ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 3, pp. 360–368. © Pleiades Publishing, Ltd., 2009.
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EXPERIMENTAL ARTICLES

Culturable Microorganisms from the Earthworm Digestive Tract

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Received January 22, 2008

Abstract—The cultured aerobic copiotrophic bacteria and fungi from food-free digestive tracts of *Aporrectodea caliginosa, Lumbricus terrestris,* and *Eisenia fetida* earthworms, soil (compost), and fresh earthworm excrements were investigated. The microorganisms were isolated on nutrient media and identified by sequencing the fragments of bacterial 16S rRNA and fungal 28S rRNA (D1/D2 domain) gene sequences with subsequent phylogenetic analysis. Bacteria isolated from the digestive tracts of earthworms belonged to the families *Aeromonadaceae, Comamonadaceae, Enterobacteriaceae, Flavobacteriaceae, Moraxellaceae, Pseudomonadaceae*, and *Sphingobacteriaceae* (*Bacteroidetes*), as well as *Actinobacteria.* For five strains, namely *Ochrobactrum* sp. 341-2 (α-*Proteobacteria*), *Massilia* sp. 557-1 (β-*Proteobacteria*), *Sphingobacterium* sp. 611-2 (*Bacteroidetes*), *Leifsonia* sp. 555-1, and a bacterium from the family *Microbacteriaceae*, isolate 521-1 (*Actinobacteria*), the similarity to known 16S rRNA sequences was 93–97%; they therefore, probably belong to new species and genera. Bacterial groups isolated from the digestive tracts of earthworms were significantly different from those isolated from soil and excrements. Some bacterial taxa occurred in different sections of *A. caliginosa* intestine and in intestines of different earthworm species; however, the overall composition of bacterial communities in these objects is different. Existence of bacterial groupings symbiotically associated with intestines is proposed. Among the fungi, *Bjerkandera adusta* and *Syspastospora parasitica* were isolated from the cleaned digestive tracts as light-colored, sterile mycelium, as well as *Geotrichum candidum, Acremonium murorum* (*A. murorum* var. *felina*), *Alternaria alternata, Aspergillus candidus, A. versicolor, Cladosporium cladosporioides, Rhizomucor racemosus, Mucor hiemalis, Fusarium* (*F. oxysporum, Fusarium* sp.), and *Penicillium* spp. These fungi survive for a long time in the earthworm's digestive environment. Investigation of the functional characteristics and role in the host organism is required to confirm the symbiotic status of the microorganisms associated with the earthworm digestive tract.

Key words: culturable bacteria, fungi, earthworms, digestive tract, symbionts.

DOI: 10.1134/S0026261709030151

Investigation of the symbiotic relations between microorganisms and invertebrates is one of the major fields of soil microbial ecology. Symbiosis implies coexistence of two or more species, with the higher organized partner containing the other one, either inside its cells or special organs, or in the digestive tract. A vast variety of soil microorganisms and animals implies various associations between them, including intestinal symbioses. For example, the symbionts of the termite's digestive tract are known, as well as their probable functions [1]. The microorganisms inhabiting the digestive tracts of other terrestrial invertebrates (diplopoda, wood lice, insect larvae or imago, and earthworms) have also been among the subjects of microbiological investigation. Electron microscopy [2–6], isolation on selective media [7–12], and molecular genetic research [13] provide direct or indirect evidence for the existence of microbial groupings differing in their composition from those inhabiting soil and associated substrates. In order to confirm the symbiotic nature of these microbial communities, their functional relations with the host should be confirmed. Thus, investigation of the composition and structure of microbial populations of invertebrate's digestive tract and of their function in the host organism is an important aspect of soil ecology. Few publications deal with the functional role of the microorganisms inhabiting earthworm intestines. Bacteria were demonstrated to participate in nitrous oxide (N_2O) production and methane oxidation; these compounds are known as greenhouse gases [14–19]. Thus, although earthworms are important components of the mesofauna and perform important functions of "ecosystem engineering" in soil, the

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taxonomic composition and functions of the microbial population are poorly understood [20].

In spite of a broad application of molecular genetic methods, which permit quantification and identification of microorganisms in their habitat, isolation on nutrient media remains important for investigation of the biochemical activity of bacteria and fungi, dinitrogen fixers, producers of vitamins, antibiotics, and enzymes, or destructors of biopolymers.

The goal of the present work was taxonomic characterization of culturable aerobic bacteria and fungi from the digestive tract of earthworms *Aporrectodea caliginosa*, *Lumbricus terrestris*, and *Eisenia fetida* and their comparison with the species inhabiting soil and associated substrates.

MATERIALS AND METHODS

Earthworms. The earthworms from different ecologo-trophic groups were investigated, namely the soil *Aporrectodea caliginosa* (Sav.) and the burrowing *Lumbricus terrestris* (L.) from the cultivated sod–podzolic soil under leguminous–gramineous vegetation (a long-term experiment of the Department of Agrochemistry, Moscow University, Moscow oblast, the Chashnikovo Soil Ecology Center). The total content of carbon and nitrogen in the soil was 1.72% and 0.13%, respectively; pH of the water extract was 5.7. *Eisenia fetida* (Sav., 1826) were collected of composting, semirotten cattle manure.

In order to remove the food (soil or compost) from the earthworm's digestive tracts, the earthworms were stored on moist filter paper or moist sterile sand for 5–7 days at 5–7°C. The cleanliness of the digestive tract was determined visually, as the presence or absence of dark particles. In the course of storage in moist environment, the earthworms usually removed food from their intestines. Only such intestines were used for isolation of associated bacteria.

Intestine isolation. The earthworms were frozen on a freezing stage (Peltier element) to –16°C and dissected immediately after defrosting. Care was taken to avoid repeated freezing–thawing.

Isolation of microorganisms. Bacterial and fungal groupings were compared from the intestines of *A. caliginosa*, *L. terrestris*, and *E. fetida* earthworms, as well as from food, intestine, and fresh excrements of *A. caliginosa*. Five isolations of microorganisms were carried out. For the isolation, mixed samples were used containing approx. 50 mg of soil (compost), intestines or their compartments (obtained from five earthworms), and fresh excrements (obtained by placing the earthworms on sterile moist filter paper and incubation in a refrigerator for several hours). Bacteria were desorbed with a DIAX 900 homogenizer (Heidolph, Germany) at 8000 rpm for 30 s or an ELMI Sky Line vortex at 3200 rpm for 2 min after formation of the vortex. For bacterial isolation, nystatin (500 mg/l) was added to suppress fungal growth. Several dilutions of each sample were plated (5 petri dishes per dilution). The R2A agarized medium contained the following (g/l): yeast extract, 0.5; peptone, 0.5; casamino acids, 0.5; glucose, 0.5; starch, 0.5; sodium pyruvate, 0.3; Na_2HPO_4, 0.3; magnesium sulfate, 0.05. The plates were incubated for 10–14 days at 18–20°C. Fungi were isolated from dilutions of the sample plated on agarized Czapek medium, at pH 4.5. Several morphologically similar colonies were used for transfers. Microbial isolates were stored at –18 or –70°C in Eppendorf test tubes with the relevant medium supplemented with 25% glycerol. Over 1000 bacterial strains and approx. 150 fungal strains were isolated.

Preparation for PCR amplification. Fungal and bacterial isolates were grown for 4 days at 30°C in 50 μl of liquid Czapek medium in 96-well plates (NUNC™, Denmark). The cultures from each cell were then collected and washed with 100 μl of PBS (phosphate-buffered saline, 137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; and 2 mM KH_2PO_4). Fungal biomass was lysed by (1) 2-h incubation with a lysing enzyme in Y1 buffer (sorbitol, 1 M; EDTA, 0.1 M; lyticase (RNA/DNA Mini Kit, Qiagen, Germany), 100 U/ml); (2) an aliquot of Y1 buffer was incubated with 100 μl of the PCR lysing solution A (67 mM Tris–HCl, pH 8.8; 16 mM $(NH_4)_2SO_4$; 5 μM β-mercaptoethanol; 6.7 mM $MgCl_2$; 6.7 μM EDTA, pH 8.0; 1.7 μl SDS; and 50 μg/ml proteinase K) [21] for 4–8 h at 55°C. Proteinase K was deactivated by heating the lysate for 10 min at 80°C. Bacteria were lysed in the PCR lysing solution A immediately after washing with PBS.

Amplification of bacterial 16S rRNA genes and fungal 28 rRNA genes (D1/D2 domain). According to the manufacturer's recommendations (Qiagen GmbH, Germany), the reaction mixture (20–21 μl) for the polymerase chain reaction (PCR) contained the following: distilled water, 9 μl; Q solution, 4 μl; 20 mM dNTPs solution (pH 8.0), 4 μl; PCR buffer (10×, with 15 mM $MgCl_2$), 2.0 μl; *Taq* DNA polymerase, 1.0 U; primers (NL-1 (upstream) [5'-GCATATCAATAAGCGGAGGAAAA-3'] and NL-4 (downstream) [5'-GGTCCGTGTTTCAAGACGG-3']), 0.12 nmol each; and fungal lysate, 2 μl or primers F27 (upstream) [5'-AGAGTTTGATCMTGGCTCAG-3'] and R1492 (downstream) [5'-TACGGYTACCTTGTTACGACTT-3'], 0. 12 nmol each; and bacterial lysate, 1 μl. Amplification conditions were as follows: initial denaturation, 95°C, 2 min; 30 cycles (denaturation, 95°C, 1 min; annealing, 50°C, 1 min; amplification, 72°C, 1.5 min); final annealing, 72°C, 10 min. The reaction was carried out in an Eppendorf 5341 thermocycler (Germany). The PCR products of ~680 bp (fungi) and 1400 bp (bacteria) were obtained by electrophoresis in 2% agarose gel in a 96-well format (Invitrogen, Germany).

Amplicon sequencing. Prior to sequencing, the PCR products were purified using the MinElute 96 UF PCR purification kit (Qiagen, Germany). Single-direc-

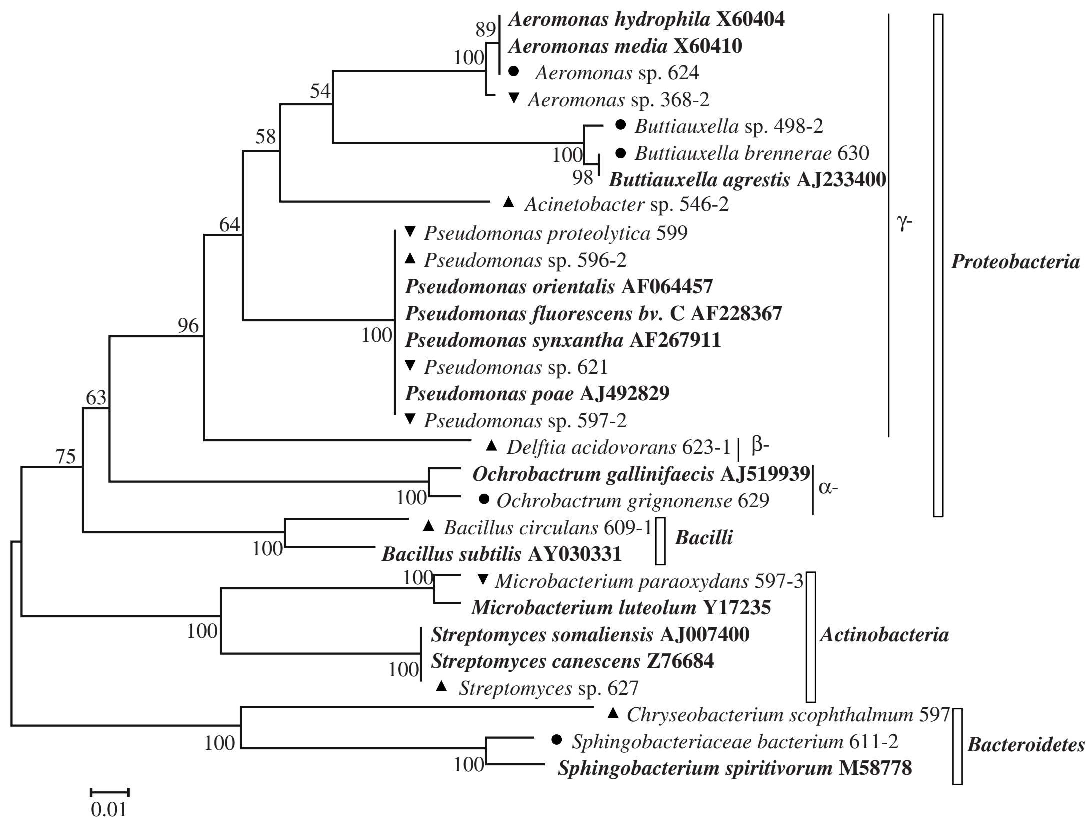

Fig. 1. Phylogenetic tree of bacterial isolates obtained from the digestive tracts of *Aporrectodea caliginosa* earthworm and of some related bacterial species (in boldface). Bacteria isolated from the anterior compartment are marked by ▲; those isolated from the posterior compartment are marked by ▼; and those isolated from both compartments are marked by ●. Aligned fragments of 16S rRNA genes contain approx. 570 nucleotides. The bootstrap level is shown at the branching points.

tional sequence was carried out with NL-4 and R1492 primers in an Eppendorf 5341 thermocycler (Germany) according to the BigDye Terminator v.1.1 Cycle Sequencing Kit protocol (Applied Biosystems, United States). The reaction mixture contained the following: Ready Reaction Premix (2.5×), 4 µl; BigDye Sequencing Buffer (5.0×), 2 µl; primer, 10 pmol; PCR product, 60 ng; deionized water, 12 µl. Reaction conditions were as follows: 25 cycles (96°C, 20 s; 50°C, 20 s; 60°C, 4 min). In case of low similarity between the fragments of bacterial ribosomal genes to the known sequences, additional sequencing of the amplicons was carried out with primers F27 and R1087. In some cases, identification based on the fragments of 16S rRNA genes is insufficient to determine the species; complete gene sequencing, together with the biochemical and morphological characterization of the isolates are required. Additional identification of microscopic fungi was carried out using the relevant manuals for each systematic group.

Phylogenetic analysis of 16S and 28S rRNA genes. The ribosomal gene fragments were identified using the GenBank BLAST software package (http://www.ncbi.nlm.nih.gko/BLAST) [22]. For phylogenetic analysis of bacterial isolates, the MEGA v. 4.1 software package was used [23]; the neighbor-joining cluster method and Kimura two-parameter algorithm were applied. The isolates exhibiting 100% similarity between their ribosomal gene fragments were analyzed and represented on a phylogenetic tree as one strain.

RESULTS

Analysis of Bacterial Communities from Soil, Digestive Tract, and Earthworm Excrements

Bacteria isolated from the anterior and posterior intestines of *Aporrectodea caliginosa*. The total number of isolates obtained was 16. Bacteria isolated from the anterior and posterior sections of *A. caliginosa* intestine belonged to 11 and 10 taxa, respectively. The isolates exhibited high similarity (99–100%) to known species and strains (Fig. 1) of the following taxa: *Proteobacteria* (classes α-, β-, and γ-), *Bacteroidetes*, *Firmicutes* (class *Bacilli*), and *Actinobacteria*. Five bacterial species were found both in the anterior and posterior intestine: *Ochrobactrum grignonense* (α-*Proteobacteria*, *Brucellaceae*), *Delftia acidovorans* (β-*Proteobacteria*), *Aeromonas* sp., *Pseudomonas* sp. (γ-*Proteobacteria*), and *Sphingobacterium* sp. (*Bacteroidetes*) (Figs. 2, 3).

Bacteria shared by soil, intestine, and fresh excrements of *Aporrectodea caliginosa*. The composition of bacteria isolated from soil, intestine, and excrements varied significantly. However, among 43 isolates from earthworm intestines and 25 obtained from fresh excrements, 9 were shared. Among bacteria isolated from soil (40 taxa) and intestine, 13 were shared; 9 of these were gram-positive, and 6 were spore-forming (class *Bacilli*); this is about 30% of the total number of taxa obtained. Comparison of bacteria from soil and from excrements revealed similarity of only 6 isolates, of which 3 were gram-positive and 3 were gram-negative. Bacteria *Brevundimonas diminuta* (α-*Proteobacteria*), *D. acidovorans* (β-*Proteobacteria*), and *Kocuria palustris* (*Actinobacteria*) were isolated from all substrates (Figs. 2, 3).

Comparison of bacteria isolated from the digestive tracts of *Aporrectodea caliginosa*, *Lumbricus terrestris*, and *Eisenia fetida* earthworms. In this experiment, bacterial groupings were compared isolated from the whole intestines of different earthworm species. The highest number of bacterial taxa (43) was isolated from *A. caliginosa* digestive tract; from *L. terrestris* and *E. fetida*, 22 and 21 taxa, respectively, were isolated. Members of the orders *Proteobacteria* (classes α-, β-, γ-), *Bacteroidetes* (classes *Flavobacteria* and *Sphingobacteria*), *Actinobacteria*, and *Firmicutes* (class *Bacilli*) were isolated from all earthworm species (Fig. 3). *B. diminuta* (α-*Proteobacteria*), *D. acidovorans* (β-*Proteobacteria*), *Aeromonas* spp., *Acinetobacter* sp. (γ-*Proteobacteria*), and *Kocuria palustris* (*Actinobacteria*) were identified; the number of taxa shared by these earthworm species is much higher (Figs. 2, 3).

Of all bacterial isolates, five exhibited relatively low similarity between the sequenced 16S rRNA gene fragments (approx. 1490 nucleotides) and the genes of known taxa (93–97%): *Ochrobactrum* sp. 341-2 (α-*Proteobacteria*), *Massilia* sp. 557-1 (β-*Proteobacteria*), *Sphingobacterium* sp. 611-2 (*Bacteroidetes*), *Leifsonia* sp. 555-1, and a member of the family *Microbacteriaceae*, isolate 521-1 (*Actinobacteria*).

Analysis of Micromycete Communities in the Earthworm's Digestive Tract and Excrements

Micromycetes were revealed in digestive tracts of the earthworms incubated without food. The temperature of their incubation had no effect on the number of fungal CFU isolated from the intestines (Table 1).

Micromycete diversity decreased with incubation time. Among the identified fungi isolated from the earthworms after 20 days of starvation, are *Bjerkandera adusta* and *Syspastospora parasitica* represented by light-colored sterile mycelia, as well as *Geotrichum candidum*, *Acremonium murorum* (*A. murorum* var. *felina*), *Alternaria alternata*, *Aspergillus candidus*, *A. versicolor*, *Cladosporium cladosporioides*, *Rhizomucor racemosus*, *Mucor hiemalis*, *Fusarium* (*F. oxysporum*, *Fusarium* sp.), and *Penicillium* spp. (Table 2). The density of fungal populations in the intestine was 10^3–10^4 CFU per air-dry intestine; this value is close to the density of fungal populations in soil mineral horizons. These fungi may be considered the ones most resistant to the conditions within a digestive tract. Their symbiotic relations with earthworms require additional research.

Table 1. Number of fungi (CFU) in the digestive tracts of *Aporrectodea caliginosa, Lumbricus terrestris*, and *Eisenia fetida* earthworms incubated for 20 days without food at different temperatures

Earthworm species	CFU/intestine $\times 10^3$	
	4°C	15°C
A. caliginosa	1.0 ± 0.4*	0.9 ± 0.4
L. terrestris	0.5 ± 0.3	1.1 ± 0.3
E. fetida	0.7 ± 0.2	1.0 ± 0.5

* $n = 3$.

DISCUSSION

In the present work, taxonomic analysis was performed of the components of aerobic bacterial and fungal groupings inhabiting earthworm digestive tracts by plating on standard nutrient media. The microorganisms were isolated from digestive tracts of earthworm cleaned of food residues and most of the microorganisms arriving from the environment; for this purpose, the earthworms were incubated without food under low temperature (5–7°C). We believe that the organisms isolated by this procedure are associated with the earthworms, rather than transient ones; the only way to obtain completely free digestive tracts is by hatching the earthworms from sterilized cocoons. Since the isolated microorganisms were grown on rich nutrient media (R2A agar for bacteria and Czapek medium for fungi), they are aerobic copiotrophs. Modern molecular genetic techniques were used for identification of bacteria and fungi, and the interpretation of our results may therefore be somewhat strict. These results confirmed our earlier suggestion that the specific microorganisms inhabiting earthworm intestines are not revealed in the environment as the dominant ones [9].

The microbial community of the earthworm's digestive tract contains bacteria of various taxa. The isolated bacteria belong to the families *Aeromonadaceae*, *Comamonadaceae*, *Enterobacteriaceae*, *Flavobacteriaceae*, *Moraxellaceae*, *Pseudomonadaceae*, *Sphingobacteriaceae* (*Bacteroidetes*), and to *Actinobacteria*. Some strains exhibited low similarity to the known taxa (93–97%) and possibly belong to new species and genera. Numerous members of new taxa may possibly be revealed by a more detailed analysis of the culturable

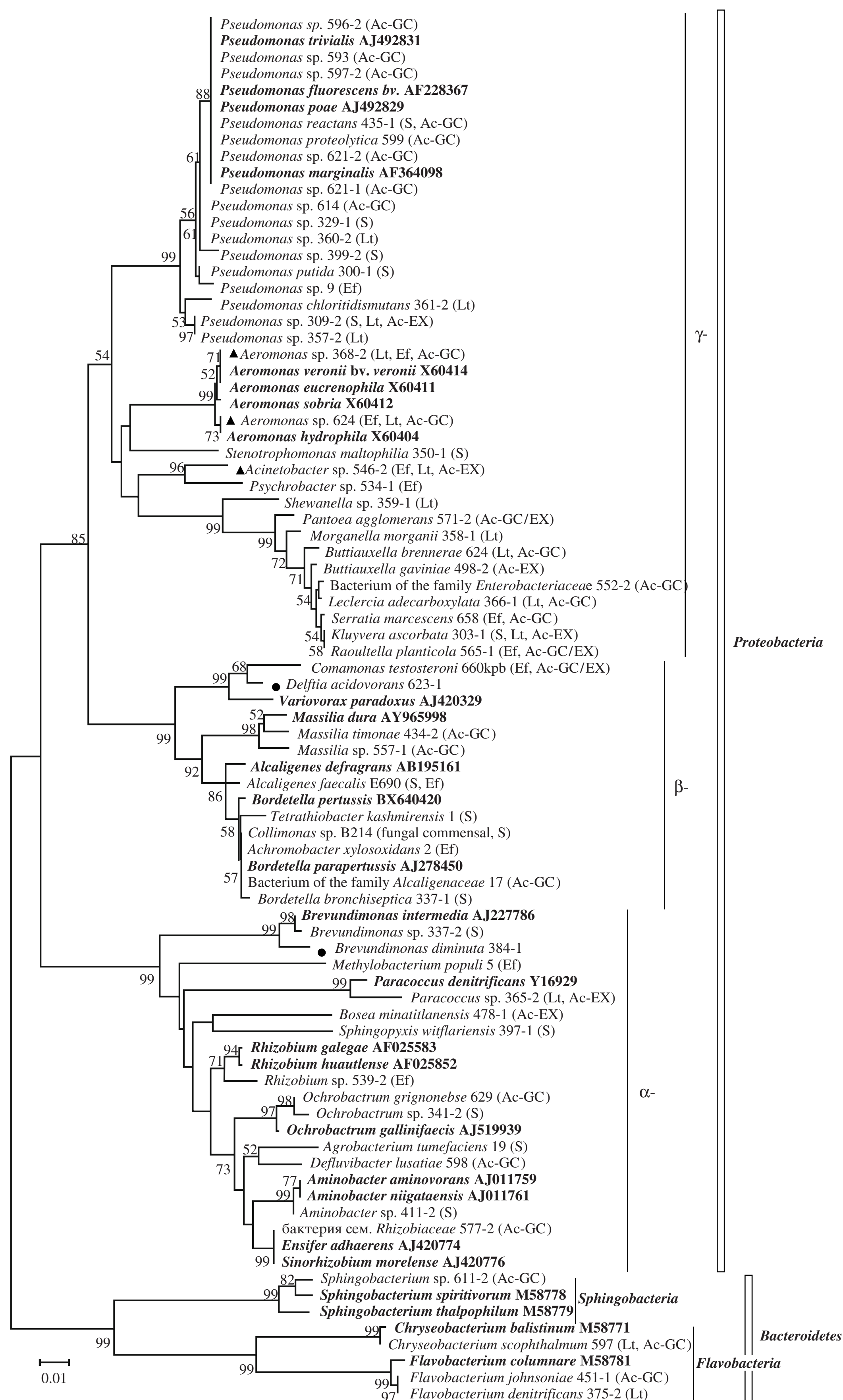

Fig. 2. Phylogenetic tree of gram-negative bacteria isolated from soil, digestive tracts, and earthworm excrements and some related bacterial species (in boldface). The source of isolation is indicated in parentheses: soil (S) *Aporrectodea caliginosa* (Ac), *Lumbricus terrestris* (Lt), and *Eisenia fetida* (Ef) earthworms, gut content (GC), gut walls (Lt, Ef), excrements (EX). Bacteria isolated from all the substrates are marked by ●; those isolated from all earthworm species are marked by ▲. Aligned fragments of 16S rRNA genes contain approx. 470 nucleotides. The bootstrap level is shown at the branching points; bootstrap values below 50% are not shown.

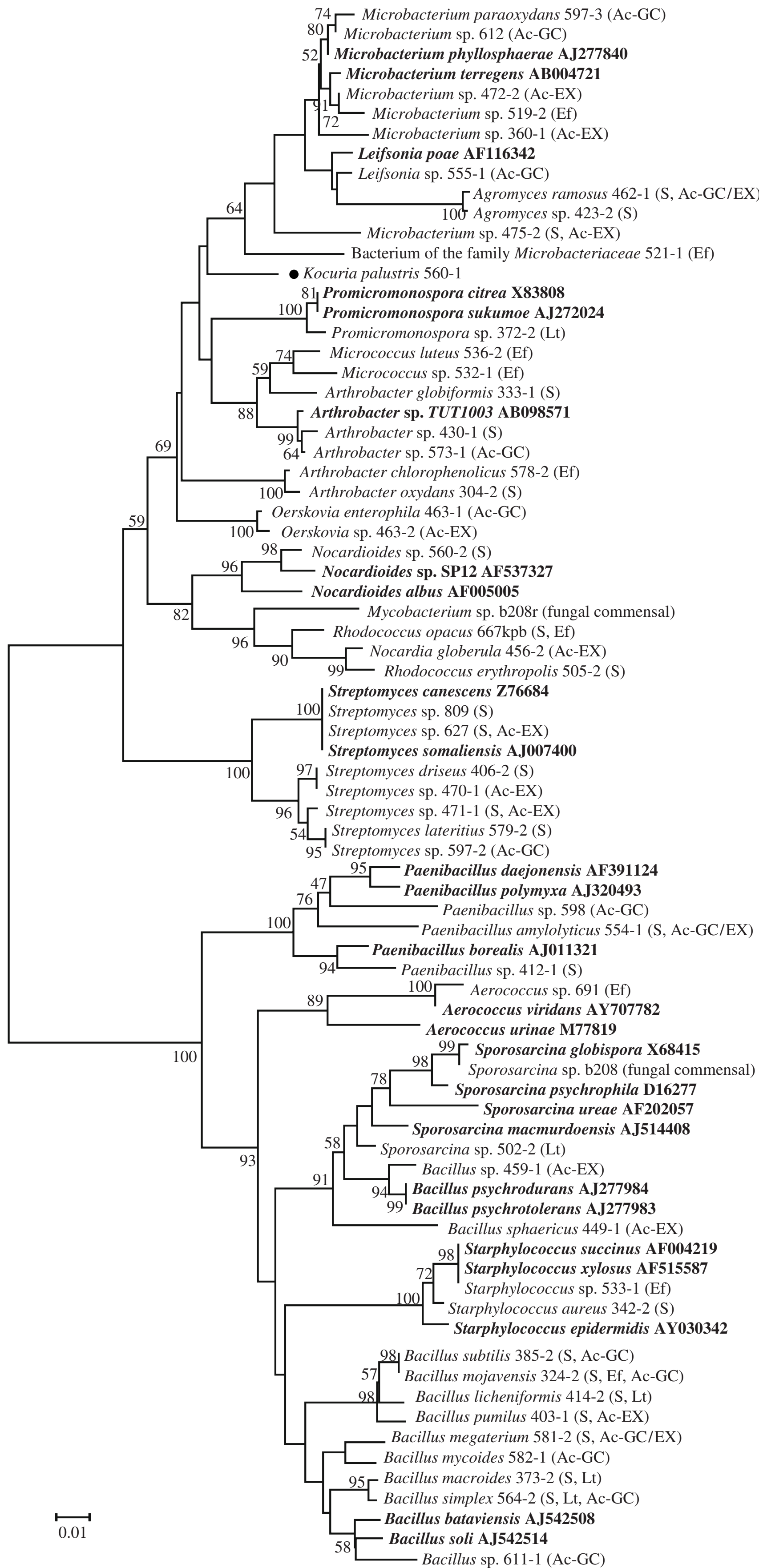

Fig. 3. Phylogenetic tree of gram-positive bacteria isolated from soil, digestive tracts, and earthworm excrements and some related bacterial species (in boldface). Bacteria isolated from all the substrates are marked by ●. Aligned fragments of 16S rRNA genes contain approx. 520 nucleotides. The bootstrap level is shown at the branching points; bootstrap values below 50% are not shown.

Table 2. Micromycetes isolated from the digestive tracts of *Aporrectodea caliginosa* earthworms incubated without soil at 4°C

Species/Genus	Term of incubation without soil, days	
	5	20
Acremonium murorum (A. murorum var. *felina)**	+	+
Acremonium sp.	+	–
Alternaria sp.	+	+
Aspergillus candidus	+	+
A. fumigatus	+	–
A. ustus	+	–
A. versicolor	+	+
Cladosporium sphaerospermum	+	+
Chrysosporium sp.	+	–
Doratomyces stemonitis	+	–
*Eupenicillium crusaceum**	+	–
Fusarium (F. oxysporum, *Fusarium* sp.*)*	+	+
Gliocladium penicilloides	+	+
Humicola grisea	+	–
*Mucor hiemalis**	+	+
*Ophiobolus herpotrichus/Setamelonomma**	+	–
Paecylomyces varioti	+	+
Penicillium (P. chrysogenum, *P. griseoroseum*, P. crustosum**, *Penicillium* spp.*)*	+	+
*Rhizomucor racemosus**	+	+
*Trichoderma viride (Hypocrea rufa)**	+	+
Verticillium (V. lateritium, V. epiphytum)*	+	–
Geotrichum candidum, *Syspastospora parasitica*, *Bjerkandera adusta*, light-colored sterile mycelium;	+	+

Notes: + indicates that the micromycete was isolated; – indicates that the micromycete wasn't isolated.
* Identified by 28S rDNA (D1/D2 domain).

bacteria (involving the application of various media and cultivation conditions, including anaerobic ones).

For the isolates from *A. caliginosa*, a number of taxa was shown to be similar to those isolated from soil, although their numbers in earthworm intestines and fresh excrements were lower. The taxonomic composition of bacteria isolated from various intestinal compartments and excrements of *A. caliginosa* was different; some bacteria, however, inhabited both intestinal compartments.

Only two bacterial taxa were common for digestive tracts of all earthowrm species under study, *Acinetobacter* sp. (related to *Acinetobacter* sp. ANT9054, *A. lwoffii*) and *Aeromonas* sp. (related to *A. jandaei*, *A. media* and *A. hydrophila*) (γ-*Proteobacteria*).

Some of the isolates belong to new taxa; most of them, however, belong to the known species classified as the facultatively associated microbiota of earthworm intestines. Though these bacteria occur in soil as well, they are probably capable of active growth in the intestine. We admit, however, that the preliminary procedures used to purify the intestines probably affected the composition of microbial communities; determination of certain physiological characteristics is required to confirm that these bacteria indeed belong to the intestinal microbiota. These characteristics include, among others, resistance to digestive enzymes and killer compounds of the earthworms [24, 25], capacity for anaerobic and facultatively anaerobic growth [26], specific enzymatic activity, and resistance to abrasive action of soil particles within earthworm intestines. Generally speaking, it is impossible to differentiate between the facultative and obligate microbial symbionts at this stage of investigation. Analysis of the earthworms grown from cocoons under controlled conditions (gnotobiotic animals supplied with sterile food and environments) may be required to confirm the role of intestine-

associated microorganisms (symbionts) for survival of the earthworms.

The data concerning the microbial populations of the earthworm digestive tract are scant and often inconsistent. This may result either from the fact that different earthworm species were investigated, inhabiting the substrates with different microbial communities, or from the nonstandard procedures used for earthworm preparation and differing isolation conditions. However, most of the works indicate the presence of specific bacterial groupings in earthworm intestines. The members of *Vibrio* [27], *Streptococcus* [11], and *Actinobacteria* [4, 28–31] are mentioned among the dominant bacteria. Tret'yakova et al. [9], found that the members of *Enterobacteriaceae, Vibrionaceae*, and *Actinobacteria* were the dominant culturable bacteria in *E. fetida*; they were not predominant in the samples of the earthworm's environment (compost). Among the bacteria isolated from *E. fetida*, 90% were rapidly growing, gram-negative, oxidase-positive fermenting bacteria identified as *Aeromonas hydrophila* [32]. Anaerobic conditions were detected in the central portion of the earthworms' digestive tract [17, 26], and the ratio of bacteria capable of anaerobic growth was higher in the intestine than in soil [26].

We have found no mention of micromycetes as permanent inhabitants of the digestive tracts of earthworms. The fungi isolated from the digestive tracts are not necessarily symbiotic because they were isolated from the environment as well. However, their prolonged survival in the intestines with the population density close to that in soil suggests a certain relationship with earthworms. We have previously demonstrated that resistance to digestion facilitates survival of some of these fungi, and their spores germinate more actively when treated with the intestinal liquid [24, 25].

To conclude, it should be mentioned that the digestive tract of earthworms acts as both a filter and a fermentor for microorganisms. Some of the microorganisms from the environment are eliminated, while resistant microorganisms grow under these conditions. Mechanical [33] and biochemical (killer, stimulating) activity of the digestive tract's environment [24, 25] are the acting agents, and antagonistic interactions between microorganisms are also possible [34].

Digestive tract of the earthworms is a specific habitat for microorganisms. A specific grouping of intestinal microorganisms exists; although its taxonomic composition is variable, it is possibly resistant against the biochemical activity and performs certain functions. Since all the animals investigated contain intestinal microbial associates, this finding is not surprising. In order to confirm the existence of symbionts in earthworms, their specific functions in the host organism should be determined.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research, projects nos. 05-04-48676a, 06-04-48557a, and 08-04-00786a, the President of the Russian Federation Leading Scientific Schools grant NSh-2227.2008.4, and the European Union Project "Biotic and Abiotic Mechanisms of TSE Infectivity Retention and Dissemination in Soil" (QLRT-2001-02493).

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