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Metatranscriptomic Evidence for Direct Interspecies Electron Transfer between *Geobacter* and *Methanothrix* Species in Methanogenic Rice Paddy Soils

Dawn E. Holmes,[a,b] Pravin M. Shrestha,[a,c] David J. F. Walker,[a] Yan Dang,[a] Kelly P. Nevin,[a] Trevor L. Woodard,[a] Derek R. Lovley[a]

Department of Microbiology, University of Massachusetts Amherst, Amherst, Massachusetts, USA[a]; Department of Physical and Biological Sciences, Western New England University, Springfield, Massachusetts, USA[b]; Energy Biosciences Institute, University of California Berkeley, Berkeley, California, USA[c]

ABSTRACT The possibility that *Methanothrix* (formerly *Methanosaeta*) and *Geobacter* species cooperate via direct interspecies electron transfer (DIET) in terrestrial methanogenic environments was investigated in rice paddy soils. Genes with high sequence similarity to the gene for the PilA pilin monomer of the electrically conductive pili (e-pili) of *Geobacter sulfurreducens* accounted for over half of the PilA gene sequences in metagenomic libraries and 42% of the mRNA transcripts in RNA sequencing (RNA-seq) libraries. This abundance of e-pilin genes and transcripts is significant because e-pili can serve as conduits for DIET. Most of the e-pilin genes and transcripts were affiliated with *Geobacter* species, but sequences most closely related to putative e-pilin genes from genera such as *Desulfobacterium*, *Deferribacter*, *Geoalkalibacter*, and *Desulfobacula*, were also detected. Approximately 17% of all metagenomic and metatranscriptomic bacterial sequences clustered with *Geobacter* species, and the finding that *Geobacter* spp. were actively transcribing growth-related genes indicated that they were metabolically active in the soils. Genes coding for e-pilin were among the most highly transcribed *Geobacter* genes. In addition, homologs of genes encoding OmcS, a *c*-type cytochrome associated with the e-pili of *G. sulfurreducens* and required for DIET, were also highly expressed in the soils. *Methanothrix* species in the soils highly expressed genes for enzymes involved in the reduction of carbon dioxide to methane. DIET is the only electron donor known to support CO_2 reduction in *Methanothrix*. Thus, these results are consistent with a model in which *Geobacter* species were providing electrons to *Methanothrix* species for methane production through electrical connections of e-pili.

IMPORTANCE *Methanothrix* species are some of the most important microbial contributors to global methane production, but surprisingly little is known about their physiology and ecology. The possibility that DIET is a source of electrons for *Methanothrix* in methanogenic rice paddy soils is important because it demonstrates that the contribution that *Methanothrix* makes to methane production in terrestrial environments may extend beyond the conversion of acetate to methane. Furthermore, defined coculture studies have suggested that when *Methanothrix* species receive some of their energy from DIET, they grow faster than when acetate is their sole energy source. Thus, *Methanothrix* growth and metabolism in methanogenic soils may be faster and more robust than generally considered. The results also suggest that the reason that *Geobacter* species are repeatedly found to be among the most metabolically active microorganisms in methanogenic soils is that they grow syntrophically in cooperation with *Methanothrix* spp., and possibly other methanogens, via DIET.

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Address correspondence to Dawn E. Holmes, dholmes@wne.edu.

D.E.H. and P.M.S. contributed equally to this work.

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Methanothrix (formerly *Methanosaeta*) species are thought to be among the most prodigious methane-producing microorganisms on Earth. This is because of the abundance of *Methanothrix* in many terrestrial soils and sediments that are important sources of atmospheric methane, and the ability of *Methanothrix* to very effectively utilize acetate, the precursor for ca. two-thirds of the methane produced in these environments (1, 2).

Methanothrix spp. also have the potential to produce methane from carbon dioxide reduction. The genomes of *Methanothrix* species cultures (1, 3) and the metagenomes of *Methanothrix* spp. in anaerobic digesters (4) contain a complete set of genes coding for the required enzymes. It is unlikely that hydrogen or formate is the electron donor for carbon dioxide reduction, because none of the four *Methanothrix* spp. available in culture can use hydrogen or formate as electron donors (2, 5–8), and *Methanothrix* species lack genes coding for membrane-bound hydrogenase complexes known to be required for hydrogen uptake into the cell by other methanogen species (1, 3, 9). However, *Methanothrix harundinacea* can reduce carbon dioxide to methane with electrons received from direct interspecies electron transfer (DIET), and DIET-derived electrons can serve as the sole energy source to support growth (4, 10). High transcript abundance for genes involved in the reduction of carbon dioxide to methane is likely to indicate that *Methanothrix* spp. are participating in DIET (4).

Although there is substantial evidence that *Methanothrix* spp. can reduce carbon dioxide to methane with electrons derived from DIET in anaerobic brewery waste digesters (4, 11, 12), it is unknown whether DIET by *Methanothrix* spp. also contributes to carbon dioxide reduction in anaerobic soils and sediments. There are substantial differences between anaerobic digesters and methanogenic soils and sediments that might influence *Methanothrix* participation in DIET. For example, brewery wastes have a relatively simple organic composition, with ethanol as the major substrate (12), and organics are provided at high rates, supporting rapid metabolic fluxes. In contrast, the primary organic substrates in freshwater methanogenic environments are composed of a much more complex assemblage of polymeric fermentable material that is only slowly degraded, and ethanol is unlikely to be an important metabolic intermediate in these environments (13). Evidence consistent with the concept of *Methanothrix* spp. receiving electrons from DIET in anaerobic soils was high expression of the *mer* gene, which encodes a key enzyme in the carbon dioxide reduction pathway, by *Methanothrix* spp. in incubations of arctic peat soils (14).

The only bacteria known to donate electrons to methanogens via DIET are *Geobacter* species (4, 10, 15). *Geobacter* species require electrically conductive pili (e-pili) and outer membrane *c*-type cytochromes to participate in DIET (4, 15–18) unless DIET is artificially stimulated with the addition of conductive materials, such as granular activated carbon, carbon cloth, or biochar (19–21). Magnetite also appears to promote DIET between *Geobacter* and *Methanosarcina* species (22), but it is likely that e-pili are still required (23). Data consistent with *Geobacter* species playing a role in DIET in terrestrial methanogenic environments were the finding that *Geobacter* spp. were among the most metabolically active bacteria in methanogenic rice paddy soils (24–26). However, not all *Geobacter* species produce the e-pili required for DIET (27, 28) and thus, more direct analysis of the potential for e-pilin expression is required.

Therefore, in order to further evaluate whether *Methanothrix* and *Geobacter* species might be participating in DIET in terrestrial methanogenic environments, we analyzed the metagenome and metatranscriptome from bacteria and archaea present in methanogenic rice paddy soils. Rice paddies were selected as the model terrestrial environment because methane generated by rice paddies accounts for ~10% of global atmospheric methane production (29, 30). The results suggest that *Methanothrix* spp.

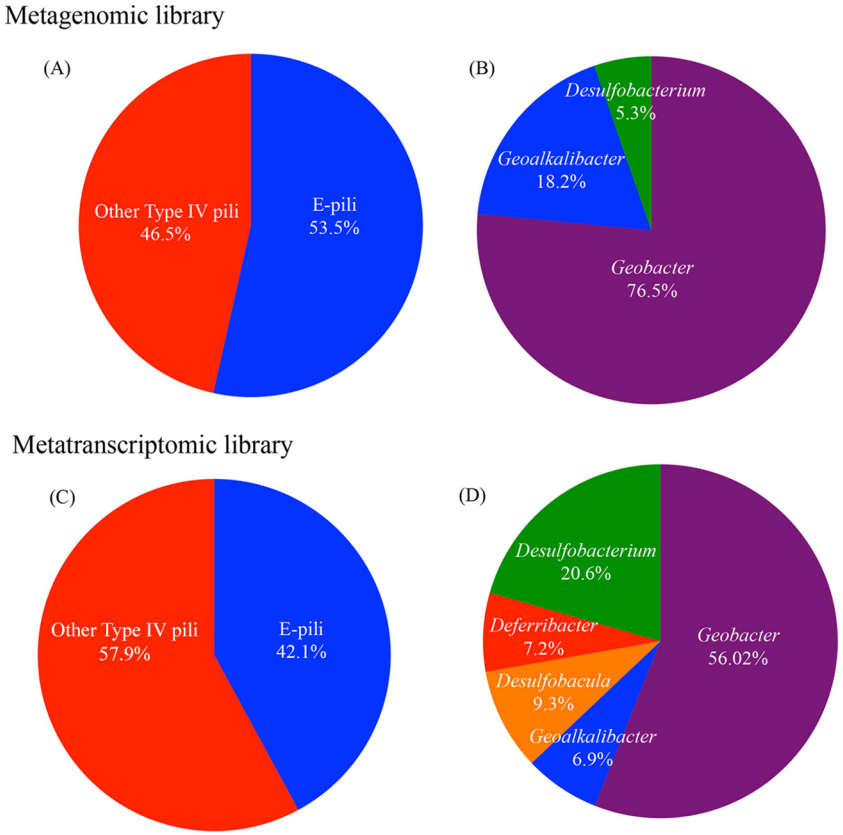

FIG 1 (A and C) Proportion of total type IV pilin reads that clustered with long type IV pili or e-pili in metagenomic (A) and metatranscriptomic (C) libraries. (B and D) Taxonomic assignment of e-pilin reads in metagenomic (B) and metatranscriptomic (D) libraries. Results represent an average of values obtained from nhmmer, BLASTx, and Bowtie2 alignments of QC-filtered merged reads.

were receiving electrons for the reduction of carbon dioxide via DIET from *Geobacter*, and possibly other microorganisms, that were actively expressing e-pilin genes.

RESULTS AND DISCUSSION

Metabolically active microorganisms expressing e-pilin genes. Analysis of quality control (QC)-filtered and merged rice paddy metagenomic and metatranscriptomic libraries showed that about half of the total pilin reads were phylogenetically related to electrically conductive *Geobacter* e-pilin gene sequences. BLASTx and Bowtie2 analyses showed that ca. 53.5% of the metagenomic reads clustered with putative e-pili (Fig. 1A and Table 1). Analysis of metatranscriptomic libraries with BLASTx and Bowtie2 algorithms also demonstrated that putative e-pili accounted for ~42% of the type IV *pilA* genes that were being actively transcribed in the rice paddy sediments (Fig. 1 and Table 1).

Most of the e-pilin genes and gene transcripts clustered within the genus *Geobacter* (Fig. 1), but sequences most closely related to e-pili from genera such as *Desulfobacterium*, *Deferribacter*, *Geoalkalibacter*, and *Desulfobacula*, were also detected (Fig. 1). The ability of microorganisms from genera outside *Geobacter* to participate in DIET has yet to be evaluated, but extracellular electron transfer has been observed in all of these genera, with the exception of *Desulfobacula*. For example, *Desulfobacterium autotrophicum* (31), several species of *Deferribacter* (32–34), and *Geoalkalibacter* (35–37) are all capable of Fe(III) reduction. In addition, *Deferribacter* spp. were enriched from sediments on current-harvesting anodes (38, 39), and *Geoalkalibacter* spp. produce high current densities in microbial fuel cells (40, 41). The capacity to produce high current densities and to participate in DIET appear to be linked in *Geobacter* species (28).

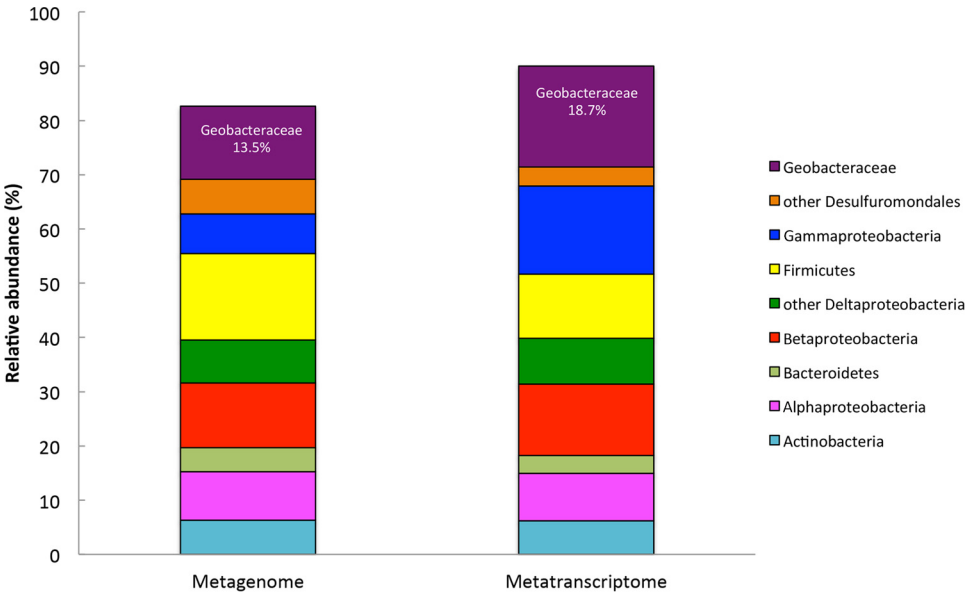

FIG 2 Phylogenetic distribution of QC-filtered merged bacterial reads (~150 bp) in metagenomic and metatranscriptomic libraries constructed with DNA or mRNA extracted from methanogenic rice paddy soils. These values represent the mean of results obtained from four different software programs (Tables S3A and B): GenomePeek, MG-RAST, BLASTx, and Kraken.

Therefore, it seems feasible that some of these organisms may also be participating in DIET in the rice paddy soil.

Analysis of the metagenomic and metatranscriptomic libraries (Fig. 2) indicated that of the bacteria harboring e-pilin sequences, *Geobacter* spp. were by far the most abundant. *Geobacteraceae* species accounted for 19% and 14% of the bacterial sequences in the metatranscriptomic and metagenomic libraries, respectively. In order to determine whether *Geobacter* spp. were metabolically active in the rice paddy soil and to further evaluate the potential significance of e-pili in *Geobacter* respiration, merged metatranscriptomic reads were mapped against the *G. sulfurreducens* genome, and $\log_2$ reads per kilobase per million (RPKM) values were determined (see Table S6 in the supplemental material).

Citrate synthase has been shown to be an indicator of *Geobacter* metabolism in the subsurface (42–44). Citrate synthase (*gltA*) was one of the most highly expressed *Geobacter* genes ($\log_2$ RPKM, 8.4) in the rice paddy soils, well above the median $\log_2$ RPKM value of 5.7 (Table S6). Other genes that are indicative of *Geobacter* growth in the subsurface, such as genes coding for ribosomal proteins, like *rpsC*, which encodes 30S ribosomal protein S3 (45), were also being actively transcribed by *Geobacter* spp. in the subsurface ($\log_2$ RPKM, 6.7) (Table S6). In addition to *rpsC*, many other growth-related genes were being significantly transcribed by *Geobacter* species: 103 genes were involved in protein synthesis, 14 genes were involved in transcription, 15 genes were involved in DNA replication, and 23 genes were involved in cell division.

The e-pilin gene ($\log_2$ RPKM, 9.1) and genes for all of the e-pilin accessory proteins (*pilA-C*, *pilB*, *pilC*, *pilD*, *pilE*, *pilM*, *pilN*, *pilO*, *pilQ*, *pilR*, *pilS*, *pilT*, *pilV*, *pilW*, and *pilY*) were highly expressed by *Geobacter* species. Transcript abundance was also high ($\log_2$ RPKM, 7.05) for genes that mapped to the *G. sulfurreducens* gene for the multiheme *c*-type cytochrome OmcS. OmcS is localized along the e-pili of *G. sulfurreducens* (46). A mutation associated with increased rates of DIET in defined cocultures greatly increases OmcS expression, and OmcS-deficient mutants are incapable of DIET (16). Thus, the high expression of genes for OmcS and e-pili are consistent with the hypothesis that *Geobacter* spp. in the rice paddy soils are participating in DIET.

Methanogens involved in DIET. Consistent with the finding that the rice paddy soil incubations were actively producing methane (~0.3 mmol methane per liter of soil per

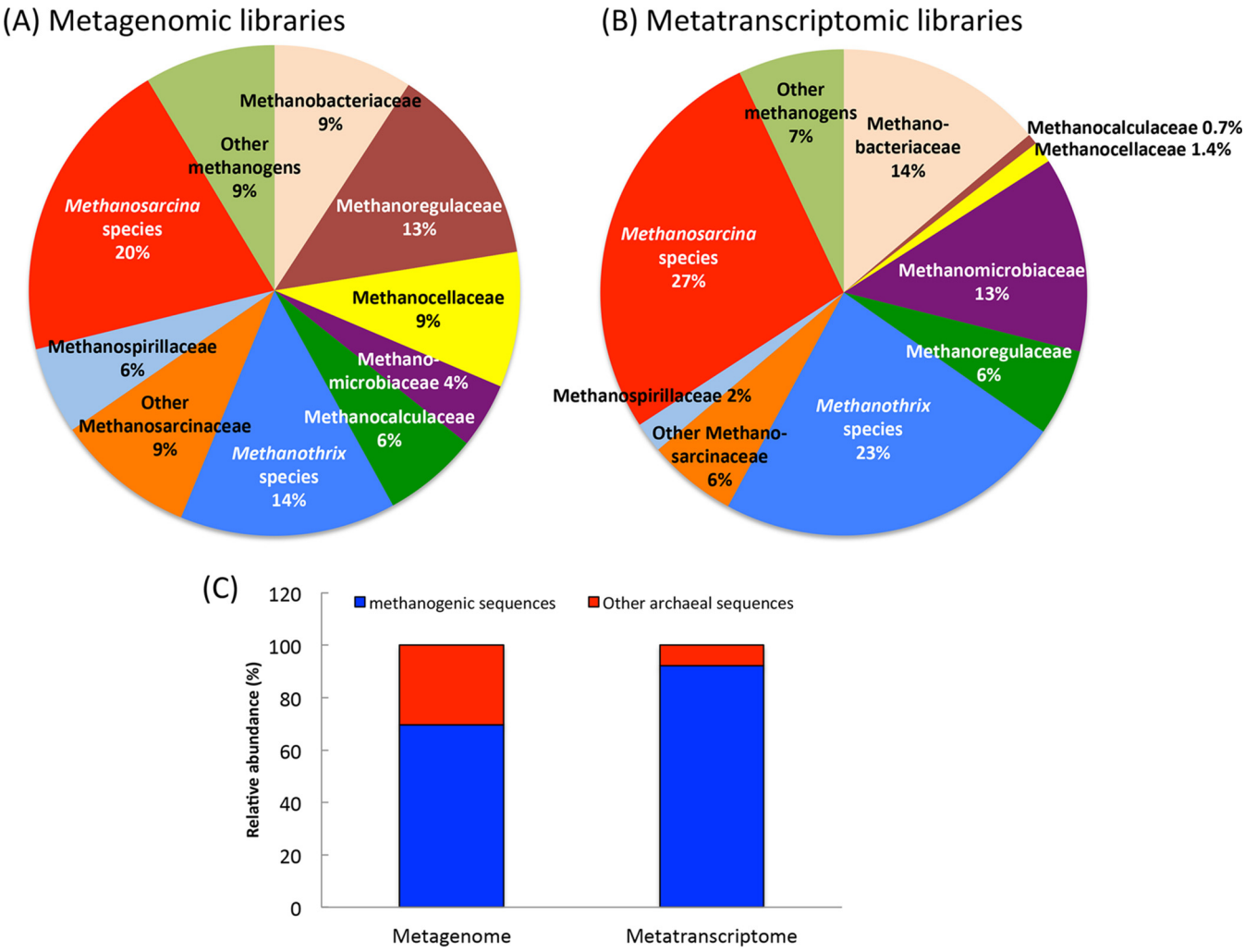

FIG 3 (A and B) Phylogenetic distribution of QC-filtered merged methanogenic reads (~150 bp) in metagenomic (A) and metatranscriptomic (B) libraries constructed with DNA or mRNA extracted from methanogenic rice paddy soils. (C) Relative abundance of methanogenic and nonmethanogenic archaeal sequences in the metagenomic and metatranscriptomic libraries. These values represent the mean of results obtained from four different software programs (Tables S3A and B): GenomePeek, MG-RAST, BLASTx, and Kraken.

day), methanogens were the predominant archaea and accounted for 70% and 92% of the archaeal sequences detected in metagenomic and metatranscriptomic libraries, respectively (Fig. 3). Similar to other studies of methanogenic communities associated with flooded rice paddy soils (47, 48), *Methanothrix* and *Methanosarcina* spp. were the most abundant methanogens and together accounted for almost half of the sequences. These results are significant because these are the only genera shown thus far to participate in DIET (4, 10, 15).

To date, no molecular strategy for definitively determining whether *Methanosarcina* species are engaged in DIET has been developed, because some *Methanosarcina* species can also use hydrogen as an electron donor for carbon dioxide reduction (49). However, as detailed in the introduction, high expression of genes that encode enzymes that are exclusively part of the pathway for the reduction of carbon dioxide to methane is considered to be diagnostic for DIET in *Methanothrix* species (4).

In order to further evaluate whether *Methanothrix* species were metabolically active and were transcribing carbon dioxide reduction genes, metatranscriptomic reads were mapped against the *Methanothrix concilii* genome, and $\log_2$ RPKM values were determined (Table S7). Similar to *Geobacter* species, *Methanothrix* species were expressing a number of genes involved in protein translation, transcription, cell division, and DNA replication, demonstrating that they were actively growing in the rice paddy soils. As

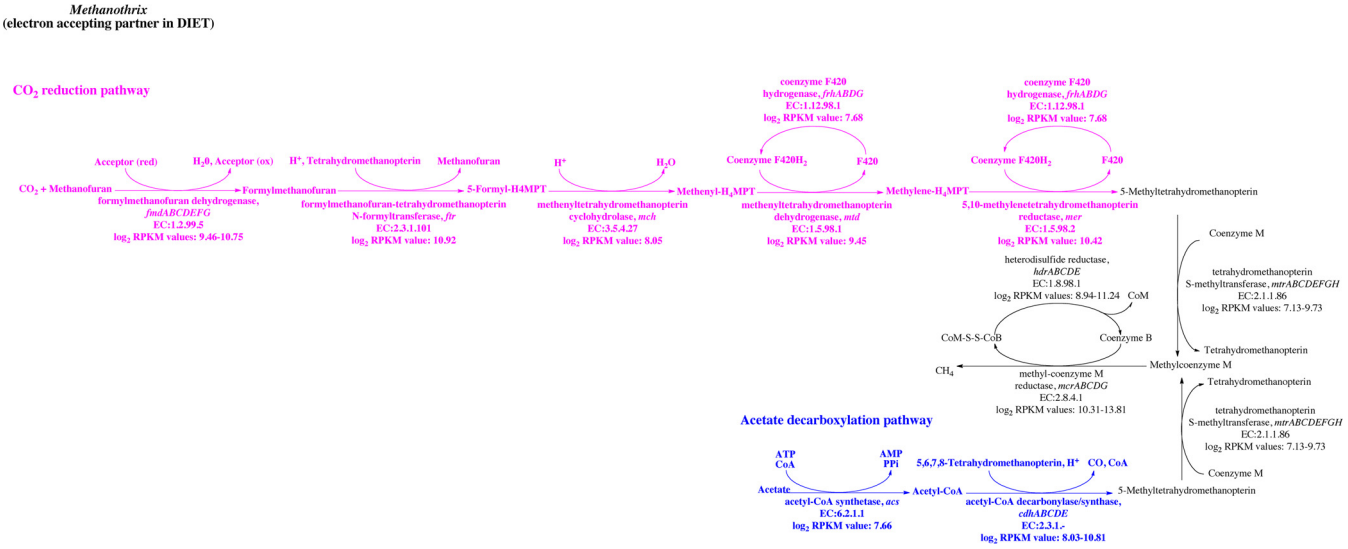

FIG 4 Enzymes involved in the carbon reduction or acetate decarboxylation pathways for methanogenesis and the $\log_2$ RPKM values from these genes when RNA-seq libraries were mapped against the *M. concilii* genome. Several proteins from these pathways are composed of multiple subunits, and the range of $\log_2$ values reflects the range of expression for all of the subunits. Further details of gene expression are provided in Table S7.

expected, *Methanothrix* species in the rice paddy soils highly expressed genes coding for enzymes involved in the conversion of acetate to methane, as well as genes coding for enzymes common to both the pathway for acetate conversion to methane and carbon dioxide reduction to methane (Fig. 4 and Table S7). However, genes coding for enzymes specifically associated with the reduction of carbon dioxide to methane were also highly expressed. In fact, $\log_2$ RPKM values from many of the genes coding for subunits from formylmethanofuran dehydrogenase (*fmd*), the first enzyme from the CO_2 reduction pathway, were higher than values from the first acetate metabolism gene, acetyl-coenzyme A (acetyl-CoA) synthetase (*acs*) (Fig. 4 and Table S7). *Methanothrix* spp. also significantly expressed all of the other genes coding for proteins from the carbon dioxide reduction pathway (*ftr*, *mch*, *mtd*, *mer*, and *frh*) in the paddy soils. These results suggest that the *Methanothrix* spp. in the rice paddy soils were actively involved in the reduction of carbon dioxide to methane, which is the methanogenic pathway that would need to be utilized by *Methanothrix* spp. accepting electrons from *Geobacter* during DIET.

Implications. The results presented here provide the first evidence, to our knowledge, that DIET may be operative in methanogenic environments other than brewery digesters. The transcriptomic data suggest that *Methanothrix* spp. were actively reducing carbon dioxide to methane, and electrons derived from DIET are the only known electron donors for carbon dioxide reduction in *Methanothrix* (4). *Geobacter* species, which are capable of donating electrons to *Methanothrix* spp. (4, 10), were also abundant, metabolically active, and transcribed genes coding for e-pilin.

Methanothrix spp. are already thought to be major contributors to global methane production because they are often the most abundant methanogens capable of utilizing acetate in many methanogenic soils and sediments (1, 26, 50–55). The finding that *Methanothrix* spp. may also produce methane from carbon dioxide by participating in DIET suggests that *Methanothrix* may play an even more important role in global methane production than previously considered.

The relative contribution of *Methanothrix* spp. versus other methanogens in the rice paddy soils to carbon dioxide reduction to methane cannot be determined from omics data alone. The metatranscriptomic data suggested that methanogens from other genera, such as *Methanobacterium*, *Methanoculleus*, and *Methanoregula*, which utilize hydrogen as an electron donor for the reduction of carbon dioxide, were also metabolically active in the rice paddy soils. Elucidation of the relative electron flow to carbon

dioxide reduction via hydrogen as an intermediary electron carrier and DIET will require much more extensive investigation with novel approaches that can quantify rates of electron flux.

The possibility that *Methanothrix* is receiving electrons from DIET is an important consideration when attempting to model the growth and activity of *Methanothrix* in soils and sediments. The growth of *Methanothrix harundinacea* receiving electrons via DIET as well as acetate in defined coculture with *G. metallireducens* is much better than the growth of *M. harundinacea* on acetate alone, suggesting that low-potential electrons that support carbon dioxide reduction enhance growth (4). Therefore, the slow growth attributed to *Methanothrix* spp. based on laboratory studies with acetate-grown cultures will not be representative for modeling the growth of *Methanothrix* spp. in terrestrial environments when *Methanothrix* spp. participate in DIET. More in-depth characterization of the physiology of *Methanothrix* growing via DIET is warranted.

MATERIALS AND METHODS

Soil incubations. Rice paddy soil incubations were conducted with soils collected from the Akaogi Farm in Westminster West, VT (http://www.ricenortheasternus.org). Soil samples were placed in sealed mason jars that were completely filled, sealed for transport to the laboratory, and stored at 16°C until use. Surface water was pumped into 5-gallon carboys with a peristaltic pump and stored at 4°C.

A soil slurry with approximately 6 kg of soil mixed with 600 ml of surface water at a ratio of 10:1 was prepared. Six replicate incubations were established in which 500 ml of this slurry and 100 ml of surface water were added to 1-liter bottles in an anaerobic chamber under an N_2 atmosphere. No organic or inorganic supplements were added to the incubations. The bottles were sealed with butyl rubber stoppers and incubated in the dark at 25°C for 25 days. When molecular samples were taken from the soil incubations, most of the Fe(III) (92% $\pm$ 7%) had been reduced, sulfate concentrations were $<$10 μM, and ca. 0.3 mmol of methane per liter of soil per day was produced.

Analytical methods. Fe(II) formation in the soil incubations was monitored over time with a ferrozine assay in a split-beam dual-detector spectrophotometer (Spectronic Genesys 2; Thermo Electron Corp., Mountain View, CA) at an absorbance of 562 nm after a 1-h extraction with 0.5 N HCl (56). The remaining Fe(III) was then converted to Fe(II) with the addition of 0.25 M hydroxylamine, and after an additional hour, Fe(II) was again measured with the ferrozine assay.

Sulfate reduction was monitored with an ion chromatograph (ICS-2100; Dionex, CA) equipped with an AS18 column under isocratic elution with 32 mM KOH as the eluent (57). Methane in the headspace of soil incubations was measured by gas chromatography with a flame ionization detector (GC-8A; Shimadzu).

Extraction of nucleic acids. For nucleic acid extraction, 1.5 g of soil was removed from each of the six bottles, suspended in RNA*later*, and stored at 4°C overnight. The soil samples were then centrifuged at 20,000 $\times$ *g* for 1 min, the supernatants were discarded, and each 1.5-g sample was divided into three 2-ml tubes, making a total of 18 tubes. Total nucleic acids were then extracted as previously described (58–60). Briefly, the pellets were mixed with equal volumes of glass beads (0.17 to 0.18 mm in diameter) and resuspended in 700 μl of precooled TPM buffer (50 mM Tris-HCl [pH 5.0], 1.7% [wt/vol] polyvinylpyrrolidone, 20 mM $MgCl_2$). Subsequently, the mixture was shaken in a bead beater (FastPrep-24 instrument; MP Biomedicals) at 5.5 ms^{-1} for 45 s. Soil and cell debris were pelleted by centrifugation at 20,000 $\times$ *g* for 1 min at 4°C, and the supernatant was transferred to a fresh tube. The pellet was suspended in 700 μl of phenol lysis buffer (5 mM Tris-HCl [pH 5.0], 5 mM Na_2EDTA, 0.1% [wt/vol] sodium dodecyl sulfate, and 6% [vol/vol] water-saturated phenol), and the lysis procedure was repeated as described above. Supernatants from the two lysis treatments were pooled.

The pooled supernatant was extracted first with water-saturated phenol, second with phenol-chloroform-isoamyl alcohol (25:24:1), and third with chloroform-isoamyl alcohol (24:1), each time using 500 μl of extractant. The resulting aqueous phase was mixed with 0.1 volume of 3 M sodium acetate (pH 5.7) and 1 volume of isopropanol, incubated at $-$80°C for 1 h, and centrifuged for 30 min at 20,000 $\times$ *g* at 4°C. The nucleic acid pellet was washed with 70% ethanol, air dried, and resuspended in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Total nucleic acids from all three aliquots of soil sample were pooled (making a total of 6 tubes) and used for DNA and mRNA isolation.

DNA isolation and Illumina metagenomic library preparation. For DNA isolation, 2 μl of RNase (Sigma, USA) was added to 98-μl aliquots of total nucleic acids extracted from six different microcosms and incubated at 37°C for 1 h. Genomic DNA was then precipitated with 100% ethanol. These 6 aliquots were then condensed down to 2 tubes by combining three samples into one. In order to ensure sufficient sequencing coverage, these 2 DNA samples were used to construct two composite multiplex shotgun metagenomic libraries with the TruSeq DNA sample preparation kit (Illumina, USA), according to the manufacturer's directions. Briefly, 1 μg of DNA was fragmented with a nebulizer (Illumina, USA), fragment ends were then repaired, and 3′ ends were adenylated. Indexed paired-end adaptors were then ligated onto the fragments and enriched with a final 10-cycle PCR.

mRNA isolation and Illumina RNA-seq library preparation. For RNA isolation, 20 μl of DNase I buffer and 5 μl of DNase I (Ambion, USA) were added to six separate tubes containing 175 μl of total nucleic acids and incubated at 37°C for 1 h. Total RNA was further purified with the RNeasy minikit (Qiagen, USA). To ensure that RNA samples were free from DNA contamination, 1 μl of each RNA sample

was used as the template for PCR with primers targeting the 16S rRNA gene. The MICROBExpress kit (Ambion) was then used to isolate mRNA from total RNA, and the Experion RNA HiSens kit (Bio-Rad) was used to confirm mRNA enrichment prior to RNA sequencing (RNA-seq) library preparation. The MICROBExpress kit was used for mRNA enrichment because previous studies have shown that it was able to successfully enrich mRNA from rice paddy soil RNA extracts (59, 60).

The TruSeq RNA sample preparation kit (Illumina) was used to prepare RNA-seq libraries, according to the manufacturer's instructions. Briefly, 100 ng of mRNA was chemically fragmented and converted into single-stranded cDNA by random-hexamer priming. Double-stranded cDNA was then synthesized, overhangs were made blunt, and 3′ ends were adenylated. Adenylated products were then ligated with individual adapters containing unique hexameric barcodes and enriched with a final 10-cycle PCR.

Both DNA- and mRNA-based Illumina libraries were assessed and quantified with the Experion DNA HiSens kit (Bio-Rad, USA) prior to sequencing. In total, eight libraries (two from DNA and six from mRNA) containing unique barcodes and representing six different soil incubations were mixed in equimolar concentrations and used for hybridization in an Illumina Hi-Seq 2000 flow cell for paired-end sequencing. Sequencing was conducted by the Deep Sequencing Core Facility at the University of Massachusetts Medical School in Worchester, MA.

Assembly of Illumina reads. All of the raw sequencing and QC-filtered data were quality checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Initial raw nonfiltered forward and reverse metagenomic and metatranscriptomic sequencing libraries contained an average of 3,913,198 and 3,302,512 reads that were ~100 bp long (Tables S1A and B). Sequences from all of the libraries were trimmed and filtered with Trimmomatic (61), with the sliding window approach set to trim bases with quality scores lower than 3, strings of 3+Ns, and reads with a mean quality score lower than 20. Bases were also cut from the start and end of reads that fell below a threshold quality of 3, and any reads smaller than 50 bp were eliminated from the library. These parameters yielded an average of 3,318,562 and 2,222,209 quality reads per metagenomic and metatranscriptomic sequencing library, respectively (Tables S1A and B).

All paired-end reads were then merged with FLASH (62), resulting in 306,024 and 1,115,233 reads with an average read length of 154 bp for the metagenomic and metatranscriptomic libraries, respectively (Tables S1A and B). After merging the QC-filtered reads, SortMeRNA (63) was used to separate all rRNA reads from nonribosomal reads in both the metagenomic and metatranscriptomic libraries. Databases used by SortMeRNA to identify all rRNA sequences included Rfam 5.8S Eukarya, Rfam 5S Archaea/Bacteria, SILVA 16S Archaea, SILVA 16S Bacteria, SILVA 23S Bacteria, SILVA 18S Eukarya, and SILVA 28S Eukarya (64, 65).

Contigs were also assembled from QC-filtered and merged metatranscriptomic and nonmerged metagenomic nonribosomal reads with SeqMan NGen (DNAStar) and MegaHit softwares (66), with an overlapping base length of 50 bp and a minimum contig length of 150 bp. Assembly of these reads yielded ~17,191 and ~7,432 contigs with average sizes of 455 and 217 bp from the metagenomic and metatranscriptomic libraries, respectively (Table S2). Approximately 22% and 17% of the metagenomic and metatranscriptomic reads, respectively, could be assembled into these contigs. The software program Prodigal (67) was then used to identify open reading frames (ORFs) within these contigs, and this analysis yielded ~12,473 and ~4,070 ORFs, respectively.

Taxonomic classification of metagenomic and metatranscriptomic reads. The phylogenetic composition of the merged QC-filtered reads (~150 bp long) was determined with 4 different software programs: NCBI BLAST-2.2.31+ standalone software (68), GenomePeek software (69), Kraken (70), and MG-RAST (71). Prior to analysis with NCBI BLAST-2.2.31+ standalone software, protein-coding reads from each of the metagenomic and metatranscriptomic libraries were grouped into clusters with CD-HIT (72) using the BLOSUM62 scoring matrix and an E value of 0.001. All BLASTx output text files were then imported into MEGAN (73) for taxonomic classification.

Merged QC-filtered reads containing both ribosomal and nonribosomal genes and transcripts were also analyzed with GenomePeek (69), Kraken (70), and MG-RAST (71), using default parameters. The minikraken database was used for taxonomic assignment by the Kraken program. Relative abundances of various archaeal and bacterial taxa were determined separately by each of these programs (Tables S3A to D) and then averaged.

The taxonomic distribution of archaeal and bacterial sequences was also determined by analysis of ORFs identified by Prodigal after assembly of metagenomic and metatranscriptomic contigs. Protein sequences were compared to the NR database with the BLASTp algorithm (74). The results obtained from this analysis were similar to those from the analysis of unassembled reads (Table S4).

Analysis and identification of type IV pilin genes. The e-pili of *Geobacter* species described to date are composed of a PilA monomer (e-pilin) that is homologous to type IVa PilA proteins found in many bacteria (75, 76). However, the e-pilin genes are phylogenetically distinct from PilA monomers that are the subunits for poorly conductive pili of other microorganisms (75, 77). Therefore, it was possible to differentiate between e-pili and long type IV *pilA* genes by alignment with previously characterized pilin genes. First, the nhmmer search function (78) was used to scan QC-filtered merged metatranscriptomic and metagenomic reads for the presence of *pilA* genes by comparison to a *pilA* nucleotide Hidden Markov Model (HMM) database built by hmmbuild in HMMER 3 (79, 80). The HMM database used for this comparison was built from an alignment of 88 different type IV *pilA* nucleotide sequences, with 36 e-pilin genes and 52 long pilin genes (Table S5).

Potential pilin genes were also identified in QC-filtered merged metagenomic and metatranscriptomic libraries with the BLASTx algorithm by comparison to protein databases built from 88 PilA protein sequences with the makeblastdb function using NCBI BLAST-2.2.31+ standalone software (68). In

TABLE 1 Number and proportion of QC-filtered merged reads from metagenomic and metatranscriptomic libraries that mapped to various nucleic acid databases determined with Bowtie2 software

Read information	Mean	SEM
No. of reads		
Metatranscriptomic reads mapped to *Geobacter* species	86,988.17	6,732.52
Metatranscriptomic reads mapped to *Methanothrix* species	84,646.18	4,169.30
Metagenomic reads mapped to *Geobacter*	27,848.18	4,232.47
Metagenomic reads mapped to *Methanothrix*	21,421.68	3,568.21
Metatranscriptomic reads mapped to metagenomic contigs	23,073.77	1,521.34
Metatranscriptomic reads mapped to unassembled metagenomic reads	27,968.10	1,910.16
Metatranscriptomic reads mapped to e-pilin genes	1,147.87	155.55
Metatranscriptomic reads mapped to long type IV pilin genes	1,278.93	325.76
Metagenomic reads mapped to e-pilin genes	334.11	10.42
Metagenomic reads mapped to long type IV pilin genes	275.27	26.25
% of e-pilin reads mapped to *Geobacter* (metatranscriptome)	66.54	2.50
% of e-pilin reads mapped to *Geobacter* (metagenome)	82.39	5.42
% of long type IV pilin reads mapped to *Geobacter* (metatranscriptome)	3.77	1.12
% of long type IV pilin reads mapped to *Geobacter* (metagenome)	4.66	0.16

addition, PilFind (81), FlaFind (82), Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan), and Motif Search (http://www.genome.jp/tools/motif/) were used to scan for the presence of type IV pilin-like motifs. All reads identified by these programs were then further screened by comparison to sequences in the NR database with the BLASTx algorithm.

Mapping mRNA reads. QC-filtered merged paired-end libraries that had rRNA removed with SortMeRNA software were used for all transcriptomic mapping analyses. For analysis of gene expression by dominant acetoclastic methanogens found in the rice paddy, mRNA reads were mapped against *Methanothrix concilii* GP-6 (accession no. NC_015416.1). *Geobacter* gene abundance was determined by mapping against the genome of *Geobacter sulfurreducens* PCA (accession no. NC_002939.1). Type IV pilin abundance was determined by mapping against a database composed of 88 different type IV pilA nucleotide sequences. The ArrayStar software (DNAStar) was used to map metatranscriptomic reads to *M. concilii*, *G. sulfurreducens*, and the *pilA* database. All mapped reads were normalized with the reads assigned per kilobase of target per million mapped reads (RPKM) method (83). The total numbers of merged QC-filtered metatranscriptomic reads that mapped to merged QC-filtered metagenomic reads, metagenomic-assembled contigs, the *G. sulfurreducens* genome, the *M. concilii* genome, or the pilin database were determined with the Bowtie2 software (84) and ArrayStar (Table 1).

Accession number(s). The metagenomics and metatranscriptomics sequence reads have been submitted to the European Nucleotide Archive (ENA) database under accession no. PRJEB15510.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00223-17.

SUPPLEMENTAL FILE 1, PDF file, 1.7 MB.

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