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Novel [NiFe]- and [FeFe]-Hydrogenase Gene Transcripts Indicative of Active Facultative Aerobes and Obligate Anaerobes in Earthworm Gut Contents▽†

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The concomitant occurrence of molecular hydrogen (H_2) and organic acids along the alimentary canal of the earthworm is indicative of ongoing fermentation during gut passage. Fermentative H_2 production is catalyzed by [FeFe]-hydrogenases and group 4 [NiFe]-hydrogenases in obligate anaerobes (e.g., *Clostridiales*) and facultative aerobes (e.g., *Enterobacteriaceae*), respectively, functional groups that might respond differently to contrasting redox conditions. Thus, the objectives of this study were to assess the redox potentials of the alimentary canal of *Lumbricus terrestris* and analyze the hydrogenase transcript diversities of H_2 producers in glucose-supplemented gut content microcosms. Although redox potentials in the core of the alimentary canal were variable on an individual worm basis, average redox potentials were similar. The lowest redox potentials occurred in the foregut and midgut regions, averaging 40 and 110 mV, respectively. Correlation plots between hydrogenase amino acid sequences and 16S rRNA gene sequences indicated that closely related hydrogenases belonged to closely related taxa, whereas distantly related hydrogenases did not necessarily belong to distantly related taxa. Of 178 [FeFe]-hydrogenase gene transcripts, 177 clustered in 12 *Clostridiales*-affiliated operational taxonomic units, the majority of which were indicative of heretofore unknown hydrogenases. Of 86 group 4 [NiFe]-hydrogenase gene transcripts, 79% and 21% were affiliated with organisms in the *Enterobacteriaceae* and *Aeromonadaceae*, respectively. The collective results (i) suggest that fermenters must cope with variable and moderately oxidative redox conditions along the alimentary canal, (ii) demonstrate that heretofore undetected hydrogenases are present in the earthworm gut, and (iii) corroborate previous findings implicating *Clostridiaceae* and *Enterobacteriaceae* as active fermentative taxa in earthworm gut content.

Earthworms are an abundant soil macrofauna and have beneficial effects on the structure and fertility of soils (18, 19, 31, 32, 36). Earthworms consume soil and thus ingest soil microbes. Ingested microbes capable of anaerobiosis can become activated due to the *in situ* conditions in the gut, which include anoxia, near neutral pH, and large amounts of organic carbon (8, 16, 24, 25, 26, 27, 37, 56, 63). The polysaccharide-rich mucus of the worm is an important source of organic carbon in the earthworm gut and can be depolymerized by exoenzymes of hydrolytic microbes in the gut (8, 37). Saccharides in the alimentary canal include glucose, galactose, maltose, mannose, and fucose and constitute potential substrates for ingested fermenters during gut passage (42, 63). In this regard, high concentrations of various organic acids such as acetate, butyrate, lactate, and succinate in the gut of earthworms are indicative of diverse ongoing fermentations (24, 63), and anaerobic *Clostridiaceae* and facultatively aerobic *Enterobacteriaceae* have been identified to be active glucose fermenters in the gut content of the earthworm (*Lumbricus terrestris*) (64). These microbial taxa are capable of producing H_2 and might therefore be linked to the *in vivo* emission of H_2 by earthworms (63).

Clostridiaceae produce H_2 to regenerate oxidized ferredoxin, a process that is generally catalyzed by [FeFe]-hydrogenases (39, 49, 58, 59). Facultative aerobes, such as *Enterobacteriaceae*, can perform mixed acid fermentation and produce H_2 during formate oxidation by the formate hydrogen lyase complex that contains a specialized group 4 [NiFe]-hydrogenase (23, 39, 49, 58, 59). The dissimilar responses of H_2-producing facultative aerobes and H_2-producing obligate anaerobes to O_2 (53) suggest that these functional groups might respond differently to contrasting redox conditions. Thus, the objectives of this study were (i) to assess the redox potentials of the alimentary canal of *Lumbricus terrestris* and (ii) to resolve fermentative H_2 producers in the gut by assessing [FeFe]- and group 4 [NiFe]-hydrogenase transcript diversities in glucose-supplemented gut content microcosms (64).

MATERIALS AND METHODS

Earthworms. Adult specimens of *L. terrestris* were purchased from ANZO (Bayreuth, Germany) and maintained in soil obtained from the meadow Trafo Wiese in Bayreuth (Germany) as described elsewhere (24).

Microsensor measurements. Anesthetized earthworms were embedded horizontally in agar as previously described (63). Microsensors and reference electrodes for the measurement of dissolved H_2 (61) and redox potential (43) were purchased from Unisense (Aarhus, Denmark) and were mounted on a micromanipulator (Märtzhäuser, Wetzlar, Germany). Redox electrodes were calibrated using quinhydrone redox buffer solutions (10 g of quinhydrone in 1 liter

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TABLE 1. Sequences and properties of [FeFe]- and group 4 [NiFe]-hydrogenase primers

Primer	Target group	Sequence[a]	Reference	Position	Deg.[b]
HydH1f	[FeFe] (*Bacteria*)	TIACITSITGYWSYCCIGSHTGG[c]	47	524[d]	192
HydH3r	[FeFe] (*Bacteria*)	CAICCIYMIGGRCAISNCAT	47	1126[d]	64
NiFe-gF	[NiFe] (*Gammaproteobacteria*)	GAYCGIRTITGYGGIATYTGYGG	This study	715[e]	32
NiFe-gR	[NiFe] (*Gammaproteobacteria*)	GTRCAIGARTARCAIGGRTC	This study	1585[e]	16
NiFe-uniF	[NiFe] (*Bacteria*/*Archaea*)	GAIMGIRTITGYGGIATHTGY	This study	715[e]	48
NiFe-uniFb	[NiFe] (*Bacteria*/*Archaea*)	GARMGIGTITGYTCICTGTGY	This study	715[e]	16
NiFe-uniR	[NiFe] (*Bacteria*/*Archaea*)	GTRCAISWIWIRCAIGGRTC	This study	1585[e]	64

[a] I, inosine. IUPAC nomenclature was used for mixed bases (13).
[b] Deg., degeneracy, i.e., the number of combinations for the degenerate primers.
[c] The published primer was shortened by one nucleotide position at the 5′ end, as this position turned out to be variable in the [FeFe]-hydrogenase genes available from the public databases.
[d] The 5′ end in the nucleic acid sequence of the *D. vulgaris* [FeFe]-hydrogenase gene (GenBank accession no. AAS96246).
[e] The 5′ end in the nucleic acid sequence of the *E. coli* group 4 [NiFe]-hydrogenase gene (GenBank accession no. AAC75763).

of buffer solution, pH 4 or pH 7). Redox potentials were calculated relative to the standard H_2 electrode. H_2 sensors were calibrated by adding defined volumes of H_2-saturated water to a defined volume of 0.9% NaCl. Radial redox potential measurements were obtained at room temperature. Alternatively, two point measurements were made at the worm surface and the worm center.

H_2 mixing ratios. The terms H_2 mixing ratio and H_2 concentration are used for H_2 in the gas phase and H_2 dissolved in the aqueous phase, respectively. Both terms are related to each other by the Bunsen solubility coefficient (αH_2 at 20°C = 0.018) and the molar volume of gases (24.056 liter mol^{-1}) according to the equation ppmv = (μmol $liter^{-1}$ × 24.056 liter mol^{-1})/0.018, where ppmv is parts per million by volume (11).

Hydrogenase assay. Earthworms were washed, killed, and dissected in an O_2-free Mecaplex chamber (Mecaplex, Switzerland) containing a 100% N_2 gas phase. A total of 19 earthworms with an average weight of 4.8 ± 1.1 g were dissected. The midgut was selected for analysis, as previous studies identified it as a region containing high *in situ* concentrations of H_2 (63). Midgut content was homogenized for 60 min with anoxic lysozyme buffer (35) and zirconium beads at a ratio of 1 ml buffer and 0.7 g beads per g (fresh weight) of gut content. Homogenized and lysed midgut content was centrifuged for 20 min at 14,000 rpm and 4°C (JA-20 rotor; Beckmann Instruments, San Ramon, CA), and the supernatant fluid was used as cell extract. An anoxic cell extract of *Sporomusa rhizae* (21) was prepared by protocols previously described (29) and served as a positive control. Hydrogenase activity was determined by monitoring the H_2-dependent increase in absorbance of benzyl viologen at 578 nm (14). Assay tubes (approximately 15 ml) contained 5 ml of anoxic assay buffer (Tris hydrochloride [50 mM; pH 7.5]) containing 0.5 mM benzyl viologen; the assay temperature was 35°C. Assay tubes were supplemented with 5 ml H_2 (100%), and tubes lacking H_2 served as controls. Protein was determined by the method of Bradford (6). Assays were initiated by injection of cell extracts.

Source of samples and nucleic acid extraction. All nucleic acids were extracted from samples of previously described glucose-supplemented *L. terrestris* gut content microcosms (see reference 64 for a detailed description of the experimental design, process data, and 16S rRNA-based community analysis of the microcosms). At the end of the microcosm experiment (i.e., at 51 h), microcosm samples were stored at −80°C until they were used for the extraction of nucleic acids for hydrogenase transcript and gene analyses. Nucleic acids were extracted by bead-beating lysis, organic solvent extraction, and precipitation (22). RNA was purified from DNA with a Qiagen RNA/DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

[FeFe]- and group 4 [NiFe]-hydrogenase primers. Previously published [FeFe]-hydrogenase primers (47) were slightly modified, and new primers for group 4 [NiFe]-hydrogenases were designed (Table 1). All primers were purchased from biomers.net (Ulm, Germany). For design of group 4 [NiFe]-hydrogenases, 184 gene sequences (see Table S1 in the supplemental material) of the large subunit from group 4 [NiFe]-hydrogenases were identified within the GenBank database (5) via a BLAST analysis (1) with *hycE* from *Escherichia coli* (GenBank accession no. AAC75763) as the search query. The gene sequences were translated *in silico* and prealigned with the ClustalW algorithm implemented in ARB software (http://www.arb-home.de, 34), and the alignment was refined manually. Primers were designed according to the consensus sequence of two highly conserved regions, designated signatures L1 (C[GS][ILV]C[AGNS]XXH) and L2 ([DE][PL]CX[AGST]CX[DE][RL]) (residues in brackets indicate variability at a given position per reference 58), using the consensus degenerate hybrid oligonucleotide (CODEHOP) strategy (Table 1) (44). Inosine nucleotides were used as less destabilizing nucleotides (60) to cover highly variable positions within the nucleic acid sequence alignment in order to reduce the degeneracy of the primers. Two different pairs of primer were designed, one targeting only group 4 [NiFe]-hydrogenases of the *Gammaproteobacteria* (primers NiFe-gF/NiFe-gR) and one targeting all group 4 [NiFe]-hydrogenases (primers NiFe-uniF/NiFe-uniFb/NiFe-uniR) (Table 1). Primer NiFe-uniFb was designed to cover [NiFe]-hydrogenase targets that were not covered by primer NiFe-uniF.

Reverse transcription-PCR and PCR. RNA was transcribed into cDNA by using random hexamers and Superscript III reverse transcriptase (SuperScript cDNA synthesis kit; Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Conditions for [FeFe]-hydrogenase PCR were as published previously (47). The optimized PCR mixture for amplification of group 4 [NiFe]-hydrogenase sequences from either microcosm-derived cDNA or DNA was 5 Prime master mix (1× concentrated; 5 Prime, Hamburg, Germany), 0.1 mg bovine serum albumin ml^{-1}, approximately 2 ng DNA μl^{-1}, and either 0.4 μM primers NiFe-gF/NiFe-gR or 2 μM primers NiFe-uniF/NiFe-uniFb/NiFe-uniR. Primers NiFe-uniF and NiFe-uniFb were premixed to a 9:1 ratio (this ratio reflects the comparative number of publicly available genes targeted by the respective primers). PCR consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of sequential regimens of 94°C for 45 s, 52.5°C or 50°C (for primers NiFe-gF/NiFe-gR and NiFe-uniF/NiFe-uniFb/NiFe-uniR, respectively) for 45 s, and 72°C for 90 s and a final extension at 72°C for 5 min.

Cloning and sequence analyses. Purified PCR products (Montage DNA gel extraction kit; Millipore, Billerica, MA) were ligated into pGEM-T vector plasmids (Promega, Mannheim, Germany) and transformed into competent cells of *E. coli* JM109 (the protocol was according to the manufacturer's instructions; Promega, Mannheim, Germany). M13 PCR (primer set M13F/M13R) was done to screen for clones containing the correct-sized insert according to a published protocol (38). Sequencing was done at Macrogen (Seoul, South Korea). Analysis of *in silico*-translated hydrogenase amino acid sequences was performed with MEGA (release beta 4.1) (55) and ARB (34) software. The term identity was used when microcosm-derived hydrogenase amino acid sequences were compared to publicly available hydrogenase amino acid sequences via a BLASTp analysis (1). The term similarity (*s*) was used when microcosm-derived hydrogenase amino acid sequences were compared to each other via the distance matrix tool implemented in ARB software (34), where *s* is equal to $1 - d$ and *d* is the dissimilarity.

Criteria for establishing hydrogenase OTUs. Amplified *in silico*-translated [FeFe]-hydrogenase amino acid sequences were assigned to operational taxonomic units (OTUs) on the basis of a sequence similarity threshold of 80%. This sequence similarity threshold was chosen on the basis of sequence similarity correlations between publicly available hydrogenase amino acid sequences and corresponding 16S rRNA gene sequences (see Supplement S1 in the supplemental material). [FeFe]-hydrogenases showing an amino acid sequence similarity of at least 80% had a high probability of belonging to organisms within the same family (see Supplement S1 in the supplemental material). Amplified group 4 [NiFe]-hydrogenase gene transcripts and gene sequences could be accurately assigned phylogenetically to host taxa and were therefore not grouped within OTUs (see Supplement S1 in the supplemental material).

Nucleotide sequence accession numbers. The sequences obtained in this study are available from the EMBL nucleotide sequence database under accession numbers FR715716 to FR715893, FR717338 to FR717357, and FR717732 to FR717817.

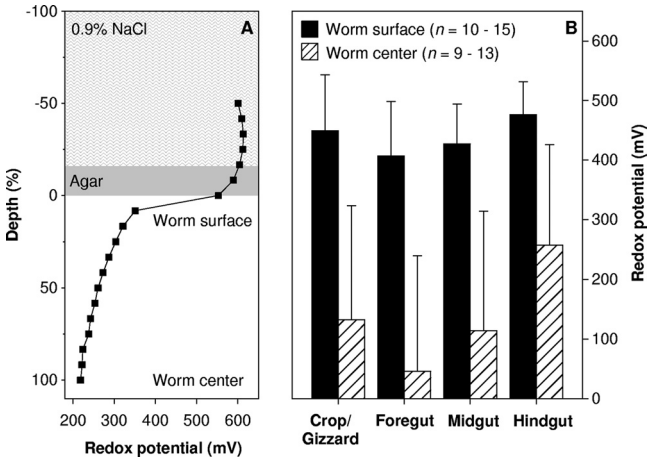

FIG. 1. *In situ* redox potentials (E_h) of a radial microsensor profile of the crop/gizzard region of *L. terrestris* (A) and *in situ* redox potentials along the alimentary canal of *L. terrestris* (B). The worm radius equals 100% (i.e., 3 mm) in panel A. The pattern shown in panel A is representative of replicate analyses, and the means of 9 to 15 replicates per region are shown in panel B (error bars indicate positive standard deviations).

RESULTS

***In situ* redox potentials and H_2 concentrations along the alimentary canal of *L. terrestris*.** Redox potentials became more negative toward the core of the alimentary canal (Fig. 1A), a result consistent with the lack of detectable O_2 in the gut of the earthworm (24, 63). Redox potentials of radial profiles of the crop/gizzard, foregut, midgut, and hindgut regions of the earthworm were similar, with potentials being several hundred mV more negative at the center of the alimentary canal than at the surface of the earthworm (Fig. 1B). Despite the similarities between average values obtained in the core of the alimentary canal, redox potentials at the core were highly variable from one individual to the next, ranging from approximately −200 mV to approximately +390 mV. The lowest average redox potentials occurred in the core of the foregut (Fig. 1B).

H_2 concentrations were highest in the center of the foregut (Table 2). However, H_2 concentrations in the center of the different gut regions were highly variable (Table 2) (63), indicating that hot spots for H_2 production in the gut are variable from worm to worm. Nonetheless, the data confirm that H_2 is produced in the gut of *L. terrestris*. In this regard, hydrogenase activities in cell extracts of midgut content ranged from 6.4 to 8.5 mU per mg protein (1 mU is defined as 1 nmol H_2 min^{-1}).

[FeFe]-hydrogenase gene transcripts in gut content microcosms. [FeFe]-hydrogenase-specific transcript PCR was performed with RNA obtained from glucose-supplemented anoxic gut content microcosms as outlined in reference 64. The 178 sequences obtained yielded 13 different OTUs on the basis of an amino acid sequence threshold similarity of 80% (Fig. 2). The majority of the *in silico*-translated sequences clustered in OTUs 1 to 3 and displayed similar identities to hydrogenase amino acid sequences from the *Clostridiales* families *Lachnospiraceae*, *Clostridiaceae*, and *Ruminococcaceae* (see Table S2 in the supplemental material). Furthermore, some of the sequences within each of the three most abundant OTUs (i.e., OTUs 1 to 3) displayed a high degree of dissimilarity (e.g., sequences H36 and H63 in OTU 1 were 14.7% dissimilar), indicating that OTUs 1 to 3 harbored sub-OTU-level [FeFe]-hydrogenase diversity (Fig. 2; see Table S2 in the supplemental material). OTUs 4 to 9, 11, and 12 were less abundant and were affiliated with hydrogenases of the *Clostridiaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, and *Ruminococcaceae* (Fig. 2). OTU 13 displayed the lowest identity to a known hydrogenase (61% to *Alkaliphilus oremlandii*) (see Table S2 in the supplemental material). Sequence H178 (OUT 10) had the highest identity to a known [FeFe]-hydrogenase gene (84% to that of *Pelobacter carbinolicus*) (see Table S2 in the supplemental material). Furthermore, sequence H178 was the only sequence that could be affiliated with the *Deltaproteobacteria* but not to the *Clostridia*.

A coverage of 97% at 80% sequence threshold similarity indicated that the 178 sequences provided good coverage of the [FeFe]-hydrogenase diversity in the gut content microcosms (see Table S3 in the supplemental material). However, the species richness estimates (see Table S3 in the supplemental material) and rarefaction curve analysis at the 80% threshold (see Fig. S1 in the supplemental material) indicated that additional less abundant hydrogenases might be present in gut contents.

[NiFe]-hydrogenase gene transcripts and genes in gut content microcosms. [NiFe]-hydrogenase transcript analysis was performed with a newly designed primer pair, NiFe-gF/NiFe-gR, targeting only group 4 [NiFe]-hydrogenases of the *Gammaproteobacteria* (Table 1). Seventy-four percent of the 86 *in silico*-translated amino acid sequences clustered with hydrogenases of diverse *Enterobacteriaceae* species and were related to HycE of *E. coli* (Fig. 3). HycE is the catalytic subunit of the hydrogenase 3 isoenzyme (HYD3) (45). HYD3, together with the formate dehydrogenase H (Fdh-H), forms a formate hydrogen lyase complex (FHL-1) that oxidizes formate derived from mixed acid fermentation and produces H_2 via the reduction of cytoplasmic protons, thus minimizing acidification (4, 46). Five percent of the sequences were in a cluster that was related to HyfG (Fig. 3). In *E. coli*, HyfG is the large subunit of a putative H_2-producing [NiFe]-hydrogenase, namely, HYD4 (2). HYD4 together with Fdh-H is proposed to form a second formate hydrogen lyase (FHL-2) that might be involved in (i) energy conservation during intracellular H_2 cycling in the presence of nitrate, nitrite, or fumarate, (ii) syntrophic growth on formate, or (iii) delivering CO_2 for carboxylation-linked processes in biosynthesis (2, 3). The highest identity (87%) of *hyfG*-like clone sequences was to a hydrogenase of *Pectobacterium atrosepticum* (see Table S4 in the supplemental mate-

TABLE 2. H_2 concentrations in center of *L. terrestris* alimentary canal

Alimentary canal section	H_2 concn (μM)[a]		
	Average	SD	Data range
Crop/gizzard	2.6	± 2.1	0.8–6.3
Foregut	4.9	± 3.2	0.6–9.1
Midgut	1.7	± 2.0	0.4–6.4
Hindgut	0.6	± 0.5	0.0–1.7

[a] H_2 concentrations were determined from 5 different worms.

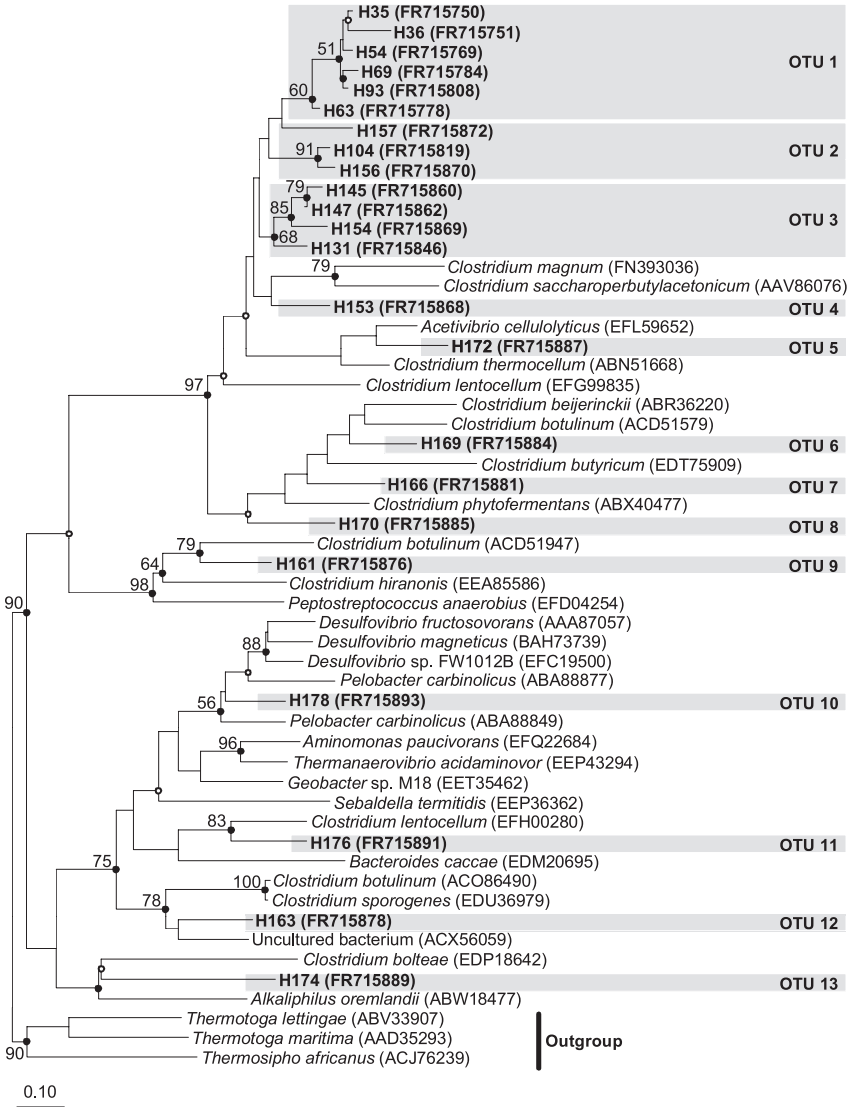

FIG. 2. Phylogenetic tree of *in silico*-translated amino acid sequences derived from [FeFe]-hydrogenase gene transcripts (boldface) and closely related sequences. GenBank accession numbers are indicated in parentheses. Sequences correspond to residues 183 to 375 of the *Desulfovibrio vulgaris* hydrogenase (GenBank accession no. AAS96246). The consensus tree was drawn on the basis of neighbor-joining, maximum parsimony, and maximum likelihood trees. Solid and open circles, nodes congruent in all three analyses and two analyses, respectively. Branch lengths were based on the neighbor-joining analysis. Bootstrap values are means from maximum parsimony trees (100 resamplings) and neighbor-joining trees (1,000 resamplings) and are displayed only for nodes supported by all three analyses and values above 50%. Sequences were affiliated within 13 different OTUs (gray boxes) on the basis of an amino acid sequence threshold similarity of 80% (see Table S2 in the supplemental material). A total of 178 sequences were analyzed; representative sequences are shown for each OTU. The bar indicates a 0.1 change per amino acid.

rial). Twenty-one percent of the sequences were affiliated with the *Aeromonadaceae*, and the closest related sequence (98 to 99% identity) was to a hydrogenase of *Aeromonas salmonicida* (Fig. 3; see Table S4 in the supplemental material). Overall high identities to group 4 [NiFe]-hydrogenases of the *Gammaproteobacteria* verified that primers NiFe-gF/NiFe-gR were specific for these hydrogenases. At a threshold similarity of 80%, both the coverage and species estimates indicated that the 86 clones provided good coverage of the diversity of *Gammaproteobacteria* group 4 [NiFe]-hydrogenases in the earthworm gut content microcosms (see Table S3 in the supplemental material).

Primers NiFe-uniF/NiFe-uniFb/NiFe-uniR (Table 1) have a higher degree of degeneracy than primers NiFe-gF/NiFe-gR and should therefore cover a broader range of group 4 [NiFe]-hydrogenases (see Materials and Methods), so they were used to detect hydrogenases that belonged not only to the *Gammaproteobacteria* but also to all other known group 4 [NiFe]-hydrogenases. However, amplification of transcript-derived cDNA from gut content microcosms failed. The relatively large background of rRNA may have hindered a proper annealing of the highly degenerated primers NiFe-uniF/NiFe-uniFb/NiFe-uniR to the hydrogenase template transcripts. Nonetheless, amplification was successful with DNA of the microcosms. Sixty percent of 20 clone sequences were affiliated with the families *Enterobacteriaceae* and *Aeromonadaceae* of the *Gam-*

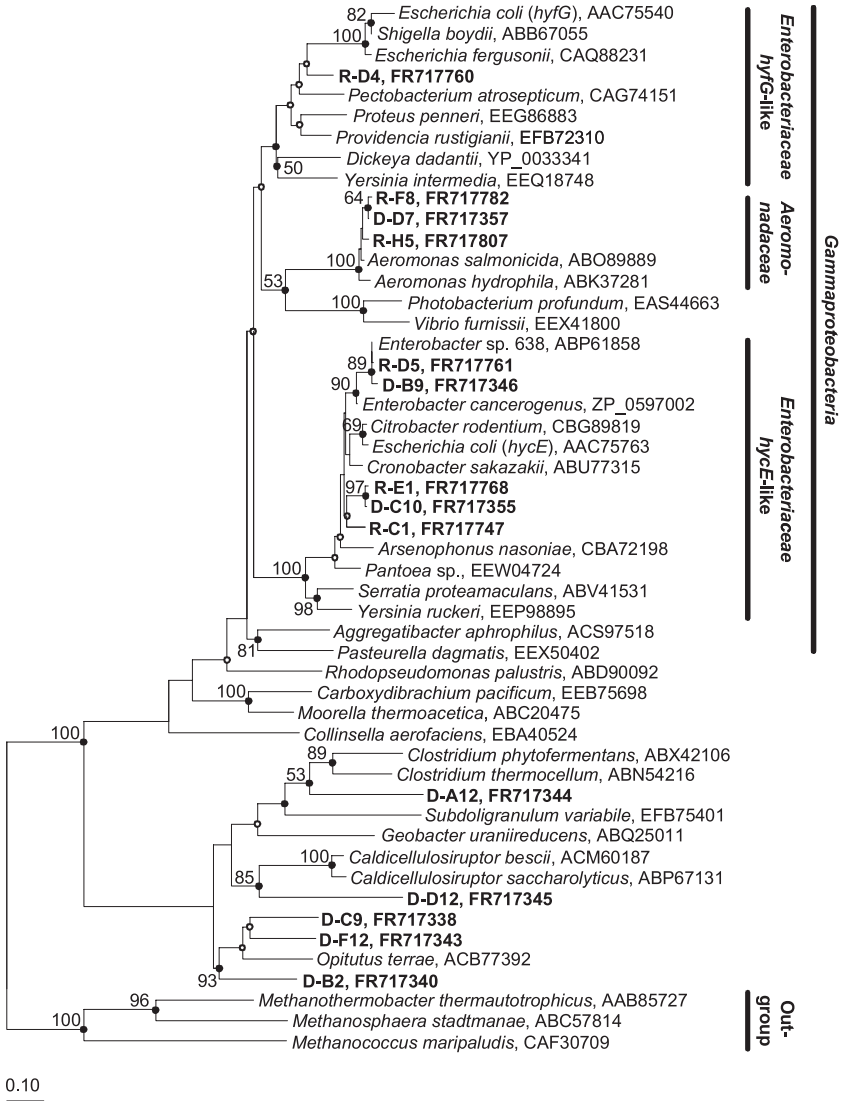

FIG. 3. Phylogenetic tree of *in silico*-translated amino acid sequences derived from [NiFe]-hydrogenase gene transcripts (boldface) and closely related sequences. GenBank accession numbers are indicated in parentheses. Sequences correspond to residues 341 to 710 of the *E. coli* hydrogenase 3 HycE protein (GenBank accession no. AAC75763). The consensus tree was drawn as described for Fig. 2. Bootstrap values are means from maximum parsimony trees (500 resamplings), maximum likelihood trees (10 resamplings), and neighbor-joining trees (1000 resamplings). A total of 106 sequences were analyzed; representative sequences are shown for each phylogenic position. The bar indicates a 0.1 change per amino acid.

maproteobacteria (Fig. 3). These sequences were also amplified at the transcript level with primers NiFe-gF/NiFe-gR (see above). One sequence was most closely related to [NiFe]-hydrogenase of *Clostridium thermocellum* (70% identity), and another was most closely related to that of *Caldicellulosiruptor saccharolyticus* (65%) (see Table S4 in the supplemental material). Six sequences that had an amino acid sequence dissimilarity of up to 26% to each other clustered next to a hydrogenase of *Opitutus terrae* (see Table S4 in the supplemental material). Although the 20 sequences were insufficient for obtaining a good coverage of group 4 [NiFe]-hydrogenase-containing taxa in the gut content microcosms, primers NiFe-uniF/NiFe-uniFb/NiFe-uniR were effective in detecting novel group 4 [NiFe]-hydrogenase genes not resolved with primers NiFe-gF/NiFe-gR.

DISCUSSION

Redox potentials and fermentative processes in the alimentary canal of *Lumbricus terrestris*. Concentrations of dissolved O_2 (a strong oxidizing agent) in the alimentary canal of *L. terrestris* are below the detection limit of oxygen microsensors (0.3 μM) (63). Furthermore, high concentrations of products indicative of anaerobic metabolism (e.g., H_2 and organic acids) are prevalent in the gut of *L. terrestris* (63). Thus, redox potentials along the alimentary canal of *L. terrestris* might be expected to be reducing, since oxidizing redox potentials can inhibit the growth of many anaerobic bacteria (7). However, average redox potentials were >0 mV in the center of the different gut sections (Fig. 1). Traces of oxygen that were below the detection limit of oxygen electrodes might have increased

the redox potentials (51). The presence of alternative inorganic electron acceptors [e.g., nitrate, nitrite, and iron(III) (63)] may also have contributed to the relatively high redox potentials in the earthworm gut. However, fermentative growth is possible at redox potentials as high as those measured in the earthworm gut (53).

Obligate anaerobes and facultative aerobes can adjust their metabolic fluxes according to environmental redox conditions (30, 33, 52). However, since redox potentials along the alimentary canal were only marginally different, other factors, such as the length of time that ingested taxa are exposed to anoxia during gut passage, might at least partly explain why different fermentations appear to occur along the alimentary canal (63). For example, butyrate fermentation, likely facilitated by ingested spore-forming clostridia that may be relatively inactive preingestion, appears to occur in the second half of the alimentary canal rather than in the crop/gizzard (63). The high variability of the measured redox potentials indicated that redox conditions were not uniform on a worm-to-worm basis and that diverse redox couples might be engaged *in situ* (53). Nevertheless, the relatively high average redox potentials likely contribute to the apparent lack of methanogenic and acetogenic activities in gut contents of earthworms (26, 27), since methanogenesis and acetogenesis are considered to require a relatively negative redox potential for optimal activity (15, 62).

H_2-producing fermenters in gut of *L. terrestris*. 16S rRNA-based stable isotope probing (16S rRNA SIP) identified members of anaerobic *Clostridiaceae* (known to produce H_2 via [FeFe]-hydrogenases during butyrate fermentation) and facultatively aerobic *Enterobacteriaceae* (known to produce H_2 via group 4 [NiFe]-hydrogenases during mixed acid fermentation [39, 49, 59]) to be active, major glucose-fermenting taxa in anoxic gut content microcosms (64). Abundant sequences that were affiliated with other obligate fermenters (*Peptostreptococcaceae*) and facultative aerobes (*Aeromonadaceae*) were also obtained in the aforementioned study but were not identified to be active glucose-utilizing taxa (64). In this regard, nearly half the CO_2 produced in glucose-supplemented microcosms was attributed to endogenous carbon sources, suggesting that taxa not directly involved in glucose utilization were nonetheless active (64). One objective of the current study was to identify taxa associated with the production of H_2 in that earlier investigation.

Seventy-four percent of the sequences obtained for group 4 [NiFe]-hydrogenases (amplified with *Gammaproteobacteria*-specific primers NiFe-gF/NiFe-gR [Table 1]) were related to HycE of *E. coli* (Fig. 3; see Table S4 in the supplemental material). The HycE-affiliated amino acid sequences had high levels of identity (94 to 98%) to those of several species of the genus *Enterobacter* (see Table S4 in the supplemental material; data not shown). This result and those obtained previously by 16S rRNA SIP (64) reinforce the likelihood that *Enterobacter* and other genera within the *Enterobacteriaceae* can contribute to the production of H_2 in the gut of earthworms. Five percent of the sequences that were obtained from transcripts of gammaproteobacterial group 4 [NiFe]-hydrogenases were related to HyfG of *E. coli* (Fig. 3; see Table S4 in the supplemental material). The *hyf* operon, including *hyfG*, has been shown to be silent under several physiological conditions, and definitive evidence for HYD4 (the *E. coli* hydrogenase that contains HyfG)-mediated H_2 production has not been obtained (50). Thus, the presence of a transcript related to *hyfG* in gut content is of special interest, although the function of such a hydrogenase remains unclear.

Aeromonadaceae is another family of facultative aerobes capable of forming H_2 during mixed acid fermentation (54). Members of the genus *Aeromonas* occur in the earthworm gut (28) and were enriched but not identified as active glucose consumers in the [^{13}C]glucose 16S rRNA SIP study (64). In the current study, 21% of the [NiFe]-hydrogenase-affiliated sequences were related to *Aeromonas* spp. (Fig. 3; see Table S4 in the supplemental material). These findings indicate that *Aeromonas* spp. can contribute to the fermentative production of H_2 in the gut of earthworms but they may be less competitive for glucose than other taxa.

Group 4 [NiFe]-hydrogenases are not restricted to the formate-hydrogen lyase complex of facultative aerobes within the *Gammaproteobacteria* (23, 58, 59). Analysis of genomic sequence information in the public database GenBank revealed the occurrence of group 4 [NiFe]-hydrogenase-related genes in several bacterial phyla (e.g., *Actinobacteria*, *Verrucomicrobia*, and *Firmicutes*, as well as *Archaea*) (see Table S1 in the supplemental material). Members of these phyla occur in the gut of earthworms (28, 64). Thus, a second [NiFe]-hydrogenase primer system (primers NiFe-uniF, NiFe-uniFb, and NiFe-uniR [Table 1]) was designed to target a broader range of hydrogenases than was possible with primers NiFe-gF/NiFe-gR (Table 1). Hydrogenases from the *Enterobacteriaceae* and *Aeromonadaceae* that were detected with primers NiFe-gF/NiFe-gR at the transcript level accounted for 60% of the sequences amplified with primers NiFe-uniF/NiFe-uniFb/NiFe-uniR at the gene level (Fig. 3; see Table S4 in the supplemental material), reinforcing the potential importance of facultative aerobes in the production of H_2 in the earthworm gut. In addition, other sequences affiliated with group 4 [NiFe]-hydrogenase genes of *Opitutus terrae* (73 to 78% amino acid sequence identity), *Clostridium thermocellum* (70%), and *Caldicellulosiruptor saccharolyticus* (65%). These organisms belong to the *Verrucomicrobia* as well as *Ruminococcaceae* and *Thermoanaerobacterales* within the *Firmicutes* (see Table S1 in the supplemental material) and are known to produce H_2 during the fermentation of polymeric substrates (10, 40, 57). Members of these taxa were not identified to be active glucose fermenters in the gut content microcosms (64) but may contribute to fermentative H_2 production via the degradation of mucus-derived substrates in the gut of earthworms. Unfortunately, detection of transcripts indicative of group 4 [NiFe]-hydrogenases failed with primers NiFe-uniF/NiFe-uniFb/NiFe-uniR (Table 1). However, the PCR efficiencies of these primers were higher with DNA and RNA extracted from a fen soil (data not shown). Thus, both primer systems for group 4 [NiFe]-hydrogenases (Table 1) are useful tools to detect group 4 [NiFe]-hydrogenases from environmental samples.

Out of 178 [FeFe]-hydrogenase-affiliated sequences amplified from RNA samples of the gut content microcosms, 177 clustered with hydrogenase-affiliated sequences of the *Clostridiales* families *Clostridiaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, and *Ruminococcaceae* (Fig. 2). On the basis of 16S rRNA SIP analysis, members of the *Clostridiaceae* were major active glucose fermenters, *Peptostreptococcaceae* were highly

abundant but not active in the consumption (i.e., assimilation) of glucose, *Lachnospiraceae* were less abundant, and *Ruminococcaceae* were not detected (64). The BLAST (1) identities of *Clostridiales*-related [FeFe]-hydrogenase-affiliated sequences were considerably lower than the BLAST identities of *Gammaproteobacteria*-related group 4 [NiFe]-hydrogenase-affiliated sequences (see Table S2 and Table S4 in the supplemental material). Furthermore, [FeFe]-hydrogenases of *Clostridia* do not cluster according to their family affiliation like group 4 [NiFe]-hydrogenases of *Gammaproteobacteria* do (47) (see Supplement S1 in the supplemental material). Thus, the amplified [FeFe]-hydrogenase-affiliated sequences cannot be affiliated unambiguously at the family level. However, on the basis of the 16S rRNA SIP analysis (64), it is likely that most of these hydrogenases belong to organisms of either the *Clostridiaceae* or the *Peptostreptococcaceae* and only to a lesser extent to organisms of the *Lachnospiraceae* and the *Ruminococcaceae*.

The diversity of [FeFe]-hydrogenases affiliated with the *Clostridia* was higher than the diversity of group 4 [NiFe]-hydrogenases affiliated with the *Gammaproteobacteria* (see Table S3 in the supplemental material). However, a high [FeFe]-hydrogenase diversity is not necessarily correlated to a high diversity of organisms that contain those hydrogenases (see Supplement S1 in the supplemental material), since multiple hydrogenases can occur in a single organism. Thus, the actual number of different [FeFe]-hydrogenase-containing families in the gut content microcosms might be less than 13 (the number of OTUs found at 80% threshold similarity).

Both the group 4 [NiFe]-hydrogenase data obtained in this study and the 16S rRNA SIP data obtained before (64) suggested that *Enterobacteriaceae* and *Aeromonadaceae* were the most abundant families of facultative aerobes in the anoxic gut content microcosms (see Fig. S2 in the supplemental material). The [FeFe]-hydrogenase-affiliated sequences had lower scores for identity to known hydrogenases than the amplified group 4 [NiFe]-hydrogenase-affiliated sequences (see Table S2 and Table S4 in the supplemental material), indicating a higher degree of novelty for the [FeFe]-hydrogenases. However, this higher novelty makes determining the affiliation of [FeFe]-hydrogenase-affiliated sequences at the family level less reliable. Nonetheless, [FeFe]-hydrogenase-affiliated sequences clustered in close proximity to hydrogenases of families that were detected with the 16S rRNA SIP analysis (namely, *Clostridiaceae*, *Lachnospiraceae*, and *Peptostreptococcaceae*) and were affiliated with the order *Clostridiales* (see Fig. S2 in the supplemental material).

Potential significance of H_2 production in gut of earthworms. The microbial production of H_2 in the alimentary canal of *L. terrestris* results in the *in vivo* emission of approximately 6 nmol H_2 g (fresh weight)$^{-1}$ h^{-1} (63). On the basis of a total earthworm biomass in terrestrial habitats of approximately 10^{12} kg (fresh weight) (17), earthworms may globally emit 0.11 Tg H_2 year^{-1}. This amount is in the range of H_2 emissions for ruminants and wetlands, which emit approximately 0.14 and 0.11 Tg H_2 year^{-1}, respectively (20). This estimate is based solely on H_2 emissions from *L. terrestris*, and it is therefore important that future studies determine how variable emissions with other species might be and to also determine if earthworm feeding guilds (19) display similar tendencies relative to the emission of H_2. The estimate nonetheless illustrates the potential magnitude of H_2 production in earthworm guts and reinforces the hypothesis that earthworms constitute a mobile source of reductant (i.e., H_2) for the microbiota in aerated soils (63). This hypothesis is further supported by the fact that H_2 concentrations at the worm surface (approximately 4 μM [63]) are 4 orders of magnitude higher than those in a solution equilibrated to tropospheric H_2 mixing ratios (0.53 ppmv [41], which corresponds to approximately 0.4 nM). H_2 at low tropospheric H_2 mixing ratios can be taken up only by organisms that have special high-affinity H_2 uptake [NiFe]-hydrogenases (apparent K_m, 40 to 370 nM; e.g., *Streptomycetes* spp. [12]), whereas the elevated H_2 concentrations in the immediate vicinity of earthworms may support the growth of bacteria that have low-affinity H_2 uptake [NiFe]-hydrogenases (apparent K_m, >800 nM; e.g., Knallgas bacteria [11, 48]).

Conclusions and limitations. The phylogenetic relationships between group 4 [NiFe]- or [FeFe]-hydrogenase gene transcripts and 16S rRNA genes were evaluated to determine if hydrogenase gene transcripts provided reliable phylogenetic information for resolving fermentative H_2-producing community members (see Supplement S1 in the supplemental material). The results indicated that closely related (≥80% amino acid sequence similarity) hydrogenases most likely belong to the same family. However, distantly related hydrogenases did not necessarily belong to different species, genera, or families. Thus, hydrogenase gene transcripts amplified from environmental samples can be adequately affiliated with a certain family only if they share at least 80% sequence similarity to a known hydrogenase.

Group 4 [NiFe]-hydrogenases of facultatively aerobic *Enterobacteriaceae* and *Aeromonadaceae*, members of which are capable of mixed acid fermentation (39), as well as [FeFe]-hydrogenases of anaerobic *Clostridiales*, members of which are capable of butyrate fermentation (9), were detected at the transcript level in anoxic glucose-supplemented gut content microcosms of the earthworm *L. terrestris*. The taxa resolved by the analysis of hydrogenase gene transcripts in the current study were in good accordance with the taxa resolved by the analysis of 16S rRNA in the preceding study (64). Furthermore, the *in situ* profiles of fermentation products (e.g., fatty acids and H_2) along the alimentary canal of *L. terrestris* are indicative of ongoing H_2-producing mixed acid and butyrate fermentations (63). These collective observations support the hypothesis that *Enterobacteriaceae* and *Clostridiaceae* are important drivers of H_2 production in the alimentary canal. However, the detection of highly novel hydrogenases that could not be unambiguously phylogenetically assigned suggests that additional taxa may also contribute to fermentative H_2 production in the alimentary canal.

These conclusions must be placed in the perspective of the experimental conditions employed. The sequence data were derived from anoxic gut content microcosms that simulated many but not all of the *in situ* conditions in the gut to which ingested soil bacteria are exposed (64) and likewise are from an experiment with only one earthworm species. It is therefore important that future studies determine if the H_2-producing fermentative processes and taxa resolved in *L. terrestris* are representative of those in different earthworm species and feeding guilds. In this regard, the availability of primers for targeting [FeFe]- or group 4 [NiFe]-hydrogenase genes (47)

(Table 1) can facilitate the detection of fermentative H_2 producers not only in the gut of earthworms but also in other habitats, such as wetlands, ruminants, and biogas plants, where the fermentative production of H_2 is trophically linked to ecosystem function.

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