APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2006, p. 1019–1026 Vol. 72, No. 2
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.2.1019–1026.2006

Nitrous Oxide Reductase Genes (*nosZ*) of Denitrifying Microbial Populations in Soil and the Earthworm Gut Are Phylogenetically Similar†

Marcus A. Horn,* Harold L. Drake, and Andreas Schramm‡

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

Received 15 September 2005/Accepted 8 November 2005

Earthworms emit nitrous oxide (N_2O) and dinitrogen (N_2). It has been hypothesized that the in situ conditions of the earthworm gut activates ingested soil denitrifiers during gut passage and leads to these in vivo emissions (M. A. Horn, A. Schramm, and H. L. Drake, Appl. Environ. Microbiol. 69:1662–1669, 2003). This hypothesis implies that the denitrifiers in the earthworm gut are not endemic to the gut but rather are regular members of the soil denitrifier population. To test this hypothesis, the denitrifier populations of gut and soil from three different sites were comparatively assessed by sequence analysis of *nosZ*, the gene for the terminal enzyme in denitrification, N_2O reductase. A total of 182 and 180 *nosZ* sequences were retrieved from gut and soil, respectively; coverage of gene libraries was 79 to 100%. Many of the *nosZ* sequences were heretofore unknown, clustered with known soil-derived sequences, or were related to N_2O reductases of the genera *Bradyrhizobium*, *Brucella*, *Dechloromonas*, *Flavobacterium*, *Pseudomonas*, *Ralstonia*, and *Sinorhizobium*. Although the numbers of estimators for genotype richness of sequence data from the gut were higher than those of soil, only one gut-derived *nosZ* sequence did not group phylogenetically with any of the soil-derived *nosZ* sequences. Thus, the phylogenies of *nosZ* from gut and soil were not dissimilar, indicating that gut denitrifiers are soil derived.

Denitrification is the reduction of nitrate or nitrite to nitrogenous gases (48) and can lead to the emission of nitrous oxide (N_2O) from soils (4, 11–14, 24). During complete denitrification, nitrate is reduced to dinitrogen (N_2) via the intermediates nitrite, nitric oxide (NO), and N_2O (48). The oxidoreductases that sequentially reduce nitrate to N_2 are termed nitrate reductase (encoded by the *narGHI* genes), nitrite reductase (encoded by *nirK* or *nirS*), NO reductase (encoded by *norBC*), and N_2O reductase (encoded by *nosZ*) (51). The genes of these enzymes have been used as molecular markers for the cultivation-independent analysis of denitrifiers in various environments, including soil (3, 32, 36–38, 45).

Earthworms emit N_2O (27, 31) and N_2 (21), and these in vivo emissions are coincident with a very abundant denitrifying population in the earthworm gut (25, 27, 31). The earthworm gut is anoxic and has a high content of water, sugars, organic and amino acids, nitrite, and ammonium, thus making it an ideal habitat for denitrifiers (22). The denitrifier population of the earthworm gut has been evaluated by cultivation methods (25, 27), and denitrifiers that occur in soil also occur in the earthworm gut (25). Analyses of 16S rRNA genes in earthworm guts and casts indicate that the microbiota in the earthworm gut is largely food derived rather than endemic (17, 18, 43). Based on these data, denitrifiers in the earthworm gut are hypothesized to be ingested soil microorganisms that are activated by the special microenvironment of the gut (22, 25). This hypothesis implies that denitrifiers in the earthworm gut are members of the soil microbial community and not endemic to the earthworm gut. If this hypothesis is valid, the species composition of denitrifiers in the earthworm gut should be equivalent to that of the soil. To test this hypothesis, the denitrifiers of soil and the earthworm gut were comparatively assessed by sequence analysis of *nosZ*.

MATERIALS AND METHODS

Field sites and sampling. Earthworms and soils were collected in September 2000 and May 2001 from a conventionally farmed field (site B), an organically farmed garden (site H), and a conventionally farmed meadow (site HW) near Bayreuth, Germany (22). Earthworms were identified by standard protocols (5) as *Aporrectodea caliginosa* (Savigny), *Allolobophora chlorotica* (Savigny), *Lumbricus terrestris* L., and *Lumbricus rubellus*. Worms were washed three times with sterile, double-distilled water, sedated, and surface sterilized with ethanol (70%) prior to extraction of gut contents by pressing worms from anterior to posterior with sterilized tweezers. Gut contents of worms from the same site were pooled to form samples of 1 g (fresh weight) each. Soil and gut contents were stored at −80°C until processed.

Denitrifiers from earthworm gut. *Dechloromonas denitrificans* (DSM 15892^T), *Flavobacterium denitrificans* (DSM 15936^T), and *Pseudomonas* sp. strain ED3 (GenBank accession number AJ318919) were cultured as previously described (20, 25) (The superscript T indicates a type strain; the 16S rRNA gene sequence of strain ED3 is 99.9% identical to that of *Pseudomonas fluorescens* [ATCC 17428]).

Extraction of nucleic acids. DNA was isolated from soil, gut contents, and denitrifiers using the FastDNA SPIN kit (BIO 101, Carlsbad, CA) according to the manufacturer's protocol, with the following modifications to minimize shearing of large DNA fragments: bead beating was omitted, and samples (each, 1 g [fresh weight]) were suspended in 500 μl lysis buffer (150 mM NaCl, 100 mM EDTA, 10 mg lysozyme ml^{-1}, pH 8.0) and incubated for 30 min at 37°C in a thermomixer (model 5436; Eppendorf, Hamburg, Germany). Samples were supplemented with 60 μg of proteinase K, incubated 30 min at 37°C, and then

* Corresponding author. Mailing address: Department of Ecological Microbiology, University of Bayreuth, Dr.-Hans-Frisch-Strasse 1-3, 95440 Bayreuth, Germany. Phone: (49) (0)921-555 642. Fax: (49) (0)921-555 793. E-mail: marcus.horn@uni-bayreuth.de.

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

‡ Present address: Department of Microbiology, University of Aarhus, 8000 Aarhus C, Denmark.

subjected to three freeze-thaw cycles (−80°C for 10 min and 60°C for 1 min). Chemical lysis yielded insufficient amounts of DNA from the soil of site HW; thus, bead beating (BIO 101 protocol) was used for soil from this site.

Primers and PCR conditions. Fragments of *nosZ* genes were amplified using primers nosZ661F (CGG CTG GGG GCT GAC CAA) and nosZ1773R (ATR TCG ATC ARC TGB TCG TT) (38). Primers PsNosZ175F (TTC ATC AAC GAC AAG GCC) and PsNosZ1144R (CGG TGG GCA GGA AGC GGT) were designed with ARB software (http://www.arb-home.de) (46) for the selective amplification of *Pseudomonas*-related *nosZ* fragments.

Primer specificity was evaluated in silico using GenBank's BLAST tool (1). PCR conditions were tested with genomic DNA from *Pseudomonas aeruginosa* (DSM 6195^T). Each amplification reaction was performed in a total volume of 50 μl and contained 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.3 mg of bovine serum albumin ml^{-1}, 10 μl of TaqM, 200 μM nucleotides, 20 pmol of each primer, 1 μl of template DNA, and 1 U of *Taq* polymerase (Eppendorf, Hamburg, Germany). Touchdown PCR was performed with a T-Gradient cycler (Biometra, Göttingen, Germany) with 5 min of denaturation at 94°C, followed by 8 (10) cycles, each consisting of 94°C for 1 min, primer annealing at 60 to 56°C (63 to 58°C) for 1 min, and extension at 72°C for 3 min; this was followed by 27 cycles with annealing at 56°C (58°C) (numbers of cycles and temperatures given in parentheses refer to amplifications performed with the primer pair PsNosZ175F-PsNosZ1144R). The final PCR extension step was at 72°C for 5 min.

Cloning, screening, and sequencing. PCR products of denitrifying earthworm gut isolates were purified by cutting out the band of expected size (1.1 kb) from a low-melting-point agarose gel (2%) with a subsequent extraction using the MiniElute gel extraction kit (QIAGEN, Hilden, Germany). PCR products of all samples were ligated into pGEM-T (Promega, Mannheim, Germany) and transformed into *Escherichia coli* JM109 (Promega) according to the manufacturer's protocol. For screening of the *nosZ* libraries, *nosZ* fragments were directly amplified from 1 μl of resuspended clones and electrophoresed on agarose gels (1%); those of the expected size (1.1 kb) were analyzed. The gut and soil clone libraries were screened by restriction fragment length polymorphism (RFLP); *nosZ* fragments were digested simultaneously with 2 U of HaeIII and RsaI (Promega) for 2 h at 37°C prior to separation on an 8% polyacrylamide gel (DCode; Bio-Rad, Richmond, CA) for 4 h at 120 V. One to 30 representative clones of each RFLP pattern per library were selected for sequencing. For the clone libraries from the denitrifying earthworm gut isolates, two clones of each library were selected for sequencing without prior RFLP analysis. The Wizard Plus Minipreps DNA Purification system (Promega, Mannheim, Germany) was used for the preparation of plasmids, and DNA was sequenced commercially at MWG Biotech (Ebersberg, Germany).

***nosZ* sequence analysis.** Sequence analysis was performed with the ARB software package (http://www.arb-home.de) (46). *nosZ* sequences and publicly available sequences overlapping in the same region were translated in silico and prealigned with the ClustalW algorithm, and the alignment was refined manually. Regions of primer binding sites were excluded from further sequence analysis. Neighbor-joining, parsimony, and maximum-likelihood algorithms were applied to sequences that were longer than 350 amino acids; a consensus tree was drawn using consistent branchings from two to three methods, while inconsistent branches were drawn as multifurcations (30). Partial sequences (<350 amino acids) were added to the consensus tree by maximum-parsimony analysis without changing the tree topology. Unless otherwise indicated, all sequence analyses were based on the in silico-translated *nosZ* sequences.

Analysis of *nosZ* genotype diversity. Percent accepted mutations matrix (PAM)-corrected distance matrices were generated from aligned amino acid sequences with ARB (http://www.arb-home.de) (46). Partial nucleic acid sequences that shared >98% sequence similarity and sequences that had the same RFLP patterns with fully sequenced *nosZ* fragments were thereby represented by the fully sequenced *nosZ* fragments. DOTUR (http://www.plantpath.wisc.edu/fac/joh/dotur.html) (39) was applied to define genotypes, based on the amino acid sequence dissimilarity of proteins derived from translated *nosZ* fragments by the furthest-neighbor method. Analyses were performed with four arbitrary, predefined genotype definitions, i.e., sequences that had a maximal dissimilarity value (D) of ≤0.49, 3.49, 7.49, or 15.49% were combined into single *nosZ* genotypes. Additional information on DOTUR is available at the publisher's website (http://www.plantpath.wisc.edu/fac/joh/DOTUR/DOTURManual.pdf).

Coverage (C) (19), which is the number of the detected genotypes relative to their expected total number in a gene library, was calculated by S-Libshuff analysis as $C = 1 - yx^{-1}$ (40), where y is the number of genotypes that occurred only once, and x is the number of clones screened. The diversity of genotypes represented in soil and gut gene libraries was analyzed by rarefaction analysis (23). Genotype richness (S), i.e., the number of different genotypes present in a gene library, was estimated by an abundance-based coverage estimator (8, 9) and Bootstrap (44), bias-corrected Chao1 (7, 9), and interpolated Jackknife (6) estimators. All calculations were done with DOTUR (39).

TABLE 1. Summary of data from amino acid analyses of in silico-translated *nosZ* fragments from denitrifier communities in agricultural soils and earthworm gut contents

Source[a]	n[b]	D[c] (%)	S[d]	C[e] (%)	Estimated no. of genotypes[f]			
					ACE	Boot	Chao	Jack
Site B								
Gut	48	0.49	21	79	40	26	30	32
		3.49	12	88	23	14	17	18
		7.49	8	98	9	9	8	9
		15.49	5	100	5	5	5	5
Soil	80	0.49	19	91	33	23	31	34
		3.49	10	96	17	12	12	14
		7.49	7	98	11	8	8	10
		15.49	4	100	4	4	4	4
Site H								
Gut	94	0.49	39	79	78	48	81	95
		3.49	24	84	66	30	84	104
		7.49	13	93	32	16	27	27
		15.49	7	98	12	8	8	10
Soil	48	0.49	22	81	32	27	28	32
		3.49	12	90	19	14	15	17
		7.49	7	94	10	7	10	13
		15.49	5	98	NA[g]	6	8	6
Site HW								
Gut	40	0.49	11	90	19	13	14	16
		3.49	7	93	22	9	12	12
		7.49	4	98	7	5	5	6
		15.49	3	100	4	3	3	4
Soil	52	0.49	11	88	21	13	26	38
		3.49	8	90	18	10	18	24
		7.49	6	98	8	7	7	8
		15.49	3	100	3	3	3	NA[g]

[a] Abbreviations of study sites are described in Materials and Methods.
[b] n, number of clones screened.
[c] D, maximal evolutionary distance (PAM) used for the assignment of genotypes with the furthest-neighbor algorithm.
[d] S, genotype richness.
[e] C, coverage (22); see Materials and Methods for details.
[f] Expressed as richness estimators; see Materials and Methods for details. Estimators: ACE, abundance-based coverage estimator; Boot, Bootstrap; Chao, bias-corrected Chao1; Jack, interpolated Jackknife.
[g] NA, not applicable.

S-Libshuff analyses (40, 42) were performed to determine the significance of differences between gene libraries of soil and those of the earthworm gut independent of an arbitrary, predefined genotype definition. For comparison of two gene libraries, Libshuff analyses plot coverage against the evolutionary distance of sequences for one gene library (homologous coverage, C_{Soil} or $C_{Gut} = 1 - y_{Soil}x_{Soil}^{-1}$ or $1 - y_{Gut}x_{Gut}^{-1}$, where the indices depict the gene library; see above for definitions of x and y) and for sequences of one gene library compared to the other (heterologous coverage, $C_{Soil/Gut}$ or $C_{Gut/Soil} = 1 - y_{Soil}x_{Gut}^{-1}$ or $1 - y_{Gut}x_{Soil}^{-1}$; see above). The coverage curves describe how well the sampling represents the entire library at various levels of evolutionary distance. Libshuff analysis calculates differences between homologous and heterologous coverage curves by a Cramer-von Mises-type statistic and compares them by a Monte Carlo test (42).

Accession numbers. All *nosZ* sequences have been deposited in the EMBL nucleotide sequences database under accession numbers AJ703894 to AJ704214 and AJ704930 to AJ704933.

RESULTS

Amplification and screening of *nosZ* fragments. High-molecular-weight genomic DNA (10 to 20 kb) was obtained from soil and earthworm gut content from three study sites and from

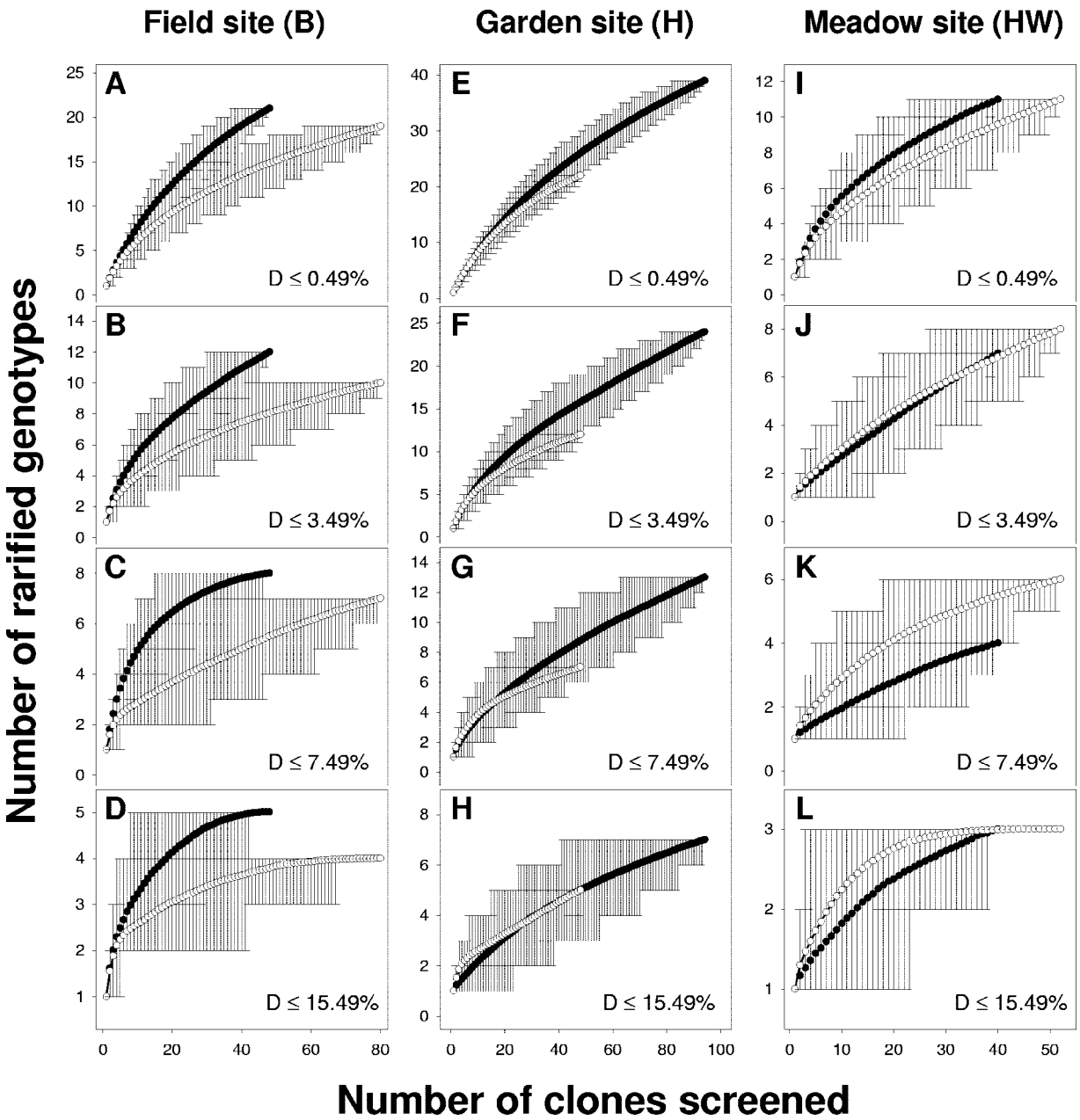
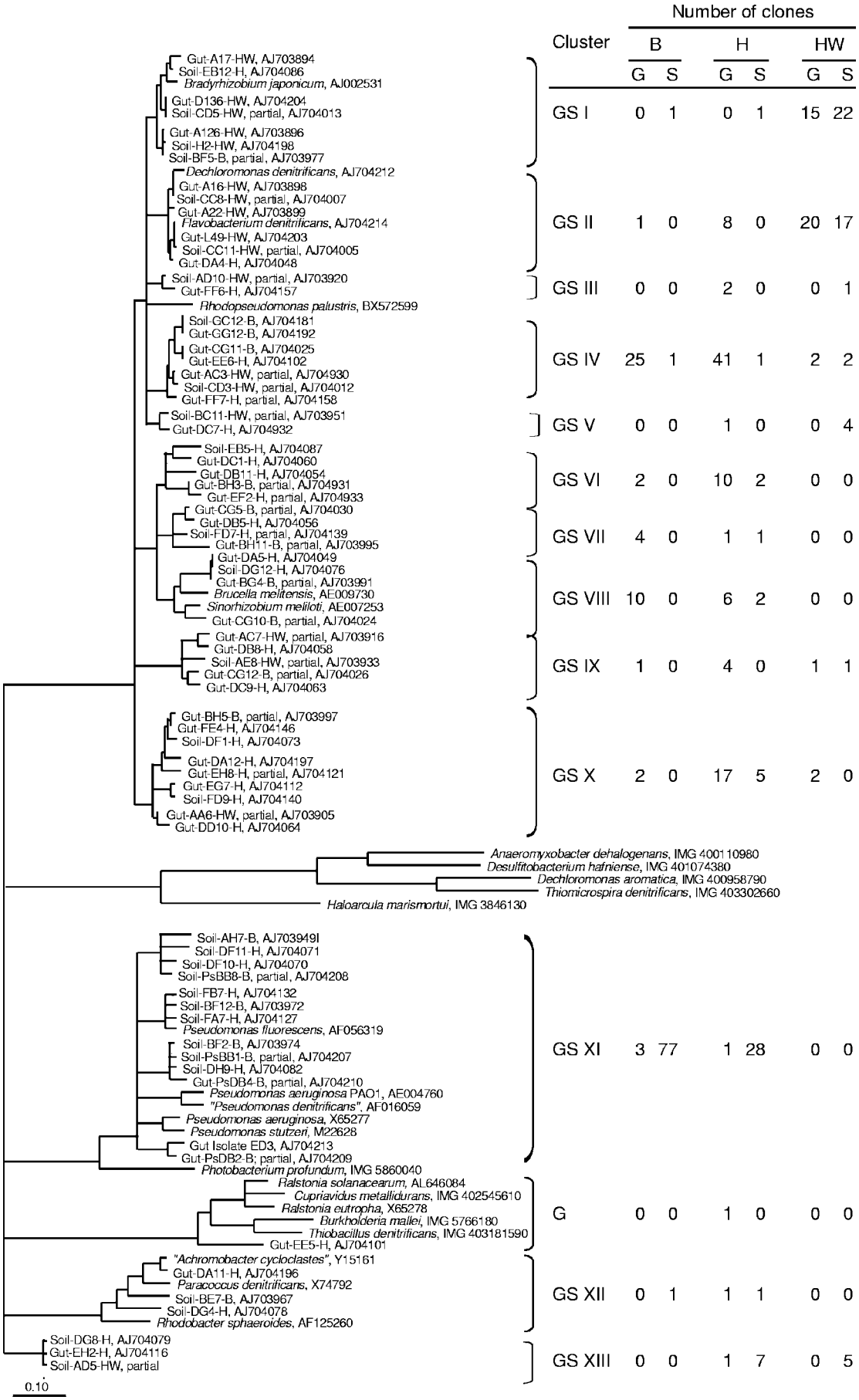


FIG. 1. Rarefaction curves with cloned *nosZ* fragments from the earthworm gut (●) and surrounding soil (○) for various evolutionary distances (*D*). Error bars represent 95% confidence intervals.

three denitrifying earthworm gut isolates, i.e., *D. denitrificans*, *F. denitrificans*, and *Pseudomonas* sp. strain ED3 (20, 25). *nosZ* gene fragments (1 to 1.1 kb) were successfully amplified from all DNA preparations. A total of 362 *nosZ*-positive clones (180 clones from soil and 182 clones from earthworm gut content) were screened by RFLP and sequenced (Table 1). The coverage varied from 79 to 100% (Table 1), indicating that the gene libraries were sufficiently sampled.

The highest DNA sequence similarity of representative *nosZ* sequences with different RFLP patterns was 99%, indicating that *nosZ* diversity was well resolved by the RFLP analysis. The gut- and soil-translated *nosZ* sequences contained 59 and 45 different genotypes at $D \leq 0.49\%$, respectively. The mean evolutionary distances among sequences from the field (B), garden (H), and meadow (HW) sites were 14.4% ± 9.0%, 12.1% ± 7.8%, and 7.0% ± 9.1%, respectively, indicating that the gut and soil sequences of site HW were more similar than those of sites B and H. Gene libraries from earthworm gut content and soil contained 40 to 94 and 48 to 80 *nosZ*-positive clones, respectively (Table 1). The number of genotypes depended on the threshold for genotype definition, i.e., it decreased with increasing evolutionary distance (0.49 to 15.49) used (Table 1). *nosZ* libraries of the garden (H) yielded the highest number of *nosZ* genotypes for soil and for gut, followed by the field (B) and



Cluster	Number of clones					
	B		H		HW	
	G	S	G	S	G	S
GS I	0	1	0	1	15	22
GS II	1	0	8	0	20	17
GS III	0	0	2	0	0	1
GS IV	25	1	41	1	2	2
GS V	0	0	1	0	0	4
GS VI	2	0	10	2	0	0
GS VII	4	0	1	1	0	0
GS VIII	10	0	6	2	0	0
GS IX	1	0	4	0	1	1
GS X	2	0	17	5	2	0
GS XI	3	77	1	28	0	0
G	0	0	1	0	0	0
GS XII	0	1	1	1	0	0
GS XIII	0	0	1	7	0	5

the meadow (HW). More *nosZ* genotypes were detected in gut than in soil libraries from sites B and H. The numbers of *nosZ* genotypes in gut and soil libraries from site HW were similar. Overall, the numbers of different genotypes detected in gut and soil libraries were similar.

Estimation of *nosZ* genotype richness in gene libraries. In most libraries, the estimated number of genotypes was similar to the number of genotypes found (Table 1). Estimated genotype richness was highest for the gut and soil libraries of the garden site (H), followed by the field site (B) and the meadow site (HW) (Table 1). Estimated genotype richness depended on the estimator used but was mostly higher for the gut libraries than for the soil libraries from sites B and H. The estimated genotype richness for the soil library of site HW was mostly higher than that of the corresponding gut library (Table 1). However, at evolutionary distances of 7.49 and 15.49%, differences were only marginal for all sites.

Rarefaction analysis. At evolutionary distance levels of $D \leq 0.49\%$, $\leq 3.49\%$, $\leq 7.49\%$, and $\leq 15.49\%$, most rarefaction curves of gut libraries were higher than those of corresponding soil libraries (Fig. 1A to G and I). Rarefaction curves of the gut and soil libraries from sites H and HW were essentially identical at $D \leq 15.49\%$ (Fig. 1H) and $D \leq 3.49$ (Fig. 1J), respectively. Rarefaction curves at $D \leq 7.49$ (Fig. 1K) and $D \leq 15.49\%$ (Fig. 1L) for libraries of site HW were higher for soil than for gut. However, differences between gut and soil rarefaction curves at all distance levels were not significant; confidence intervals overlapped. Confidence intervals of gut and soil curves tended to separate for site B only (Fig. 1A to D). The collective rarefaction analyses (Fig. 1) indicated that the *nosZ* diversities detected in soil and gut were similar.

S-Libshuff analysis. Two gene libraries are assumed to be different if their homologous and heterologous coverage curves differed significantly (42). For *nosZ* libraries of the field (B) and garden (H) sampling sites, C_{Gut} was significantly different from $C_{\text{Gut/Soil}}$ only at an evolutionary distance of $<12\%$ and 5%, respectively (see Table S1 and Fig. S1 in the supplemental material). In contrast, C_{Gut} was not significantly different from $C_{\text{Gut/Soil}}$ for the meadow site (HW) at all evolutionary distances (see Table S1 in the supplemental material), indicating that the detected *nosZ* genotypes in gut and soil were similar for site HW. Homologous and heterologous coverage curves differed significantly between soil *nosZ* libraries and between gut libraries from the three soils, indicating differences in the detected *nosZ* genotypes between the three sampling sites.

Phylogenetic analysis. *nosZ* phylogenetic trees that were generated from truncated (1.1-kb) and full-length (approximately 2-kb), in silico-translated *nosZ* sequences of pure cultures retrieved from the EMBL database (http://www.ebi.ac.uk) had congruent topologies (data not shown), indicating that the 1.1-kb *nosZ* fragments were sufficient for phylogenetic analyses. Thus, all sequences were truncated to 1.1 kb for the following analyses of the newly retrieved *nosZ* fragments.

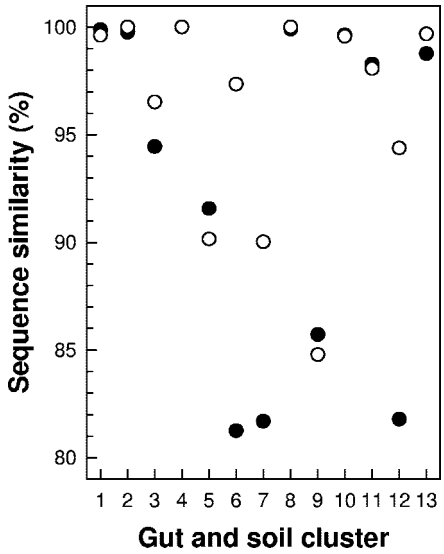


FIG. 3. Pairwise comparisons of *nosZ* (●) and in silico-translated protein (○) sequences from gut and soil. Only the highest values obtained within a cluster are shown. The similarity of a translated *nosZ* protein sequence is plotted as 1 − D (as a percentage), where *D* is the PAM-corrected evolutionary distance.

Translated *nosZ* sequences from soil and the earthworm gut did not cluster with marine sequences, but some sequences clustered with translated *nosZ* sequences derived from a Michigan soil (45; see Fig. S2 in the supplemental material), indicating that terrestrial denitrifier populations differed from those found in marine habitats. The analysis revealed 14 phylogenetically distinct *nosZ* clusters, 13 of which contained both gut- and soil-derived sequences (Fig. 2, GS clusters I to XIII). The mean evolutionary distance of translated *nosZ* sequences within a cluster was $6.3\% \pm 5.5\%$. Some of the gut-derived sequences were nearly identical (99 to 100% sequence similarity) (Fig. 3) to soil-derived sequences. Only one cluster (the G cluster) contained a single gut-derived sequence but no soil-derived sequence. *nosZ* sequences that occurred universally in all three of the gut libraries also occurred in at least one of the soil libraries (e.g., GS clusters II and IV).

All clusters contained hitherto-unknown *nosZ* genotypes, especially clusters I, IV, and XI (Fig. 2; see Fig. S2 in the supplemental material). Genotypes of GS clusters I and II were abundant in gut and soil libraries, whereas genotypes of GS clusters IV and X were abundant only in gut libraries. Genotypes of *Pseudomonas*-related GS cluster XI were abundant in soil libraries (sites B and H) (Fig. 2), demonstrating

FIG. 2. Unrooted maximum-likelihood tree of representative *nosZ* fragments (approximately 350 amino acids). Branching that was not reproduced with neighbor-joining and parsimony algorithms is drawn as multifurcations. Short sequences of 100 to 260 amino acids are termed "partial" and were added to the tree without changing the topology. The nomenclature for the sequences obtained in this study is as follows: soil or gut, retrieved from soil or the earthworm gut; identification code; and study site H (garden), HW (meadow), or B (field) (see Materials and Methods). Sequences generated with the new primers PsNosZ175F and PsNosZ1144R are indicated by the sequence identifier "Ps." Accession numbers are provided. G, gut; S, soil. The bar represents an estimated sequence dissimilarity of 10%. Sequences of some other studies (37, 38) were not considered, due to the small overlap (76 amino acids) with fragments from this study.

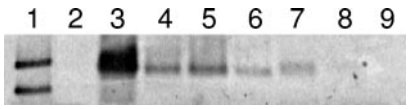


FIG. 4. PCR amplification of *Pseudomonas*-related *nosZ* from site B with serial dilutions of template DNA. Lanes: 1, marker (1.0 and 0.75 kb); 2, negative control (water); 3, positive control (*Pseudomonas aeruginosa*); 4 to 6, soil DNA at a dilution of 10^0, 10^{-1}, and 10^{-2}, respectively; 7 to 9, gut DNA at a dilution of 10^0, 10^{-1}, and 10^{-2}, respectively.

that the general primer pair nosZ662F-nosZ1772R (38) worked well for the amplification of *Pseudomonas*-related *nosZ* sequences. However, such sequences were not detected in the gut libraries that were constructed with this primer pair, suggesting that the earthworm gut contained a lower number of *Pseudomonas*-related *nosZ* targets than soil. New primers (PsNosZ175F and PsNosZ1144R) for the amplification of soil *Pseudomonas*-related *nosZ* sequences (i.e., GS cluster XI) were designed to test this possibility. The new primers yielded 1-kb *Pseudomonas*-related *nosZ* fragments from both soil and gut (Fig. 2 and 4). The field site (site B) yielded the highest number of *Pseudomonas*-related *nosZ* fragments, and the relative detectable abundance of these fragments was evaluated. *Pseudomonas*-related *nosZ* fragments were detected in soil and gut DNA extracts at 100- and 10-fold dilutions, respectively (Fig. 4), confirming that more *Pseudomonas*-related *nosZ* sequences could be detected in soil than in the earthworm gut at this sampling site.

The *nosZ* sequences of the denitrifying earthworm gut isolates were assessed to determine whether some of the previously unknown *nosZ* sequences retrieved from the earthworm gut and the soil could be assigned to organisms. Translated *nosZ* sequences of the denitrifying earthworm gut isolates (i.e., *D. denitrificans*, *F. denitrificans*, and *Pseudomonas* sp. strain ED3) fell into GS clusters II and XI and were highly similar to translated *nosZ* sequences retrieved from gut and soil in this study (Fig. 2). The translated *nosZ* sequence of *D. denitrificans* was also highly similar (>99%) to translated *nosZ* sequences from a Michigan soil (45). Translated *nosZ* sequences from soil and the earthworm gut clustered with translated *nosZ* sequences of *Achromobacter*, *Flavobacterium*, and *Paracoccus* and the proteobacteria *Bradyrhizobium*, *Dechloromonas*, *Pseudomonas*, and *Sinorhizobium*, indicating a possible occurrence of these genera in soil and the earthworm gut.

Comparison of 16S rRNA gene and *nosZ* similarity. The comparative phylogeny of *nosZ* and the 16S rRNA gene of *nosZ*-containing denitrifiers was assessed to determine if *nosZ* could be used for the identification of denitrifying species. Pairwise similarities of sequences retrieved from denitrifying isolates from this study and public databases ranged from 37 to 99% and from 60 to 100% for *nosZ* and 16S rRNA genes, respectively, indicating that the 16S rRNA is more conserved than *nosZ* (Fig. 5). Denitrifiers that shared <48% *nosZ* similarity (or 68% protein similarity of translated *nosZ*) always shared <97% 16S rRNA gene similarity. Thus, *nosZ* sequences with a similarity of <48% (or 68% protein similarity of translated *nosZ*) to any *nosZ* of cultured denitrifiers indicate a hitherto-unknown denitrifying species.

Approximately 90% of any two denitrifiers that shared >97% 16S rRNA sequence similarity shared >65% *nosZ* similarity (equivalent to 90% protein similarity of translated *nosZ*) (Fig. 5). However, the scattering of data points (Fig. 5) indicates horizontal gene transfer of *nosZ*, making it difficult to predict 16S rRNA gene similarities and consequently new species based on *nosZ* similarities.

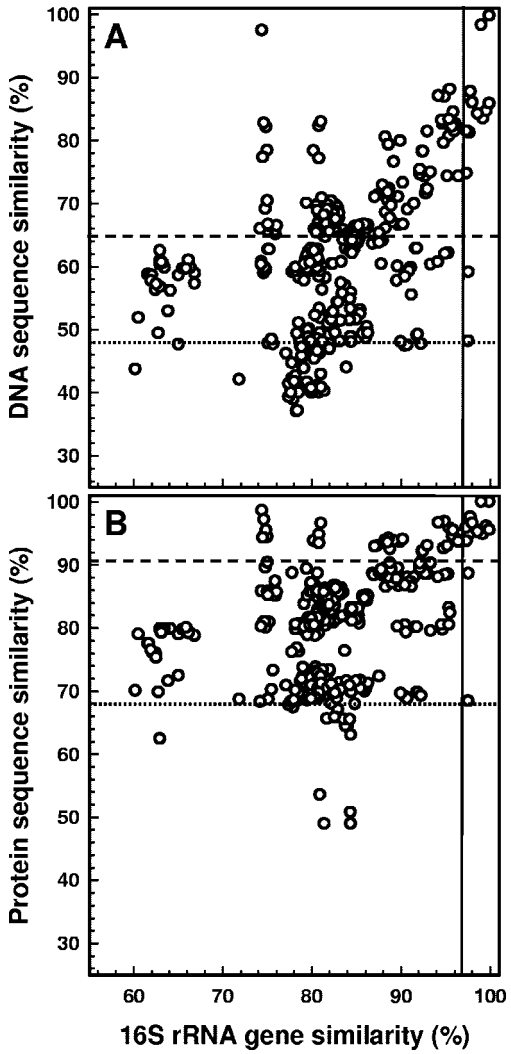


FIG. 5. Correlation of 16S rRNA gene similarity with *nosZ* (A) and in silico-translated *nosZ* sequence similarity (B) of pure cultures. The similarity of the translated *nosZ* protein sequence is plotted as $1 - D$ (as a percentage), where D is the PAM-corrected evolutionary distance. The solid lines indicate 16S rRNA gene threshold values for species delineation, and the dotted lines indicate threshold values below which sequences are indicative of novel denitrifier species. The dashed lines represent the expression $1 - Q$, where Q is the 90% quantile of pairwise sequence comparisons with a 16S rRNA similarity of >97%.

DISCUSSION

Earthworms emit denitrification-derived N_2O (27, 31) and N_2 (21). Earthworm gut content has a greater capacity to denitrify and produce N_2O than soil (25, 27). The occurrence of a highly active, gut-specific denitrifier population or the activation of soil denitrifiers during gut passage could theoretically account for the emission of N gases by the earthworm. Most known denitrifiers,

including four out of five earthworm gut isolates (25), reduce N_2O to N_2 (52, 53); furthermore, the emission of nitrate-derived N_2 by earthworms (21) suggests that *nosZ*-containing microbiota are functionally active in the earthworm gut. Thus, *nosZ* was selected as a marker for the comparative analysis of denitrifier diversity in gut and soil. The 59 different *nosZ* genotypes detected in the gut represented a significantly higher phylogenetic diversity of denitrifiers than was detected in previous cultivation-based studies of gut content (25) or cast (18), which collectively yielded nine denitrifiers. If certain *nosZ* sequences occurred universally in all gut libraries but were absent from soil libraries, such sequences would be evidence that the earthworm gut harbors endemic denitrifiers. However, such sequences were not detected. In contrast, identical *nosZ* sequences were found in gut and soil, and all but 1 (out of 182) gut-derived sequences grouped phylogenetically with those obtained in the soil libraries (Fig. 2). The remaining single gut-derived sequence (Fig. 2, G cluster) was also similar to that of common soil bacteria, i.e., *Ralstonia solanacearum* and *Ralstonia eutropha* (50); this genus has also previously been found in earthworm gut, cast, and soil (18, 25).

The absence of an endemic, earthworm gut-specific denitrifier population is in agreement with previous studies. The passage of ingested matter through the earthworm takes <20 h (2), presumably too short a time to sustain a gut-specific population in the gut lumen; in fact, such a population would require an unrealistically high growth rate. The earthworm gut has relatively little compartmentalization (16), and a gut-specific microbiota might be restricted to the gut wall. However, cell densities of earthworm gut wall-associated microbes are low (26, 49), their association with the gut wall appears to be opportunistic (18, 29, 35, 43), and the gut wall contributes only marginally to the production of N_2O (25) and N_2 (21). No evidence for an endemic, gut-specific microbiota has been obtained by cultivation or 16S rRNA gene-based analyses (18, 29, 35, 43).

Based on the above considerations, a quantitatively significant, gut-specific denitrifier population in the gut lumen or at the gut wall is unlikely. Thus, the proposal that the origin of the denitrifiers in the earthworm gut is ingested soil would seem to be a given. However, in general, the detected *nosZ* diversity of gut exceeded that of soil. For example, for site H, up to 22 different *nosZ* genotypes were detected in soil compared to 39 different *nosZ* genotypes in the earthworm gut (Table 1, site H). Coverage tended to be higher for soil libraries than for gut libraries (Table 1) and thus cannot account for this discrepancy. Selective feeding on microbe-rich particles, e.g., rhizosphere soil (15), and the proposed activation during gut passage might explain the slightly higher *nosZ* diversity in the gut libraries of sites B and H. The activation of microorganisms increases cell volume and genetic material (33). Active cells might also have increased lysis efficiency, which in comparison to inactive microorganisms would make them easier to detect by molecular methods.

Although pseudomonads are common in soil and can be readily cultured from the earthworm gut (25, 34), *Pseudomonas*-related *nosZ* sequences were only detected in the soil samples of the field site (B) but not in the corresponding gut samples when the general primer pair nosZ662F-nosZ1772R was used (Fig. 2). Although *Pseudomonas*-related *nosZ* fragments were obtained from both soil and gut samples from site B with newly designed *Pseudomonas*-specific primers PsNosZ175F and PsNosZ1144R (Fig. 2), semiquantitative PCR indicated that numbers of *Pseudomonas*-related *nosZ* targets in soil DNA extract were higher than in gut DNA extracts (Fig. 4) and, consequently, that the cell numbers of *Pseudomonas*-related denitrifiers were higher in soil than in the earthworm gut. *Gammaproteobacteria*, of which the genus *Pseudomonas* is a member, are abundant in the food source of *Lumbricus* but are not detected in anterior parts of the gut (41), and the number of *Pseudomonas*-related species decreases during gut passage (10, 47). Soil pseudomonads might be selectively digested during passage through the earthworm (10, 41), and cells with a high volume (e.g., cells that are highly active) are more likely to be mechanically disrupted in the gizzard of earthworms than are smaller cells (e.g., cells that are dormant or inactive) (16). The assumed digestion of soil pseudomonads during gut passage is consistent with the low detected *Pseudomonas*-related *nosZ* diversity in the gut compared to that of soil.

Libshuff analyses revealed no significant differences between *nosZ* gene libraries from gut and soil for the meadow site (HW) (see Table S1 in the supplemental material), while gut and soil libraries from the field (B) and garden (H) sites were only significantly different at a *nosZ* evolutionary distance of <12 and 5%, respectively (see Fig. S1 in the supplemental material). However, even a 12% evolutionary distance is not a major qualitative difference for *nosZ* (Fig. 5) and other functional genes such as the dissimilatory sulfite reductase genes (*dsrAB*), the ammonia monooxygenase gene (*amoA*), and the particulate methane monooxygenase gene (*pmoA*). PAM-corrected amino acid sequence dissimilarity of *nosZ*, *dsrAB*, *amoA*, and *pmoA* from organisms of the same genus ranges from 0 to 32% (mean, 8%), 2 to 26% (mean, 12%), 0 to 13% (mean, 6%), to 0 to 2% (mean 1%), respectively (data not shown), indicating that *nosZ* is the least-conserved marker of these four genes, apparently subjected to horizontal gene transfer. The mean PAM-corrected dissimilarity of *dsrAB*, *amoA*, and *pmoA* from organisms of the same genus equals 9%, and only ammonium oxidizers that show more than 15% *amoA* dissimilarity are always different species (28). The equivalent value for *nosZ* would be 32%, although 12% might be a more realistic threshold (Fig. 5). Consequently, a *nosZ* dissimilarity of 5 or 12% does not prove the occurrence of different denitrifying species; in addition, the obvious existence of extensive horizontal gene transfer leaves some uncertainty about the identity of the *nosZ*-carrying organisms. Nevertheless, the collective *nosZ* analyses (Table 1; Fig. 2, 3, and 5) demonstrated that the *nosZ* gene pool of the denitrifying populations in gut and soil had no significant differences. However, there is still some uncertainty about whether the denitrifier species composition in gut and soil is also similar.

In conclusion, the present study lends support to the hypothesis that the high denitrification potential of the earthworm gut (22, 25, 27, 31) and the concomitant in vivo emission of both N_2O (27) and N_2 (21) are not due to an earthworm gut-specific denitrifier population but due to the activation of ingested soil denitrifiers during gut passage.

ACKNOWLEDGMENTS

Support for this study was provided by the Deutsche Forschungsgemeinschaft (DFG DR310/2-1).

We thank Achim Schmalenberger and Steffen Kolb for provision of *dsrAB* and *pmoA* alignments, respectively, and Erik Malzahn for technical assistance.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Barley, K. P.** 1961. The abundance of earthworms in agricultural land and their possible significance in agriculture. Adv. Agron. **13:**249–268.
3. **Braker, G., and J. M. Tiedje.** 2003. Nitric oxide reductase (*norB*) genes from pure cultures and environmental samples. Appl. Environ. Microbiol. **69:**3476–3483.
4. **Bremner, J. M.** 1997. Sources of nitrous oxide in soils. Nutr. Cycling Agroecosys. **49:**7–16.
5. **Brohmer, P.** 1984. Fauna von Deutschland, 16. Auflage. Quelle und Meyer, Heidelberg, Germany.
6. **Burnham, K. P., and W. S. Overton.** 1979. Robust estimation of population size when capture probabilities vary among animals. Ecology **60:**927–936.
7. **Chao, A.** 1984. Non-parametric estimation of the number of classes in a population. Scand. J. Stat. **11:**265–270.
8. **Chao, A., and S. M. Lee.** 1992. Estimating the number of classes via sample coverage. J. Am. Stat. Assoc. **87:**210–217.
9. **Chao, A., M. C. Ma, and M. C. K. Yang.** 1993. Stopping rules and estimation for recapture debugging with unequal failure rates. Biometrika **80:**193–201.
10. **Clegg, C. D., J. M. Anderson, H. M. Lappin-Scott, J. D. van Elsas, and J. M. Jolly.** 1995. Interaction of a genetically modified *Pseudomonas fluorescens* with the soil-feeding earthworm *Octolasion cyaneum* (Lumbricidae). Soil Biol. Biochem. **27:**1423–1429.
11. **Colliver, B. B., and T. Stephenson.** 2000. Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers. Biotech. Adv. **18:**219–232.
12. **Conrad, R.** 1995. Soil microbial processes involved in production and consumption of atmospheric trace gases, p. 207–250. *In* J. G. Jones (ed.), Advances in microbial ecology, vol. 14. Plenum Press, New York, N.Y.
13. **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.
14. **Davidson, E. A.** 1991. Fluxes of nitrous oxide and nitric oxide from terrestial ecosytems, p. 219–235. *In* J. E. Rogers and W. B. Whitman (ed.), Microbial production and consumption of greenhouse gases. American Society for Microbiology, Washington, D.C.
15. **Doube, B. M., and G. G. Brown.** 1998. Life in a complex community: functional interactions between earthworms, organic matter, microorganisms, and plants, p. 179–211. *In* C. A. Edwards (ed.), Earthworm ecology. St. Lucie Press, Boca Raton, Fla.
16. **Edwards, C. A., and P. J. Bohlen.** 1996. Biology and ecology of earthworms, 3rd ed. Chapman & Hall, London, United Kingdom.
17. **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.
18. **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.
19. **Good, I. J.** 1958. The population frequency of species and the estimation of the population parameters. Biometrics **40:**237–246.
20. **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.
21. **Horn, M. A., R. Mertel, M. Gehre, M. Kästner, and H. L. Drake.** 2006. In vivo emission of dinitrogen by earthworms via denitrifying bacteria in the gut. Appl. Environ. Microbiol. **72:**1013–1018.
22. **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.
23. **Hurlbert, S. H.** 1971. The nonconcept of species diversity: a critique and alternative parameters. Ecology **52:**577–586.
24. **Hutchinson, G. L.** 1995. Biosphere-atmosphere exchange of gaseous N oxides, p. 219–236. *In* R. Lal, J. Kimble, E. Levine, and B. A. Stewart (ed.), Soils and global change. CRC Press, Inc., Boca Raton, Fla.
25. **Ihssen, J., M. A. Horn, C. Matthies, A. Gößner, A. Schramm, and H. L. Drake.** 2003. N_2O-producing microrganisms in the gut of the earthworm *Aporrectodea caliginosa* are indicative of ingested soil bacteria. Appl. Environ. Microbiol. **69:**1655–1661.
26. **Jolly, J. M.** 1993. Scanning electron microscopy of the gut microflora of two earthworms: *Lumbricus terrestris* and *Octolasion cyaneum*. Microb. Ecol. **26:**235–245.
27. **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.
28. **Koops, H.-P., U. Purkhold, A. Pommerening-Röser, G. Timmermann, and M. Wagner.** 2003. The lithoautotrophic ammonia-oxidizing bacteria. *In* M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (ed.), The prokaryotes: an evolving electronic resource for the microbiological community, 3rd ed., release 3.13. Springer-Verlag, New York, N.Y. [Online.] http://141.150.157.117:8080/prokPUB/index.htm.
29. **Lee, K. E.** 1985. Earthworms. Academic Press, Sydney, Australia.
30. **Ludwig, W., O. Strunk, S. Klugbauer, N. Klugbauer, M. Weizenegger, J. Neumaier, M. Bachleitner, and K.-H. Schleifer.** 1998. Bacterial phylogeny based on comparative sequence analysis. Electrophoresis **19:**554–568.
31. **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.
32. **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.
33. **Oliver, J. D.** 1993. Formation of viable but nonculturable cells, p. 239–272. *In* S. Kjelleberg (ed.), Starvation in Bacteria. Plenum Press, New York, N.Y.
34. **Palleroni, N. J.** 1999. Introduction to the family Pseudomonadaceae. *In* M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (ed.), The prokaryotes: an evolving electronic resource for the microbiological community, 3rd ed., release 3.13. Springer-Verlag, New York, N.Y. [Online.] http://141.150.157.117:8080/prokPUB/index.htm.
35. **Parle, J. N.** 1963. Microorganisms in the intestine of earthworms. J. Gen. Microbiol. **31:**1–13.
36. **Rich, J. J., R. S. Heichen, P. J. 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.
37. **Rösch, C., A. Mergel, and H. Bothe.** 2002. Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Appl. Environ. Microbiol. **68:**3818–3829.
38. **Scala, D. J., and L. J. Kerkhof.** 1999. Diversity of nitrous oxide reductase (*nosZ*) genes in continental shelf sediments. Appl. Environ. Microbiol. **65:** 1681–1687.
39. **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.
40. **Schloss, P. D., B. R. Larget, and J. Handelsman.** 2004. Integration of microbial ecology and statistics: a test to compare gene libraries. Appl. Environ. Microbiol. **70:**5485–5492.
41. **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.
42. **Singleton, D. R., M. A. Furlong, S. L. Rathbun, and W. B. Whitman.** 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl. Environ. Microbiol. **67:**4374–4376.
43. **Singleton, D. R., P. F. Hendrix, D. C. Coleman, and W. B. Whitman.** 2003. Identification of uncultured bacteria tightly associated with the intestine of the earthworm *Lumbricus rubellus* (Lumbricidae; Oligochaeta). Soil Biol. Biochem. **35:**1547–1555.
44. **Smith, E. P., and G. vanBelle.** 1984. Non-parametric estimation of species richness. Biometrics **40:**119–129.
45. **Stres, B., I. Mahne, G. Avgustin, and J. M. Tiedje.** 2004. Nitrous oxide reductase (*nosZ*) gene fragments differ between native and cultivated Michigan soils. Appl. Environ. Microbiol. **70:**301–309.
46. **Strunk, O., O. Gross, B. Reichel, M. May, S. Hermann, N. Stuckmann, B. Nonhoff, M. Lenke, T. Ginhart, A. Vilbig, T. Ludwig, A. Bode, K.-H. Schleiffer, and W. Ludwig.** ARB: a software environment for sequence data. Department of Microbiology, Technische Universität München, Munich, Germany. [Online.] http://www.mikro.biologie.tu-muenchen.de.
47. **Thorpe, I. S., K. Killham, J. I. Prosser, and L. A. Glover.** 1993. Novel method for the study of the population-dynamics of a genetically modified microorganism in the gut of the earthworm *Lumbricus terrestris*. Biol. Fertil. Soils **15:**55–59.
48. **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, N.Y.
49. **Vinceslas-Akpa, M., and M. Loquet.** 1995. Observation in situ de la microflore lieé au tube digestif de *Eisenia fetida andrei* (Lumbricidae). Eur. J. Soil Biol. **31:**101–110.
50. **Yabuuchi, E., Y. Kosako, I. Yano, H. Hotta, and Y. Nishiuchi.** 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. Microbiol. Immunol. **39:**897–904.
51. **Zumft, W. G.** 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. **61:**533–616.
52. **Zumft, W. G.** 1992. The denitrifying prokaryotes, p. 554–582. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer Verlag, New York, N.Y.
53. **Zumft, W. G., and H. Körner.** 1997. Enzyme diversity and mosaic gene organization in denitrification. Antonie Leeuwenhoek **71:**43–58.