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Anaerobic Consumers of Monosaccharides in a Moderately Acidic Fen▽†

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16S rRNA-based stable isotope probing identified active xylose- and glucose-fermenting *Bacteria* and active *Archaea*, including methanogens, in anoxic slurries of material obtained from a moderately acidic, CH_4-emitting fen. Xylose and glucose were converted to fatty acids, CO_2, H_2, and CH_4 under moderately acidic, anoxic conditions, indicating that the fen harbors moderately acid-tolerant xylose- and glucose-using fermenters, as well as moderately acid-tolerant methanogens. Organisms of the families *Acidaminococcaceae*, *Aeromonadaceae*, *Clostridiaceae*, *Enterobacteriaceae*, and *Pseudomonadaceae* and the order *Actinomycetales*, including hitherto unknown organisms, utilized xylose- or glucose-derived carbon, suggesting that highly diverse facultative aerobes and obligate anaerobes contribute to the flow of carbon in the fen under anoxic conditions. Uncultured *Euryarchaeota* (i.e., *Methanosarcinaceae* and *Methanobacteriaceae*) and *Crenarchaeota* species were identified by 16S rRNA analysis of anoxic slurries, demonstrating that the acidic fen harbors novel methanogens and *Crenarchaeota* organisms capable of anaerobiosis. Fermentation-derived molecules are conceived to be the primary drivers of methanogenesis when electron acceptors other than CO_2 are absent, and the collective findings of this study indicate that fen soils harbor diverse, acid-tolerant, and novel xylose-utilizing as well as glucose-utilizing facultative aerobes and obligate anaerobes that form trophic links to novel moderately acid-tolerant methanogens.

Wetlands harbor approximately 30% of the global reserves of soil carbon; they are characterized by highly dynamic O_2 gradients and temporarily negligible concentrations of inorganic electron acceptors, such as sulfate or nitrate (e.g., 39, 43, 50); and they are one of the most important global sources of CH_4 (2). Wetlands account for about 20% of the global annual emission of the greenhouse gas CH_4 (19), which increased by 70% between 1970 and 2004 (42). CH_4 is the expected end product of the mineralization of organic matter when CO_2 is the sole electron acceptor (37). The organic matter of peat consists of 20 to 50% lignin and lignin-derived substances (e.g., lignocellulose [48]). Lignocellulose is the main component of the cell walls of plant tissue (1, 35) and consists of cellulose, hemicellulose, and lignin (1). The main components of hemicellulose are the polymers xylan (which consists mainly of xylose) and cellulose (which consists mainly of glucose). Xylose and glucose are released via the hydrolysis of xylan and cellulose by extracellular enzymes of primary fermenters (26). Hence, xylose and glucose are conceived to be intermediates in the "intermediary ecosystem metabolism" of methanogenic wetlands (i.e., in the metabolic processes that precede methanogenesis in wetlands), which can be converted by fermenters to organic acids (e.g., acetate), alcohols, CO_2, and H_2. Acetate and H_2-CO_2 are major substrates for methanogenesis (7, 37, 50, 51). Thus, fermentation is the prerequisite for the production of CH_4.

Although 16S rRNA gene pools of a few acidic wetlands have been evaluated (8, 21, 27), the microbial community composition of active fermenters involved in the carbon flow of fens is still largely unresolved. Since fermenters do not form monophyletic groups, it is not possible to accurately identify fermenters based on 16S rRNA gene sequences alone. Although methanogens in acidic wetlands have been assessed by 16S rRNA gene and methyl coenzyme M reductase alpha (*mcrA*) subunit analyses (e.g., see references 13, 14, and 18), the active fraction of methanogens has thus far not been identified. The objectives of this study were to employ different technologies to identify (i) xylose- and glucose-utilizing fermenters, (ii) intermediates in the carbon flow to methane, and (iii) active, moderately acid-tolerant *Archaea* in a northern temperate acidic fen.

MATERIALS AND METHODS

Sampling site and sampling. The Schlöppnerbrunnen fen is located in the Lehstenbach catchment in the Fichtelgebirge, in northeastern Bavaria, Germany (50°07′53″N, 11°52′51″E), and is 700 m above sea level. The fen emits up to approximately 530 μmol CH_4 m^{-2} h^{-1} (S. Goldberg, K.-H. Knorr, and G. Gebauer, unpublished data). Norway spruce (*Picea abies*) is the dominant tree of the catchment. Thirty percent of the catchment is covered by fens or intermittent seeps. The annual precipitation varies between 900 and 1,160 mm $year^{-1}$, and the average annual temperature range is 5 to 8°C (43). Schlöppnerbrunnen is completely overgrown by *Molinia caerulea*, *Eriophorum vaginatum*, *Carex canescens*, and *Juncus effusus* (43). The fen soil is a fibric histosol type, with a pH of approximately 5. Soil samples were taken on 2 November 2005 from the upper 20 cm of the fen, transported in air-tight plastic bags, and stored at 2°C until processed (within approximately 2 h). The dry weight of the collected soil was approximately 15% (wt/wt).

Incubations for stable isotope probing. An anoxic mineral salt solution was prepared using a modified Hungate technique (20). The sterile anoxic mineral solution contained mineral salts [12.6 mg/liter $(NH_4)_2SO_4$; 13.5 mg/liter $CaCl_2 \cdot 2H_2O$; 10 mg/liter $MgCl_2 \cdot 6H_2O$; 4 mg/liter KH_2PO_4; and 10 mg/liter $FeCl_2 \cdot 4H_2O$; modified from the solution described in ref. 28] and trace ele-

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ments [15 mg/liter nitrilotriacetic acid; 5 mg/liter $MnSO_4 \cdot 1H_2O$; 1 mg/liter $FeSO_4 \cdot 7H_2O$; 1 mg/liter $CoCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$; 1 mg/liter $ZnSO_4 \cdot H_2O$; 0.1 mg/liter $CuSO_4 \cdot H_2O$; 2 mg/liter $AlK(SO_4)_2 \cdot 12H_2O$; 0.1 mg/liter H_3BO_3; and 0.1 mg/liter $Na_2MoO_4 \cdot 2H_2O$]. The solution was boiled and subsequently cooled under a 100% N_2 gas phase. After the solution was autoclaved, the pH was adjusted to 4.5 with HCl, and a filter-sterilized (0.2-μm-pore size) vitamin solution (10 ml per liter) containing 0.02 mg/liter biotin, 0.02 mg/liter folic acid, 0.1 mg/liter pyridoxine-HCl, 0.05 mg/liter thiamine-HCl, 0.05 mg/liter riboflavin, 0.05 mg/liter niacin, 0.05 mg/liter DL-Ca-pantothenic acid, 0.001 vitamin B_{12}, 0.05 mg/liter *p*-aminobenzoic acid, and 0.05 mg/liter lipoic acid was added anoxically. A soil slurry was prepared by adding 690 ml of the sterile anoxic mineral salt solution to 275 g (fresh weight) of fen soil in a sterile screw-cap, butyl rubber-stoppered flask (1.1 liter), and the gas phase was adjusted to 100% N_2 in order to maximize anaerobic activities and minimize incubation times. Fen soil was subsequently preincubated without any substrates to minimize the amount of electron acceptors other than CO_2. The preincubation was performed in an end-over-end shaker for 8 days in the dark, until nitrate and sulfate were depleted and most of the Fe^{3+} was converted to Fe^{2+} (based on Fe^{2+} reaching a stable, maximum end concentration). The slurry was divided into 11 butyl rubber-stoppered flasks (350 ml) in an anaerobic chamber (Mecaplex, Switzerland) under an N_2 atmosphere. [^{13}C]xylose or [^{13}C]glucose as well as [^{12}C]xylose or [^{12}C]glucose was provided as a supplement from sterile, anoxic stock solutions to a final concentration of approximately 0.4 mM. Controls were unsupplemented slurries. Three replicates were performed for both the ^{12}C-labeled substrate-containing slurry and the control slurry. Substrates were refed when their concentration was below 0.2 mM. The flasks were incubated at 15°C in the dark. Gases (H_2, CO_2, and CH_4), monosaccharides, alcohols, and organic acids were analyzed.

Analytical techniques. The moisture content of the fen soil was determined by weighing the soil before and after it was dried at 60°C for 72 h. pH was measured with a model U457-S7/110 combination pH electrode (Ingold, Steinbach, Germany). Nitrate, sulfate, and Fe^{2+} were measured spectrophotometrically (46, 47, 49). Gases were measured with a 5980 series II model gas chromatograph, with thermal conductivity and flame ionization detection, and with a 3396 series II model integrator (Hewlett Packard, Palo Alto, CA). Monosaccharides, organic acids, and alcohols were analyzed with a 1090 series II high-performance liquid chromatograph with a refractive index detector (Hewlett Packard, Palo Alto, CA [29, 36]).

Nucleic acid extraction. RNA was extracted from 0.5 g of soil by bead-beating lysis, organic solvent extraction, and precipitation (16). The purification of RNA from coextracted DNA was performed with a Qiagen RNA/DNA mini-kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA was extracted from fen material with a Power Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA), according to the manufacturer's protocol.

Separation of ^{13}C- and ^{12}C-labeled RNA. A gradient solution (buoyant density, 1.78 ± 0.005 g ml^{-1}) of cesium trifluoroacetate (83.3%), gradient buffer (pH 8; 100 mM Tris; 100 mM KCl; 1 mM EDTA; 13.5%), and formamide (3.2%) was added to 500 ng of RNA and filled into 13-mm-by-51-mm polyallomer Quick Seal tubes (Beckman). RNA fractions were resolved by equilibrium centrifugation (~130,000 × *g* at 20°C for 62 h) (11, 32). Fractions (400 μl) were collected with syringes with 21-gauge needles. The densities of the gradient solutions of all fractions were determined by weighing them at 25°C. The fractionated RNA was recovered by the addition of an equal volume of isopropanol to the sample and centrifugation at 13,000 × *g* (at 4°C for 15 min). The RNA pellet was washed with 70% ice-cold ethanol, air dried, and dissolved in 20 μl of diethyl pyrocarbonate-treated water.

Reverse transcriptase PCR and PCR. Reverse transcription of RNA was performed with random hexamers and SuperScript II reverse transcriptase according to the manufacturer's protocol (Invitrogen, Karlsruhe, Germany). cDNA was amplified by the bacterial and archaeal 16S rRNA-specific primer sets 27F/907RM (30) and ARC4F/915R (17), respectively. PCR was performed in a PeqLab Primus 96 model thermal cycler (PeqLab, Erlangen, Germany). A denaturation step of 5 min at 95°C was performed at the beginning of PCR amplifications. Denaturation and elongation were performed at 95°C (5 min) and 72°C (90 s), respectively. For the primer 27F/907RM, a 4-cycle pre-PCR procedure with annealing at 40°C (1 min) was performed, followed by 30 cycles with an annealing temperature at 50°C (for 30 s). A step of 31 PCR cycles with annealing at 55°C (50 s) was used for the primer ARC4F/915R. For denaturing gradient gel electrophoresis (DGGE) analysis, PCR was performed with the primers GM5-clamp/907RM for *Bacteria* and 344Fa-Clamp/915R for *Archaea,* for 33 cycles at an annealing temperature of 56°C (18, 40). PCR with the primer M13F/M13R was performed per the published protocol (38).

DGGE. 16S rRNA genes were analyzed by DGGE (18, 40) using a PhorU system (Ingeny, Goes, The Netherlands) on 6% polyacrylamide gels, with a denaturant gradient from 35 to 70% (100% denaturant was 42% [wt/vol] urea and 40% [vol/vol] formamide). Electrophoresis was performed for 18 h at 100 V and 60°C. Gels were stained with SYBR Green (Invitrogen, Karlsruhe, Germany), visualized on a fluorescence imager unit (Storm 860; GE Healthcare, Piscataway, NJ), and analyzed with ImageQuant 5.0 software (GE Healthcare, Piscataway, NJ).

T-RFLP analysis. For terminal restriction fragment length polymorphism (T-RFLP) analysis, PCR was performed with primers 27F and ARC915R labeled with IRD700 (MWG, Ebersberg, Germany). T-RFLP was performed per the published protocol (31). Amplified and IRD700-labeled bacterial cDNA were restricted with mung bean nuclease (New England Biolabs, Frankfurt am Main, Germany) to remove single-stranded DNA and minimize the formation of pseudo-terminal restriction fragments (pseudo-T-RFs) (12). The restricted cDNA was purified with Millipore PCR_{96} cleanup plates (Millipore Corporation, Bedford, MA) and digested with the restriction endonuclease MspI (Fermentas, St. Leon-Rot, Germany). Gel electrophoresis was performed with a NEN model 4300 DNA analyzer (Licor, Lincoln, NE). The polyacrylamide gel consisted of 15 g of urea, 3.75 ml of 40% acrylamide-bis solution (37.5:1; 2.6% C; Bio-Rad, Hercules, CA), 12 ml of 2.5× Tris-borate-EDTA buffer (AppliChem GmbH, Darmstadt, Germany), and 3.25 ml of double-distilled H_2O. For the stabilization of the comb region of the gel, a bind-silane solution (1:1 bind-silane [PlusOne; GE Healthcare, Piscataway, NJ] and 10% acetic acid) was applied to the glass plates. The gel was poured according to the manufacturer's protocol. Electrophoresis was performed for 4 h at 1,200 V and 45°C. Gels were analyzed with GelQuest (Sequentix, Klein Raden, Germany).

Cloning, sequencing, phylogenetic analysis, and in silico T-RFLP analysis. PCR products were ligated in a pGEM-T Easy vector and transformed into *Escherichia coli* JM109 competent cells according to the manufacturer's protocol (Promega, Madison, WI). Clones were screened for the correct insert with M13F/M13R (38). RFLP analysis of cloned bacterial and archaeal 16S rRNA genes was performed with the restriction enzymes HaeIII (Fermentas, St. Leon-Rot, Germany) and TacI (New England BioLabs, Ipswich, MA), respectively. One representative sequence for each of the different RFLP patterns was obtained commercially from Macrogen (Seoul, South Korea). Sequences were analyzed with ARB software (34) and WU-BLAST2 (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were aligned with FastAligner software (included in ARB software) (http://www.arb-home.de [34]), and the alignment was refined manually. Tree calculations were based upon 16S rRNA sequences (700 to 800 bp) and performed using parsimony, neighbor-joining, and maximum likelihood methods. Consensus trees were drawn using consistent branching of all three methods (33). The outgroups *Thermocrinis albus* (EBI accession no. AJ278895) and *Thermoplasma acidophilum* (EBI accession no. M20822) were used for calculations of the bacterial and archaeal trees, respectively. In silico T-RFs were calculated with T-RFcut software (44) implemented in ARB and compared to experimentally obtained T-RFs to associate T-RFs with 16S rRNA sequences.

Nucleotide sequence accession numbers. 16S rRNA sequences obtained in this study were deposited in the European Bioinformatics Institute (http://www.ebi.ac.uk) under the accession numbers AM712557 to AM712594, AM773593, AM773619, AM773938, AM773944, AM773947, AM773964, AM773974, AM773982, AM773991, and AM773995.

RESULTS

Effects of xylose and glucose on fermentation and methanogenesis. Anoxic slurries of fresh fen soil were preincubated for 8 days to ensure that electron acceptors other than CO_2 were consumed prior to the addition of monosaccharides. CH_4 was produced at rates of 16 ± 0.01 μmol day^{-1} $g_{dw}{}^{-1}$ (where g_{dw} is grams [dry weight]) in unsupplemented controls after the preincubation period, indicating that (i) electron acceptors other than CO_2 had been consumed or were not inhibiting methanogenesis and (ii) fermentative microbes would very likely be the primary consumers of monosaccharides after the preincubation period. Xylose and glucose were initially consumed within 5 and 4 days, respectively (Fig. 1). The cumulative amounts of xylose and glucose that were consumed within 14 days were 18 μmol $g_{dw}{}^{-1}$ and 20 μmol $g_{dw}{}^{-1}$, respectively.

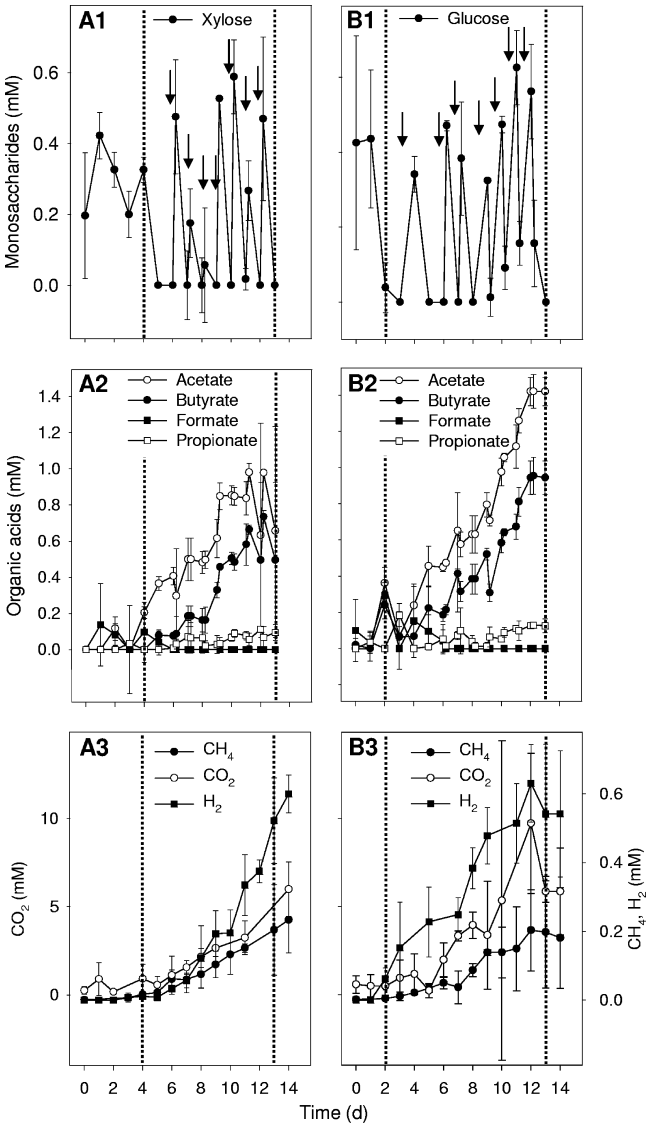

FIG. 1. Effects of supplemental xylose (A1) and glucose (B1) on the production of organic acids (A2 and B2) and gases (A3 and B3) by anoxic fen slurries ($n = 3$). Concentrations were corrected with values obtained from control slurries (i.e., unsupplemented anoxic slurries). Gases are given in mM, i.e., mmol produced per liter of liquid phase. Arrows indicate the addition of monosaccharides. Dotted lines indicate early and late sampling time points for RNA gradients.

Formate accumulated transiently up to approximately 2 μmol g_{dw}^{-1} and was consumed. In xylose- and glucose-supplemented fen slurries, acetate accumulated to approximately 4.4 and 9.5 μmol g_{dw}^{-1}, respectively, and butyrate to approximately 3.3 and 6.3 μmol g_{dw}^{-1}, respectively (Fig. 1A2 and B2), indicating that fermentation occurred in the slurries. Approximately 40 μmol CO_2 g_{dw}^{-1} was produced in both treatments (Fig. 1A3 and B3). H_2 and CH_4 accumulated in both the xylose-supplemented slurry (approximately 4.0 μmol g_{dw}^{-1} and 1.6 μmol g_{dw}^{-1}, respectively; Fig. 1A3) and the glucose-supplemented slurry (approximately 3.6 μmol g_{dw}^{-1} and 1.2 μmol g_{dw}^{-1}, respectively; Fig. 1B3). H_2 was not detected in unsupplemented slurries. At day 5 of the xylose experiment, 75% of the electrons were recovered in fermentation products (acetate, butyrate, formate, and CO_2 [27%]) and 1% in CH_4; at day 12, electron recoveries from fermentation products (acetate, butyrate, propionate, and CO_2 [50%]) and CH_4 approximated 89% and 2%, respectively. At day 3 of the glucose experiment, 70% of the electrons were recovered from fermentation products (acetate, butyrate, propionate, and CO_2 [37%]) and 0.4% from CH_4; at day 12, electron recoveries from fermentation products (acetate, butyrate, propionate, and CO_2 [48%]) and CH_4 approximated 98% and 2%, respectively. Since the bulk of recovered electrons were accounted for in fermentation products, it can be concluded that fermentation was significant during electron flow.

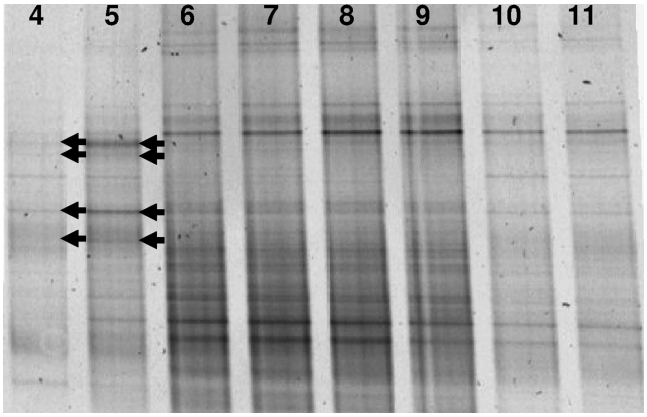

FIG. 2. DGGE profile of fractions 4 to 11 of bacterial RNA gradients obtained from the [^{13}C]xylose experiment at day 13. Numbers at the top of the lanes correspond to fractions 4 to 11. Arrows identify bands that occur only in the "heavy" fractions (4 and 5) or are more intensive than in the "light" fractions (6–11).

Fingerprints of xylose and glucose consumers. Isopycnic centrifugations to separate ^{13}C- and ^{12}C-labeled RNA were performed with RNA extracted after days 2 and 4 (early time points, before monosaccharides were completely consumed; Fig. 1A1 and B1) from xylose- and glucose-supplemented slurries, respectively, and at day 13 (a late time point) for both slurries. The buoyant densities of all fractions (1–12) followed a linear gradient from 1.84 to 1.76 g ml^{-1}, indicating that the isopycnic centrifugation was successful. The buoyant densities of fractions 4 and 5 ("heavy") and 10 ("light") were 1.816 to 1.81 g ml^{-1} and 1.768 g ml^{-1}, respectively, indicating that these fractions contained [^{13}C]RNA and [^{12}C]RNA, respectively (32). DGGE profiles of bacterial 16S rRNA fragments amplified from "heavy" fractions 4 and 5 were different from those of "light" fractions 6 to 11 (Fig. 2 and data not shown). These results indicate that certain bacterial 16S rRNAs in the "heavy" fractions were distinct from those of the "light" fractions and that a sufficient amount of ^{13}C label was incorporated into the RNA to allow a successful separation of [^{13}C]RNA from [^{12}C]RNA at the early and late time points.

T-RFLP analysis of density-resolved "heavy" bacterial rRNA in fraction 4 obtained from the incubations with [^{13}C]xylose and [^{13}C]glucose at early and late time points showed up to 31 T-RFs (Fig. 3), indicating that (i) diverse microorganisms had metabolized the labeled substrates and (ii) a sufficient amount of ^{13}C label was incorporated into the RNA at the early time point. In

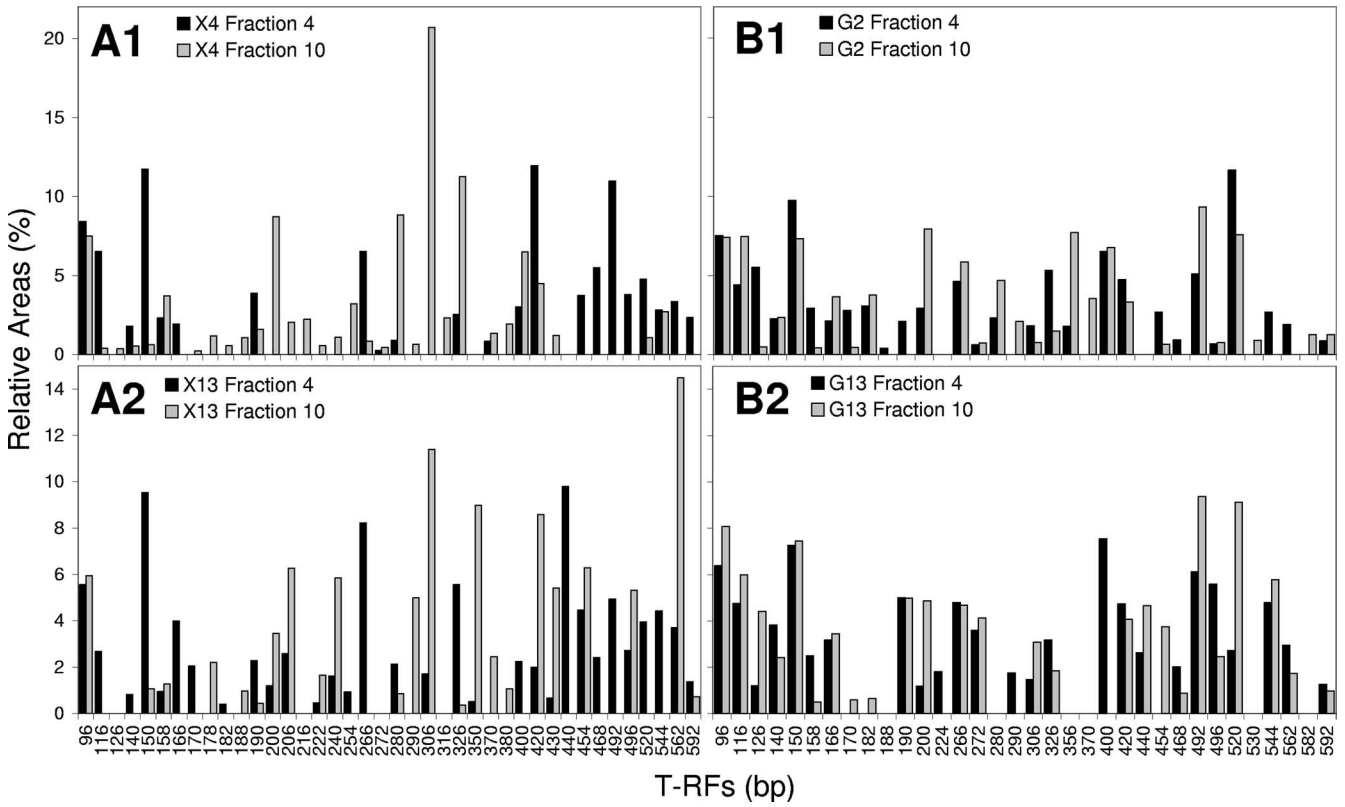

FIG. 3. T-RFLP profiles of bacterial cDNA of "heavy" fraction 4 and "light" fraction 10 obtained from the [^{13}C]xylose experiment (A) or the [^{13}C]glucose experiment (B) at days 2 (A1), 4 (B1), and 13 (A2 and B2).

the [^{13}C]xylose experiment, T-RFLP profiles of the heavy fraction yielded a higher relative abundance of 18 T-RFs (Fig. 3A1 and A2, T-RFs 116, 140, 150, 166, 170, 190, 266, 326, 420, 440, 454, 468, 492, 496, 520, 544, 562, and 592 bp; these T-RFs refer to those detected at day 4 and/or 13) than the T-RFLP profiles of the "light" fraction (Fig. 3A1 and A2). Eleven of these T-RFs (116, 140, 150, 166, 190, 266, 468, 492, 520, 544, and 592 bp) occurred at both day 4 and day 13, and three of these T-RFs (166, 266, and 440 bp) became more prominent by day 13. In the [^{13}C]glucose experiment, T-RFLP profiles of the "heavy" fraction yielded a higher relative abundance of 15 T-RFs (Fig. 3B1 and B2, 126, 140, 150, 158, 170, 190, 224, 326, 400, 420, 468, 496, 520, 544, and 562 bp; these T-RFs refer to those detected at day 2 and/or 13) than the T-RFLP profiles of the "light" fraction. Five of these T-RFs (158, 326, 420, 468, and 562 bp) occurred at both day 2 and day 13, and three of these T-RFs (140, 290, and 496 bp) became more prominent by day 13.

Phylogenetic analysis of active prokaryotic populations. 16S rRNA gene libraries of the "heavy" RNA fractions were generated to elucidate the phylogenetic identity of anaerobic xylose and glucose consumers. A total of 190 clones were screened by RFLP. Coverage of gene libraries as determined by RFLP analysis ranged from 86% to 96% (see Fig. S1 in the supplemental material). Fourteen different operational taxonomic units (OTUs; i.e., different RFLP patterns) were detected in the "heavy" fractions that were obtained from the early time points of the xylose and glucose incubations. Ten and six different OTUs were retrieved from the "heavy" RNA of the xylose and of the glucose incubations, respectively, at day 13 (data not shown). These 16S rRNA sequences were affiliated with classes *Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria*, as well as with phyla *Actinobacteria*, *Acidobacteria*, and *Clostridia* (Fig. 4), indicating that phylogenetically diverse bacteria metabolized xylose and glucose in the moderately acidic fen soil slurries.

T-RFs detected in RNA density gradients from the [^{13}C]xylose experiment were assigned to the cloned 16S rRNA genes either from fraction 4 or from direct 16S rRNA gene analysis (Table 1 and data not shown). T-RFs indicative of *Acidobacteriaceae* (96 and 116 bp), *Acidothermaceae* (140 bp), *Sphingomonadaceae* and *Aeromonadaceae* (both 150 bp), *Pseudomonadaceae* (492 bp), and *Enterobacteriaceae* and *Aeromonadaceae* (both 496 bp), as well as from *Clostridiaceae* (520 bp) organisms, were identified in the "heavy" fraction at the early time point (Fig. 3A1). These T-RFs had a lower relative abundance at the late sampling time point (Fig. 3A2). Relative abundance levels of T-RFs indicative of *Syntrophobacteraceae*, *Rhodobiaceae* (both 166 bp), and *Acidobacteriaceae* (266 bp) species were higher at day 13 than at day 4 (Fig. 3A1 and A2). The 440-bp T-RF indicative of *Rhodospirillaceae* and *Chlorobiaceae* organisms occurred only at day 13 (Fig. 3A2). T-RFs detected in RNA density gradients from the [^{13}C]glucose experiment were assigned to families *Sphingomonadaceae* and *Aeromonadaceae* (both 150 bp) and to the class *Clostridia* (520 bp). Relative abundance levels of such T-RFs were already higher in the "heavy" fraction than in the "light" fraction after 2 days (Fig.

FIG. 4. Consensus phylogenetic tree of bacterial 16S rRNA sequences obtained in this study (bold). Accession numbers are provided after sequence identifiers, and the relative abundance levels (percentages) of OTUs in gene libraries are given in parentheses. Codes: X4 and X13, sequences retrieved from "heavy" fractions at days 4 and 13, respectively, of the [^{13}C]xylose experiment; G2 and G13, sequences retrieved from "heavy" fractions at days 2 and 13, respectively, of the [^{13}C]glucose experiment; B, sequences obtained by direct 16S rRNA analysis of fen soil; n.d., not detected. Scale bar represents 10% sequence dissimilarity. The outgroup is *Thermocrinis albus* (accession no. AJ278895).

TABLE 1. Phylogenetic affiliations of representative 16S rRNA gene sequences and corresponding T-RFs[a]

Organism affiliation[b]		Sequence,[c] accession no. (no. of sequences)	Organism with the closest related sequence (accession no.)	Similarity (%)	Closest cultured relative (accession no.)	Similarity (%)	T-RF length (bp)
Class	Family						
Alphaproteobacteria	*Sphingomonadaceae*	X4A5, AM712585 (2)	*Sphingomonas* sp. (AF385529)	92	*Sphingomonas echinoides* (AJ012461)	95	150
	Rhodobiaceae	B2-16, AM773982 (3)	Uncultured bacterium (AJ583166)	95	*Roseospirillum parvum* (AJ011919)	90	166
	Rhodospirillaceae	B2-8, AM773974	Uncultured bacterium (AJ295647)	96	*Skermanella parooensis* (X90760)	90	440
Deltaproteobacteria	*Syntrophobacteraceae*	B1-92, AM773964	Uncultured bacterium (AJ306805)	97	*Syntrophobacter wolinii* (X70905)	96	166
Gammaproteobacteria	*Enterobacteriaceae*	X13A3, AM712574 (2)	*Shigella sonnei* (X80726)	>99	*Shigella flexneri* (X96963)	>99	496
	Aeromonadaceae	G2A6, AM712567	Bacterium H4 (AY345546)	99	*Tolumonas auensis* (X92889)	98	496
	Aeromonadaceae	G2A13, AM712565	Uncultured bacterium (AJ318149)	92	*Tolumonas auensis* (X92889)	93	150
	Pseudomonadaceae	X13A11, AM712542 (2)	*Pseudomonas* sp. (AF098466)	100	*Pseudomonas putida* (AJ308312)	99	492
Actinobacteria	*Acidothermaceae*	X4A11, AM712581	Uncultured actinomycete (Z73383)	94	*Acidothermus cellulolyticus* (X70635)	90	140
Acidobacteria	*Acidobacteriaceae*	X4A10, AM712580 (16)	Uncultured bacterium (AY043842)	93	*Acidobacterium capsulatum* (D26171)	92	96
	Acidobacteriaceae	B1-69, AM773947	Uncultured bacterium (AJ000986)	94	*Acidobacterium capsulatum* (D26171)	93	116
	Acidobacteriaceae	B2-33, AM773995 (2)	Uncultured bacterium (AF523892)	97	*Acidobacterium capsulatum* (D26171)	94	266
Sphingobacteria	*Flexibacteraceae*	B1-26, AM773619	Uncultured bacterium (AY218620)	85	*Flexibacter canadensis* (M62793)	86	166
Clostridia	*Clostridiaceae*	G13A6, AM712563 (5)	Uncultured bacterium (AY212768)	>99	*Clostridium puniceum* (X71857)	>99	520
	Acidaminococcaceae	G13A4, AM712561 (2)	Uncultured bacterium (AY524568)	95	*Acetonema longum* (M61919)	89	290
Chlorobia	*Chlorobiaceae*	B1-60, AM773938	Uncultured bacterium (AF445728)	89	*Chlorobium limicola* (Y101640)	78	440

[a] The phylogenetic affiliations of representative 16S rRNA gene sequences and the corresponding T-RFs are those of organisms shown in Fig. 3 and 4.
[b] Sequence length, ~700 to 800 bp.
[c] For an explanation of sequence identifiers, see the legend to Fig. 4.

3B1), indicating that these community members had consumed [^{13}C]glucose during the initial incubation period. The relative abundance levels of these T-RFs decreased during the incubation period (Fig. 3B2). The relative abundance levels of T-RFs indicative of *Enterobacteriaceae* and *Aeromonadaceae* species (both 496 bp) were higher at day 13 than at day 2. The T-RF indicative of members of the family *Acidaminococcaceae* (290 bp) occurred only at day 13 (Fig. 3B2).

An archaeal 16S rRNA gene library was generated from RNA preparations after 13 days of incubation, to identify po-

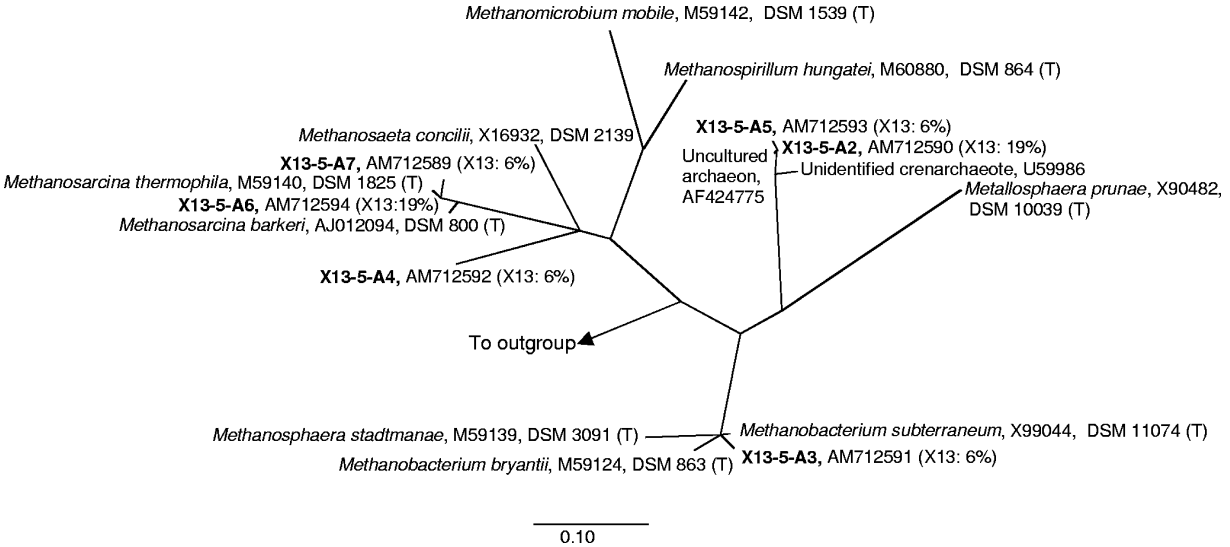

FIG. 5. Consensus phylogenetic tree of archaeal 16S rRNA sequences (bold) obtained at day 13 of the [^{13}C]xylose experiment. Accession numbers are provided after sequence identifiers, and relative abundance levels (percentages) of OTUs in gene library X13 are given in parentheses. Scale bar represents 10% sequence dissimilarity. The outgroup is *Thermoplasma acidophilum* (accession no. M20822).

tentially active *Archaea*. Sixteen clones were screened by RFLP, and six representative clones were sequenced (Fig. 5). Archaeal 16S rRNA sequences clustered with the 16S rRNA sequences of *Methanobacterium subterraneum* (accession no. X99044; 95% sequence similarity), *Methanosarcina thermophila* (accession no. M59140; 98% sequence similarity), and *Methanosarcina barkeri* (accession no. AJ012094; 97% sequence similarity) and the phylum *Crenarchaeota* group I.3 (94 to 99% sequence similarity [Fig. 5]).

DISCUSSION

Most of the phylogenetic groups detected by RNA-based stable isotope probing (RNA-SIP) in this study (Table 1 and Fig. 3 and 4) are widespread in peatlands (e.g., 8, 21, 27). Species of *Acidobacteria* accounted for 23%, *Deltaproteobacteria* for 17%, and *Alphaproteobacteria* for 16% of the 16S rRNA gene sequences detected in soil from the Ljubljana Marsh (Slovenia) and are hypothesized to represent the most abundant phylogenetic groups there (27). The main groups of 16S rRNA gene sequences retrieved from an acidic sphagnum peat bog (pH 3.9 to 4.5) in western Siberia were affiliated with organisms of *Acidobacteria*, *Alphaproteobacteria*, *Verrucomicrobia*, *Actinobacteria*, and *Deltaproteobacteria* (8). Thus, *Acidobacteria* species are readily detectable and, hence, potentially abundant in peatlands. Indeed, many sequences obtained in the present study from "heavy" RNA during the SIP were affiliated with *Acidobacteria* (Table 1, Fig. 4). However, little is known about their function in the flow of carbon in wetlands. The nearest cultured relative (based on 16S rRNA gene similarities) of the *Acidobacteria* sequences detected was *Acidobacterium capsulatum*, an organism that can use xylose and glucose as the sole carbon sources at moderately acidic pH levels (3–6) but that grows only aerobically (23). The relative abundance of a 266-bp T-RF indicative of *Acidobacterium* sp. increased in "heavy" RNA during the incubation of fen material with [^{13}C]xylose (Fig. 3A1 and A2). Such findings suggest that certain *Acidobacteriaceae* species survived for a minimum of 13 days and/or utilized xylose under anoxic conditions.

A novel facultative, moderately acid-tolerant (growth at pH 4 to 7) species within the family *Rhodospirillaceae* was isolated from a mesotrophic Siberian fen (45). Sequences affiliated with *Rhodospirillaceae* were retrieved from the "heavy" fraction of RNA in the [^{13}C]glucose experiment. A T-RF indicative of such organisms (440 bp; Table 1, Fig. 3 and 4) had higher relative abundance levels after prolonged incubation in both the [^{13}C]glucose and [^{13}C]xylose experiments.

The relative abundance of *Actinomycetes*-related T-RFs (140 bp; Fig. 3 and 4) is consistent with the occurrence of anaerobic and facultative *Actinomycetes* species (15). Other recognized families that are capable of hexose and/or pentose fermentation include *Acidaminococcaceae* (obligate anaerobes), *Aeromonadaceae* (facultative aerobes), *Clostridiaceae* (obligate anaerobes), and *Enterobacteriaceae* (facultative aerobes) (4, 9, 15). Sequences indicative of these organisms were detected by RNA-SIP (Fig. 3 and 4). Some members of the family *Acidaminococcaceae* are capable of glucose fermentation and production of acetate (10, 22). For example, the acetogen *Acetonema longum* converts glucose stoichiometrically to acetate via the acetyl-coenzyme A pathway (10, 22). 16S rRNA sequences retrieved from the "heavy" RNA of the [^{13}C]glucose experiment affiliated with *A. longum* and T-RFs indicative of such sequences had higher relative abundance levels at the late time point (Fig. 3B1 and B2), indicating that members of the family *Acidaminococcaceae* are likely involved in the metabolism of glucose in anoxic fen slurries. No sequences affiliated with *A. longum* were retrieved from "heavy" RNA in the [^{13}C]xylose experiment (Fig. 3A1 and A2), an observation that agrees with the fact that *A. longum* is unable to use xylose (22).

Most of the 16S rRNA sequences retrieved from the "heavy" RNA of the [^{13}C]glucose and [^{13}C]xylose incubations were affiliated with the genus *Clostridium*. Species of this genus are capable of the production of acetate, butyrate, and H_2/CO_2 from glucose and xylose (e.g., see reference 9) and occur in acidic bogs (e.g., see reference 28), which is consistent with the fermentation products observed with monosaccharide-supplemented fen slurries (Fig. 1) and with the high relative abundance level of T-RFs indicative of *Clostridia* organisms in "heavy" RNA (520 bp; Fig. 3A1, A2, and B1). A significant drift of clostridial [^{12}C]RNA molecules into the "heavy" fractions is unlikely because clostridial species have a relatively low G+C content (9, 15).

Products indicative of mixed acidic and butyrate fermentations (i.e., formate, acetate, butyrate, CO_2, and H_2) were formed in xylose- and glucose-supplemented fen slurries. *Shigella* and *Tolumonas* species are known to ferment sugars (4). *Shigella*- and *Tolumonas*-related RNAs were labeled with [^{13}C]xylose and [^{13}C]glucose (Fig. 3 and 4), indicating that these monosaccharides were degraded by these organisms in anoxic fen slurries. Sequences closely related to those of *Shigella sonnei* and *Tolumonas auensis* were detected (Table 1). *S. sonnei* is a facultative, acid-resistant member of the family *Enterobacteriaceae* that utilizes mixed acid fermentation to convert monosaccharides to formate, acetate, and ethanol without the production of gases (4). Formate, acetate, and ethanol are also fermentation products of *T. auensis*, which is a facultative member of the family *Aeromonadaceae* (4).

T-RFs of 150 bp, indicative of the family *Sphingomonadaceae*, had a high relative level of abundance in the [^{13}C]RNA-dominated fractions of the xylose and glucose experiments at the early time points (Fig. 3 and 4). The nearest cultured relative (based on 16S rRNA gene similarities) of the detected *Sphingomonadaceae* sequences was *Sphingomonas echinoides* (95% similarity; Table 1), indicating that a potentially new genus of the family *Sphingomonadaceae* was detected. Species of *Sphingomonas* grow on monosaccharides (e.g., xylose and glucose) under oxic conditions (5), and one genus within the family *Sphingomonadaceae* (*Zymomonas* sp.) ferments monosaccharides (e.g., glucose [5]). Thus, it is hypothesized that new species of this family assimilated xylose- and glucose-derived carbon in anoxic fen slurries.

The acidic fen slurries produced amounts of CH_4 that were similar to those of another acidic peat (0.4 to 1.7 μmol g_{dw}^{-1} day^{-1}) from the same region in the Fichtelgebirge (18). The transient production and subsequent consumption of formate were concomitant to the accumulation of CH_4 (Fig. 1), suggesting that formate was a precursor of CH_4. These results indicate that fermentation processes and methanogenesis were active in anoxic fen slurries and were trophically linked. RNA of *Methanobacteriaceae*, *Methanosarcinaceae*, and *Crenarcha-*

eota organisms was detected at day 13 in the [^{13}C]xylose experiment, indicating that the anoxic fen slurries harbor active acid-tolerant methanogens and *Crenarchaeota* species. DNA but not RNA of *Methanobacteriaceae* or *Methanosarcinaceae* species has also been detected in other acidic wetlands (e.g., 6, 18, 25). *Methanosarcinaceae* species reduce acetate to CH_4 (3), indicating that a net consumption of acetate might have occurred during the [^{13}C]xylose experiment. Knowledge about the physiology of mesophilic *Crenarchaeota* species is incomplete, mainly because only a single mesophilic crenarchaeal isolate (i.e., an aerobic, autotrophic ammonia oxidizer) has been cultivated (24). Detection of the phylum *Crenarchaeota* group 1.3 RNA in fen slurries after 13 days of anoxic incubation (Fig. 5) is consistent with the widespread occurrence of this crenarchaeal group in anoxic habitats (e.g., flooded soils and sediments) (41) and suggests that members of this crenarchaeal group are capable of anaerobiosis.

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