

At 23 days post supplementation, higher relative abundances of Methanocellaceae-affiliated mcrA sequences and Methanobacteriaceae-affiliated 16S rRNA gene sequences were obtained from 'heavy' fractions of $^{13}$C-formate-supplemented microcosms than from those of $^{12}$C-formate-supplemented microcosms (Tables 1 and 2). In contrast, at 18 days post supplementation, higher relative abundances of Methanobacteriaceae-, Methanocellaceae- and Methanosarcinaceae-affiliated 16S rRNA gene sequences were obtained from 'heavy' fractions of $^{13}$CO$_2$-supplemented microcosms than those of $^{12}$CO$_2$-supplemented microcosms (Table 2). Relative abundances of Methanosarcinaceae-affiliated mcrA sequences were also higher at 18 days post supplementation in 'heavy' fractions of $^{13}$CO$_2$-supplemented microcosms than those of $^{12}$CO$_2$-supplemented microcosms (Table 1). At the end of incubation, Methanoregula-affiliated mcrA sequences were marginally higher in 'heavy' fractions of $^{13}$CO$_2$- and $^{13}$C-formate-supplemented microcosms than those of $^{12}$CO$_2$- and $^{12}$C-formate-supplemented microcosms (Table 1). Methanocella paludicola, Methanosarcina mazei, Methanosarcina barkeri, Methanobacterium formicicum, and Methanoregula boonei were the cultivated species most closely related to labeled mcrA and 16S rRNA gene phylotype (Fig. 5 and 6).
Bacterial diversity. A total of 393 bacterial 16S rRNA gene sequences and 69 fhs sequences were analyzed. Family-level coverage of 16S rRNA gene sequences and fhs approximated 94% and 93%, respectively. Twenty-eight of the 58 detected bacterial 16S rRNA family-level phylotypes did not have cultured representatives (Supplemental Fig. S2). Bacterial 16S rRNA gene sequences were affiliated with the phyla Proteobacteria (32% of total), Acidobacteria (28% of total), Actinobacteria (27% of total), Firmicutes (1% of total), Planctomycetes (1% of total), Verrucomicrobia (1% of total), Bacteroidetes (<1% of total), Chloroflexi (<1% of total), Spirochaetes (<1% of total), and unclassified taxa (8% of total) (Table 3, Supplemental Table S1, Supplemental Fig. S2).

Fifteen species-level fhs phylotypes were detected and affiliated with the families Phyllobacteriaceae (36% of total), Acetobacteraceae (34% of total), Rhodobacteraceae (10% of total), Verrucomicrobiaceae (9% of total), Oceanospirillaceae (3% of total), Hyphomicrobiaceae (1% of total), Hyphomonadaceae (1% of total), Thermoanaerobacteraceae (1% of total), Veillonellaceae (1% of total), 1 novel family (1% of total), and 1 unclassified family (3% of total, all of which were affiliated with “Candidatus Pelagibacter sp.”) (Supplemental Fig. S3, Supplemental Table S2). None of the 15 detected species-level fhs phylotypes had cultured representatives. Fhs sequences affiliated with Phyllobacteriaceae (36% of total) and Acetobacteraceae (34% of total) were the most abundant fhs phylotypes obtained from both [13C]-formate and [12C]-formate treatments. These fhs phylotypes were most closely related to Mesorhizobium loti (67-83% fhs amino acid similarity) and Granulibacter bethesdensis (67-82% fhs amino acid similarity) (Supplemental Fig. S3). Two fhs phylotypes detected in formate-pulsed microcosms were related to Sporomusa ovata (72% fhs amino acid
similarity) and *Moorella thermoacetica* (74% fhs amino acid similarity) (Supplemental Fig. S3), indicating that the fen soil harbors organisms belonging to the monophyletic acetogenic genera *Moorella* and *Sporomusa* (17, 18, 20, 89).

**Formate- and CO$_2$-consuming Bacteria.** Higher relative abundances of *Acetobacteraceae*- and *Rhodospirillaceae*-affiliated 16S rRNA gene sequences (*Alphaproteobacteria*) were obtained from ‘heavy’ fractions of [${}^{13}$C]-formate-supplemented microcosms than from those of [${}^{12}$C]-formate-supplemented microcosms (Supplemental Table S1), indicating that organisms of these phylotypes assimilated formate. The relative abundances of *Acidimicrobiaceae*-affiliated 16S rRNA gene sequences (*Actinobacteria*) was marginally higher in ‘heavy’ fractions of [${}^{13}$C]-formate-supplemented microcosms than in those of [${}^{12}$C]-formate-supplemented microcosms (Supplemental Table S1), indicating that organisms of this phylotype could have assimilated a marginal amount of formate. In contrast, higher relative abundances of *Conexibacteraceae*- and *Solirubrobacteraceae*-affiliated 16S rRNA gene sequences (*Actinobacteria*) were obtained from ‘heavy’ fractions of [${}^{13}$CO$_2$]-supplemented microcosms than from those of [${}^{12}$CO$_2$]-supplemented microcosms (Supplemental Table S1), indicating that these phylotypes assimilated CO$_2$. The relative abundance of *Thermomonosporaceae*-affiliated 16S rRNA gene sequences (*Actinobacteria*) was marginally higher in ‘heavy’ fractions of [${}^{13}$CO$_2$]-supplemented microcosms than in those of [${}^{12}$CO$_2$]-supplemented microcosms (Supplemental Table S1), indicating that this phylotype could have assimilated a marginal amount of CO$_2$. None of the labeled taxa are known to contain acetogens. The cultivated species most closely related to the labeled bacterial 16S rRNA phylotype were *Rhodovastum atsumiense* (90-97% 16S rRNA gene similarity), *Acidimicrobium ferrooxidans* (87-91% 16S rRNA gene similarity), *Rhodocista centenaria* (88-93% 16S rRNA gene similarity).
Formate and CO$_2$ constitute potential trophic links to both methanogenesis and acetogenesis (20, 34, 95). Both formate and CO$_2$ experimentally stimulated methanogenesis, whereas only formate stimulated acetate synthesis. CO$_2^-$ dependent stimulation of methanogenesis suggests that endogenously available CO$_2$ limited methane production under the experimental conditions of this study. Whether such limitation occurs in situ is unknown, but the findings underscore the importance that CO$_2$ availability might have for optimal methanogenesis. The availability of CO$_2$ can affect the metabolic capacities of certain acetogens (20). That CO$_2$ did not appear to stimulate acetate production suggests that endogenous reductant available for acetogenesis was limiting. However, this speculation must be qualified since (a) endogenously produced acetate was likely not restricted to acetogenesis (i.e., acetate can be produced by non-acetogens [20]) and (b) acetoclastic methanogenesis appeared to be stimulated in CO$_2$ treatments, thus resulting in a consumption of acetate that might mask acetate production.

**Taxa associated with formate- and CO$_2$-enhanced methanogenesis.** The collective labeling data indicated that *Methanobacteriaceae* and *Methanocellaceae* assimilated formate-derived carbon and that *Methanobacteriaceae*, *Methanocellaceae*, and *Methanosarcinaceae* assimilated CO$_2$-derived carbon. The
labeling of the same taxa in both formate and CO$_2$ treatments is consistent with the
capacity of many methanogens to utilize both formate and CO$_2$ (34).

*M. formicicum* was the most closely related cultured species to labeled
*Methanobacteriaceae*-affiliated phylotypes (89-95% *mcrA* amino acid similarity, 89-
93% 16S rRNA gene similarity) (Fig. 5 and 6). Certain members of the family
*Methanobacteriaceae* can utilizes CO$_2$, H$_2$, and formate for methane production (9).

[¹³C]-formate yielded labeling of *Methanobacteriaceae*-affiliated phylotypes whereas
¹³CO$_2$ yielded marginal labeling of these phylotypes (Tables 1 and 2), indicating that
growth of fen *Methanobacteriaceae*-related species was more robust with
supplemental formate than with supplemental CO$_2$ and endogenous reductant.

*M. paludicola* was the most closely related cultured species to labeled
*Methanocellaceae*-affiliated phylotypes in both formate and CO$_2$ treatments (79-83%
*mcrA* amino acid similarity, 86-97% 16S rRNA gene similarity) (Fig. 5 and 6). *M.
paludicola* was isolated from rice paddy soil as the first cultured species within ‘Rice
Cluster I,’ is a mesophilic methanogen capable of utilizing formate, CO$_2$, and H$_2$, and
can use acetate as a source of carbon (78). The detection of novel labeled
*Methanocella*-related *mcrA* and 16S rRNA gene sequences in formate and CO$_2$
treatments (Tables 1 and 2) indicated that novel species of *Methanocella* used
formate and CO$_2$ for methane production and possibly acetate as a source of carbon
in anoxic fen microcosms.

*M. mazei* (88-96% *mcrA* amino acid similarity, 93-98% 16S rRNA gene
similarity) and *M. barkeri* (87-96% *mcrA* amino acid similarity, 94-98% 16S rRNA
gene similarity) were the most closely related cultured species to labeled
*Methanosarcinaceae*-affiliated phylotypes (Fig. 5 and 6). *M. mazei* and *M. barkeri*
utilize acetate and H$_2$-CO$_2$ but do not utilize formate (34). Formate is not known to
be utilized by species of *Methanosarcina* (34). *Methanosarcina*-related phylotypes were labeled in $^{13}$CO$_2$-supplemented microcosms in which the disappearance of acetate was concomitant to the production of methane but were not labeled in $[^{13}\text{C}]$-formate supplemented microcosms in which $^{13}$C-enriched acetate accumulated (Tables 1 and 2). Certain *Methanosarcina* species may not be able to utilize acetate under certain conditions (51, 62). Although *Methanosarcina*-affiliated phylotypes could have theoretically assimilated $^{13}$CO$_2$-produced $[^{13}\text{C}]$-acetate in the $^{13}$CO$_2$ treatment, there was no evidence for the stimulation of acetate production in CO$_2$ treatments and the $[^{13}\text{C}]$-formate treatment in which labeled acetate was produced did not yield labeling of *Methanosarcina*-affiliated phylotypes. It thus seems likely that *Methanosarcina*-related phylotypes used CO$_2$ for methane production at the expense of endogenous reductant.

*M. boonei* was the most closely related cultured species to the marginally labeled unclassified methanogenic family (81-90% *mcrA* amino acid similarity, 95-98% 16S rRNA gene similarity) (Fig. 5 and 6). *M. boonei* was isolated from an acidic bog, grows by hydrogenotrophic methanogenesis, requires small amounts of acetate as a source of carbon, and cannot utilize formate (7). On the other hand, *Methanoregula formicica* can use formate and H$_2$ for methanogenesis (92). The late marginal labeling (Table 1) of *M. boonei*-related phylotypes might have occurred by assimilation of $[^{13}\text{C}]$-formate, $[^{13}\text{C}]$-formate-derived $^{13}$CO$_2$ and/or $[^{13}\text{C}]$-acetate formed by acetogens.

**Acetoclastic methanogenesis.** Although supplemental acetate does not stimulate methanogenesis in anoxic Schlöppnerbrunnen fen microcosms (38, 90), methane production was concomitant to the disappearance of acetate in CO$_2$-supplemented microcosms (Fig. 3). Acetate can be (a) used as a source of carbon
by hydrogenotrophic methanogens such as *M. paludicola* (78), (b) utilized by acetoclastic methanogens like *Methanosaetaceae* (34), or (c) oxidized in a syntrophic partnership (33). The greatest production of methane in CO$_2$ treatments was coincident with the disappearance of acetate (Fig. 3). The increased concentration of CO$_2$ or the periodic increase in pH (i.e., up to approximately 5) could have enhanced acetoclastic methanogenesis in CO$_2$ treatments. For example, acetoclastic methanogenesis by *M. barkeri* MS does not occur at low pH (e.g., pH 4.5) (62). Furthermore, the estimated Gibbs free energy for the apparent acetoclastic methanogenesis in the CO$_2$ treatments was exergonic (Fig. 4B), and *mcrA* sequences related to *Methanosaetaceae* (a taxon that can only use acetate for methanogenesis [34]) were more abundant when acetate was being consumed (Fig. 3, Table 1). Although the syntrophic oxidation of acetate is well documented for high temperature habitats, its occurrence at low temperatures is less well understood (33, 69). Acetoclastic methanogenesis is thermodynamically more favorable than syntrophic acetate oxidation at 15°C in sediments of Lake Kinneret (Israel) (69). Thus, it is likely that the consumption of acetate was at least partially linked to acetoclastic methanogenesis and that *Methanosaetaceae*-affiliated methanogens were participants in this consumption. The inability of supplemental acetate to stimulate methanogenesis (38, 90) suggests that acetoclastic methanogens cannot utilize substantially more acetate than that produced endogenously or that supplemental acetate is toxic (60).

**Formate-dependent acetogenesis.** The concomitant formate-dependent stimulation of the production of methane and acetate (Fig. 2), the enrichment of $^{13}$C in acetate in $[^{13}$C$_2$]-formate treatments, and the estimated exergonic Gibbs free energies for the apparent formate-driven acetogenesis and methanogenesis (Fig. 4A)
suggest that acetogens and methanogenes competed for supplemental formate. The most closely related cultured species to the labeled *Acetobacteraceae* - and *Rhodospirillaceae*-affiliated phylotypes, and the marginally labeled *Acidimicrobiaceae*-affiliated phylotypes are not known to be capable of acetogenesis but some are capable of either anaerobic phototrophic growth with organic acids or anaerobic growth via the reduction of iron (11, 26, 37). The high relative abundances of *Phyllobacteriaceae* - and *Acetobacteraceae*-affiliated sequences in *fhs* libraries (Supplemental Table S2) indicated that these taxa might have been involved in formate-dependent processes. *Fhs* sequences most closely affiliated with the acetogens *S. ovata* and *M. thermoacetica* (17, 18, 20, 89) were detected in formate-pulsed microcosms. Although species of *Sporomusa* and *Moorella* have not been previously isolated from acidic fens, species of these genera have been isolated from various soils (17, 18, 20, 89). *Spirochaetaceae* and *Holophagaceae* contain acetogenic species (8, 56), and *Spirochaetacea* - and *Holophagaceae*-affiliated 16S rRNA gene sequences were likewise detected. Two novel family-level phylotypes were also detected within the *Clostridia*, a class that contains many acetogenic clostridial species (20). However, a labeling of sequences affiliated with these acetogen-containing taxa was not apparent.

The capacity of acetogens to utilize very diverse organic compounds (18, 20) might result in a very limited assimilation of formate-derived carbon with dissimilation (i.e., the conservation of energy) being the primary purpose of formate utilization. Alternatively, the extent of replication of acetogens at the expense of formate might have been inadequate relative to detectable labeling, a possibility reinforced by the relatively poor thermodynamics of formate-coupled acetogenesis (Fig. 4A). In addition, the large uncultured diversity of detected 16S rRNA gene-based phylotypes
and the broad distribution of fhs in prokaryotic taxa (35, 53) suggests that highly diverse previously uncultivated fhs-containing taxa are likely present in fen soil and might therefore have not been efficiently targeted with the fhs primers. In this regard, a recent study in which new fhs primers were developed for accessing acetogens in the rumen (a) found that the majority of retrieved fhs sequences were affiliated with non-acetogenic taxa and (b) identified potential acetogens that were not closely related to known acetogens (35). These results highlight the difficulty in targeting highly novel acetogens with fhs primers designed from the currently available sequences in public databases. It is thus possible that hitherto unknown acetogens were involved in the acetogenic consumption of supplemental formate and that the fhs analysis failed to detect them.

Many acetogens are non-monophyletic, i.e., are phylogenetically distributed with non-acetogens in the same genera (18, 20), a factor complicating their assessment by standard 16S rRNA gene analysis. Several organisms originally described as non-acetogens have been later discovered to be acetogenic (e.g., Clostridium glycolicum [20, 49]), thus raising the question as to whether any of the detected non-acetogenic taxa might contain heretofore unknown acetogenic capabilities. The recent isolations of taxonomically and physiologically novel acetogens such as Alkalibaculum bacchi (3) and Moorella perchloratireducens (4) illustrate the existence of hitherto unknown acetogens in various ecosystems. In addition, certain archaea (i.e., Methanosarcina acetivorans and Archaeoglobus fulgidus) are capable of carbon monoxide-dependent acetogenesis in pure culture (36, 55), suggesting that archaeal taxa might have participated in the formation of acetate. However, achaeal formate-driven acetogenesis has not been documented, and an obvious labeling of a non-methanogenic archaea was not detected.
General diversity and additional CO$_2$- and formate-metabolizing taxa. A high degree of novelty was detected in the 16S rRNA gene sequence dataset (Figure 6, Supplemental Fig. S2). Most of the detected bacterial 16S rRNA gene sequences were affiliated with the phyla Proteobacteria and Acidobacteria (Table 3), taxa that have been observed to be dominant in other boggy soils (15, 40, 43). The detection of the phyla Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Planctomycetes, Spirochaetes, and Verrucomicrobia is likewise consistent with the occurrence of these taxa in other fens and bogs (15, 40, 43). Previous studies have documented the occurrence of Crenarchaeota in the fen Schlöppnerbrunnen (32, 90), and half of the detected archaeal 16S rRNA phylotypes were affiliated with this phylum (Table 2). The Crenarchaeota was earlier thought to contain only obligate thermophiles (28) but has since been shown to contain mesophiles (e.g., 84), including "Candidatus N. maritimus" (42) to which 11% of the detected archaeal 16S rRNA gene sequences were distantly affiliated (Table 2, Fig. 6). Additional distantly related cultured genera of the detected crenarchaeotal phylotypes grow anaerobically with peptides and sulphur (e.g., Thermofilum, Staphylothermus [28]) (Fig. 6). It is likely that Crenarchaeota-affiliated organisms have anaerobic physiological capabilities not currently represented in cultured crenarchaeotal taxa.

Certain 16S rRNA gene sequences detected in $[^{13}\text{C}]$- or $[^{12}\text{C}]$-formate treatments were affiliated with Acidimicrobiaceae, Sinobacteraceae, Rhodocyclaceae, and Holophagaceae, taxa capable of organic acid-coupled anaerobic growth and the reduction of electron acceptors other than CO$_2$ (11, 12, 23, 27). The reduction of alternative electron acceptors in fen soil microcosms during the pre-incubation period support previous studies that have identified different anaerobic metabolic activities in fen soil (57, 71). The nearest cultured species of labeled
Conexibacteraceae and Solirubrobacteraceae (i.e., C. woesei and S. solii, respectively) and marginally labeled Thermomonosporaceae (i.e., A. formosensis) are not known to be capable of anaerobic growth (41, 45, 66), thus raising questions about the metabolic potentials these taxa might have under anoxic conditions. *Rhodospirillum rubrum* of the family Rhodospirillaceae contains formate-hydrogen lyase (26), and the labeling of Rhodospirillaceae-affiliated taxa in formate treatments suggest that they might have been involved in the apparent production of H$_2$ from formate.

Approximately one-fifth of the apparent formate-derived reductant was accounted for in propionate. Propionate can be formed anaerobically by *Desulfobulbus propionicus* from acetate, CO$_2$, and H$_2$ via the reversal of syntrophic propionate oxidation (50). The reductive formation of propionate from acetate, CO$_2$, and H$_2$ is associated with rice roots and might be linked to organisms with physiological properties similar to *D. propionicus* (e.g., ethanol fermentation or sulfate reduction) or to syntrophic propionate oxidizers that can also catalyze the synthesis of propionate from acetate, CO$_2$, and H$_2$ (13). The $\Delta$G values for this reaction with rice roots ranged from -15 to -38 kJ mol$^{-1}$ propionate (13). A minor fraction of the 16S rRNA gene sequences from the $[^{13}$C]-formate treatment were closely related to the Desulfuromonadaceae-affiliated ethanol fermenter *Pelobacter propionicus* (96.4-96.9% similarity) (Supplemental Table S1, Supplemental Fig. S2), an organism with overlapping physiological properties to *D. propionicus* (80). Furthermore, 16S rRNA gene sequences related to the Syntrophobacteraceae-affiliated syntrophic propionate oxidizer *Syntrophobacter wolinii* (94.3%) (6) were detected in CO$_2$ treatments (Supplemental Table S1, Supplemental Fig. S2). The detection of these taxa in fen soils (32, 43, 57) and the availability of acetate, CO$_2$, and H$_2$ in formate treatments
suggest that propionate production might have been catalyzed by microorganisms capable of reductive propionate formation.

**Conclusions, limitations, and future perspectives.** Detected labeling patterns indicated that methanogens and non-methanogens concomitantly assimilated the same substrate. Likewise, methanogens and acetogens appeared to concomitantly dissimilate formate. These observations indicate that differing taxa competed for the same substrate under experimental conditions. The daily pulsing of low concentrations of formate was designed to achieve adequate labeling without grossly exceeding in situ relevant concentrations of formate (47). However, although pH was periodically controlled, temporal fluctuations in pH due to the formation of fatty acids yielded changing conditions not fully representative of in situ conditions. Furthermore, because of potential biases introduced by stable isotope probing and the different labeling patterns of mcrA sequences and methanogen-derived 16S rRNA gene sequences, one cannot exclude the possibility that taxa that were unlabeled or only marginally labeled were more involved in substrate utilization than was indicated by the labeling patterns (i.e., assimilation may not have been tightly linked to dissimilation for all dissimilating taxa). In this regard, endogenous organic carbon rather than supplemental formate or CO\(_2\) was likely a major source of cell carbon, since highly oxidized one-carbon substrates are less than ideal for most taxa relative to biomass synthesis (i.e., more reduced carbonaceous substrates are preferred by heterotrophs). Nonetheless, within the constraints of these limitations, the current study resolved methanogenic taxa potentially involved in the emission of methane from the acidic fen Schlöppnerbrunnen, and extended previous findings on the acetogenic potentials of this fen (90). Although acetogenic taxa were found in the fhs and 16S rRNA gene libraries, a labeling of a known acetogenic taxa was not
detected. It thus remains uncertain (a) which microbes were responsible for the apparent conversion of formate to acetate, and (b) if dissimilation of formate to acetate was concomitant with the acetogenic assimilation of formate. That formate-derived \( H_2 \) accumulated in anoxic microcosms suggested that \( H_2 \) production exceeded the \( H_2 \)-consuming capacities of hydrogenotrophic methanogens. This speculation is consistent with the fact that cultivated \( H_2 \)-forming fermenters, a likely origin of the apparent formate-hydrogen lyase activity in fen soil, can outnumber cultivated \( H_2 \)-consuming methanogens in soil from the fen Schlöppnerbrunnen by a factor of approximately 1,000 (19, 90). The apparent concomitant occurrence of methanogenesis and formate-hydrogen lyase activity suggest that certain methanogenic taxa might have utilized formate-derived \( H_2 \) for a source of reductant. Current studies focus on determining potential differences between formate- and \( H_2 \)-utilizing methanogenic taxa, identifying the formate-hydrogen lyase-associated taxa, and further resolving the fen acetogens, a functional group that has remained difficult to characterize at the level of the taxa.

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**FIGURE LEGENDS**

**Figure 1.** Phylogenetic correlation plots of 16S rRNA gene sequence similarities and amino acid sequence similarities of *mcrA* (A) or *fhs* (B). Seventy-nine *mcrA* and 238 *fhs* sequences are plotted. The vertical solid lines that intersect the horizontal axes at 97% and 87.5% 16S rRNA gene sequence similarities identify species- and family-level phylotype thresholds, respectively. The horizontal dotted and dashed lines that intersect the left vertical axes represent the 90% quantile of pairwise comparisons of *mcrA*- (A) or *fhs*- (B) encoded amino acid sequence similarity and 16S rRNA gene sequence similarity.

**Figure 2.** Effect of formate on the production of organic acids and gases in anoxic fen soil microcosms at 15°C. Panels: A, formate; B, CO$_2$; C, acetate; D, methane; E, propionate; F, H$_2$. CO$_2$ in formate treatments (Panel B) is the combined CO$_2$ from the bicarbonate pulses and CO$_2$ derived from the apparent conversion of formate to H$_2$ and CO$_2$. Symbols: empty symbols, unsupplemented controls; grey symbols, [^{12}C]-formate treatments; filled symbols, [^{13}C]-formate treatments. Inserts show cumulative gas concentrations. Values are the means of triplicate microcosms and the error bars indicate standard deviations.

**Figure 3.** Effect of CO$_2$ on the production of organic acids and gases in anoxic fen microcosms at 15°C. Panels: A, CO$_2$; B, acetate; C, methane; D, propionate. Symbols: empty symbols, unsupplemented controls; grey symbols, $^{12}$CO$_2$ treatments; filled symbols, $^{13}$CO$_2$ treatments. Inserts show cumulative gas concentrations.
Values are the means of triplicate microcosms and the error bars indicate standard deviations.

**Figure 4.** Estimated Gibbs free energies (ΔG) in the [13C]-formate (A) and 13CO₂ (B) treatments shown in Fig. 2 and Fig. 3, respectively. Values are the means of triplicate microcosms and the error bars indicate standard deviations. Symbols and lines: filled circles, ΔG for formate-dependent methanogenesis; filled squares, ΔG for formate-dependent acetogenesis; filled triangles, ΔG for acetate-dependent methanogenesis; dashed line, acetate; non-dashed line, methane in the aqueous phase.

**Figure 5.** Phylogenic neighbor-joining tree of representative species-level amino acid sequences encoded by mcrA retrieved from formate and CO₂ treatments and of reference sequences. Values next to the branches represent the percentages of replicate trees (>50%) in which the associated taxa clustered together in the bootstrap test (1,000 bootstraps). Dotes at nodes indicate the confirmation of tree topology by Maximum Likelihood and Maximum Parsimony calculations with the same data set. Bar indicates a 0.1 estimated change per amino acid. Codes: 09, 18, and 23 indicate the number of days of incubation post supplementation; 12K, 12CO₂ treatment; 12F, [12C]-formate treatment; 13K, 13CO₂ treatment; 13F, [13C]-formate treatment. Symbols: filled stars, labeled phylotypes; empty stars, marginally labeled phylotypes.

**Figure 6.** Phylogenic neighbor-joining tree of representative family-level archaeal 16S rRNA gene sequences retrieved from formate- and CO₂-treatments and
reference sequences. Values next to the branches represent the percentages of replicate trees (>50%) in which the associated taxa clustered together in the bootstrap test (10,000 bootstraps). Dotes at nodes indicate the confirmation of tree topology by Maximum Likelihood and Maximum Parsimony calculations with the same data set. Quotation marks indicate non-validated taxa (22). Bar indicates a 0.1 estimated change per amino acid. *E. coli* (X80725) was used as outgroup. Codes: 18 and 23 indicate the number of days of incubation post supplementation; 12K, $^{12}$CO$_2$ treatment; 12F, $[^{12}$C]-formate treatment; 13K, $^{13}$CO$_2$ treatment; 13F, $[^{13}$C]-formate treatment. Symbols: filled stars, labeled phylotypes; empty stars, marginally labeled phylotypes.
Table 1. Taxonomic identities and relative abundances of mcrA sequences

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<td>2.2</td>
<td>-</td>
<td>2.1</td>
</tr>
<tr>
<td>Novel species phylotype 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.3</td>
<td>43.2</td>
<td>44.4</td>
</tr>
<tr>
<td>Novel species phylotype 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Novel species phylotype 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>2.2</td>
</tr>
<tr>
<td>Methanomicrobiaceae,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Novel species phylotype 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.7</td>
<td>-</td>
<td>6.7</td>
</tr>
<tr>
<td>Novel species phylotype 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.1</td>
<td>6.8</td>
<td>15.6</td>
</tr>
<tr>
<td>Methanosetaecae,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanosaeta concillii</td>
<td>11.1</td>
<td>2.3</td>
<td>6.7</td>
</tr>
<tr>
<td>Methanosarcinae,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanosarcina mazei</td>
<td>17.8</td>
<td>38.6</td>
<td>15.6</td>
</tr>
<tr>
<td>Unclassified family,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanoregula boonei</td>
<td>15.6</td>
<td>9.1</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total sequences</td>
<td>45</td>
<td>44</td>
<td>45</td>
</tr>
</tbody>
</table>

<sup>a</sup> - not detected.

<sup>b</sup> Sequences were considered to be novel at the species level when the mcrA sequence similarity was <85.7% to that of the next cultured species (Fig. 1A).
Table 2. Class- and family-level identities and relative abundances of archaeal 16S rRNA gene sequences

<table>
<thead>
<tr>
<th>Taxonomic level (class, family)</th>
<th>Relative abundance of sequences (%)</th>
<th>$^{12}$C and $^{13}$C treatments (18 days)</th>
<th>$[^{12}$C]-formate and $[^{13}$C]-formate treatments (23 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$^{12}$C</td>
<td>$^{13}$C</td>
</tr>
<tr>
<td>Methanobacteria,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanobacteriaceae</td>
<td></td>
<td>2.4</td>
<td>12.8</td>
</tr>
<tr>
<td>Methanomicrobia,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanocellaceae</td>
<td></td>
<td>9.6</td>
<td>16.7</td>
</tr>
<tr>
<td>Methanosarcinaceae</td>
<td></td>
<td>15.7</td>
<td>56.4</td>
</tr>
<tr>
<td>Unclassified family$^a$</td>
<td></td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Unclassified Euryarchaeota$^b$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Novel family phylotype 1$^c$</td>
<td></td>
<td>4.8</td>
<td>-$^d$</td>
</tr>
<tr>
<td>Novel family phylotype 2$^c$</td>
<td></td>
<td>1.2</td>
<td>-</td>
</tr>
<tr>
<td>Unclassified Crenarchaeota$^b$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unclassified family$^a$</td>
<td></td>
<td>26.5</td>
<td>-</td>
</tr>
<tr>
<td>Novel family phylotype 3$^c$</td>
<td></td>
<td>22.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Novel family phylotype 4$^c$</td>
<td></td>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>Novel family phylotype 5$^c$</td>
<td></td>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>Novel family phylotype 6$^c$</td>
<td></td>
<td>1.2</td>
<td>-</td>
</tr>
<tr>
<td>Novel family phylotype 7$^c$</td>
<td></td>
<td>9.6</td>
<td>11.5</td>
</tr>
<tr>
<td>Total archaeal sequences:</td>
<td></td>
<td>83</td>
<td>74</td>
</tr>
</tbody>
</table>

$^a$ Closest related cultivated species: *Methanoregula boonei* (CP0007800), 95-98% 16S rRNA gene similarity.

$^b$ Family-level phylotypes listed underneath do not necessarily belong to the same class.
Sequences were considered to be novel at the family level when the 16S rRNA gene sequence similarity was <87.5% to that of the next cultured species (92).

- not detected.

Closest related cultivated species: “Candidatus Nitrosopumilus maritimus” (CP000866), 88-89% 16S rRNA gene similarity.
Table 3. Classes and relative abundances of bacterial 16S rRNA gene sequences

<table>
<thead>
<tr>
<th>Class</th>
<th>Relative abundance of sequences (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{12}\text{C}$</td>
</tr>
<tr>
<td></td>
<td>[CO$_2$] and [CO$_2$] treatments (18 days)</td>
</tr>
<tr>
<td>Acidobacteria</td>
<td>35.2</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>21.6</td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td>22.7</td>
</tr>
<tr>
<td>Bacteroidia</td>
<td>-</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>3.4</td>
</tr>
<tr>
<td>Clostridia</td>
<td>1.1</td>
</tr>
<tr>
<td>Deltaproteobacteria</td>
<td>3.4</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>-</td>
</tr>
<tr>
<td>Holophagae</td>
<td>2.3</td>
</tr>
<tr>
<td>Ktedonobacteria</td>
<td>-</td>
</tr>
<tr>
<td>Opitutae</td>
<td>1.1</td>
</tr>
<tr>
<td>Planctomyce</td>
<td>1.1</td>
</tr>
<tr>
<td>Sphingobacteria</td>
<td>-</td>
</tr>
<tr>
<td>Spirochaetes</td>
<td>-</td>
</tr>
<tr>
<td>Verrucomicrobiae</td>
<td>1.1</td>
</tr>
<tr>
<td>Unclassified&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.8</td>
</tr>
<tr>
<td>Total bacterial sequences:</td>
<td>88</td>
</tr>
</tbody>
</table>

<sup>a</sup> Detailed analyses of bacterial sequences are provided in Supplemental Table S1.

<sup>b</sup> 16S rRNA gene sequences were assigned to unclassified taxa when the sequence similarity was <78.4% to that of the next cultured species (92). Thus, these novel phylotypes could represent novel phyla.

<sup>c</sup> -, not detected.
Fig. 1

![Scatter plot showing the relationship between 16S rRNA gene sequence similarity and amino acid sequence similarity.](image-url)
Fig. 2

Organic acids (µmol g\text{soil \text{dw}}^{-1})

Gases (µmol g\text{soil \text{dw}}^{-1})

Time (days)
Fig. 3
Fig. 4

Acetate (µM) vs. CH₄ (µM) over time (days) with changes in Δ∆∆∆G (kJ mol⁻¹ acetate or CH₄).