

# Competing Formate- and Carbon Dioxide-Utilizing Prokaryotes in an Anoxic Methane-Emitting Fen Soil<sup>∇†</sup>

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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<sub>2</sub> as a potential secondary substrate. The objective of this study was to resolve potential differences between anaerobic formate- and CO<sub>2</sub>-utilizing prokaryotes of this fen by stable isotope probing. Anoxic soil microcosms were pulsed daily with low concentrations of [<sup>13</sup>C]formate or <sup>13</sup>CO<sub>2</sub> (i.e., [<sup>13</sup>C]bicarbonate). Taxa were evaluated by assessment of 16S rRNA genes, *mcrA* (encoding the alpha-subunit of methyl-coenzyme M reductase), and *fhs* (encoding formyltetrahydrofolate synthetase). Methanogens, acetogens, and formate-hydrogen lyase-containing taxa appeared to compete for formate. Genes affiliated with *Methanocellaceae*, *Methanobacteriaceae*, *Acetobacteraceae*, and *Rhodospirillaceae* were <sup>13</sup>C enriched (i.e., labeled) in [<sup>13</sup>C]formate treatments, whereas genes affiliated with *Methanosarcinaceae*, *Conexibacteraceae*, and *Solirubrobacteraceae* were labeled in <sup>13</sup>CO<sub>2</sub> treatments. [<sup>13</sup>C]acetate was enriched in [<sup>13</sup>C]formate treatments, but labeling of known acetogenic taxa was not detected. However, several phylotypes were affiliated with acetogen-containing taxa (e.g., *Sporomusa*). *Methanosaetaceae*-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. *Crenarchaeota* constituted half of the detected archaeal 16S rRNA phylotypes. The results highlight the unresolved microbial diversity of the fen Schlöppnerbrunnen, suggest that differing taxa competed for the same substrate, and indicate that *Methanocellaceae*, *Methanobacteriaceae*, *Methanosarcinaceae*, and *Methanosaetaceae* were linked to the production of methane, but they do not clearly resolve the taxa responsible for the apparent conversion of formate to acetate.

Methane is the second most important greenhouse gas (76), and its atmospheric concentration has increased to approximately 1,775 ppb (39) (evaluation from 2005). Wetlands contribute 27 to 53% (i.e., 160 to 314 Tg of 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% 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* and *Clostridiaceae*) in wetland soils can produce organic acids, alcohols, molecular hydrogen (H<sub>2</sub>), and carbon dioxide (CO<sub>2</sub>) from potential breakdown products of biopolymers (i.e., glucose, xylose, and *N*-acetylglucosamine) (32, 90). Organic acids and alcohols are further metabolized to H<sub>2</sub> and CO<sub>2</sub> by secondary fermenters (e.g., *Syntrophobacteraceae* [6]). The terminal stage of the methanogenic food web is catalyzed by methanogens (e.g., *Methanomicrobiaceae* and *Methanosarcinaceae*) that collectively convert formate, acetate, methanol, and H<sub>2</sub>-CO<sub>2</sub> to methane (1, 19, 34, 64, 95).

Several studies have demonstrated that wetland soils contain complex prokaryotic communities (10, 15, 40, 43), a finding consistent with the aforementioned network of trophically linked processes that yield methane. The methanogenic community of the fen Schlöppnerbrunnen in southeast Germany is composed of *Methanobacteriaceae*, *Methanomicrobiaceae*, *Methanosaetaceae*, and *Methanosarcinaceae* (32, 90). Formate is a significant driver of methanogenesis under experimental conditions and might be derived from the fermentation of monosaccharides such as glucose and *N*-acetylglucosamine; it also stimulates acetogenesis (i.e., the reductive synthesis of acetate from CO<sub>2</sub> via the acetyl-coenzyme A [CoA] Wood-Ljungdahl pathway [21]) (32, 90). The periodic occurrence of up to 0.65 mM formate in fen pore water (47) reinforces the likelihood that formate is a relevant *in situ* substrate for fen methanogens and other competing prokaryotic taxa. Dissimi-

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lation of formate also yields CO<sub>2</sub>, 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<sub>2</sub>-utilizing prokaryotic taxa in soil from the fen Schlöppnerbrunnen by stable isotope probing.

## MATERIALS 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 references 32 and 71). The pH of fen pore water approximates 4.5, and formate concentrations in fen pore water can range from 0 to 0.65 mM (47). Three soil cores of 0- to 20-cm depth were taken in July 2008 (4 to 5 m apart) with a soil corer, transported in airtight sterile plastic bags, and stored on ice until processed within 6 h of sampling.

**Anoxic microcosms.** The three soil cores were homogenized together; the homogenized fen soil had an 83.5% water content. Thirty-five grams (fresh weight) of homogenized soil was placed in sterile 500-ml infusion flasks (Merck ABS, Dietikon, Switzerland) and diluted with 125 ml of 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<sub>2</sub> (100%). Anoxic solutions were prepared by using modified Hungate techniques (14).

A 15-day anoxic preincubation 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 of formate per microcosm, yielding approximately 0.3 to 0.6 mM formate in the aqueous phase (i.e., 9.7 to 17.4 μmol of formate g soil<sub>dw</sub><sup>-1</sup> [where dw is dry weight]) 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 [<sup>13</sup>C]formate (99 atom% <sup>13</sup>C) or sodium [<sup>12</sup>C]formate (i.e., unlabeled formate with a natural abundance of <sup>13</sup>C). Additional microcosms were pulsed daily with approximately 160 μmol of CO<sub>2</sub> (from a filter-sterilized solution of sodium [<sup>13</sup>C]bicarbonate or sodium [<sup>12</sup>C]bicarbonate) per microcosm, yielding approximately 1.0 to 3.1 mM CO<sub>2</sub> in the combined aqueous and gas phases (i.e., 28.9 to 87.1 μmol of CO<sub>2</sub> g soil<sub>dw</sub><sup>-1</sup>) during the incubation. The CO<sub>2</sub> that was pulsed was from a filter-sterilized solution of either sodium [<sup>13</sup>C]bicarbonate (99 atom% <sup>13</sup>C) or sodium [<sup>12</sup>C]bicarbonate (i.e., unlabeled bicarbonate with a natural abundance of <sup>13</sup>C). Control microcosms lacked supplemental formate or CO<sub>2</sub>. The purpose of the <sup>13</sup>CO<sub>2</sub> treatment was twofold: (i) to control for potential cross-feeding (i.e., labeling of microorganisms by assimilation of [<sup>13</sup>C]formate-derived <sup>13</sup>CO<sub>2</sub>) and (ii) to assess taxa capable of utilizing CO<sub>2</sub> (i.e., assimilating CO<sub>2</sub> at the expense of endogenous reductant). Two additional safeguards against CO<sub>2</sub> cross-feeding were taken: (i) formate treatments were pulsed daily with 192 μmol of <sup>12</sup>CO<sub>2</sub> (i.e., sodium [<sup>12</sup>C]bicarbonate, yielding 1.9 to 4.4 mM CO<sub>2</sub> in the combined aqueous and the gas phases, equivalent to 53.7 to 122.0 μmol of CO<sub>2</sub> g soil<sub>dw</sub><sup>-1</sup>) per microcosm, and (ii) the gas phases of microcosms were exchanged with sterile N<sub>2</sub> (100%) before substrate pulsing was initiated and every subsequent fourth day. For exchanging the gas phase with N<sub>2</sub>, microcosms were evacuated under sterile conditions for 30 min at approximately -800 mbar, after which the gas phase was replaced with sterile N<sub>2</sub> (100%). This procedure was repeated after 15 min. Microcosms were then flushed with sterile N<sub>2</sub> (100%) for 20 min. The pH was adjusted every fourth day to approximately pH 4.5 with anoxic sterile 5 M 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 of 1.725 g ml<sup>-1</sup>) containing a cesium chloride solution (buoyant density of 1.881 g ml<sup>-1</sup>; 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 (Beckman Coulter, Fullerton, CA). 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 prob-

lem 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 h [Vti 65.2 vertical rotor; Beckman Coulter, Fullerton, CA]) and fractionated. The buoyant densities of the gradient solution and fractions (see Fig. S1A in the supplemental material) 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 (see Fig. S1A) were measured with a Quanti-iT PicoGreen Assay Kit (Invitrogen, Karlsruhe, Germany).

**PCR conditions and cloning.** cDNA was amplified with the following primer sets: *mcrA*f (5'-TAYGAYCARATHGGYT-3') and *mcrA*r (5'-ACRTTCATN GCRTARTT-3') for *mcrA* (83), FTHFSf (5'-TTYACWGGHGAYTTCCATG C-3') and FTHFSr (5'-GTATTGDGTYTTRGCCATACA-3') for *fhs* (53), 27f (5'-AGAGTTTGATCMTGGCTC-3') and 907r (5'-CCGTCAATTCMTTTRA GT-3') for bacterial 16S rRNA genes (52), and Arc21Fa (5'-TCCGGTTGATC CYGSCRG-3') (38) and Arc915 (5'-GTGCTCCCCCGCAATTCCT-3') (74) for archaeal 16S rRNA genes. Amplification of *mcrA* was as described previously (59) with the following modifications: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and elongation at 72°C for 45 s, with a terminal elongation step at 72°C for 5 min. *fhs* was amplified as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and elongation at 72°C for 70 s, with a terminal elongation step at 72°C for 5 min. Bacterial 16S rRNA genes were amplified as follows: initial denaturation at 95°C for 5 min, followed by five precycles of denaturation at 95°C at 60 s, annealing at 40°C for 60 s, and elongation at 72°C for 60 s and then by 30 subsequent cycles of denaturation at 95°C for 30 s, annealing at 43°C for 30 s, and elongation at 72°C for 5 s, with terminal elongation step at 72°C for 5 min. Archaeal 16S rRNA genes were amplified as described previously (32) with the following modifications: initial denaturation at 95°C for 5 min, followed by 33 cycles of denaturation at 95°C for 50 s, annealing at 55°C for 50 s, and elongation at 72°C for 110 s, with a terminal elongation step 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<sup>-1</sup>, 4 μM (*mcrA* and *fhs*) or 0.6 μM (16S rRNA genes) of each primer, 0.6 U of *Taq* DNA polymerase, 0.2 mM each deoxynucleoside triphosphate (dNTP), and 2.6 mM (*mcrA*) or 3.6 mM (*fhs* or 16S rRNA genes) MgCl<sub>2</sub>.

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 a previously 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 [<sup>13</sup>C]formate-supplemented fen microcosms was evaluated with terminal restriction fragment length polymorphism (TRFLP) analysis. PCR was performed with fluorescently labeled primers *mcrA*f-DY681 and *mcrA*r (Biomers GmbH, Ulm, Germany) (83). Gel-purified DNA (Montage DNA Gel Extraction Kit, Millipore Corp., Bedford, MA) 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 (22). DNA was then cleaved at specific restriction sites with 2 units of each restriction enzyme, *MspI* and *RsaI* (New England BioLabs, Frankfurt/Main, Germany). Remaining DNA was quantified with a Quanti-iT PicoGreen Assay Kit, and TRFLP analysis was performed as described previously (32).

The 210-bp terminal restriction fragments of heavy fractions increased in relative intensity (see Fig. S1B in the supplemental material), 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 [<sup>13</sup>C]formate-, [<sup>12</sup>C]formate-, <sup>13</sup>CO<sub>2</sub>-, and <sup>12</sup>CO<sub>2</sub>-supplemented microcosms to identify active consumers of formate and CO<sub>2</sub>. 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 [<sup>13</sup>C]formate-supplemented microcosms and fraction 5 of [<sup>12</sup>C]formate-supplemented microcosms after 23 days of incubation with formate.

**Sequence analyses and identification of phylotypes.** All sequences were analyzed 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 the Ribosomal Database Project (RDP) Classifier at a confidence threshold

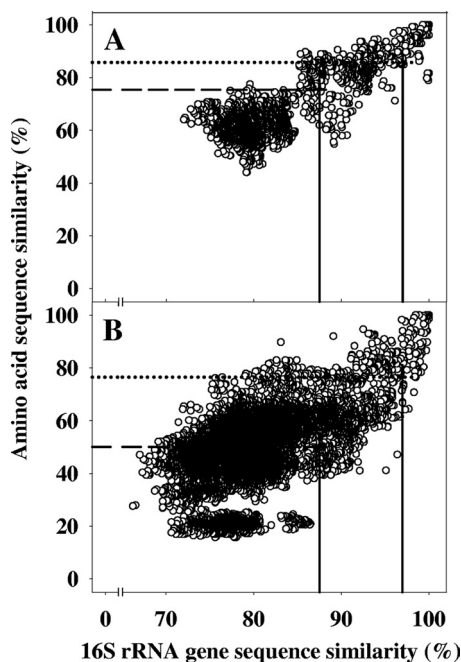


FIG. 1. Phylogenetic correlation plots of 16S rRNA gene sequence similarities and amino acid sequence similarities of *mcrA* (A) and *fhs* (B). Seventy-nine *mcrA* and 238 *fhs* sequences are plotted. The vertical solid lines that intersect the horizontal axes at 97% and 87.5% 16S rRNA gene sequence 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*-encoded (A) or *fhs*-encoded (B) amino acid sequence similarity and 16S rRNA gene sequence similarity.

of 80% (88), aligned with SINA Webaligner, and merged with the latest 16S rRNA gene database from the SILVA homepage ([www.arb-silva.de](http://www.arb-silva.de)) (72). Sequences of 16S rRNA genes were assigned to novel family-level phylotypes if they had <87.5% similarity to the next cultured taxon (93). Sequences of *mcrA* and *fhs* were translated *in silico* and aligned with reference sequences obtained from MegaBLAST using the ClustalW algorithm implemented in ARB software. Coverages were calculated by a previously 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* strain SANAE; for *fhs*, 100% similarity filter and 351 valid amino acids between positions 134 and 486 of *fhs* of *Clostridium difficile* strain 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 sequences 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 *mcrA*-encoded amino acid sequences of 85.7% and 75.4%, respectively, and bacterial *fhs*-encoded amino acid sequences of 76.4% and 50.0%, respectively (Fig. 1).

Phylogenetic 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 paludicola* SANAE. *fhs* trees used either a 100% similarity filter and 197 valid amino acids between positions 138 and 335 of *fhs* of *C. difficile* 630 or a 100% similarity filter and 175 valid amino acids between positions 292 and 468 of *fhs* of *C. 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 positions 103 and 894 of the 16S rRNA sequence of *E. coli* ATCC 11775. Bacterial 16S rRNA gene sequence trees

used a 100% similarity filter and 412 valid nucleotide positions between positions 311 and 745 of the 16S rRNA sequence of *E. coli* ATCC 11775. Calculated trees contained a bootstrap test with 100 to 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  $^{13}\text{C}$  treatment was higher than its relative abundance in the gene library from heavy fraction 4 of the  $^{12}\text{C}$  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 ( $\Delta G$ ) 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).  $\Delta G^\circ$  values were calculated from the standard Gibbs energies of formation ( $\Delta G_f^\circ$ ) at the pH of the liquid phase (that varied from pH 4.2 to 6.3) and utilized to calculate  $\Delta G$  values for the following reactions:  $4 \text{HCOO}^- + 4 \text{H}^+ \rightarrow \text{CH}_4 + 3 \text{CO}_2 + 2 \text{H}_2\text{O}$  ( $\Delta G^\circ$  varied from  $-161$  to  $-208 \text{ kJ mol}^{-1} \text{CH}_4$ );  $\text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{CH}_4 + \text{CO}_2$  ( $\Delta G^\circ$  varied from  $-44$  to  $-50 \text{ kJ mol}^{-1} \text{CH}_4$ );  $4 \text{HCOO}^- + 3 \text{H}^+ \rightarrow \text{CH}_3\text{COO}^- + 2 \text{CO}_2 + 2 \text{H}_2\text{O}$  ( $\Delta G^\circ$  varied from  $-121$  to  $-157 \text{ kJ mol}^{-1}$  acetate). Estimated concentrations of  $\text{H}^+$  and estimated  $\Delta G_f^\circ$  for  $\text{H}^+$  are based on the pH of the liquid phase (i.e., the  $\Delta G_f^\circ$  for  $\text{H}^+$  at pH 4.2 to 6.3 was  $-23.9$  to  $-35.8 \text{ kJ mol}^{-1}$ , respectively [61]).

**Analytical techniques.** Dry weight of soil was determined by weighing soil before and after drying at 60°C for 72 h. 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, CA) 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 an InLab R422 pH electrode (InLab Semi-Micro; Mettler Toledo, Giessen, Germany). Organic acids were determined by high-performance liquid chromatography (1090 series II with UV detector; Hewlett Packard, Palo Alto, CA) (91).  $\text{H}_2$  was measured with a gas chromatograph (5890 series II with a thermal conductivity detector; Hewlett-Packard, Palo Alto, CA) (48).  $\text{CO}_2$  and methane were separated with a HayeSep-D column (2m by 1/8 in; SRI Instruments, Torrance, CA) and analyzed with a flame ionization detector (SRI Instruments, Torrance, CA). 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 of soil (dry weight) (g soil $_{\text{dw}}^{-1}$ ). 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  $^{13}\text{C}$  content of acetate was determined by liquid chromatography coupled to isotope ratio mass spectrometry (Finnigan LC IsoLink; Thermo Fisher Scientific Inc., Waltham, MA) (46). Acetate was separated from other organic compounds by high-performance liquid chromatography, oxidation and acid/catalyst reagents (ammonium peroxodisulfate, phosphoric acid, and silver nitrate) were added, and organic compounds were oxidized to  $\text{CO}_2$  in an oxidation reactor at 100°C (46).  $\text{CO}_2$  of the liquid phase was 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 [ $^{13}\text{C}$ ]formate and sodium [ $^{13}\text{C}$ ]bicarbonate were obtained from Campro Scientific (Berlin, Germany). In this study, no distinction is made between  $\text{CO}_2$  and its carbonate forms.

**Nucleotide sequence accession numbers.** The sequences obtained in this study are available from the EMBL nucleotide sequence database under accession numbers FR725451 to FR725861 (*mcrA*), FR725862 to FR725930 (*fhs*), FR732102 to FR732501 (bacterial 16S rRNA genes), FR744942 to FR745247 (archaeal 16S rRNA genes), and FR745248 (*mcrA* from *M. limicola* DSM 2279).

## RESULTS

**Effect of supplemental formate and  $\text{CO}_2$  on product profiles.** Alternative electron acceptors [i.e., approximately 103  $\mu\text{mol}$  of iron(III) and 15  $\mu\text{mol}$  of sulfate g soil $_{\text{dw}}^{-1}$ ] in fen microcosms were reduced during 15 days of anoxic preincubation prior to supplementation of substrates. Sulfate was not detected, and iron(II) reached a stable end concentration at the end of the preincubation period. Nitrate was not detected during the pre-

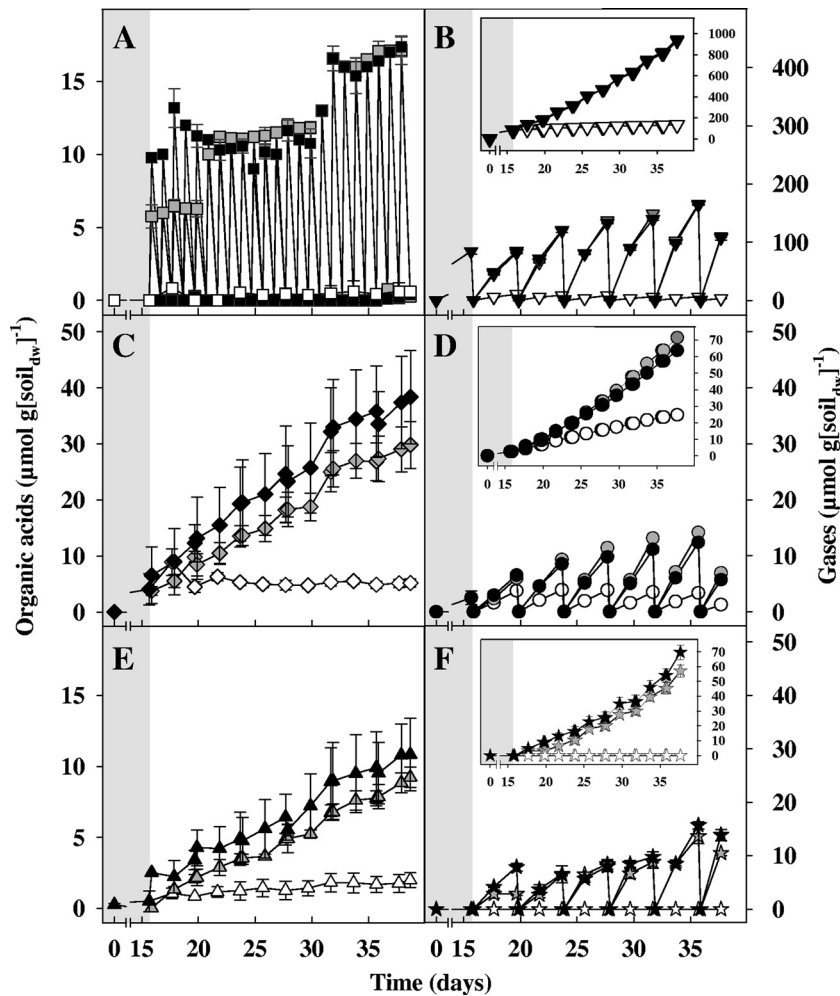


FIG. 2. Effect of formate on the production of organic acids and gases in anoxic fen soil microcosms at 15°C. Data are for formate (A), CO<sub>2</sub> (B), acetate (C), methane (D), propionate (E), and H<sub>2</sub> (F). In panel B, CO<sub>2</sub> in formate treatments represents the combined CO<sub>2</sub> from the bicarbonate pulses and CO<sub>2</sub> derived from the apparent conversion of formate to H<sub>2</sub> and CO<sub>2</sub>. Symbols: empty symbols, unsupplemented controls; gray symbols, [<sup>12</sup>C]formate treatments; black symbols, [<sup>13</sup>C]formate treatments. Insets show cumulative gas concentrations. Values are the means of triplicate microcosms, and the error bars indicate standard deviations.

incubation period (detection limit was 0.13  $\mu\text{mol}$  of nitrate  $\text{g soil}_{\text{dw}}^{-1}$ ). Concentrations of methane, acetate, and CO<sub>2</sub> approximated 2.5, 4, and 85  $\mu\text{mol}$   $\text{g soil}_{\text{dw}}^{-1}$ , respectively, at the end of the preincubation period. A total of approximately 25  $\mu\text{mol}$  methane  $\text{g soil}_{\text{dw}}^{-1}$ , 5  $\mu\text{mol}$  acetate  $\text{g soil}_{\text{dw}}^{-1}$ , and 2  $\mu\text{mol}$  propionate  $\text{g soil}_{\text{dw}}^{-1}$  were produced in the subsequent 23 days in unsupplemented controls (Fig. 2). Formate and H<sub>2</sub> remained below 1  $\mu\text{mol}$   $\text{g soil}_{\text{dw}}^{-1}$  in unsupplemented controls. Traces of butyrate, isobutyrate, and methyl butyrate were detected after 15 days in all treatments and controls (data not shown).

Each formate pulse was essentially consumed within 24 h (Fig. 2A). In total, an additional 63  $\mu\text{mol}$  of H<sub>2</sub>  $\text{g soil}_{\text{dw}}^{-1}$ , 43  $\mu\text{mol}$  of methane  $\text{g soil}_{\text{dw}}^{-1}$ , 29  $\mu\text{mol}$  of acetate  $\text{g soil}_{\text{dw}}^{-1}$ , and 8  $\mu\text{mol}$  of propionate  $\text{g soil}_{\text{dw}}^{-1}$  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<sub>2</sub> 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 <sup>13</sup>C in the [<sup>13</sup>C]formate and [<sup>12</sup>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 <sup>13</sup>C enrichment of [<sup>13</sup>C]formate-derived acetate was mostly in the methyl carbon.

An additional 20  $\mu\text{mol}$  of methane  $\text{g soil}_{\text{dw}}^{-1}$  and 1.7  $\mu\text{mol}$  of propionate  $\text{g soil}_{\text{dw}}^{-1}$  were detected in CO<sub>2</sub> treatments compared to unsupplemented controls (Fig. 3), indicating that CO<sub>2</sub> stimulated the production of these compounds. Supplemental CO<sub>2</sub> also appeared to stimulate the consumption of endogenously produced acetate (i.e., acetate produced before addition of CO<sub>2</sub>), and the production of methane increased during the disappearance of acetate (Fig. 3B and C).

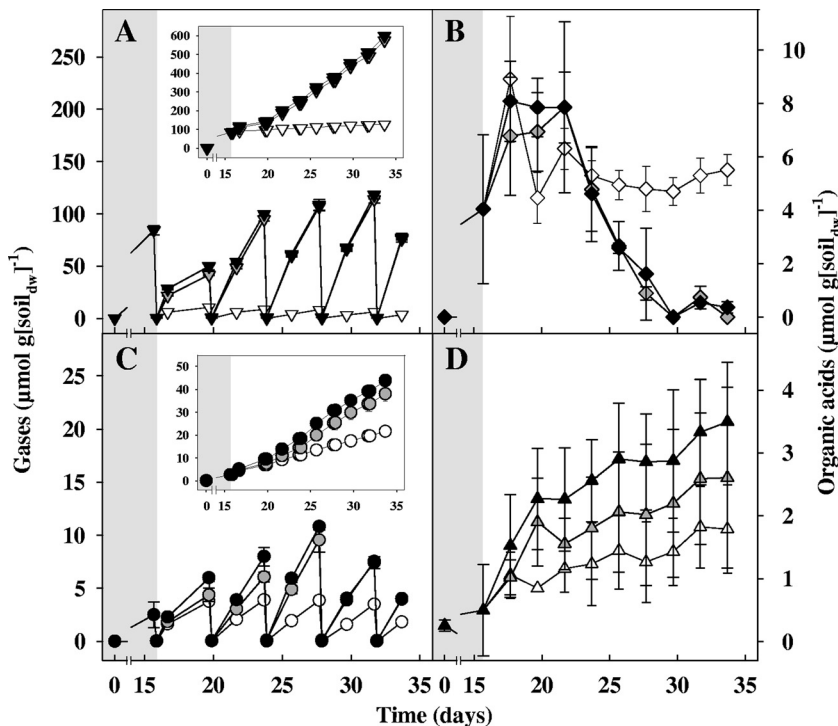


FIG. 3. Effect of CO<sub>2</sub> on the production of organic acids and gases in anoxic fen microcosms at 15°C. Data are for CO<sub>2</sub> (A), acetate (B), methane (C), and propionate (D). Symbols: empty symbols, unsupplemented controls; gray symbols, <sup>12</sup>CO<sub>2</sub> treatments; black symbols, <sup>13</sup>CO<sub>2</sub> treatments. Insets show cumulative gas concentrations. Values are the means of triplicate microcosms and the error bars indicate standard deviations.

Product profiles of <sup>13</sup>C and <sup>12</sup>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, based on the amount of product per gram of soil<sub>dw</sub> (values in parentheses are the percentage of reductant theoretically recovered from formate-derived reductant), approximately 39 μmol of methane (54%), 33 μmol of acetate (46%), 70 μmol of H<sub>2</sub> (24%), and 9 μmol of propionate (22%) were produced from 287 μmol of [<sup>13</sup>C]formate, whereas approximately 47 μmol of methane (70%), 25 μmol of acetate (38%), 57 μmol of H<sub>2</sub> (21%), and 7 μmol of propionate (18%) were produced from 267 μmol of [<sup>12</sup>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 <sup>13</sup>C and <sup>12</sup>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 [<sup>13</sup>C]formate-supplemented microcosms averaged -104 kJ mol<sup>-1</sup> CH<sub>4</sub> and -42 kJ mol<sup>-1</sup> acetate, respectively (Fig. 4A). The estimated Gibb's free energy of the apparent acetoclastic methanogenesis in <sup>13</sup>CO<sub>2</sub>-supplemented microcosms averaged -64 kJ mol<sup>-1</sup> CH<sub>4</sub> (Fig. 4B).

**Formate- and CO<sub>2</sub>-consuming methanogens.** A total of 365 *mcrA* sequences and 306 archaeal 16S rRNA gene sequences

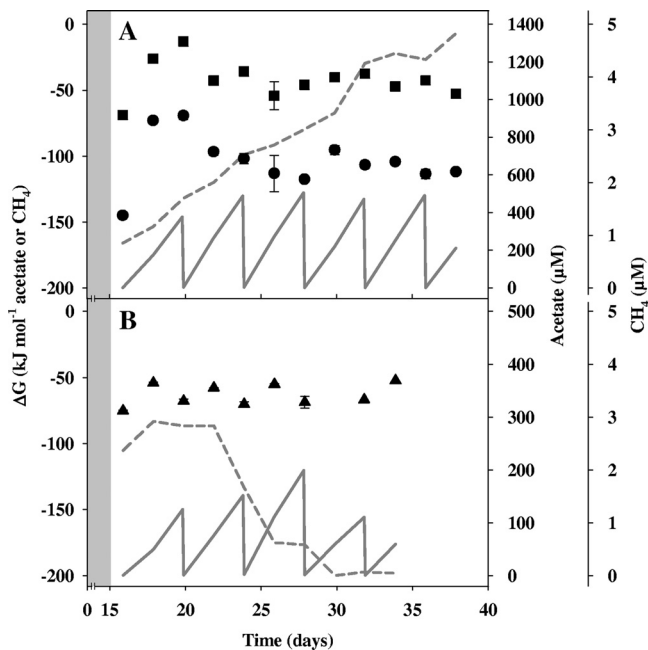


FIG. 4. Estimated Gibbs free energies ( $\Delta G$ ) in the [<sup>13</sup>C]formate (A) and <sup>13</sup>CO<sub>2</sub> (B) treatments shown in Fig. 2 and Fig. 3, respectively. Values are the means of triplicate microcosms, and the error bars indicate standard deviations. Symbols: filled circles,  $\Delta G$  for formate-dependent methanogenesis; filled squares,  $\Delta G$  for formate-dependent acetogenesis; filled triangles,  $\Delta G$  for acetate-dependent methanogenesis; dashed line, acetate; solid line, methane in the aqueous phase.

TABLE 1. Taxonomic identities and relative abundances of *mcrA* sequences

Taxonomic identity (class, family, and genus and species)	Relative abundance of sequence (%) by treatment and time posttreatment <sup>a</sup>							
	Labeled-CO <sub>2</sub> treatment				Labeled-formate treatment			
	9 days		18 days		9 days		23 days	
	<sup>12</sup> C	<sup>13</sup> C	<sup>12</sup> C	<sup>13</sup> C	<sup>12</sup> C	<sup>13</sup> C	<sup>12</sup> C	<sup>13</sup> C
<i>Methanobacteria</i>								
<i>Methanobacteriaceae</i>								
<i>Methanobacterium formicicum</i>	—	—	—	2.1	2.3	8.7	6.3	6.5
<i>Methanomicrobia</i>								
<i>Methanocellaceae</i>								
<i>Methanocella paludicola</i>	2.2	—	—	2.1	—	—	—	—
Novel species phylotype 1 <sup>b</sup>	33.3	43.2	44.4	45.8	32.6	56.5	35.4	65.2
Novel species phylotype 2 <sup>b</sup>	2.2	—	—	—	9.3	4.3	—	—
Novel species phylotype 3 <sup>b</sup>	—	—	2.2	—	2.3	—	2.1	—
<i>Methanomicrobiaceae</i>								
Novel species phylotype 4 <sup>b</sup>	6.7	—	6.7	2.1	4.7	6.5	6.3	2.2
Novel species phylotype 5 <sup>b</sup>	11.1	6.8	15.6	14.6	11.6	8.7	10.4	8.7
<i>Methanosaetaceae</i>								
<i>Methanosaeta concilii</i>	11.1	2.3	6.7	—	7.0	—	2.1	—
<i>Methanosarcinaceae</i>								
<i>Methanosarcina mazei</i>	17.8	38.6	15.6	20.8	11.6	2.2	27.1	2.2
Unclassified family								
<i>Methanoregula boonei</i>	15.6	9.1	8.9	12.5	18.6	13.0	10.4	15.2
Total no. of sequences	45	44	45	48	43	46	48	46

<sup>a</sup> —, not detected.

<sup>b</sup> Sequences were considered to be novel at the species level when the *mcrA* sequence was <85.7% identical to that of the next cultured species (Fig. 1A).

were analyzed from [<sup>13</sup>C]formate-, [<sup>12</sup>C]formate-, <sup>13</sup>CO<sub>2</sub>-, and <sup>12</sup>CO<sub>2</sub>-supplemented microcosms. The percentage of methanogens and nonmethanogens in the 16S rRNA gene library approximated 67% and 33%, respectively. Family-level coverage of *mcrA* and archaeal 16S rRNA genes was >99%. *mcrA* sequences were affiliated with the *Methanocellaceae* (48% of total), *Methanosarcinaceae* (17% of total), *Methanomicrobiaceae* (15% of total), *Methanosaetaceae* (4% of total), and *Methanobacteriaceae* (3% of total) families and with 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), two unclassified families (11% and 1% of total were affiliated with “*Candidatus Nitrosopumilus maritimus*” and *Methanoregula boonei*, respectively), and seven novel family-level phylotypes (23% of total) (Table 2). At 9 days postsupplementation, 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 [<sup>13</sup>C]formate-supplemented microcosms than from those of [<sup>12</sup>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 <sup>13</sup>CO<sub>2</sub>-supplemented microcosms than from those of <sup>12</sup>CO<sub>2</sub>-supplemented microcosms (Table 1), indicating that organisms of these phylotype were early assimilators of CO<sub>2</sub>.

At 23 days postsupplementation, higher relative abundances of *Methanocellaceae*-affiliated *mcrA* sequences and *Methano-*

*bacteriaceae*-affiliated 16S rRNA gene sequences were obtained from heavy fractions of [<sup>13</sup>C]formate-supplemented microcosms than from those of [<sup>12</sup>C]formate-supplemented microcosms (Tables 1 and 2). In contrast, at 18 days postsupplementation, higher relative abundances of *Methanobacteriaceae*-, *Methanocellaceae*-, and *Methanosarcinaceae*-affiliated 16S rRNA gene sequences were obtained from heavy fractions of <sup>13</sup>CO<sub>2</sub>-supplemented microcosms than from those of <sup>12</sup>CO<sub>2</sub>-supplemented microcosms (Table 2). Relative abundances of *Methanosarcinaceae*-affiliated *mcrA* sequences were also higher at 18 days postsupplementation in heavy fractions of <sup>13</sup>CO<sub>2</sub>-supplemented microcosms than in those of <sup>12</sup>CO<sub>2</sub>-supplemented microcosms (Table 1). At the end of incubation, *Methanoregula*-affiliated *mcrA* sequences were marginally higher in heavy fractions of <sup>13</sup>CO<sub>2</sub>- and [<sup>13</sup>C]formate-supplemented microcosms than in those of <sup>12</sup>CO<sub>2</sub>- and [<sup>12</sup>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 phylotypes (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 (see Fig. S2 in the supplemental material). 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), *Sp-*

TABLE 2. Class- and family-level identities and relative abundances of archaeal 16S rRNA gene sequences

Taxonomic identity (class and family)	Relative abundance of sequence (%) by treatment (time postsupplementation)			
	Labeled-CO <sub>2</sub> treatment (18 days)		Labeled- formate treatment (23 days)	
	<sup>12</sup> C	<sup>13</sup> C	<sup>12</sup> C	<sup>13</sup> C
<i>Methanobacteria</i>				
<i>Methanobacteriaceae</i>	2.4	12.8	14.9	33.8
<i>Methanomicrobia</i>				
<i>Methanocellaceae</i>	9.6	16.7	41.9	40.8
<i>Methanosarcinaceae</i>	15.7	56.4	10.8	5.6
Unclassified family <sup>a</sup>	1.2	1.3	1.4	1.4
Unclassified <i>Euryarchaeota</i> <sup>b</sup>				
Novel family phylotype 1 <sup>c</sup>	4.8	— <sup>d</sup>	2.7	—
Novel family phylotype 2 <sup>c</sup>	1.2	—	1.4	—
Unclassified <i>Crenarchaeota</i> <sup>b</sup>				
Unclassified family <sup>c</sup>	26.5	—	8.1	8.5
Novel family phylotype 3 <sup>c</sup>	22.9	1.3	4.1	5.6
Novel family phylotype 4 <sup>c</sup>	2.4	—	—	—
Novel family phylotype 5 <sup>c</sup>	2.4	—	4.1	—
Novel family phylotype 6 <sup>c</sup>	1.2	—	2.7	4.2
Novel family phylotype 7 <sup>c</sup>	9.6	11.5	8.1	—
Total no. of archaeal sequences:	83	74	78	71

<sup>a</sup> Closest related cultivated species, *Methanoregula boonei* (CP0007800), with 95 to 98% 16S rRNA gene similarity.

<sup>b</sup> Family-level phylotypes listed underneath do not necessarily belong to the same class.

<sup>c</sup> Sequences were considered to be novel at the family level when the 16S rRNA gene sequence was <87.5% identical to that of the next cultured species (92).

<sup>d</sup> —, not detected.

<sup>e</sup> Closest related cultivated species, "*Candidatus* Nitrosopumilus maritimus" (CP000866), with 88 to 89% 16S rRNA gene similarity.

*rochaetes* (<1% of total), and unclassified taxa (8% of total) (Table 3; see also Table S1 and Fig. S2 in the supplemental material).

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), one novel family (1% of total), and one unclassified family (3% of total, all of which were affiliated with "*Candidatus* Pelagibacter sp.") (see Fig. S3 and Table S2 in the supplemental material). 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 [<sup>13</sup>C]formate and [<sup>12</sup>C]formate treatments. These *fhs* phylotypes were most closely related to *Mesorhizobium loti* (67 to 83% *fhs* amino acid similarity) and *Granulibacter bethesdensis* (67 to 82% *fhs* amino acid similarity) (see 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) (see Fig. S3), indicating that the fen soil harbors organisms belonging to the monophyletic acetogenic genera *Moorella* and *Sporomusa* (17, 18, 21, 89).

**Formate- and CO<sub>2</sub>-consuming Bacteria.** Higher relative abundances of *Acetobacteraceae*- and *Rhodospirillaceae*-affiliated 16S rRNA gene sequences (*Alphaproteobacteria*) were obtained from heavy fractions of [<sup>13</sup>C]formate-supplemented microcosms than from those of [<sup>12</sup>C]formate-supplemented microcosms (see Table S1 in the supplemental material), 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 [<sup>13</sup>C]formate-supplemented microcosms than in those of [<sup>12</sup>C]formate-supplemented microcosms (see Table S1 in the supplemental material), 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 <sup>13</sup>CO<sub>2</sub>-supplemented microcosms than from those of <sup>12</sup>CO<sub>2</sub>-supplemented microcosms (see Table S1), indicating that these phylotypes assimilated CO<sub>2</sub>. The relative abundance of *Thermomonosporaceae*-affiliated 16S rRNA gene sequences (*Actinobacteria*) was marginally higher in heavy fractions of <sup>13</sup>CO<sub>2</sub>-supplemented microcosms than in those of <sup>12</sup>CO<sub>2</sub>-supplemented microcosms (see Table S1), indicating that this phylotype could have assimilated a marginal amount of CO<sub>2</sub>. 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 to 97% 16S rRNA gene similarity), *Acidimicrobium ferrooxidans* (87 to 91% 16S rRNA gene similarity), *Rhodocista centenaria* (88 to 93% 16S rRNA gene similarity), *Conexibacter woesei* (84 to 94% 16S rRNA gene similarity), *Solirubrobacter soli* (85 to 95% 16S rRNA gene similarity), and *Actinomadura formosensis* (85 to 99% 16S rRNA gene similarity) (see Fig. S2).

## DISCUSSION

Formate and CO<sub>2</sub> constitute potential trophic links to both methanogenesis and acetogenesis (21, 34, 95). Both formate and CO<sub>2</sub> experimentally stimulated methanogenesis, whereas only formate stimulated acetate synthesis. CO<sub>2</sub>-dependent stimulation of methanogenesis suggests that endogenously available CO<sub>2</sub> 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<sub>2</sub> availability might have for optimal methanogenesis. The availability of CO<sub>2</sub> can affect the metabolic capacities of certain acetogens (21). That CO<sub>2</sub> did not appear to stimulate acetate production suggests that the endogenous reductant available for acetogenesis was limiting. However, this speculation must be qualified since (i) endogenously produced acetate was likely not restricted to acetogenesis (i.e., acetate can be produced by nonacetogens [21]) and (ii) acetoclastic methanogenesis appeared to be stimulated in CO<sub>2</sub> treatments, thus resulting in a consumption of acetate that might mask acetate production.

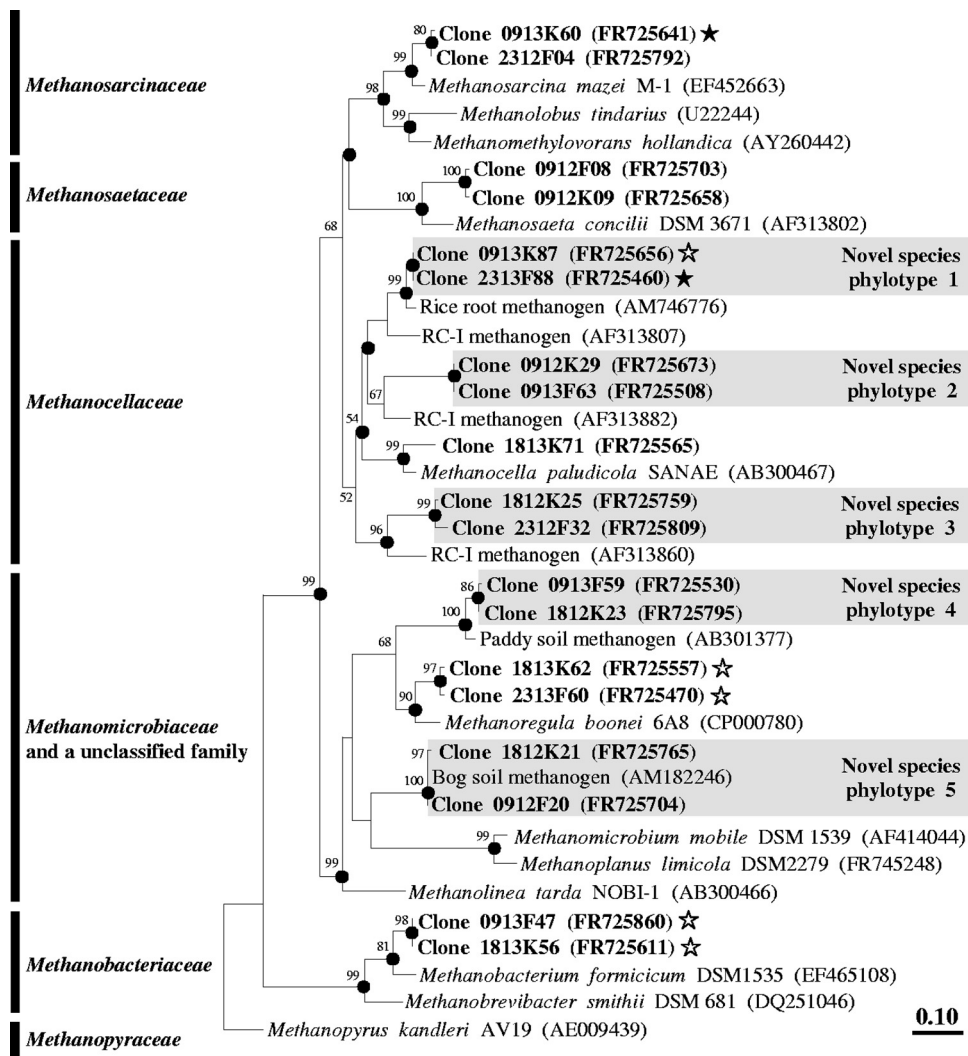


FIG. 5. Phylogenetic neighbor-joining tree of representative species-level amino acid sequences encoded by *mcrA* retrieved from formate and CO<sub>2</sub> treatments and of reference sequences. Values next to the branches represent the percentages of replicate trees (>50%) in which the associated taxa clustered together in the bootstrap test (1,000 bootstraps). Dots 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. Clones are identified, in order, by the number of days postsupplementation (09, 18, or 23) the type of treatment (12K, <sup>12</sup>C<sub>2</sub> treatment; 12F, [<sup>12</sup>C]formate treatment; 13K, <sup>13</sup>C<sub>2</sub> treatment; 13F, [<sup>13</sup>C]formate treatment) and clone number. Accession numbers are given in parentheses. Symbols: filled stars, labeled phylotypes; empty stars, marginally labeled phylotypes.

**Taxa associated with formate- and CO<sub>2</sub>-enhanced methanogenesis.** The collective labeling data indicated that *Methanobacteriaceae* and *Methanocellaceae* assimilated formate-derived carbon and that *Methanobacteriaceae*, *Methanocellaceae*, and *Methanosarcinaceae* assimilated CO<sub>2</sub>-derived carbon. The labeling of the same taxa in both formate and CO<sub>2</sub> treatments is consistent with the capacity of many methanogens to utilize both formate and CO<sub>2</sub> (34).

*Methanobacterium formicicum* was the most closely related cultured species to labeled *Methanobacteriaceae*-affiliated phylotypes (89 to 95% *mcrA* amino acid similarity and 89 to 93% 16S rRNA gene similarity) (Fig. 5 and 6). Certain members of the family *Methanobacteriaceae* can utilize CO<sub>2</sub>, H<sub>2</sub>, and formate for methane production (9). [<sup>13</sup>C]-formate yielded labeling of *Methanobacteriaceae*-affiliated phylotypes whereas

<sup>13</sup>C<sub>2</sub> 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<sub>2</sub> and endogenous reductant.

*Methanocella paludicola* was the most closely related cultured species to labeled *Methanocellaceae*-affiliated phylotypes in both formate and CO<sub>2</sub> treatments (79 to 83% *mcrA* amino acid similarity and 86 to 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<sub>2</sub>, and H<sub>2</sub> 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<sub>2</sub> treatments (Tables 1 and 2) indicated that novel species of *Methanocella* used formate and



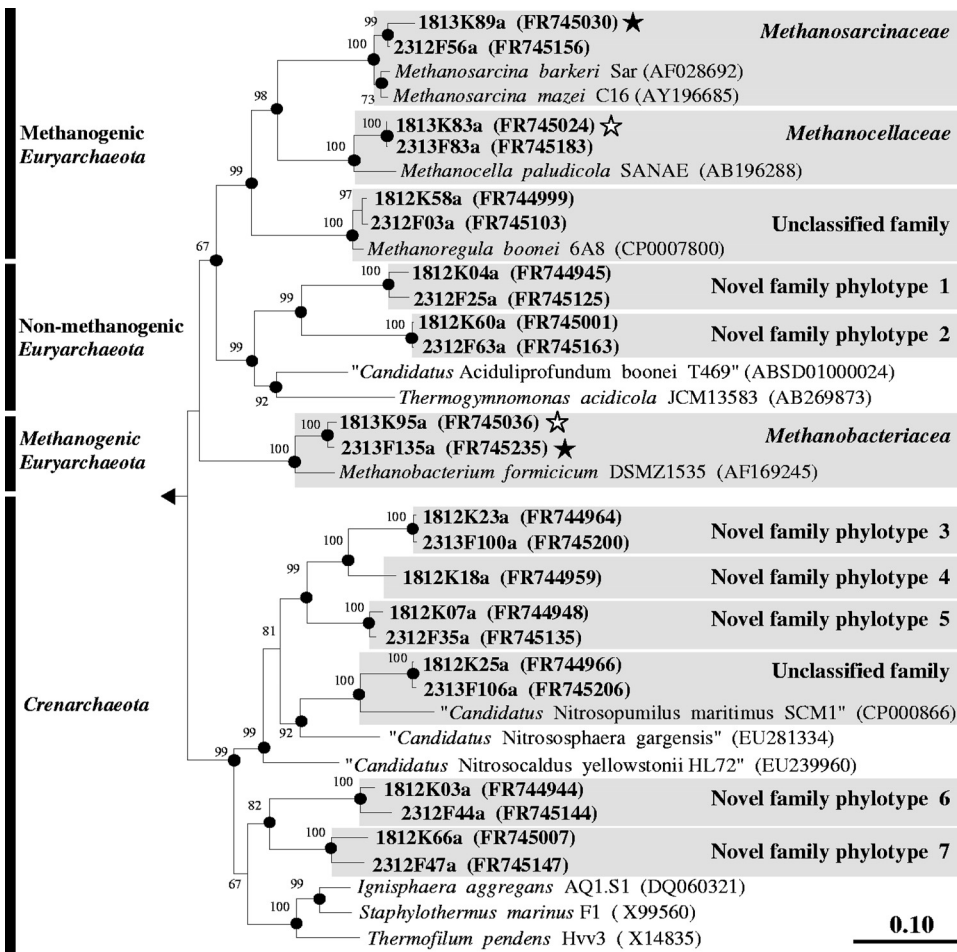


FIG. 6. Phylogenetic neighbor-joining tree of representative family-level archaeal 16S rRNA gene sequences retrieved from formate and CO<sub>2</sub> treatments and reference sequences. Values next to the branches represent the percentages of replicate trees (>50%) in which the associated taxa clustered together in the bootstrap test (10,000 bootstraps). Dots at nodes indicate the confirmation of tree topology by maximum-likelihood and maximum-parsimony calculations with the same data set. Quotation marks indicate provisional taxa (J. P. Euzéby, List of Prokaryotic Names with Standing in Nomenclature [http://www.bacterio.cict.fr/number.html]). Bar indicates a 0.1 estimated change per amino acid. *E. coli* (X80725) was used as an outgroup. Clones are identified, in order, by the number of days postsupplementation (18 or 23) the type of treatment (12K, <sup>12</sup>CO<sub>2</sub> treatment; 12F, [<sup>12</sup>C]formate treatment; 13K, <sup>13</sup>CO<sub>2</sub> treatment; 13F, [<sup>13</sup>C]formate treatment) and clone number, followed by “a” to indicate archaeal sequence. Accession numbers are given in parentheses. Symbols: filled stars, labeled phylotypes; empty stars, marginally labeled phylotypes.

CO<sub>2</sub> for methane production and possibly acetate as a source of carbon in anoxic fen microcosms.

*Methanosarcina mazei* (88 to 96% *mcrA* amino acid similarity, 93 to 98% 16S rRNA gene similarity) and *Methanosarcina barkeri* (87 to 96% *mcrA* amino acid similarity, 94 to 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<sub>2</sub>-CO<sub>2</sub> but do not utilize formate (34). Formate is not known to be utilized by species of *Methanosarcina* (34). *Methanosarcina*-related phylotypes were labeled in <sup>13</sup>CO<sub>2</sub>-supplemented microcosms in which the disappearance of acetate was concomitant to the production of methane but were not labeled in [<sup>13</sup>C]formate-supplemented microcosms in which <sup>13</sup>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 <sup>13</sup>CO<sub>2</sub>-produced [<sup>13</sup>C] acetate in the <sup>13</sup>CO<sub>2</sub> treatment, there was no evidence for the stimulation of acetate production in CO<sub>2</sub> treatments, and the [<sup>13</sup>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<sub>2</sub> for methane production at the expense of endogenous reductant.

*Methanoregula boonei* was the most closely related cultured species to the marginally labeled unclassified methanogenic family (81 to 90% *mcrA* amino acid similarity, 95 to 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<sub>2</sub> for methanogenesis (92). The late marginal labeling (Table 1) of *M. boonei*-related

TABLE 3. Classes and relative abundances of bacterial 16S rRNA gene sequences

Class	Relative abundance of sequence (%) by treatment (time postsupplementation) <sup>a</sup>			
	Labeled-CO <sub>2</sub> treatment (18 days)		Labeled-formate treatment (23 days)	
	<sup>12</sup> C	<sup>13</sup> C	<sup>12</sup> C	<sup>13</sup> C
<i>Acidobacteria</i>	35.2	23.5	26.9	18.2
<i>Actinobacteria</i>	21.6	38.2	26.9	20.2
<i>Alphaproteobacteria</i>	22.7	19.6	16.3	40.4
<i>Bacteroidia</i>	—	—	—	1.0
<i>Betaproteobacteria</i>	3.4	—	10.6	5.1
<i>Clostridia</i>	1.1	1.0	—	2.0
<i>Deltaproteobacteria</i>	3.4	2.9	1.0	1.0
<i>Gammaproteobacteria</i>	—	—	—	2.0
<i>Holophagae</i>	2.3	2.0	1.9	4.0
<i>Ktedonobacteria</i>	—	—	1.0	1.0
<i>Opitutae</i>	1.1	—	—	—
<i>Planctomycea</i>	1.1	1.0	1.0	1.0
<i>Sphingobacteria</i>	—	—	1.0	—
<i>Spirochaetes</i>	—	2.0	—	—
<i>Verrucomicrobiae</i>	1.1	—	1.9	—
Unclassified <sup>b</sup>	6.8	9.8	11.5	4.0
Total no. of bacterial sequences	88	102	104	99

<sup>a</sup> Detailed analyses of bacterial sequences are provided in Table S1 in the supplemental material. —, not detected.

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

phylotypes might have occurred by assimilation of [<sup>13</sup>C]-formate, [<sup>13</sup>C]-formate-derived <sup>13</sup>CO<sub>2</sub> and/or [<sup>13</sup>C]-acetate formed by acetogens.

**Acetoclastic methanogenesis.** Although supplemental acetate does not stimulate methanogenesis in anoxic Schlössnerbrunnen fen microcosms (38, 90), methane production was concomitant to the disappearance of acetate in CO<sub>2</sub>-supplemented microcosms (Fig. 3). Acetate can be used as a source of carbon by hydrogenotrophic methanogens such as *M. paludicola* (78), utilized by acetoclastic methanogens like *Methanosaetaceae* (34), or oxidized in a syntrophic partnership (33). The greatest production of methane in CO<sub>2</sub> treatments was coincident with the disappearance of acetate (Fig. 3). The increased concentration of CO<sub>2</sub> or the periodic increase in pH (i.e., up to approximately 5) could have enhanced acetoclastic methanogenesis in CO<sub>2</sub> 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<sub>2</sub> 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 and 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 <sup>13</sup>C in acetate in [<sup>13</sup>C]formate treatments, and the estimated exergonic Gibbs free energies for the apparent formate-driven acetogenesis and methanogenesis (Fig. 4A) suggest that acetogens and methanogens 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 (see Table S2 in the supplemental material) indicated that these taxa might have been involved in formate-dependent processes. *fhs* sequences most closely affiliated with the acetogens *S. ovata* and *Moorella thermoacetica* (17, 18, 21, 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, 21, 89). *Spirochaetaceae* and *Holophagaceae* contain acetogenic species (8, 56), and *Spirochaetaceae*- 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, 21). 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, 21) 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) suggest that highly diverse previously uncultivated *fhs*-containing taxa are likely present in fen soil and therefore might not have 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 found that the majority of retrieved *fhs* sequences were affiliated with nonacetogenic taxa and 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 nonmonophyletic; i.e., they are phylo-

genetically distributed with nonacetogens in the same genera (18, 20, 21), a factor complicating their assessment by standard 16S rRNA gene analysis. Several organisms originally described as nonacetogens have been later discovered to be acetogenic (e.g., *Clostridium glycolicum* [20, 21, 49]), thus raising the question as to whether any of the detected nonacetogenic 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, archaeal formate-driven acetogenesis has not been documented, and an obvious labeling of a nonmethanogenic archaea was not detected.

**General diversity and additional CO<sub>2</sub>- and formate-metabolizing taxa.** A high degree of novelty was detected in the 16S rRNA gene sequence data set (Fig. 6; see also Fig. S2 in the supplemental material). 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 phylogenotypes 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 “*Ca. Nitrosopumilus maritimus*” (42) to which 11% of the detected archaeal 16S rRNA gene sequences were distantly affiliated (Table 2 and Fig. 6). Additional distantly related cultured genera of the detected crenarchaeotal phylogenotypes grow anaerobically with peptides and sulfur (e.g., *Thermofilum* and *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 [<sup>13</sup>C]formate or [<sup>12</sup>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<sub>2</sub> (11, 12, 23, 27). The reduction of alternative electron acceptors in fen soil microcosms during the preincubation period supports 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. soli*, 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 treat-

ments suggest that they might have been involved in the apparent production of H<sub>2</sub> 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<sub>2</sub>, and H<sub>2</sub> via the reversal of syntrophic propionate oxidation (50). The reductive formation of propionate from acetate, CO<sub>2</sub>, and H<sub>2</sub> 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<sub>2</sub>, and H<sub>2</sub> (13). The ΔG values for this reaction with rice roots ranged from –15 to –38 kJ mol<sup>-1</sup> propionate (13). A minor fraction of the 16S rRNA gene sequences from the [<sup>13</sup>C]formate treatment were closely related to the *Desulfuromonadaceae*-affiliated ethanol fermenter *Pelobacter propionicus* (96.4 to 96.9% similarity) (see Table S1 and Fig. S2 in the supplemental material), an organism with physiological properties that overlap those of *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<sub>2</sub> treatments (see Table S1 and Fig. S2). The detection of these taxa in fen soils (32, 43, 57) and the availability of acetate, CO<sub>2</sub>, and H<sub>2</sub> 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 nonmethanogens 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 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<sub>2</sub> 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 taxon was not detected. It thus remains uncertain which microbes were responsible for the apparent conversion of formate to acetate and

whether dissimilation of formate to acetate was concomitant with the acetogenic assimilation of formate. That formate-derived H<sub>2</sub> accumulated in anoxic microcosms suggested that H<sub>2</sub> production exceeded the H<sub>2</sub>-consuming capacities of hydrogenotrophic methanogens. This speculation is consistent with the fact that cultivated H<sub>2</sub>-forming fermenters, a likely origin of the apparent formate-hydrogen lyase activity in fen soil, can outnumber cultivated H<sub>2</sub>-consuming methanogens in soil from the Schlöppnerbrunnen fen by a factor of approximately 1,000 (19, 90). The apparent concomitant occurrence of methanogenesis and formate-hydrogen lyase activity suggests that certain methanogenic taxa might have utilized formate-derived H<sub>2</sub> for a source of reductant. Current studies focus on determining potential differences between formate- and H<sub>2</sub>-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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