Applied and Environmental Microbiology, Sept. 2010, p. 6205–6214 Vol. 76, No. 18
0099-2240/10/$12.00 doi:10.1128/AEM.01373-10

Effect of Earthworm Feeding Guilds on Ingested Dissimilatory Nitrate Reducers and Denitrifiers in the Alimentary Canal of the Earthworm▽†

Peter S. Depkat-Jakob, Maik Hilgarth, Marcus A. Horn, and Harold L. Drake*

Department of Ecological Microbiology, University of Bayreuth, 95440 Bayreuth, Germany

Received 9 June 2010/Accepted 19 July 2010

The earthworm gut is an anoxic nitrous oxide (N_2O)-emitting microzone in aerated soils. *In situ* conditions of the gut might stimulate ingested nitrate-reducing soil bacteria linked to this emission. The objective of this study was to determine if dissimilatory nitrate reducers and denitrifiers in the alimentary canal were affected by feeding guilds (epigeic [*Lumbricus rubellus*], anecic [*Lumbricus terrestris*], and endogeic [*Aporrectodea caliginosa*]). Genes and gene transcripts of *narG* (encodes a subunit of nitrate reductase and targets both dissimilatory nitrate reducers and denitrifiers) and *nosZ* (encodes a subunit of N_2O reductase and targets denitrifiers) were detected in guts and soils. Gut-derived sequences were similar to those of cultured and uncultured soil bacteria and to soil-derived sequences obtained in this study. Gut-derived *narG* sequences and *narG* terminal restriction fragments (TRFs) were affiliated mainly with Gram-positive organisms (*Actinobacteria*). The majority of gut- and uppermost-soil-derived *narG* transcripts were affiliated with *Mycobacterium* (*Actinobacteria*). In contrast, *narG* sequences indicative of Gram-negative organisms (*Proteobacteria*) were dominant in mineral soil. Most *nosZ* sequences and *nosZ* TRFs were affiliated with *Bradyrhizobium* (*Alphaproteobacteria*) and uncultured soil bacteria. TRF profiles indicated that *nosZ* transcripts were more affected by earthworm feeding guilds than were *nosZ* genes, whereas *narG* transcripts were less affected by earthworm feeding guilds than were *narG* genes. *narG* and *nosZ* transcripts were different and less diverse in the earthworm gut than in mineral soil. The collective results indicate that dissimilatory nitrate reducers and denitrifiers in the earthworm gut are soil derived and that ingested *narG*- and *nosZ*-containing taxa were not uniformly stimulated in the guts of worms from different feeding guilds.

Earthworms have a profound impact on the structure and fertility of soils and occur in three feeding guilds (6, 17). Epigeic earthworms (e.g., *Lumbricus rubellus*) feed preferentially on litter, live above the mineral soil, rarely form burrows, and take up minor amounts of soil. Anecic earthworms (e.g., *Lumbricus terrestris*) feed on organic residues, build deep vertical burrows into the mineral soil, and ingest medium amounts of soil. Endogeic species (e.g., *Aporrectodea caliginosa*) build predominantly horizontal burrows and ingest large amounts of mineral soil and humified material.

An important feature of the earthworm relative to its ecological function is its alimentary canal, an anatomical structure that constitutes a transient mobile anoxic microzone for ingested soil microbial biomes in aerated soils (16). The abundances of cultivable fermenters and nitrate reducers are up to three orders of magnitude higher in the earthworm gut than in preingested soil (16, 32, 33, 34). The dissimilatory reduction of nitrate (i.e., the reduction of nitrate to nitrite or ammonium) is often facilitated by fermentative organisms (70) and occurs in habitats subject to anoxia and a high level of availability of organic carbon (71), conditions characteristic of the earthworm gut (13, 16, 29, 33, 75). Denitrification (i.e., the reduction of nitrate or nitrite to a nitrogenous gas), the dissimilatory reduction of nitrate, and fermentations appear to occur in the earthworm gut, and nitrous oxide (N_2O), dinitrogen (N_2), and molecular hydrogen (H_2) are emitted *in vivo* (29, 31, 32, 34, 42, 75, 76). On a dry weight basis, earthworms can emit much larger amounts of N_2O than soils (12, 29, 31, 32, 34, 42, 75, 76). The burrowing activities and feeding habits of earthworms, as well as *in situ* soil conditions, can influence the emission of nitrogenous gases from soils inhabited by earthworms (3, 5, 34, 40, 42, 54).

Denitrification is carried out by several oxidoreductases, i.e., dissimilatory nitrate reductase (encoded by either *narGHI* or *napAB*), nitrite reductase (encoded by *nirK* and *nirS*), NO reductase (encoded by *norBC*), and N_2O reductase (encoded by *nosZ*) (50, 77). Dissimilatory nitrate reducers also possess nitrate reductases, most of which are encoded by *narGHI* (45). Thus, denitrifiers and dissimilatory nitrate reducers can be evaluated by analyzing *narG* (11, 14, 20, 26, 46, 49), whereas only denitrifiers are targeted by analyzing *nosZ* (30, 52, 58, 65, 66, 67, 76).

The capacity of earthworms to emit nitrogenous gases is primarily linked to denitrifiers in the alimentary canal (30, 31, 34, 42, 76). However, cultured dissimilatory nitrate reducers outnumber cultured denitrifiers in gut contents (32), suggesting that dissimilatory nitrate reducers might influence *in vivo* emission of N_2O by their capacity to compete with denitrifiers for nitrate. Active nitrate-reducing taxa in the earthworm alimentary canal have not been identified, and although there is evidence indicating that the composition of gut taxa might be

* Corresponding author. Mailing address: Department of Ecological Microbiology, University of Bayreuth, 95440 Bayreuth, Germany. Phone: (49) (0)921-555640. Fax: (49) (0)921-555793. E-mail: HLD@Uni-Bayreuth.De.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 23 July 2010.

influenced by earthworm feeding guilds (1, 39, 69), the potential effect of feeding guilds on the diversity and activities of nitrate-reducing taxa remains largely unknown. Thus, the structural gene markers *narG* and *nosZ* were used in this study to elucidate dissimilatory nitrate reducers and denitrifiers in the earthworm alimentary canal of three earthworm feeding guilds and surrounding soils at both gene and gene transcript levels. The two hypotheses addressed were as follows: (i) soil-derived dissimilatory nitrate reducers and denitrifiers are activated in the earthworm alimentary canal; and (ii) earthworm feeding guilds influence this activation.

MATERIALS AND METHODS

Field sites and sampling. Soils and specimens of *A. caliginosa*, *L. terrestris*, and *L. rubellus* were collected in spring 2007 (*narG* libraries), late summer 2008 (*nosZ* libraries), and early winter 2008 (*narG* and *nosZ* transcript libraries; terminal restriction fragment length polymorphism [TRFLP] analyses) from the meadow Trafo Wiese near Bayreuth, Germany, that is described elsewhere (29). Worms were collected and identified by standard protocols (7, 33, 42, 55). Soil samples were taken from the surface (uppermost soil, including decaying organic material) and from a depth of 15 cm (mineral soil). Worms and soils were stored in the dark for 1 h at 5°C before use.

Extraction of nucleic acids, reverse transcription of RNA, and amplification of *narG* and *nosZ*. Earthworms (6 to 18 specimens per worm species) were washed, sedated with CO_2, sacrificed by brief immersion in 70% ethanol, and dried under oxic conditions (33, 34). Alimentary canals were dissected, and RNA and DNA were coextracted from an approximately 0.5-g sample by bead-beating lysis, organic solvent extraction, and precipitation (27). The separation of RNA and DNA was performed with a Qiagen RNA/DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The absence of DNA in RNA fractions was indicated by the inability to gain a *narG* or *nosZ* PCR product under the conditions described below. Reverse transcription of RNA into cDNA was performed with a SuperScript Vilo cDNA synthesis kit (Invitrogen, Carlsbad, CA) using approximately 50 ng of RNA per 20 μl of reaction mixture and random hexamers for 10 min at 25°C, 120 min at 42°C, and 5 min at 85°C with a TGradient cycler (Biometra, Göttingen, Germany). PCR amplification was performed with primers narG1960f/narG2650r (5′-TAY GTS GGS CAR GAR AA-3′; 5′-TTY TCR TAC CAB GTB GC-3′ [49]) and nosZF/nosZR (5′-CGC TGT TCI TCG ACA GYC AG-3′; 5′-ATG TGC AKI GCR TGG CAG AA-3′ [52]) (Biomers GmbH, Ulm, Germany). Each 25-μl PCR mixture contained 10 μl of 5 Prime MasterMix (×2.5) (5 Prime, Hamburg, Germany), 2 μl of bovine serum albumin (30 μg μl^{-1}), 1 μl of additional magnesium chloride (25 mM), 1.5 μl of each primer (100 pmol μl^{-1} for *narG* and 10 pmol μl^{-1} for *nosZ*), 8 ml of double-distilled H_2O, and 1 μl each of DNA and cDNA, respectively. Each PCR was carried out with an initial denaturation (95°C, 8 min). Terminal elongation was for 10 min at 72°C; denaturation and elongation of each PCR and each cycle were performed at 95°C (1 min) and 72°C (2 min), respectively. For *narG*, the annealing temperature was lowered stepwise from 56°C to 52°C in 8 precycles, followed by 35 cycles with the annealing temperature at 52°C. *narG* PCR products were not obtained with cDNA samples, and conditions for cDNA were switched to 45 cycles with the annealing temperature at 58°C. For *nosZ*, the annealing temperature was lowered stepwise from 58°C to 52°C in 12 precycles, followed by 30 cycles with the annealing temperature at 52°C.

Cloning, sequencing, and sequence analysis. *narG* and *nosZ* PCR products were electrophoresed on agarose gel (1%), excised, purified with a Montage gel extraction kit (Millipore Corp., Billerica, MA) according to the manufacturer's protocol, and ligated into pGEM-T vectors (Promega, Mannheim, Germany). Competent cells of *Escherichia coli* JM109 were transformed with the vector (protocol per manufacturer's instructions; Promega, Mannheim, Germany). *narG* and *nosZ* inserts were amplified with primers M13uni (5′-GTA AAA CGA CGG CCA G-3′) and M13rev (5′-CAG GAA ACA GCT ATG ACC-3′) (43). The conditions were 10 min for denaturation at 95°C and 35 cycles with 1 min at 95°C, 45 s at 50°C, and 90 s at 72°C. The final elongation step was 10 min at 72°C. Aliquots of the PCR product were electrophoresed on agarose gel (1%) to check for inserts of the right length. Clones with the correct insert were chosen randomly and selected for sequencing at Macrogen (Seoul, South Korea). Analyses of *narG* and *nosZ* sequences were performed with MEGA4 (68) and BLAST (http://blast.ncbi.nlm.nih.gov/). DOTUR 1.53 was used for defining species level genotypes (i.e., operational taxonomic units [OTUs]) (59). Except for TRFLP analysis, all phylogenic and statistical analyzes were performed with amino acid sequences obtained by translation of *narG* and *nosZ* sequences. Threshold dissimilarity values of 41% and 14% of amino acid sequences were used for defining species level OTUs for *narG* and *nosZ*, respectively (48). These threshold dissimilarity values were chosen because comparative sequence analyses of *narG* and *nosZ* with 16S rRNA genes indicated that these values correspond with 90% probability to a 16S rRNA sequence similarity of ≥97% (48), a conservative estimate for species level differentiation (64). DOTUR 1.53 was used for assessing coverage, richness (average of the richness estimators Chao1, ACE, jackknife, and bootstrap with a standard deviation), and diversity (Shannon-Weaver diversity index, Simpson's diversity index, Simpson's reciprocal index). Evenness was calculated as the Shannon-Weaver diversity index divided by the natural logarithm of the calculated genotype number (51) at species level OTU threshold dissimilarity values of 41% and 14% for *narG* and *nosZ*, respectively.

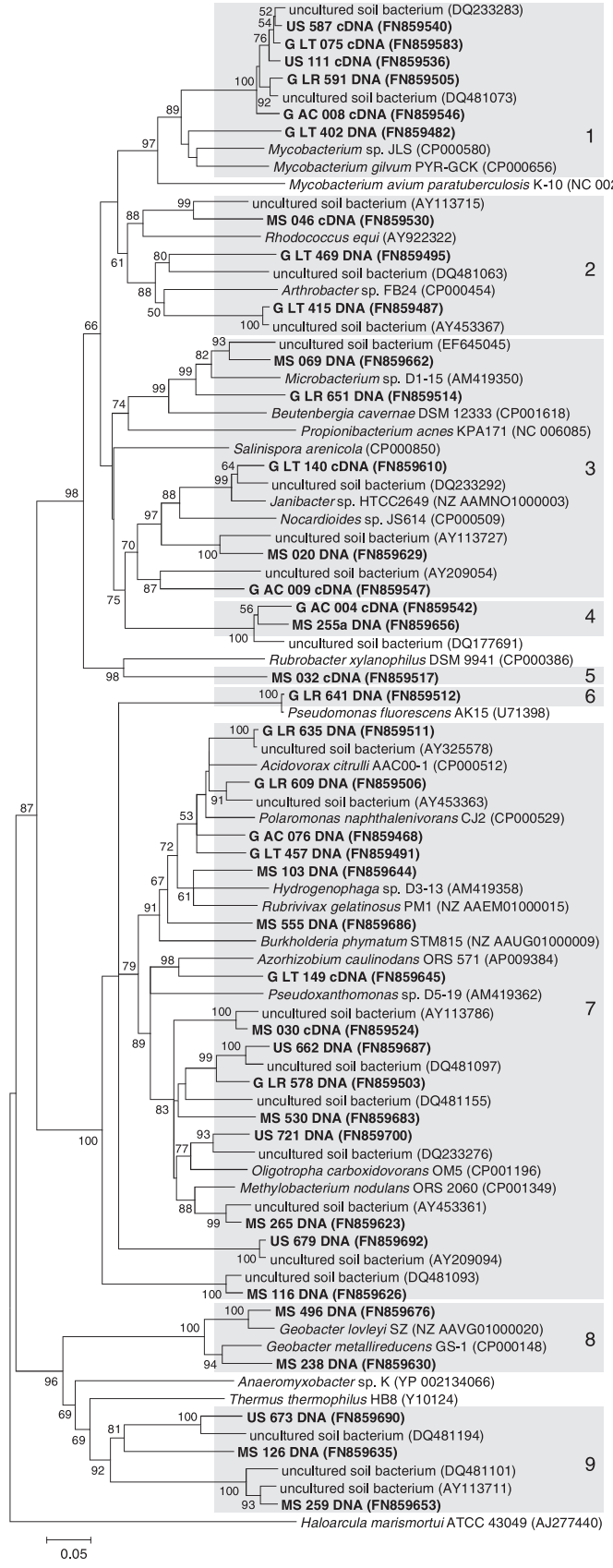
Phylogenetic analysis. Phylogenetic trees were calculated from aligned amino acid sequences (ClustalW; alignment was manually refined) with MEGA4 (68) using the neighbor-joining method (56) with a bootstrap test (10,000 replicates) (22). All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option).

TRFLP analysis. *narG* and *nosZ* gene fragments were amplified with the fluorescently labeled primers narG1960f-DY681 and nosZF-DY681 (Biomers GmbH, Ulm, Germany), respectively. Triplicate PCRs were carried out for each sample. PCR products were run on an agarose gel (1%). Gel-purified DNA (Montage gel extraction kit; Millipore Corp., Billerica, MA) was digested with mung bean nuclease (New England Biolabs, Frankfurt/Main, Germany) according to the manufacturer's protocol to reduce the probability of single-stranded DNA causing pseudoterminal restriction fragments (18). PCR products were purified (Montage gel extraction kit) and digested with the restriction enzyme BanI (New England Biolabs, Frankfurt/Main, Germany) or MaeIII (Roche Diagnostics GmbH, Mannheim, Germany) for *narG* and with HhaI (New England Biolabs, Frankfurt/Main, Germany) for *nosZ* according to the manufacturers' protocol but with 3 units and a 16-h digestion step in a reaction volume of 10 μl. TRFLP analysis was performed on a Nen 4300 DNA analyzer (Li-Cor, Lincoln, NE). The polyacrylamide gel consisted of 15 g of urea (Roche Diagnostics GmbH, Mannheim, Germany), 3.75 ml of 40% acrylamide–bis solution (37.5:1; Bio-Rad, Hercules, CA), 6 ml of Tris-borate-EDTA buffer (54 g of Tris, 27.5 g of boric acid, 20 ml of 0.5 M EDTA [pH 8.0], and double-distilled H_2O to 1,000 ml), and 3.25 ml of double-distilled H_2O. A bind-silane solution (1:1 ratio of bind-silane [PlusOne; GE Healthcare, Piscataway, NJ] and 10% acetic acid) was applied to the glass plates for stabilization of the comb region of the gel. Electrophoresis was performed for 3 h at 1,500 V and 45°C. Gels were analyzed with GelQuest (version 2.6.3; SequentiX, Klein Raden, Germany). Mean values and standard deviations were calculated from triplicate analyses of each sample. All terminal restriction fragments (TRFs) with a relative fluorescence of >3% for at least one sample were used for analysis, and their collective relative fluorescences were set as 100%. TRFs were assigned to genotypes by *in silico* analysis (MEGA4 [68]). TRFLP analysis of the *narG* transcript with BanI mainly yielded TRFs that were too small to evaluate and were dissimilar to *narG* TRFs (data not shown). Principal-component analyses of *narG* and *nosZ* TRFs were performed with RapidMiner.

Nucleotide sequence accession numbers. The sequences obtained in this study are available from the EMBL nucleotide sequence database under accession numbers FN859458 to FN859704 (*narG*), FN859705 to FN859774 (*nosZ*), and FN859874 to FN859960 (*nosZ*).

RESULTS

Diversity and phylogeny of *narG* and *narG* transcripts. A collective total of 247 *narG* fragments and *narG* transcript fragments were retrieved from alimentary canal and soil samples, forming 9 species level OTUs at an amino acid sequence dissimilarity of 41% (48) (Fig. 1). This species level cutoff value is very conservative, and the real number of species is assumed to be higher, as evidenced by the large number of known species affiliated with certain OTUs (e.g., OTUs 3 and 7 [Fig. 1]). The 139 *narG* sequences (82 from soil, 57 from gut) were distributed in 8 OTUs. Soil- and gut-derived gene sequences were each distributed in 5 OTUs. The 108 *narG* transcripts (26 from soil, 82 from gut) were distributed in 4 OTUs. Soil- and gut-derived transcripts were each distributed in 3 OTUs.



	No. of sequences										
	DNA					cDNA					
	Earthworms			Soils		Earthworms			Soils		
	AC	LT	LR	MS	US	AC	LT	LR	MS	US	Sum
1	1	2	4	1	1	17	47	2	1	7	83
2	5	3	0	4	2	1	0	0	3	0	18
3	3	0	4	4	2	2	6	0	6	0	27
4	0	1	3	3	0	1	3	0	0	1	12
5	0	0	0	0	0	0	1	0	1	0	2
6	0	0	1	0	0	0	0	0	0	0	1
7	13	11	6	40	12	0	2	0	7	0	91
8	0	0	0	5	0	0	0	0	0	0	5
9	0	0	0	7	1	0	0	0	0	0	8
	22	17	18	64	18	21	59	2	18	8	247

FIG. 1. Phylogenetic tree of representative *narG* sequences obtained from earthworms and soil and the related *narG* sequences. Sequences obtained in this study are in bold, and accession numbers are in parentheses. The tree is based on 215 translated amino acids. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) are shown next to the branches (values below 50% are masked) (22). The table shows the origin of sequences in each cluster (shaded text) as calculated with DOTUR 1.53. Abbreviations: G, gut; AC, *A. caliginosa*; LT, *L. terrestris*; LR, *L. rubellus*; MS, mineral soil; US, uppermost soil.

TABLE 1. Estimated genotypes, coverage percentages, and diversity indices of *narG* and *nosZ* amino acid sequences from the alimentary canals of earthworms and from soils[a]

Gene	Library	No. of sequences	No. of OTUs	Coverage (%)	Diversity indices			
					Richness	H′[b]	Evenness	1/D[c]
narG[d]	DNA, guts	57	5	100	5 ± 0	1.30	0.81	2.95
	DNA, soils	82	5	100	5 ± 0	1.16	0.72	2.33
	cDNA, guts	82	3	100	3 ± 0	0.55	0.50	1.45
	cDNA, soils	26	3	100	3 ± 0	1.06	0.97	3.00
	DNA, total	139	8	99.3	8 ± 0	1.41	0.68	2.67
	cDNA, total	108	4	100	4 ± 0	0.82	0.59	1.80
	All *narG* sequences	247	9	99.6	10 ± 0	1.57	0.71	3.75
nosZ[e]	DNA, guts	70	11	98.6	12 ± 1	1.53	0.64	2.75
	DNA, soils	19	8	84.2	10 ± 1	1.84	0.88	6.33
	cDNA, guts	39	9	92.3	11 ± 1	1.74	0.79	4.72
	cDNA, soils	29	13	72.4	20 ± 3	2.08	0.81	5.64
	DNA, total	89	15	95.5	17 ± 1	1.78	0.66	3.23
	cDNA, total	68	18	83.8	41 ± 13	2.15	0.74	5.68
	All *nosZ* sequences	157	26	92.4	66 ± 48	2.15	0.66	4.30

[a] All calculations were carried out with DOTUR 1.53 and are based on amino acid sequences as described in Materials and Methods.
[b] H′, Shannon-Weaver diversity index.
[c] 1/D, reciprocal Simpson's diversity index.
[d] *narG* at a species level cutoff value of 41% dissimilarity of amino acid sequences.
[e] *nosZ* at a species level cutoff value of 14% dissimilarity of amino acid sequences.

Coverage for all *narG* libraries approximated 100% (Table 1). The estimated richness of all *narG* sequences (i.e., 10 OTUs) was only slightly higher than that of the 9 species level OTUs (Table 1 and Fig. 1). The predicted numbers of OTUs for gene and transcript sequences were 8 and 4, respectively. Diversity indices (e.g., Shannon-Weaver) for *narG* differed minimally between gut-derived sequences and soil-derived sequences (Table 1). In contrast, diversity indices for *narG* transcripts were higher for soil-derived sequences than for gut-derived sequences.

narG sequences were affiliated with sequences of Gram-negative genera (e.g., *Acidovorax*, *Azorhizobium*, *Oligotropha*, *Methylobacterium*, *Geobacter*, and *Thermus* [OTUs 6 to 9]) and Gram-positive genera (i.e., *Actinobacteria*; e.g., *Mycobacterium*, *Rhodococcus*, *Arthrobacter*, *Microbacterium*, *Beutenbergia*, *Janibacter*, and *Rubrobacter* [OTUs 1 to 5]) that are common to soils (Fig. 1). Most (i.e., 233 out of 247) *narG* sequences had the highest levels of similarity to those of uncultured soil bacteria and occurred in shared OTUs (i.e., OTUs that contained both soil- and gut-derived sequences), suggesting that *narG* sequences retrieved from the earthworm gut originated primarily from ingested soil bacteria.

Most *narG* sequences from soil were affiliated with OTUs of Gram-negative bacteria (mainly OTU 7), whereas *narG* sequences from the gut occurred more evenly in OTUs of both Gram-negative bacteria and *Actinobacteria* (Fig. 1). OTU 1 (*Mycobacterium*) contained the majority of *narG* transcripts, and sequences obtained from the gut and uppermost soil were dominant. Mineral soil-derived *narG* transcripts clustered mainly with OTUs 2, 3, and 7.

TRF patterns of *narG* and *narG* transcripts. A total of 13 *narG* TRFs were obtained (Fig. 2A). TRFs of the three earthworm feeding guilds were highly overlapping (i.e., were more similar than dissimilar), with the bulk of the sequences occurring in TRFs 91 bp (primarily indicative of OTUs 2 and 3), 104 bp (primarily indicative of OTU 3), 458 bp (OTU 7), and 632 bp (could not be assigned to an OTU) (Fig. 2A; see also Table S1 in the supplemental material). *narG* TRFs from mineral and uppermost soils were dissimilar (Fig. 2A and 3A). Certain dominant TRFs in mineral soil (i.e., TRFs 168 bp, 190 bp, and 243/245 bp, all indicative of OTU 7) were apparent only in *A. caliginosa*. Significant differences between *narG* TRFs from crop/gizzard and those from gut were not apparent. Of the assignable *narG* sequences, those of *Actinobacteria* were dominant in all three feeding guilds and moist, uppermost soils, whereas Gram-negative bacteria were dominant in drier mineral soils and were also detected in the gut of the endogeic earthworm *A. caliginosa*.

A total of 15 *narG* transcript TRFs were obtained (Fig. 4A). *narG* transcript TRFs from all three feeding guilds were similar, whereas *narG* transcript TRFs from mineral and uppermost soils were dissimilar (Fig. 3B and 4A). TRF 357 bp (OTU 1; see Table S2 in the supplemental material) was dominant in all three feeding guilds and in uppermost soils. In contrast, the most dominant TRFs in mineral soils were 573 bp (OTU 7), 560 bp (could not be assigned), and 127 bp (OTU 7). TRF patterns corroborated the sequence data and revealed a high level of prevalence of OTU 1 *narG* transcripts in gut and uppermost-soil libraries, whereas sequences from mineral soil were mostly affiliated with OTUs 2, 3, and 7. Gut samples from *A. caliginosa* and *L. terrestris* showed highly similar *narG* transcript TRF patterns, whereas TRF 477 bp (OTU 4) was significant only in the gut *of L. rubellus* (Fig. 4A).

Diversity and phylogeny of *nosZ* and *nosZ* transcripts. A collective total of 157 *nosZ* fragments and *nosZ* transcript fragments were retrieved from alimentary canal and soil samples, forming 26 species level OTUs at an amino acid sequence dissimilarity of 14% (48) (Fig. 5). The 89 *nosZ* sequences (19

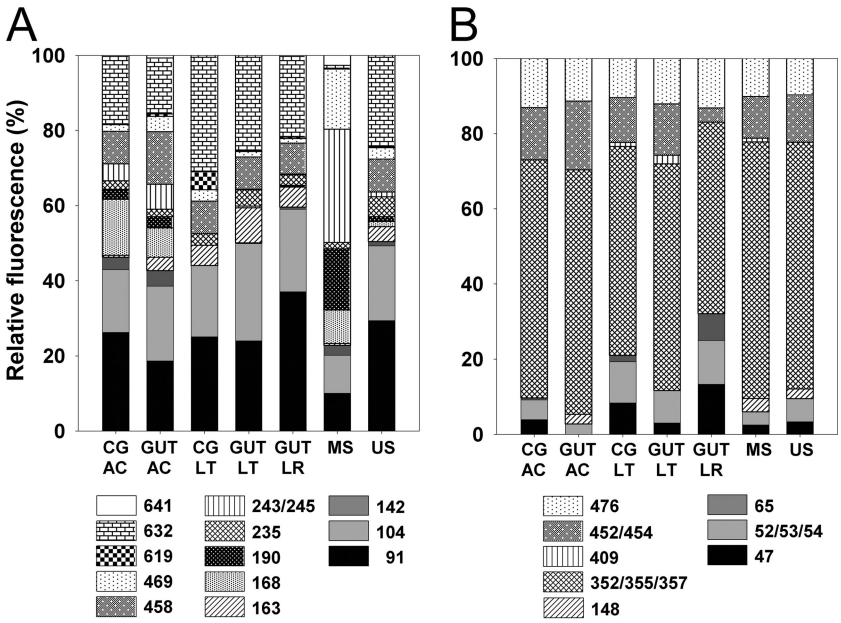


FIG. 2. TRFLP analysis of earthworm alimentary canal- and soil-derived *narG* and *nosZ* sequences. (A) *narG* (digestion was with BanI). (B) *nosZ* (digestion was with HhaI). Abbreviations: CG, crop/gizzard; AC, *A. caliginosa*; LT, *L. terrestris*; LR, *L. rubellus*; MS, mineral soil; US, uppermost soil. The legend shows the lengths of the TRFs. Shown are mean values ($n = 3$).

from soil, 70 from gut) were distributed among 15 OTUs. Soil- and gut-derived gene sequences were distributed among 8 and 11 OTUs, respectively. The 68 *nosZ* transcripts (29 from soil, 39 from gut) were distributed among 18 OTUs. Soil- and gut-derived transcripts were distributed among 13 and 9 OTUs, respectively.

Coverages for soil and earthworm *nosZ* libraries ranged from approximately 72.4% to 84.2% and 92.3% to 98.6%, respectively, with the collective coverage of all sequences approximating 92% (Table 1). The estimated richness of all *nosZ* sequences yielded 66 OTUs, a much larger number than the 26 species level OTUs (Table 1 and Fig. 5). The predicted numbers of OTUs for gene and transcript sequences were 17 and 41, respectively. Diversity indices of *nosZ* and *nosZ* transcripts were lower for the earthworm gut than for soil (Table 1), suggesting that a restricted rather than a general stimulation of soil-derived denitrifiers occurred in the earthworm alimentary canal.

nosZ sequences were affiliated with *Alphaproteobacteria* (e.g., genera *Bradyrhizobium*, *Oligotropha*, *Sinorhizobium*, and *Paracoccus*), *Betaproteobacteria* (e.g., genera *Bordetella*, *Cupriavidus*, and "*Lutiella*"), and *Pseudomonas stutzeri* A1501, with most sequences having the highest similarity to *nosZ* sequences from uncultured soil bacteria. OTUs 1 and 11 were dominant in *nosZ* sequences, whereas OTUs 1 and 3 were dominant in *nosZ* transcripts. The closest relatives of OTUs 1, 3, and 11 were *Bradyrhizobium japonicum* USDA 110 and two uncultured soil bacteria, respectively. Most (i.e., 134 out of 157) *nosZ* sequences from gut and soil occurred in shared OTUs, suggesting that *nosZ* sequences retrieved from the earthworm gut originated primarily from ingested soil bacteria.

TRF patterns of *nosZ* and *nosZ* transcripts. A total of 8 *nosZ* TRFs (Fig. 2B) and 10 *nosZ* transcript TRFs (Fig. 4B) were obtained. For gut-derived TRFs, gene level TRF 352/355/357 bp (of which OTUs 1, 3, and 11 contained the highest numbers of sequences [see Table S3 in the supplemental material]) was dominant (51 to 65% relative fluorescences) in all worm species (Fig. 2B), whereas this TRF at the transcript level was variable (61%, 33%, and 2% relative fluorescences for *A. caliginosa*, *L. terrestris*, and *L. rubellus*, respectively) (Fig. 4B). In contrast, gut-derived TRF 52/54 bp (of which OTUs 1, 3, and 11 contained the highest numbers of sequences [see Table S3]) was less significant at the gene level (3 to 12% relative fluorescences) than at the transcript level (15%, 28%, and 60% relative fluorescences for *A. caliginosa*, *L. terrestris*, and *L. rubellus*, respectively). Gut-derived TRF 47 bp (of which OTU 3 contained the highest number of sequences [see Table S3]) yielded higher relative fluorescences at the transcript level (10 to 24% relative fluorescences) than at the gene level (0 to 13% relative fluorescences). TRF 258 bp (OTU 23) was detected only in gut-derived transcripts from *L. rubellus* (10% relative fluorescence) and *L. terrestris* (1% relative fluorescence). Although minimal differences occurred between crop/gizzard- and gut-derived gene level TRFs obtained from *L. terrestris* and *A. caliginosa*, the relative fluorescence of TRF 47 bp was higher for that derived from crop/gizzard (Fig. 2B and 3C). The collective data indicate that TRF patterns of *nosZ* and *nosZ* transcripts are distinct and that gut-derived TRF patterns from *L. rubellus* are different from those from the other earthworm species (Fig. 3C).

The two soil samples were similar at the gene level but differed at the transcript level, especially in the case of TRF 47 bp, which yielded a high relative fluorescence (37%) in mineral soil, and TRF 52/54 bp, which yielded a high relative fluorescence (31%) in the uppermost soil (Fig. 2B and 4B). *nosZ* transcript profiles of both soils were more similar to those from the guts of *A. caliginosa* and *L. terrestris* than to the *nosZ* transcript profile from the gut of *L. rubellus* (Fig. 3C).

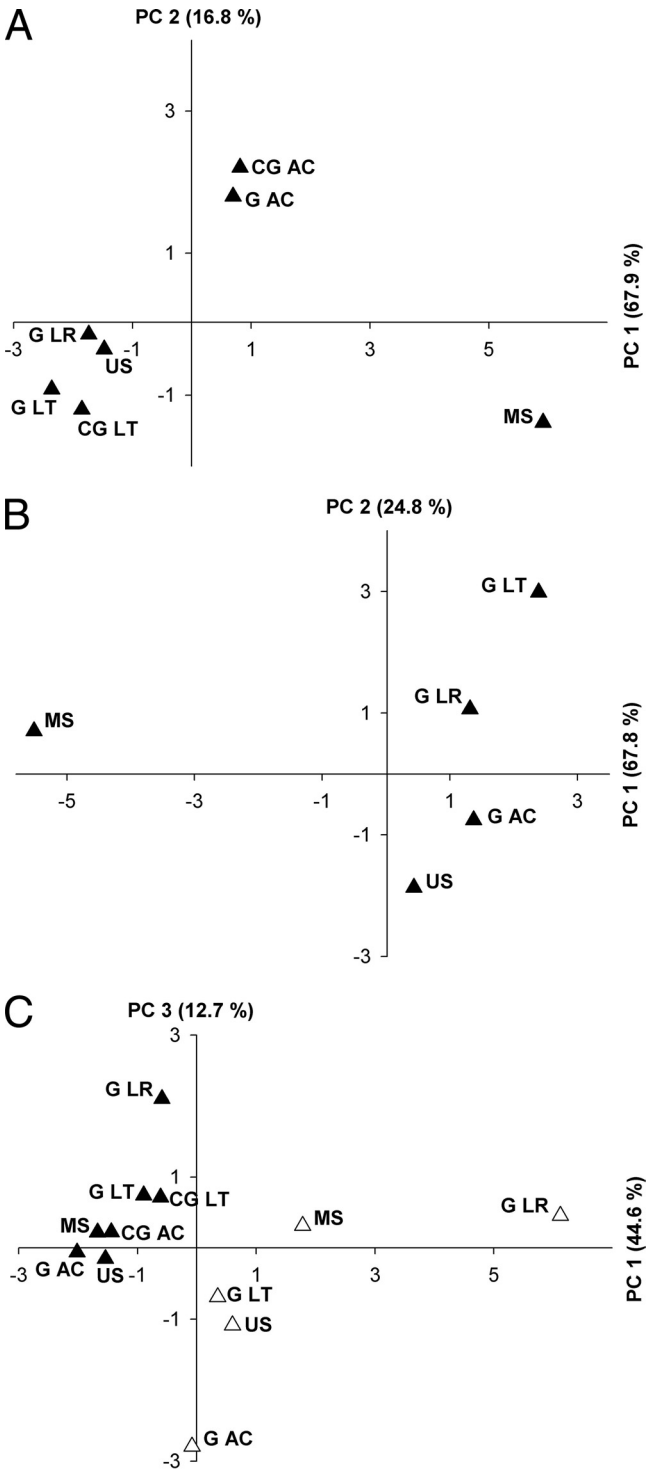


FIG. 3. Principal-component analyses of TRFs. (A) *narG* TRFs (digestion was with BanI). A variance of 84.7% is covered by the *x* (67.9%) and *y* (16.8%) axes. (B) *narG* transcript TRFs (digestion was with MaeIII). A variance of 92.6% is covered by the *x* (67.8%) and *y* (24.8%) axes. (C) *nosZ* TRFs (filled symbols) and *nosZ* transcript TRFs (open symbols). Digestion was with HhaI. A variance of 57.3% is covered by the *x* (44.6%) and *y* (12.7%) axes. Abbreviations: G, gut; CG, crop/gizzard; AC, *A. caliginosa*; LT, *L. terrestris*; LR, *L. rubellus*; MS, mineral soil; US, uppermost soil.

DISCUSSION

Guts of the earthworm species evaluated in this study have high capacities to reduce nitrate to nitrite and nitrogenous gases (29, 31, 32, 34, 42, 75, 76). The detection of *narG* and *nosZ* transcripts in earthworm alimentary canals (Fig. 1 and 5) corroborate these capacities, and the phylogenic affiliations of the detected transcripts suggest that dissimilatory nitrate reducers and denitrifiers are active in the earthworm gut.

Selective stimulation. *narG* and *nosZ* transcripts were different and less diverse in the earthworm gut than in mineral soil (Fig. 3, 4), suggesting that ingested *narG*- and *nosZ*-containing taxa were not uniformly stimulated by the *in situ* conditions of the earthworm gut. Detected community compositions can differ between the earthworm gut and preingested soil (19, 36), and bacteria isolated from earthworm casts can be more capable of reducing nitrate than bacteria isolated from bulk soil (24). Based on these collective observations together with the assumed soil-derived origin of *narG*- and *nosZ*-containing taxa detected in the present and previous studies (30, 76), one might speculate that a selective activation of ingested microbiota rather than a substantial *in situ* growth of ingested microbiota occurs during gut passage and that this activation might enhance the cultivability of certain microorganisms (16, 32, 33, 34). Although this speculation is reinforced by the fact that total cell counts increase only marginally if at all during gut passage (60, 61, 74), this speculation must be qualified, as certain ingested taxa (e.g., large pseudomonads [60, 61, 74]) are subject to degradation during gut passage. Thus, since certain ingested taxa decrease in number due to degradation, it seems likely that other ingested taxa are subject to at least a minimum amount of replication during gut passage. Otherwise, cell numbers of ingested material would decrease during gut passage. Anoxia and nitrate induce denitrification by model denitrifiers (e.g., *P. stutzeri*, *Ralstonia* [renamed *Wautersia*] *eutropha*, and *B. japonicum* [2, 49, 72]) and also regulate the dissimilatory reduction of nitrate to nitrite by *E. coli* (4). Thus, a potential stimulation of microbes capable of reducing nitrate is likely linked to the *in situ* conditions of the earthworm alimentary canal, conditions that include anoxia, large amounts of nitrate and nitrite, high moisture content, a nearly neutral pH, and high-quality organic compounds such as sugars and amino acids (13, 15, 16, 29, 33, 75).

Phylogeny of *narG*. *narG* and *narG* transcripts are related to those of actinobacterial and proteobacterial taxa common to soils (Fig. 1, 2A, and 4A). Indeed, *narG* sequences related to those detected in this study have been retrieved from soil (11, 14, 20, 49). Species of detected genera have been cultured from earthworm gut contents and casts (9, 24, 28, 32, 37, 69). Most *narG* transcripts retrieved from earthworm guts were affiliated with *Mycobacterium* (Fig. 1 and 4A). *Mycobacterium*-related *narG* sequences have been retrieved from soil (14, 49), species of *Mycobacterium* can reduce nitrate to nitrite (73), and mycobacterial species and other *Actinobacteria* occur in earthworm guts and casts and might be associated with gut walls (9, 23, 24, 37, 69). Furthermore, the percentages of *Actinobacteria*-related 16S rRNA genes can be higher in earthworm gut and cast than in soil (24, 47), accentuating the likelihood that certain taxa are stimulated during gut passage. *Mycobacterium tuberculosis* reduces nitrate to nitrite via a *narG*-containing

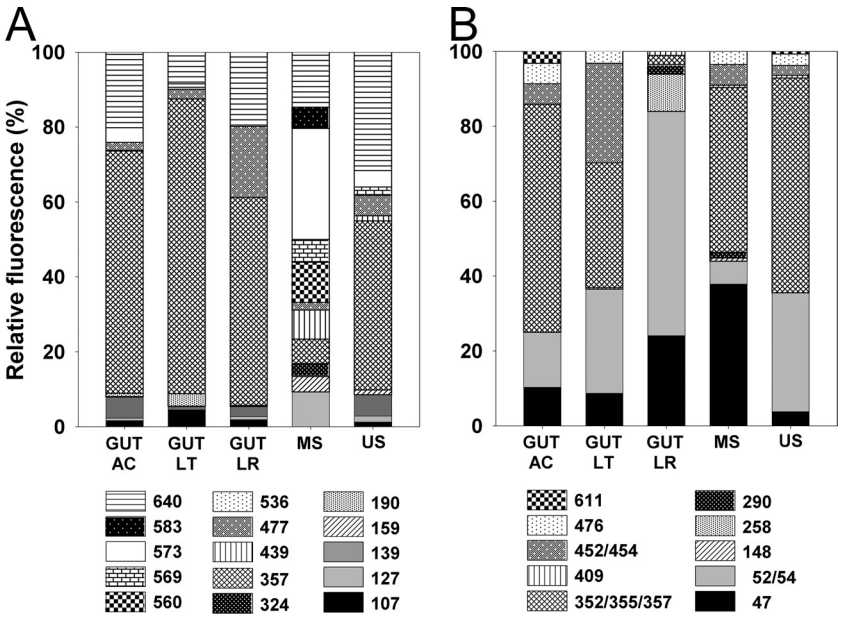


FIG. 4. TRFLP analysis of earthworm alimentary canal- and soil-derived *narG* transcripts and *nosZ* transcripts. (A) *narG* (digestion was with MaeIII). (B) *nosZ* (digestion was with HhaI). Abbreviations: CG, crop/gizzard; AC, *A. caliginosa*; LT, *L. terrestris*; LR, *L. rubellus*; MS, mineral soil; US, uppermost soil. The legend shows the lengths of the TRFs. Shown are mean values ($n = 3$).

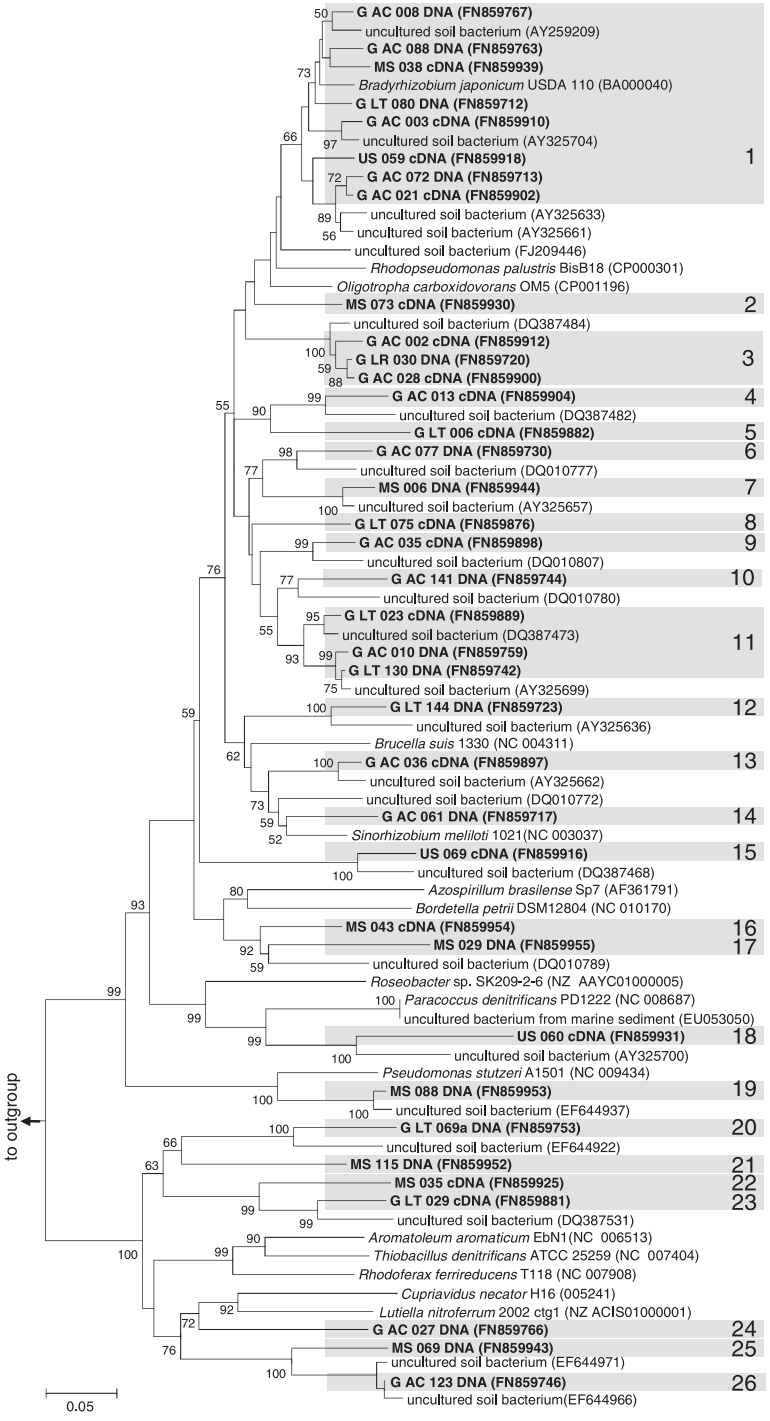
nitrate reductase that is constitutive, i.e., expressed during aerobic growth in the absence of nitrate or nitrite (63, 73). The *narGHJI* operon of *M. tuberculosis* mediates the assimilatory reduction of nitrate (41), but the nitrate reductase encoded by this operon might also dissimilate nitrate (63, 73). The detection of *Mycobacterium*-related *narG* transcripts in the earthworm gut and wet, uppermost soil suggests that the encoded nitrate reductase is important for the dissimilation and/or assimilation of nitrate. *narG* transcripts retrieved from N_2O-emitting *Chironomus plumosus* larvae are affiliated with Gram-negative bacteria rather than *Mycobacterium* (65).

Actinobacterial *narG*s and *narG* transcripts were dominant in earthworm guts (Fig. 1, 2, and 4; see also Tables S1 and S2 in the supplemental material). Many *Actinobacteria* reduce nitrate, but very few known actinobacterial species are capable of denitrification (21, 25, 38, 62). Thus, *Actinobacteria* likely compete with gammaproteobacterial denitrifiers for nitrate in the earthworm gut and might thereby affect *in vivo* emission of nitrogenous gases.

Phylogeny of *nosZ*. *nosZ* and *nosZ* transcripts affiliated mostly with denitrifying proteobacterial genera common to soil (e.g., *Bradyrhizobium* and *Pseudomonas*) (Fig. 5) (77). *nosZ* sequences related to these genera have been retrieved from soils (20, 30, 52, 66, 76) and earthworm guts (30, 76). Most *nosZ* sequences and *nosZ* TRFs from the earthworm gut were closely affiliated with *B. japonicum* USDA 110 (Fig. 2B, 4B, and 5), a facultative soil denitrifier that can form symbiotic dinitrogen-fixing associations with soybean roots (2). *B. japonicum* lacks *narG* and reduces nitrate via a less oxygen-sensitive *nap*-encoded nitrate reductase (2, 44). *B. japonicum*-related *nosZ* sequences have been retrieved from soil (20, 30, 52, 66, 76) and the earthworm gut (30, 76). In addition, *Bradyrhizobium*-related 16S rRNA gene sequences have been retrieved from the earthworm gut wall (69). Thus, *Bradyrhizobium*-related species might be important members of the transient denitrifying community in the earthworm gut. The capacity of *B. japonicum* to utilize atmospheric N_2O (i.e., 0.34 ppm of N_2O) (57) demonstrates that related species might be capable of not only the production but also the efficient consumption of N_2O.

Effect of earthworm feeding guilds. *A. caliginosa* consumes large amounts of mineral soil (17). That some *narG* TRFs in the gut of *A. caliginosa* were also present in mineral soil but not apparent in the gut of *L. terrestris* or *L. rubellus* is consistent with the tendency of the latter two species to not consume large amounts of mineral soil. *nosZ*-related community composition also varied among the three feeding guilds (Fig. 2B, 3C, and 4B). Feeding guilds of earthworms can influence 16S rRNA gene diversity of gut biota (19, 36, 37, 69), underscoring the importance that feeding guilds might have on the composition of *narG*- and *nosZ*-containing taxa in the earthworm gut. As noted above, the disruption of large bacteria during gut passage might contribute to the selective occurrence of smaller ingested bacteria in the gut (8, 60, 61, 74,). Fluids that are toxic to certain bacteria are released into the gut lumen of *A. caliginosa* (10, 35). Such toxic fluids might be dependent on the feeding guild and contribute to the differences detected in the present study. Thus, the factors that might contribute to potential feeding guild-dependent differences in gut biota are complex.

It should be noted that the *narG* and *nosZ* primers used in the current study have limitations relative to resolving nitrate-reducing and denitrifying taxa. For example, *napA* encodes a subunit of a nitrate reductase that is widely spread among bacteria but is not targeted by *narG* primers (45, 53). In addition, nitrous oxide reductases of Gram-positive denitrifiers are not covered by the *nosZ* primers. The constraints of these considerations notwithstanding, the collective findings of the



	No. of sequences										
	DNA					cDNA					
	Earthworms			Soils		Earthworms			Soils		
	AC	LT	LR	MS	US	AC	LT	LR	MS	US	Sum
1	18	20	3	4	3	7	1	3	9	3	71
2	0	0	0	0	0	1	0	0	3	0	4
3	0	0	2	0	2	7	3	4	1	1	20
4	0	0	0	0	0	3	0	0	0	0	3
5	0	0	0	0	0	0	1	0	0	0	1
6	1	0	0	0	0	0	0	0	0	0	1
7	0	1	0	2	0	0	0	0	0	0	3
8	0	0	0	0	0	0	1	0	0	0	1
9	0	0	0	0	0	1	0	0	0	0	1
10	1	1	0	0	0	0	1	0	0	0	3
11	5	4	1	1	0	2	1	0	1	1	16
12	1	1	0	0	0	0	0	0	1	0	3
13	0	0	0	0	0	2	0	0	0	1	3
14	1	0	0	0	0	0	0	0	0	0	1
15	0	0	0	0	0	0	0	0	0	1	1
16	0	0	0	0	0	0	0	0	1	0	1
17	0	0	0	1	0	0	0	0	0	0	1
18	0	0	0	0	0	0	0	0	0	1	1
19	0	0	0	2	0	0	0	0	0	1	3
20	1	2	0	0	0	0	0	0	1	0	4
21	0	0	0	1	0	0	0	0	0	0	1
22	0	0	0	0	0	0	0	0	1	0	1
23	0	0	0	0	0	0	1	0	0	0	1
24	2	0	0	0	0	0	0	0	0	0	2
25	2	0	0	1	0	0	0	0	0	0	3
26	1	2	0	2	0	0	0	0	2	0	7
	33	31	6	14	5	23	9	7	20	9	157

FIG. 5. Phylogenetic tree of representative *nosZ* sequences obtained from earthworms and soil and the related *nosZ* sequences. Sequences obtained in this study are in bold, and accession numbers are in parentheses. The tree is based on 212 translated amino acids. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) are shown next to the branches (values below 50% are masked) (22). The table shows the origin of sequences in each cluster (shaded text) as calculated with DOTUR 1.53. The outgroup was *Haloarcula marismortui* ATCC 43049 (AY5962197). Abbreviations: G, gut; AC, *A. caliginosa*; LT, *L. terrestris*; LR, *L. rubellus*; MS, mineral soil; US, uppermost soil.

present study indicate that the dissimilatory nitrate reducers and denitrifiers in the earthworm gut are largely soil derived and that these soil-derived functional groups are subject to a postingestion stimulation that is influenced by the earthworm feeding guild. These general conclusions must nonetheless be weighed against the fact that only a single species was evaluated per feeding guild, thus pointing toward the need to extend these observations to other earthworm species. The detection

of *narG* and *nosZ* transcripts that were closely related to sequences of uncultured soil bacteria (Fig. 1 and 5) suggest that novel uncultured *narG*- and *nosZ*-containing taxa contribute to the capacity of ingested soil biota to reduce nitrate and produce nitrogenous gases during gut passage, likewise emphasizing the need to bring such organisms into culture so that their response to the *in situ* conditions of the gut can be assessed at the cellular level.

ACKNOWLEDGMENTS

Support for this study was provided by the Deutsche Forschungsgemeinschaft (DR310/4-1) and the University of Bayreuth.

REFERENCES

1. **Aira, M., F. Monroy, and J. Dominguez.** 2009. Changes in bacterial numbers and microbial activity of pig slurry during transit of epigeic and anecic earthworms. J. Hazard. Mater. **162:**1404–1407.
2. **Bedmar, E. J., E. F. Robles, and M. J. Delgado.** 2005. The complete denitrification pathway of the symbiotic, nitrogen-fixing bacterium *Bradyrhizobium japonicum*. Biochem. Soc. Trans. **33:**141–144.
3. **Bertora, C., P. C. J. van Vliet, E. W. J. Hummelink, and J. W. van Groenigen.** 2007. Do earthworms increase N_2O emissions in ploughed grassland? Soil Biol. Biochem. **39:**632–640.
4. **Bonnefoy, V., and J. A. DeMoss.** 1994. Nitrate reductases in *Escherichia coli*. Antonie Van Leeuwenhoek **66:4**7–56.
5. **Borken, W., S. Grundel, and F. Beese.** 2000. Potential contribution of *Lumbricus terrestris* L. to carbon dioxide, methane and nitrous oxide fluxes from a forest soil. Biol. Fertil. Soils **32:**142–148.
6. **Bouché, M. B.** 1977. Lumbricid strategies, p. 122–132. *In* U. Lohm and T. Persson (ed.), Soil organisms as components of ecosystems. Ecological Bulletins, vol. 25. Oikos Editorial Office, Lund University, Lund, Sweden.
7. **Brohmer, P.** 1984. Fauna von Deutschland, 16th ed. Quelle and Meyer, Heidelberg, Germany.
8. **Brown, G. G.** 1995. How do earthworms affect microfloral and faunal community diversity? Plant Soil **170:**209–231.
9. **Byzov, B. A., T. Y. Nechtitaylo, B. K. Bumazhkin, A. V. Kurakov, P. N. Golyshin, and D. G. Zvyagintsev.** 2009. Culturable microorganisms from the earthworm digestive tract. Microbiology **78:**360–368.
10. **Byzov, B. A., N. V. Khomyakov, S. A. Kharin, and A. V. Kurakov.** 2007. Fate of soil bacteria and fungi in the gut of earthworms. Eur. J. Soil Biol. **43:**149–156.
11. **Chèneby, D., S. Hallet, M. Mondon, F. Martin-Laurent, J. C. Germon, and L. Philippot.** 2003. Genetic characterization of the citrate reducing community based on *narG* nucleotide sequence analysis. Microb. Ecol. **46:**113–121.
12. **Conrad, R.** 1996. Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). Microbiol. Rev. **60:**609–640.
13. **Daniel, O., and J. M. Anderson.** 1992. Microbial biomass and activity in contrasting soil materials after passage through the gut of the earthworm *Lumbricus rubellus* Hoffmeister. Soil Biol. Biochem. **24:**465–470.
14. **Deiglmayr, K., L. Philippot, D. Tscherko, and E. Kandeler.** 2006. Microbial succession of nitrate-reducing bacteria in the rhizosphere of *Poa alpina* across a glacier foreland in the Central Alps. Environ. Microbiol. **8:**1600–1612.
15. **Drake, H. L., and M. A. Horn.** 2006. Earthworms as a transient heaven for terrestrial denitrifying microbes: a review. Eng. Life Sci. **6:**261–265.
16. **Drake, H. L., and M. A. Horn.** 2007. As the worm turns: the earthworm gut as a transient habitat for soil microbial biomes. Annu. Rev. Microbiol. **61:**169–189.
17. **Edwards, C. A., and P. J. Bohlen.** 1996. Biology and ecology of earthworms, 3rd ed. Chapman & Hall, London, United Kingdom.
18. **Egert, M., and M. W. Friedrich.** 2003. Formation of pseudo-terminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. Appl. Environ. Microbiol. **69:**2555–2562.
19. **Egert, M., S. Marhan, B. Wagner, S. Scheu, and M. W. Friedrich.** 2004. Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris* L. (Oligochaeta: Lumbricidae). FEMS Microbiol. Ecol. **48:**187–197.
20. **Enwall, K., L. Philippot, and S. Hallin.** 2005. Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization. Appl. Environ. Microbiol. **71:**8335–8343.
21. **Eschbach, M., H. Möbitz, A. Rompf, and D. Jahn.** 2003. Members of the genus *Arthrobacter* grow anaerobically using nitrate ammonification and fermentation processes: anaerobic adaption of aerobic bacteria abundant in soil. FEMS Microbiol. Lett. **223:**227–230.
22. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
23. **Fischer, O. A., L. Matlova, J. Bart. L. Dvorska, P. Svastova, P. du Maine, I. Melicharek, M. Bartos, and I. Pavlik.** 2003. Earthworms (Oligochaeta, Lumbricidae) and mycobacteria. Vet. Microbiol. **91:**325–338.
24. **Furlong, M. A., D. R. Singleton, D. C. Coleman, and W. B. Whitman.** 2002. Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm *Lumbricus rubellus*. Appl. Environ. Microbiol. **68:**1265–1279.
25. **Goodfellow, M.** 1992. The family Nocardiaceae, p. 1188–1213. *In* A. Balows, H. Trüper, M. Dworkin, W. Harer, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York, NY.
26. **Gregory, L. G., P. L. Bond, D. J. Richardson, and S. Spiro.** 2003. Characterization of a nitrate-respiring bacterial community using the nitrate reductase gene (*narG*) as a functional marker. Microbiology **149:**229–237.
27. **Griffiths, R. I., A. S. Whiteley, A. G. O'Donnell, and M. J. Bailey.** 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA-based microbial community composition. Appl. Environ. Microbiol. **66:**5488–5491.
28. **Horn, M. A., J. Ihssen, C. Matthies, A. Schramm, G. Acker, and H. L. Drake.** 2005. *Dechloromonas denitrificans* sp. nov., *Flavobacterium denitrificans* sp. nov., *Paenibacillus anaericanus* sp. nov., and *Paenibacillus terrae* strain MH72, N_2O-producing bacteria isolated from the gut of the earthworm *Aporrectodea caliginosa.* Int. J. Syst. Evol. Microbiol. **55:**1255–1265.
29. **Horn, M. A., A. Schramm, and H. L. Drake.** 2003. The earthworm gut: an ideal habitat for ingested N_2O-producing microorganisms. Appl. Environ. Microbiol. **69:**1662–1669.
30. **Horn, M. A., H. L. Drake, and A. Schramm.** 2006. Nitrous oxide reductase genes (*nosZ*) of denitrifying microbial populations in soil and the earthworm gut are phylogenetically similar. Appl. Environ. Microbiol. **72:**1019–1026.
31. **Horn, M. A., R. Mertel, M. Kästner, M. Gehre, and H. L. Drake.** 2006. In vivo emission of dinitrogen by earthworms via denitrifying bacteria in the gut. Appl. Environ. Microbiol. **72:**1013–1018.
32. **Ihssen, J., M. A. Horn, C. Matthies, A. Gößner, A. Schramm, and H. L. Drake.** 2003. N_2O-producing microorganisms in the gut of the earthworm *Aporrectodea caliginosa* are indicative of ingested soil bacteria. Appl. Environ. Microbiol. **69:**1655–1661.
33. **Karsten, G., and H. L. Drake.** 1995. Comparative assessment of the aerobic and anaerobic microfloras of earthworm guts and forest soils. Appl. Environ. Microbiol. **61:**1039–1044.
34. **Karsten, G. R., and H. L. Drake.** 1997. Denitrifying bacteria in the earthworm gastrointestinal tract and in vivo emission of nitrous oxide (N_2O) by earthworms. Appl. Environ. Microbiol. **63:**1878–1882.
35. **Khomyakov, N. V., S. A. Kharin, T. Y. Nechitailo, P. N. Golyshin, A. V. Kurakov, B. A. Byzov, and D. G. Zvyagintsev.** 2007. Reaction of microorganisms to the digestive fluid of earthworms. Microbiology **76:**45–54.
36. **Knapp, B. A., J. Seeber, S. M. Podmirseg, E. Meyer, and H. Insam.** 2008. Application of denaturing gradient gel electrophoresis for analysing the gut microflora of *Lumbricus rubellus* Hoffmeister under different feeding conditions. Bull. Entomol. Res. **98:**271–279.
37. **Knapp, B. A., S. M. Podmirseg, J. Seeber, E. Meyer, and H. Insam.** 2009. Diet-related composition of gut microbiota of *Lumbricus rubellus* as revealed by a molecular fingerprinting technique and cloning. Soil Biol. Biochem. **41:**2299–2307.
38. **Kocur, M., W. E. Kloos, and K.-H. Schleifer.** 1992. The genus *Micrococcus*, p. 1301–1311. *In* A. Balows, H. Trüper, M. Dworkin, W. Harer, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York, NY.
39. **Krištůfek, V., K. Ravasz, and V. Pizl.** 1992. Changes in densities of bacteria and microfungi during gut transit in *Lumbricus rubellus* and *Aporrectodea caliginosa* (Oligochaeta, Lumbricidae). Soil Biol. Biochem. **24:**1499–1500.
40. **Lubbers, I. M., L. Brussaard, W. Otten, and J. W. van Groenigen.** Earthworm-induced N mineralization in fertilized grassland increases both N_2O emission and crop-N uptake. Eur. J. Soil Sci., in press.
41. **Malm, S., Y. Tiffert, J. Micklinghoff, S. Schultze, I. Joost, I. Weber, S. Horst, B. Ackermann, M. Schmidt, W. Wohlleben, S. Ehlers, R. Geffers, J. Reuther, and F.-C. Bange.** 2009. The roles of the nitrate reductase NarGHJI, the nitrite reductase NirBD and the response regulator GlnR in nitrate assimilation of *Mycobacterium tuberculosis*. Microbiology **155:**1332–1339.
42. **Matthies, C., A. Griesshammer, M. Schmittroth, and H. L. Drake.** 1999. Evidence for involvement of gut-associated denitrifying bacteria in emission of nitrous oxide (N_2O) by earthworms obtained from garden and forest soils. Appl. Environ. Microbiol. **65:**3599–3604.
43. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
44. **Moreno-Vivián, C., P. Cabello, M. Martínez-Luque, R. Blasco, and F. Castillo.** 1999. Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. J. Bacteriol. **181:**6573–6584.
45. **Morozkina, E. V., and R. A. Zvyagilskaya.** 2007. Nitrate reductases: structure, functions, and effect of stress factors. Biochemistry (Mosc.) **72:**1151–1160.
46. **Mounier, E., S. Hallet, D. Cheneby, E. Benizri, Y. Gruet, C. Nguyen, S. Piutti, C. Robin, S. Slezack-Deschaumes, F. Martin-Laurent, J. C. Germon,**

and L. Philippot. 2004. Influence of maize mucilage on the diversity and activity of the denitrifying community. Environ. Microbiol. **6:**301–312.
47. **Nechitaylo, T. Y., M. M. Yakimov, M. Godinho, K. N. Timmis, E. Belogolova, B. A. Byzov, A. V. Kurakov, D. L. Jones, and P. N. Golyshin.** 2010. Effect of the earthworm *Lumbricus terrestris* and *Aporrectodea caliginosa* on bacterial diversity in soil. Microb. Ecol. **59:**574–587.
48. **Palmer, K., H. L. Drake, and M. A. Horn.** 2009. Genome-derived criteria for assigning environmental *narG* and *nosZ* sequences to operational taxonomic units of nitrate reducers. Appl. Environ. Microbiol. **75:**5170–5174.
49. **Philippot, L., S. Piutti, F. Martin-Laurent, S. Hallet, and J. C. Germon.** 2002. Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. Appl. Environ. Microbiol. **68:**6121–6218.
50. **Philippot, L.** 2002. Denitrifying genes in bacterial and archaeal genomes. Biochim. Biophys. Acta **1577:**355–376.
51. **Pielou, E. C.** 1977. Mathematical ecology, p. 385. Wiley Interscience, New York, NY.
52. **Rich, J. J., R. S. Heichen, J. P. Bottomley, K. Cromack, and D. D. Myrold.** 2003. Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. Appl. Environ. Microbiol. **69:**5974–5982.
53. **Richardson, D. J., B. C. Berks. D. A. Russell, S. Spiro, and C. J. Taylor.** 2001. Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. Cell. Mol. Life Sci. **58:**165–178.
54. **Rizhiya, E., C. Bertora, P. C. J. van Vliet, P. J. Kuikman, J. H. Faber, and J. W. van Groenigen.** 2007. Earthworm activity as a determinant for N_2O emission from crop residue. Soil Biol. Biochem. **39:**2058–2069.
55. **Römbke, J., A. M. Breure, C. Mulder, and M. Rutgers.** 2005. Legislation and ecological quality assessment of soil: implementation of ecological indication systems in Europe. Ecotoxicol. Environ. Saf. **62:**201–210.
56. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
57. **Sameshima-Saito, R., K. Chiba, J. Hirayama, M. Itakura, H. Mitsui, S. Eda, and K. Minamisawa.** 2006. Symbiotic *Bradyrhizobium japonicum* reduces N_2O surrounding the soybean root system via nitrous oxide reductase. Appl. Environ. Microbiol. **72:**2526–2532.
58. **Scala, D. J., and L. J. Kerkhof.** 1998. Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments. FEMS Microbiol. Lett. **162:**61–68.
59. **Schloss, P. D., and J. Handelsman.** 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. **71:**1501–1506.
60. **Schönholzer, F., D. Hahn, and J. Zeyer.** 1999. Origins and fate of fungi and bacteria in the gut of *Lumbricus terrestris* L. studied by image analysis. FEMS Microbiol. Ecol. **28:**235–248.
61. **Schönholzer, F., D. Hahn, B. Zarda, and J. Zeyer.** 2002. Automated image analysis and in situ hybridization as tools to study bacterial populations in food resources, gut and cast of *Lumbricus terrestris* L. J. Microbiol. Methods **48:**53–68.
62. **Shoun, H., M. Kano, I. Baba, N. Takaya, and M. Matsuo.** 1998. Denitrification by actinomycetes and purification of dissimilatory nitrate reductase and azurin from *Streptomyces thioluteus*. J. Bacteriol. **180:**4413–4415.
63. **Sohaskey, C. D., and L. G. Wayne.** 2003. Role of *narK2X* and *narGHJI* in hypoxic upregulation of nitrate reduction by *Mycobacterium tuberculosis*. J. Bacteriol. **185:**7247–7256.
64. **Stackebrandt, E., and J. Ebers.** 2006. Taxonomic parameters revisited: tarnished gold standards. Microbiol. Today **33:**152–155.
65. **Stief, P., M. Poulsen, L. P. Nielsen, H. Brix, and A. Schramm.** 2009. Nitrous oxide emission by aquatic macrofauna. Proc. Natl. Acad. Sci. U. S. A. **106:**4296–4300.
66. **Stres, B., I. Mahne, G. Augustin, and J. M. Tiedje.** 2004. Nitrous oxidoreductase (*nosZ*) gene fragments differ between native and cultivated Michigan soils. Appl. Environ. Microbiol. **70:**301–309.
67. **Stres, B., T. Danevcic, L. Pal, M. M. Fuka, L. Resman, S. Leskovec, J. Hacin, D. Stopar, I. Mahne, and I. Mandic-Mulec.** 2008. Influence of temperature and soil water content on bacterial, archaeal and denitrifying microbial communities in drained fen grassland soil microcosms. FEMS Microbiol. Ecol. **66:**110–122.
68. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.
69. **Thakuria, D., O. Schmidt, D. Finan, D. Egan, and F. M. Doohan.** 2010. Gut wall bacteria of earthworms: a natural selection process. ISME J. **4:**357–366.
70. **Tiedje, J. M.** 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium, p. 179–243. *In* A. J. B. Zehnder (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, New York, NY.
71. **Tiedje, J. M., A. J. Sexstone, D. D. Myrold, and J. A. Robinson.** 1982. Denitrification: ecological niches, competition and survival. Antonie Van Leeuwenhoek **48:**569–583.
72. **van Spanning, R. J. M., D. J. Richardson, and S. T. Ferguson.** 2007. Introduction to the biochemistry and molecular biology of denitrification, p. 3–20. *In* H. Bothe, S. J. Ferguson, and W. E. Newton (ed.), Biology of the nitrogen cycle, 1st edition. Elsevier, Amsterdam, Netherlands.
73. **Weber, I., C. Fritz, S. Ruttkowski, A. Kreft, and F.-C. Bange.** 2000. Anaerobic nitrate reductase (*narGHJI*) activity of *Mycobacterium bovis* BCG *in vitro* and its contribution to virulence in immunodeficient mice. Mol. Microbiol. **35:**1017–1025.
74. **Wolter, C., and S. Scheu.** 1999. Changes in bacterial numbers and hyphal length during the gut passage through *Lumbricus terrestris* (Lumbricidae, Oligochaeta). Pedobiologia **43:**891–900.
75. **Wüst, P. K., M. A. Horn, and H. L. Drake.** 2009. In situ hydrogen and nitrous oxide as indicators of concomitant fermentation and denitrification in the alimentary canal of the earthworm *Lumbricus terrestris*. Appl. Environ. Microbiol. **75:**1852–1859.
76. **Wüst, P. K., M. A. Horn, G. Henderson, P. H. Janssen, B. H. Rehm, and H. L. Drake.** 2009. Gut-associated denitrification and in vivo emission of nitrous oxide by the earthworm families Megascolecidae and Lumbricidae in New Zealand. Appl. Environ. Microbiol. **75:**3430–3436.
77. **Zumft, W. G.** 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. **61:**533–536.