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ORIGINAL ARTICLE

Clostridiaceae and *Enterobacteriaceae* as active fermenters in earthworm gut content

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The earthworm gut provides ideal *in situ* conditions for ingested heterotrophic soil bacteria capable of anaerobiosis. High amounts of mucus- and plant-derived saccharides such as glucose are abundant in the earthworm alimentary canal, and high concentrations of molecular hydrogen (H_2) and organic acids in the alimentary canal are indicative of ongoing fermentations. Thus, the central objective of this study was to resolve potential links between fermentations and active fermenters in gut content of the anecic earthworm *Lumbricus terrestris* by 16S ribosomal RNA (rRNA)-based stable isotope probing, with [^{13}C]glucose as a model substrate. Glucose consumption in anoxic gut content microcosms was rapid and yielded soluble organic compounds (acetate, butyrate, formate, lactate, propionate, succinate and ethanol) and gases (carbon dioxide and H_2), products indicative of diverse fermentations in the alimentary canal. *Clostridiaceae* and *Enterobacteriaceae* were users of glucose-derived carbon. On the basis of the detection of 16S rRNA, active phyla in gut contents included *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospirae*, *Planctomycetes*, *Proteobacteria*, *Tenericutes* and *Verrucomicrobia*, taxa common to soils. On the basis of a 16S rRNA gene similarity cutoff of 87.5%, 82 families were detected, 17 of which were novel family-level groups. These findings (a) show the large diversity of soil taxa that might be active during gut passage, (b) show that *Clostridiaceae* and *Enterobacteriaceae* (fermentative subsets of these taxa) are selectively stimulated by glucose and might therefore be capable of consuming mucus- and plant-derived saccharides during gut passage and (c) indicate that ingested obligate anaerobes and facultative aerobes from soil can concomitantly metabolize the same source of carbon.
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Introduction

Earthworms can be the major macrofauna of soil and can have profound effects on the structure and fertility of soils (Lee, 1985; Edwards and Bohlen, 1996; Lavelle *et al.*, 1997; Makeschin, 1997). The earthworm gut is oxygen (O_2) limited and provides ideal *in situ* conditions for ingested heterotrophic soil bacteria capable of anaerobiosis (Horn *et al.*, 2003; Drake and Horn, 2007). Exoenzymes produced by ingested microorganisms stimulate the degradation of complex organic matter and thus enhance the ability of the worm to assimilate nutrients through a mutualistic digestive system (Brown *et al.*, 2000).

The earthworm alimentary canal contains high amounts of mucus- and plant-derived saccharides (Martin *et al.*, 1987; Trigo *et al.*, 1999). Glucosamine, galactosamine, glucose, galactose, mannose and fucose are components of mucopolysaccharides from *Lumbricus* sp. (Rahemtulla and Lovtrup, 1975) and occur in hydrolyzed alimentary canal contents (Wüst *et al.*, 2009a). The total concentration of monosaccharide equivalents in the aqueous phase of alimentary canal contents can be >100 mM, with many monosaccharides such as glucose approximating 10 mM or more (Horn *et al.*, 2003; Drake and Horn, 2007; Wüst *et al.*, 2009a). The occurrence of such high amounts of saccharides and the anoxia of the worm gut should favor fermentation *in situ*. Indeed, high concentrations of organic acids and molecular hydrogen (H_2) occur in the alimentary canal (Horn *et al.*, 2003; Wüst *et al.*, 2009a). For example, over 30 mM of organic acids (for example, acetate, lactate, succinate, butyrate, propionate and formate) occur in the aqueous phase of the midgut of *Lumbricus terrestris* (Wüst *et al.*, 2009a), indicating that the midgut harbors a high diversity of fermentative processes and associated taxa, which might include both facultative aerobes and obligate

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anaerobes. Furthermore, living earthworms emit denitrification-derived nitrous oxide (N_2O), which can be concomitant to the emission of H_2 (Karsten and Drake, 1997; Matthies *et al.*, 1999; Wüst *et al.*, 2009a, b). Although the occurrence of H_2 and organic acids is evidence for *in situ* fermentation, the fermentative microorganisms in the alimentary canal are unknown. Indeed, although diverse bacteria are present in the earthworm gut (Karsten and Drake, 1997; Furlong *et al.*, 2002; Ihssen *et al.*, 2003; Singleton *et al.*, 2003; Horn *et al.*, 2005; Byzov *et al.*, 2009), active bacterial taxa remain unresolved.

On the basis of these collective findings, we hypothesized that diverse obligate anaerobes and facultative aerobes ferment saccharides in the earthworm alimentary canal. The central objective of this study was to resolve potential links between fermentations and active fermenters in gut content of the anecic earthworm *L. terrestris* by 16S ribosomal RNA (rRNA)-based stable isotope probing, with [^{13}C]glucose as a model substrate.

Materials and methods

Earthworms and soil

Adult *L. terrestris* L. were purchased from ANZO (Bayreuth, Germany) and maintained in soil at 15 °C for ~5 days. Soil was obtained from the meadow Trafo Wiese in Bayreuth (Germany), which is described elsewhere (Horn *et al.*, 2003). Worms had an average weight of 4.2 ± 0.7 g ($n = 45$). Gut transit times (Hartenstein *et al.*, 1981) were evaluated to determine whether the fitness and feeding activities of purchased earthworms were similar to that of earthworms collected in the field. In brief, the procedure determines the length of time required for Coomassie Brilliant Blue R-stained soil to pass through the earthworm (Hartenstein *et al.*, 1981). Average gut transit times approximated 11 h, which is similar to that of earthworms collected in the field (Hartenstein and Amico, 1983).

Anoxic gut content microcosms

Earthworms were washed with sterile water, killed by brief immersion in 70 °C water and dissected under anoxic conditions in an O_2-free chamber (Mecaplex, Grenchen, Switzerland) containing a gas phase of 100% dinitrogen (N_2). Midgut and hindgut contents were pooled. A dilution of gut contents was necessary for obtaining adequate samples for chemical and molecular analyses. Gut content was homogenized (1:10 w/v) with sterile anoxic sodium phosphate buffer (pH 7) that contained (in grams per liter) 1.90 $NaH_2PO_4 \cdot H_2O$ and 3.36 $Na_2HPO_4 \cdot 2H_2O$. Tubes (27 ml) containing ~8 ml gut homogenates were sealed with rubber stoppers and aluminum caps and flushed with sterile N_2 (100%). Either [^{12}C]glucose or [^{13}C]glucose was added from sterile anoxic stock solutions (100 mM) to a final concentration of ~10 mM. Tubes lacking supplemental substrates served as controls. Tubes were incubated upright in the dark at 15 °C. Headspace and liquid phase were sampled with sterile syringes. Samples for nucleic acid extraction were stored at −80 °C. Values are the mean values of triplicate experiments.

Analytical techniques

Moisture content was determined by weighing gut content before and after drying at 60 °C for 72 h. pH was measured with a U457-S7/110 combination pH electrode (Ingold, Steinbach, Germany). Carbon dioxide (CO_2) and H_2 were measured with a 5890 series II gas chromatograph equipped with a thermal conductivity detector (Hewlett-Packard, Palo Alto, CA, USA; Küsel and Drake, 1995). The concentrations of gases represent the total in both the gas and liquid phases, and are shown per unit volume of the liquid phase (as are the soluble compounds). Soluble organic compounds were analyzed with a 1090 series II high performance liquid chromatograph (Hewlett-Packard; Wüst *et al.*, 2009a).

Nucleic acid extraction

RNA and DNA were coextracted by bead-beating lysis, organic solvent extraction and precipitation (Griffiths *et al.*, 2000). The purification of RNA from DNA was performed with a Qiagen RNA/DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Separation of ^{13}C- and ^{12}C-labeled RNA

A gradient solution (buoyant density 1.785 g ml^{-1}) of cesium trifluoroacetate (buoyant density 2.0 g ml^{-1}; 83.8%), gradient buffer (pH 8; 100 mM Tris; 100 mM KCl; 1 mM EDTA; 13.5%) and formamide (3.2%) was added to 500 ng RNA and filled into OptiSeal Tubes (Beckmann, Fullerton, CA, USA). All gradients were set up with the same gradient solution to minimize potential differences that might otherwise occur in the labeling patterns. ^{13}C- and ^{12}C-labeled RNA were separated by isopycnic centrifugation (130 000 × *g* at 20 °C for 67 h; vertical rotor, VTi 65.2, Beckmann; Lüders *et al.*, 2004) and fractions (450 µl each) were collected (Figure 1). The densities of the gradient solutions were determined by weighing at 25 °C. RNA was precipitated as described (Degelmann *et al.*, 2009). RNA concentrations were determined using a Quant-iT RiboGreen RNA Assay Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol, and RNA was stored at −80 °C.

Reverse transcription PCR

Reverse transcription of RNA was performed with random hexamers and Superscript III reverse transcriptase (SuperScript VILO cDNA Synthesis Kit; Invitrogen) according to the manufacturer's protocol.

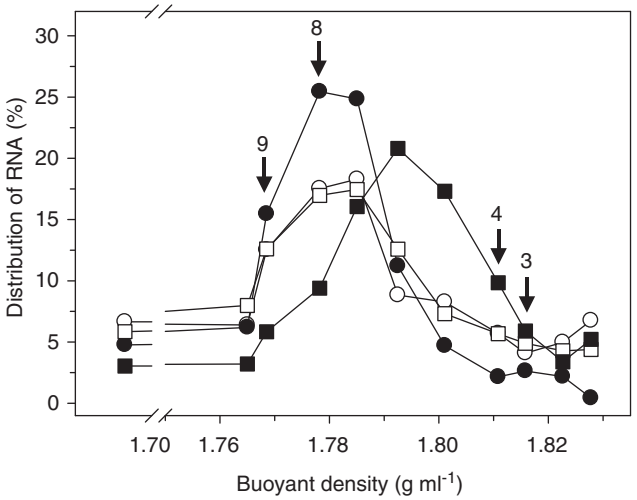


Figure 1 Distribution of RNA in gradient fractions of glucose treatments. Arrows with fraction numbers indicate 'heavy' (3 and 4) and 'light' (8 and 9) fractions, which were chosen for further analyses. RNA was measured in all gradient fractions of one replicate. Symbols: empty symbols, RNA extracted at the start of incubation; filled symbols, RNA extracted at 51 h of incubation; circles, [^{12}C]glucose treatment; squares, [^{13}C]glucose treatment.

PCR and cloning
Complementary DNA was amplified by the bacterial 16S rRNA gene-specific primer set 27F/907RM (Lane, 1991) using a 5 Prime mastermix (5 Prime, Hamburg, Germany). PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 4 cycles at 95 °C for 60 s, at 40 °C for 60 s and at 72 °C for 90 s, and 30 subsequent cycles at 95 °C for 60 s, 50 °C for 30 s, and 72 °C for 90 s. The final elongation was at 72 °C for 5 min. Purified PCR products from fractions three and four that represented labeled RNA ('heavy' fractions; buoyant density 1.809–1.817 g ml^{-1}) were pooled and fractions eight and nine that represented unlabeled RNA ('light' fractions; buoyant density 1.767–1.779 g ml^{-1}) were also pooled (Figure 1). PCR products were ligated into pGEM-T vector plasmids (Promega, Mannheim, Germany), and competent cells of *Escherichia coli* JM109 were transformed (Promega; protocol as per manufacturer's instructions). Clones with the correct insert were determined with M13 PCR (primer set M13F/M13R) according to published protocol (Messing, 1983) and selected for sequencing at Macrogen (Seoul, South Korea).

Sequence analyses
Analysis of 16S rRNA complementary DNA sequences (~880 bp) was performed with MEGA (http://www.megasoftware.net; release Beta 4.1.; Tamura *et al.*, 2007) and ARB (http://www.arb-home.de; version 2005; Ludwig *et al.*, 2004). Chimeric sequences were identified by RDP Chimera Check 2.7 (http://rdp8.cme.msu.edu; Cole *et al.*, 2003) and excluded from further analyses. The number of sequences per clone library ranged between 81 and 96. BLASTn 2.2.21 was applied for comparing sequences to those in public databases (Zhang *et al.*, 2000). Sequences were assigned to taxonomic groups using the RDP Classifier with a confidence threshold of 80% (Wang *et al.*, 2007). Sequences were aligned using the SINA Webaligner (http://www.arb-silva.de) and imported into the latest 16S rRNA gene-based database obtained from the SILVA homepage (Pruesse *et al.*, 2007). Sequences were assigned to a novel family-level group on the basis of a 16S rRNA gene similarity <87.5% to the next cultivated species (Yarza *et al.*, 2008; Schellenberger *et al.*, 2010). Coverages based on family level were calculated as described (Good, 1958; Schloss *et al.*, 2004). The detected bacterial diversity based on family level was analyzed by rarefaction analysis (Hurlbert, 1971). Phylogenetic trees were calculated using AxML and the neighbor-joining method (Saitou and Nei, 1987), which included a bootstrap test with 10 000 replicates (Felsenstein, 1985). A taxon was considered to be labeled when its abundance in the clone library from the heavy fractions of the [^{13}C]glucose treatment was higher than its abundance in the clone library from the heavy fractions of the [^{12}C]glucose treatment.

Nucleotide sequence accession numbers
The sequences obtained in this study are available from the EMBL nucleotide sequence database under accession numbers FN659069–FN659775.

Results

Effect of supplemental glucose on fermentation
Initial concentrations of glucose approximated 1 mM in unsupplemented anoxic gut content microcosms (Figure 2a). The slight initial increase in glucose concentrations that occurred was indicative of the hydrolysis of polysaccharides in gut contents (Wüst *et al.*, 2009a). The detection of glucose in unsupplemented controls and the detection of cellobiose in all microcosms (Table 1) confirm the occurrence of endogenous saccharides in midgut and hindgut contents (Wüst *et al.*, 2009a). In this regard, as gut contents were diluted 1–10 for the preparation of microcosms (see Materials and methods), the initial concentration of 1 mM glucose in unsupplemented microcosms indicated that the *in situ* concentration of glucose in gut contents approximated 10 mM.

Glucose was completely consumed within 48 h in glucose-supplemented microcosms and within 24 h in unsupplemented microcosms. Consumption of supplemental glucose was relatively linear and rapid. Product profiles of [^{12}C]- and [^{13}C]glucose incubations were nearly identical (Figure 2, Table 1), indicating that microbial activities were similar in both treatments. Approximately 20 mM CO_2 and 8 mM H_2 accumulated in incubations with supplemental glucose, whereas ~7 mM CO_2 and 0.6 mM H_2 accumulated in unsupplemented microcosms (Figures 2b and c), suggesting that supplemental

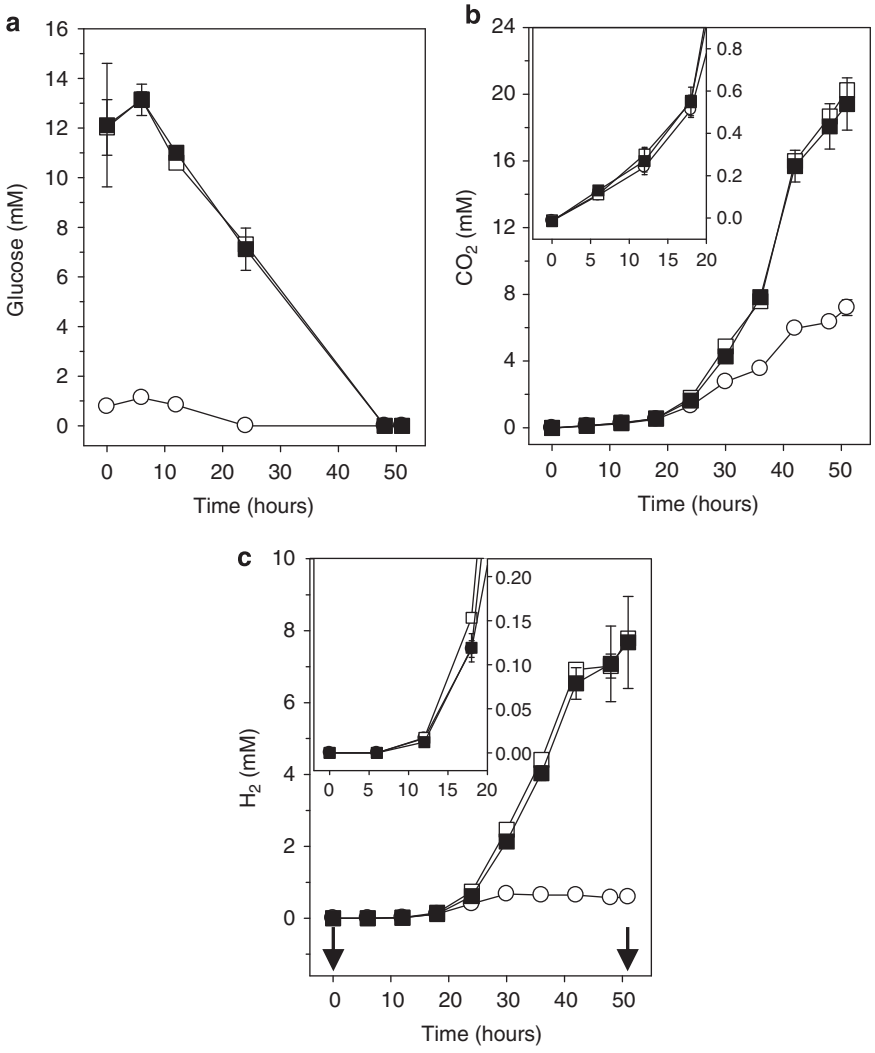


Figure 2 Effect of supplemental glucose (**a**) on the production of CO_2 (**b**) and H_2 (**c**). The symbols indicate the means of triplicates; error bars indicate s.d.. Arrows indicate the time when samples were taken for RNA stable isotope probing analyses. Insets in (**b**) and (**c**) are enlargements of the initial incubation period. Symbols: ■, results from [^{13}C]glucose treatment; □, results from [^{12}C]glucose treatment; ○, results from unsupplemented control.

glucose decreased the ratios between CO_2 and H_2 production. However, it cannot be excluded that use of alternative electron acceptors consumed a portion of the H_2 formed from either endogenous matter or supplemental glucose. CO_2 was produced without apparent delay, whereas the detection of H_2 was minimal during the initial incubation period.

All incubations started at a pH of ~7.3. pH values in glucose-supplemented microcosms at 51 h of incubation were lower than those in unsupplemented microcosms (6.9 and 7.5, respectively), which was consistent with the enhanced production of organic acids (Table 1). Acetate, butyrate, lactate, propionate, succinate and ethanol were end products in glucose-supplemented microcosms. Up to 1 mM of formate was transiently produced in glucose-supplemented microcosms, indicating that formate was an intermediate product during the degradation of glucose. Lactate, formate and succinate occurred as transient products in unsupplemented microcosms, whereas acetate, propionate and ethanol were end products. Acetate was the dominant organic acid detected in all treatments in terms of concentration. Minor concentrations (that is, <0.06 mM) of products indicative of amino-acid fermentation (that is, isobutyrate and DL-methylbutyrate; Nanninga and Gottschal, 1985; Gottschalk, 1988) were only detected in unsupplemented incubations (data not shown).

At 51 h of incubation, 72% and 60% of supplemental carbon were recovered in fermentation products of [^{12}C]- and [^{13}C]glucose incubations, respectively (based on amounts of CO_2 and soluble organic compounds detected; Figure2 and Table 1; percentages were calculated after subtracting concentrations in unsupplemented controls). The unrecovered carbon could have been dissimilated to undetected products or assimilated and perhaps

Table 1 Effect of supplemental glucose on the formation of soluble organic compounds in gut content microcosms[a]

Supplement	*Time (h)*	*Soluble organic compound (mM)*							
		Acetate	*Lactate*	*Butyrate*	*Formate*	*Succinate*	*Propionate*	*Ethanol*	*Cellobiose*
None	0	0.1 ± 0.0	0.2 ± 0.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	6	0.2 ± 0.0	0.2 ± 0.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0.0
	12	0.2 ± 0.0	0.2 ± 0.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	24	1.3 ± 0.4	0.4 ± 0.2	0 ± 0	0.3 ± 0.1	0.1 ± 0.0	0 ± 0	0.5 ± 0.5	0 ± 0
	48	2.6 ± 0.4	0.1 ± 0.0	0 ± 0	0 ± 0	0.1 ± 0.0	0 ± 0	0.3 ± 0.1	0 ± 0
	51	4.0 ± 0.2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.5 ± 0.0	0.5 ± 0.0	0 ± 0
[^{12}C]glucose	0	0.2[b]	0.2[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0.2[b]
	6	0.2 ± 0.0	0.2 ± 0.0	0 ± 0	0.1 ± 0.1	0 ± 0	0 ± 0	0 ± 0	0.2 ± 0.0
	12	0.3 ± 0.1	0.2 ± 0.0	0 ± 0	0.1 ± 0.1	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0.0
	24	1.9 ± 0.2	0.6 ± 0.2	0 ± 0	1.0 ± 0.3	0.1 ± 0.0	0 ± 0.1	0.2 ± 0.4	0.1 ± 0.0
	48	5.9 ± 0.6	3.7 ± 0.1	1.0 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0 ± 0	2.1 ± 0.1	0 ± 0
	51	8.9 ± 0.4	4.3 ± 0.0	1.5 ± 0.1	0 ± 0	0.6 ± 0.0	0.5 ± 0.0	4.2 ± 1.1	0 ± 0
[^{13}C]glucose	0	0.2[b]	0.2[b]	0[b]	0[b]	0[b]	0[b]	0[b]	0.2[b]
	6	0.2 ± 0.0	0.1 ± 0.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0.0
	12	0.3 ± 0.0	0.2 ± 0.0	0 ± 0	0.1 ± 0.1	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0.0
	24	1.6 ± 0.4	0.5 ± 0.2	0 ± 0	1.1 ± 0.3	0 ± 0	0 ± 0	0.4 ± 0.2	0.1 ± 0.0
	48	5.5 ± 1.5	3.4 ± 1.0	0.8 ± 0.3	0.5 ± 0.2	0.4 ± 0.2	0 ± 0	1.7 ± 0.6	0 ± 0
	51	8.0 ± 1.9	3.9 ± 0.8	1.5 ± 0.6	0.1 ± 0.1	0.5 ± 0.2	0.3 ± 0.0	3.6 ± 1.0	0 ± 0

[a]Unless otherwise stated, values are the means of triplicates (± s.d.).
[b]No s.d. is given because two replicates were analyzed, and the value is the average for the replicates.

converted to intracellular storage polymers. Highest percentages of supplemental ^{12}C- and ^{13}C-carbon were recovered in CO_2 (19% and 16%, respectively), lactate (18% and 16%, respectively) and acetate (14% and 9%, respectively). Approximately 74% and 63% of supplemental electrons were recovered in fermentation products of [^{12}C]- and [^{13}C]glucose incubations, respectively. The highest percentages of electrons derived from supplemental [^{12}C]- and [^{13}C]glucose were recovered in lactate (18% and 15%, respectively), ethanol (18% and 15%, respectively) and butyrate (both 17%).

Active bacteria in gut contents

A total of 707 16S rRNA complementary DNA sequences were analyzed from 'heavy' and 'light' fractions of [^{12}C]- and [^{13}C]glucose incubations (Table 2). Family-level coverage approximated 93% for sequences obtained at the beginning of incubation and 97% for sequences obtained after 51 h of incubation. Sequences were assigned to the phyla *Proteobacteria* (34.5%), *Firmicutes* (32.4%), *Actinobacteria* (20.9%), *Planctomycetes* (4.0%), *Bacteroidetes* (3.5%), *Acidobacteria* (1.6%), *Verrucomicrobia* (1.0%), *Chloroflexi* (0.3%), *Gemmatimonadetes* (0.3%), *Nitrospirae* (0.3%), *Tenericutes* (0.1%) and *Cyanobacteria* (0.1%), or were defined as unclassified *Bacteria* (0.8%; Table 2; percentages reflect the relative amount of a given taxon). In total, 82 families were detected, 17 of which were defined as novel family-level groups on the basis of a 16S rRNA gene similarity cutoff of 87.5% (Yarza *et al.*, 2008; Schellenberger *et al.*, 2010).

The rarefaction curve for sequences obtained at the start of incubation was significantly higher than the rarefaction curve for sequences obtained after 51 h of incubation (Figure 3). This result indicates that a subset of the total taxa was enriched during incubation, thus yielding a lower detected relative bacterial diversity. Sequences obtained at the start of incubation were assigned to 79 families, 17 of which were novel family-level groups, indicating a high and partially unknown diversity of microorganisms in the earthworm gut. Approximately one-third of these 79 families were affiliated with *Actinobacteria*, a phylum from which diverse family-level taxa have been isolated from soil (Joseph *et al.*, 2003). The detection of 20 families (which included two novel family-level groups) at 51 h of incubation under anoxic conditions suggests that organisms of these families were capable of anaerobiosis.

Dominant users of glucose-derived carbon

RNA obtained from [^{13}C]glucose treatments became enriched in ^{13}C on the basis of the shift of RNA toward the heavy fractions at the end of the 51 h incubation, a shift not observed with [^{12}C]glucose treatments (Figure 1). Sequences obtained from the 'heavy' fractions of 51 h samples from [^{13}C]glucose treatments were assigned to families *Aeromonadaceae*, *Bacillaceae*, *Clostridiaceae*, *Enterobacteriaceae*, '*Lachnospiraceae*' and '*Peptostreptococcaceae*' (Table 2; quotation marks indicate nonvalidated taxa; Euzéby, 2010). *Bacillaceae* and *Clostridiaceae* are spore forming, and it is thus noteworthy that gut passage can enhance the germination of spores (Fischer *et al.*, 1997).

Table 2 Phylogenetic distribution of 16S rRNA cDNA sequences obtained from [^{12}C]- and [^{13}C]glucose treatments[a]

Phylogenetic affiliation (total relative abundance of phylum)		*Relative abundance of sequences (%)*							
Phylum	*Order, family*	*[^{12}C]glucose*				*[^{13}C]glucose*			
		t_o		t_{51}		t_o		t_{51}	
		L	*H*	*L*	*H*	*L*	*H*	*L*	*H*
Acidobacteria (1.6)	*Acidobacteriales,*								
	Acidobacteriaceae	3.7	1.1	—	—	6.3	1.2	—	—
Actinobacteria (20.9)	Novel group 4[b]	—	—	—	—	1.0	1.2	—	—
	Novel group 6[b]	4.9	1.1	—	1.1	2.1	1.2	—	—
	Novel group 7[b]	—	—	—	—	1.0	—	—	—
	Novel group 8[b]	—	—	—	—	—	2.3	—	—
	Novel group 17[b]	1.2	—	—	—	—	—	—	—
	Acidimicrobiales,								
	Acidimicrobiaceae	1.2	—	—	—	—	2.3	—	—
	Iamiaceae	—	—	—	—	2.1	1.2	—	—
	Actinomycetales,								
	Novel group 5[b]	—	—	—	—	—	1.2	—	—
	Actinosynnemataceae	—	1.1	—	—	—	—	—	—
	Beutenbergiaceae	—	—	—	—	1.0	—	—	—
	Cellulomonadaceae	1.2	1.1	—	—	1.0	—	—	—
	Cryptosporangiaceae	—	1.1	—	—	1.0	—	1.1	—
	Frankiaceae	—	2.2	—	—	—	1.2	—	—
	Intrasporangiaceae	—	2.2	—	—	1.0	—	—	—
	Microbacteriaceae	2.5	9.0	—	—	5.2	4.7	—	—
	Micrococcaceae	1.2	1.1	—	—	—	—	—	—
	Micromonosporaceae	—	2.2	—	—	—	2.3	1.1	—
	Mycobacteriaceae	—	3.4	—	—	—	2.3	—	—
	Nakamurellaceae	—	2.2	—	—	2.1	4.7	—	—
	Nocardiaceae	1.2	2.2	—	1.1	—	1.2	1.1	—
	Nocardioidaceae	6.2	4.5	—	—	8.3	8.1	—	—
	Propionibacteriaceae	—	—	—	—	2.1	1.2	1.1	—
	Pseudonocardiaceae	2.5	4.5	—	—	—	4.7	—	—
	Sporichthyaceae	2.5	3.4	—	—	1.0	—	—	—
	Streptomycetaceae	1.2	—	—	—	—	—	—	—
	Rubrobacterales,								
	Novel group 1[b]	2.5	5.6	—	—	1.0	2.3	—	—
	Rubrobacteraceae	2.5	3.4	—	—	3.1	2.3	—	—
	Solirubrobacterales,								
	Patulibacteraceae	—	—	—	1.1	—	1.2	—	—
Bacteroidetes (3.5)	*Flavobacteriales,*								
	Flavobacteriaceae	11.1	2.2	—	—	5.2	4.7	—	—
	Sphingobacteriales,								
	Chitinophagaceae	—	—	—	—	—	1.2	—	—
	Flexibacteraceae	—	—	—	—	2.1	1.2	—	—
	Novel group 16[b]	—	—	—	—	1.0	—	—	—
Chloroflexi (0.3)	Novel group 14[b]	—	1.1	—	—	—	1.2	—	—
Cyanobacteria (0.1)	Family I	—	—	—	—	—	1.2	—	—
Firmicutes (32.4)	*Bacillales,*								
	Bacillaceae	1.2	1.1	6.6	5.7	1.0	—	5.7	3.4
	Incertae Sedis XII	—	1.1	—	—	1.0	—	—	—
	'*Paenibacillaceae*'	—	—	6.6	4.6	2.1	—	5.7	—
	Clostridiales,								
	Clostridiaceae	1.2	1.1	27.5	25.3	—	—	9.1	50.6
	'*Lachnospiraceae*'	—	—	1.1	2.3	—	—	—	2.2
	'*Peptostreptococcaceae*'	—	—	16.5	17.2	1.0	—	51.1	4.5
	Veillonellaceae	—	—	—	1.1	—	—	—	—
Gemmatimonadetes (0.3)	*Gemmatimonadales,*								
	Gemmatimonadaceae	—	1.1	—	—	1.0	—	—	—
Nitrospirae (0.3)	*Nitrospirales,*								
	Nitrospiraceae	—	—	—	—	1.0	1.2	—	—
Planctomycetes (4.0)	Novel group 15[b]	—	—	—	—	1.0	—	—	—
	Planctomycetales,								
	Planctomycetaceae	7.4	7.9	—	—	4.2	10.5	1.1	—
Proteobacteria (34.5)									
Alphaproteobacteria	*Caulobacterales,*								
	Caulobacteraceae	—	—	—	—	1.0	—	—	—

Table 2 (Continued)

Phylogenetic affiliation (total relative abundance of phylum)		*Relative abundance of sequences (%)*							
Phylum	*Order, family*	*[12C]glucose*				*[13C]glucose*			
		t_0		t_{51}		t_0		t_{51}	
		L	*H*	*L*	*H*	*L*	*H*	*L*	*H*
	Rhizobiales,								
	Beijerinckiaceae	1.2	—	—	—	—	—	—	—
	Bradyrhizobiaceae	4.9	3.4	—	—	6.3	5.8	2.3	—
	Hyphomicrobiaceae	2.5	1.1	—	—	4.2	2.3	—	—
	Methylobacteriaceae	1.2	—	—	—	—	—	—	—
	Methylocystaceae	1.2	1.1	—	—	—	—	—	—
	Phyllobacteriaceae	—	3.4	—	—	—	—	—	—
	Xanthobacteraceae	1.2	1.1	—	—	1.0	—	—	—
	Rhodobacterales,								
	Rhodobacteraceae	2.5	3.4	—	—	—	—	—	—
	Rhodospirillales,								
	Acetobacteraceae	1.2	2.2	—	—	—	—	—	—
	Rhodospirillaceae	1.2	5.6	—	—	4.2	4.7	1.1	—
Betaproteobacteria	*Burkholderiales,*								
	Alcaligenaceae	—	1.1	—	—	—	—	—	—
	Incertae sedis 5	—	—	—	—	2.1	1.2	—	—
	Oxalobacteraceae	—	—	—	—	—	—	1.1	—
Gammaproteobacteria	*Aeromonadales,*								
	Aeromonadaceae	1.2	2.2	14.3	20.7	1.0	2.3	9.1	4.5
	Alteromonadales,								
	Shewanellaceae	—	—	—	—	1.0	—	—	—
	Enterobacteriales,								
	Enterobacteriaceae	2.5	—	26.4	18.4	1.0	—	9.1	34.8
	Legionellales,								
	Legionellaceae	—	—	—	—	—	1.2	—	—
	Pseudomonadales,								
	Pseudomonadaceae	—	—	—	—	—	2.3	—	—
	Xanthomonadales,								
	Sinobacteraceae	—	—	—	—	2.1	—	—	—
	Xanthomonadaceae	1.2	—	—	—	1.0	—	—	—
Deltaproteobacteria	Novel group 9[b]	3.7	1.1	—	1.1	—	1.2	—	—
	Novel group 10[b]	—	1.1	—	—	—	—	—	—
	Unclassified group 1[c]	6.2	1.1	—	—	2.1	3.5	—	—
	Myxococcales,								
	Cystobacteraceae	—	1.1	1.1	—	1.0	—	—	—
	Polyangiaceae	6.2	—	—	—	5.2	1.2	—	—
	Novel group 2[b]	—	—	—	—	1.0	2.3	—	—
	Novel group 3[b]	2.5	—	—	—	—	—	—	—
Tenericutes (0.1)	Unclassified group 2[d]	—	—	—	—	—	1.2	—	—
Verrucomicrobia (1.0)	Unclassified group 3[e]	—	2.2	—	—	1.0	1.2	—	—
	Opitutales,								
	Opitutaceae	1.2	—	—	—	—	—	—	—
	Verrucomicrobiales,								
	Novel group 11[b]	—	—	—	—	—	1.2	—	—
	Subdivision 3	—	—	—	—	1.0	—	—	—
WS3 (0.1)	Incertae sedis	—	—	—	—	1.0	—	—	—
Unclassified *Bacteria* (0.8)	Novel group 12[b]	—	1.1	—	—	1.0	—	—	—
	Novel group 13[b]	2.5	—	—	—	1.0	1.2	—	—

Abbreviations: cDNA, complementary DNA; H, 'heavy' fractions (fractions 3 and 4; Figure 1); L, 'light' fractions (fractions 8 and 9; Figure 1); rRNA, ribosomal RNA; t_0, at the start of incubation; t_{51}, at 51 h of incubation.
[a]Sequences were assigned to families using the RDP Classifier (Wang *et al.*, 2007) and Blast (Zhang *et al.*, 2000). Number of sequences in the eight clone libraries ranged between 81 and 96 per library. Quotation marks indicate nonvalidated taxa (Euzéby, 2010).
[b]Novel family-level group based on a 16S rRNA gene similarity <87.5% (Yarza *et al.*, 2008).
[c]Next cultivated species: Candidatus '*Entotheonella palauensis*' (AF130847; 86.2–90.9% 16S rRNA gene similarity).
[d]Next cultivated species: Candidatus '*Lumbricincola* sp. Lr-C2' (FM165584; 90.2% 16S rRNA gene similarity).
[e]Next cultivated species: Candidatus '*Xiphinematobacter rivesi*' (AF217461; 89.5–90.3% 16S rRNA gene similarity).

In [13C]glucose treatments, the relative abundances of sequences affiliated with *Clostridiaceae* and *Enterobacteriaceae* were higher in the 'heavy' fractions than in the 'light' fractions, indicating that organisms of these families were dominant users of glucose-derived carbon. The closest related cultivated

species of labeled Clostridiaceae sequences were *Clostridium beijerinckii*, '*C. chromoreductans*', '*C. corinoforum*' and *C. puniceum* (Figure 4a). The closest related cultivated species of labeled *Enterobacteriaceae*-sequences were *Buttiauxella spp.*, *Kluyvera cryocrescens*, *Pantoea agglomerans*, *Raoultella spp.* and *Serratia fonticola* (Figure 4b). Many closest related species of sequences affiliated with *Firmicutes* and *Gammaproteobacteria* have been recovered from soil, water, intestines or feces of animals (Figures 4a and b).

Discussion

Glucose was conceived in this study to be a model trophic link in the mutualistic digestive system of earthworms (Brown *et al.*, 2000). Glucose is one of the most efficient substrates for the conservation of energy and growth (Reid, 2005). Thus, the high availability of glucose and other saccharides in earthworm gut (Horn *et al.*, 2003; Wüst *et al.*, 2009a) provides ingested soil microbes with a nutritional oasis. The organic acid product profile in glucose-supplemented incubations of gut content (Table 1) was similar to that detected along the alimentary canal (Wüst *et al.*, 2009a) and also to that of high-dilution most probable number of tubes of tryptic soy broth inoculated with earthworm gut contents (Ihssen *et al.*, 2003).

Fermentative processes and general diversity in gut contents

The detection of lactate as a transient intermediate and propionate as an end product in unsupplemented incubations (Table 1) indicates that lactate might have been consumed by propionic acid bacteria (Gottschalk, 1988). However, equal amounts of propionate were formed in unsupplemented and glucose-supplemented incubations, suggesting that propionate fermentation was not stimulated by supplemental glucose and that propionate fermenters in the earthworm gut prefer carbon sources other than glucose or lactate. Indeed, sequences related to *Propionibacteriaceae* were detected before and after incubation of earthworm gut content with [^{13}C]glucose, but were not labeled (Table 2), indicating that *Propionibacteriaceae*-related species did not significantly assimilate carbon from supplemental glucose.

Nearly half of the CO_2 produced in glucose-supplemented treatments could be attributed to endogenous sources of carbon (Figure 2b), a result consistent with previous studies showing that gut contents are rich in readily available sources of organic carbon (Brown *et al.*, 2000; Horn *et al.*, 2003; Wüst *et al.*, 2009a). Thus, microorganisms in gut contents consumed endogenous sources of carbon in addition to supplemental glucose. Although most of the organic carbon consumed during fermentation is dissimilated (that is, not assimilated; Gottschalk, 1988), the rapid dissimilation of supplemental glucose was concomitant to the labeling of rRNA, indicating that taxa capable of anaerobiosis in gut contents were poised to respond quickly to increased nutrient availability and assimilated organic carbon.

Active phyla identified by 16S rRNA analysis of earthworm gut contents included *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospirae*, *Planctomycetes*, (*Alpha-*, *Beta-*, *Gamma-* and *Delta-*) *Proteobacteria*, *Tenericutes* and *Verrucomicrobia*, taxa common to soils (Table 2). *Alpha-*, *Beta-* and *Gamma-Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes* are potentially dominant taxa in the earthworm gut, as determined on the basis of 16S rRNA gene analyses (Furlong *et al.*, 2002; Singleton *et al.*, 2003; Knapp *et al.*, 2009). Furthermore, species related to *Aeromonadaceae*, *Comamonadaceae*, *Enterobacteriaceae*, *Flavobacteriaceae*, *Moraxellaceae*, '*Paenibacillaceae*', *Pseudomonadaceae*, *Rhodocyclaceae*, *Sphingobacteriaceae* and *Actinobacteria* have been isolated from the alimentary canals of earthworms (Ihssen *et al.*, 2003; Horn *et al.*, 2005; Byzov *et al.*, 2009; Knapp *et al.*, 2009). These findings show the diverse taxa that might be metabolically active in the earthworm gut, many of which might participate in polymer hydrolysis *in situ* (Martin-Carnahan and Joseph, 2005; Bernadet and Bowman, 2006; Priest, 2009).

Sequences obtained before incubation were assigned to 14 phyla, whereas sequences obtained after incubation with glucose were assigned to only 4 phyla, with Firmicutes and Proteobacteria being dominant (Table 2). *Clostridiaceae* and *Enterobacteriaceae* were the labeled families (Table 2).

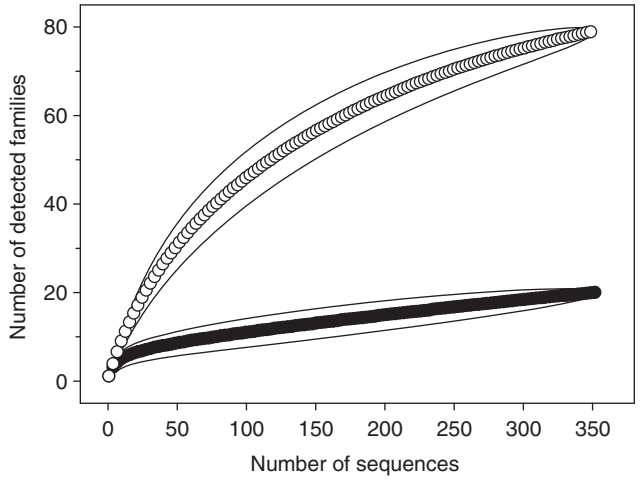
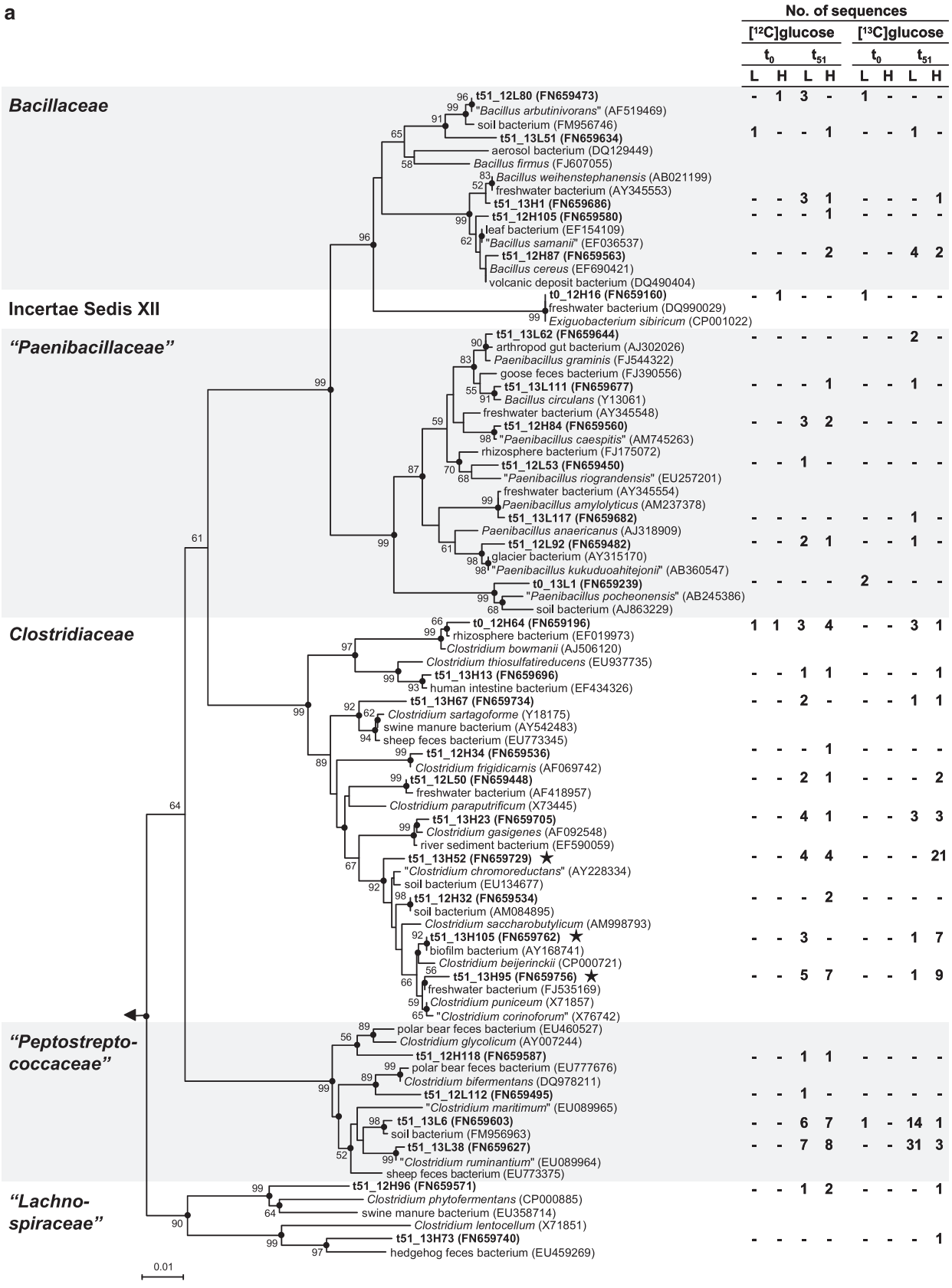


Figure 3 Rarefaction curves of 16S rRNA complementaryDNA sequences were obtained at the start of incubation (empty circles) and after 51 h of incubation (filled circles). Each curve is based on the total sequences obtained from [^{12}C]glucose- and [^{13}C]glucose-supplemented incubations. Solid lines enclosing the symbols indicate the 95% confidence intervals.

a


No. of sequences
[12C]glucose
[13C]glucose
t0
t51
L H L H L H L H
Bacillaceae
t51_12L80 (FN659473) - 1 3 - 1 - - -
"Bacillus arbutinivorans" (AF519469)
soil bacterium (FM956746)
t51_13L51 (FN659634) 1 - - 1 - - 1 -
aerosol bacterium (DQ129449)
Bacillus firmus (FJ607055)
Bacillus weihenstephanensis (AB021199)
freshwater bacterium (AY345553)
t51_13H1 (FN659686) - - 3 1 - - - 1
t51_12H105 (FN659580) - - - 1 - - - -
leaf bacterium (EF154109)
"Bacillus samanii" (EF036537)
t51_12H87 (FN659563) - - - 2 - - 4 2
Bacillus cereus (EF690421)
volcanic deposit bacterium (DQ490404)
Incertae Sedis XII
t0_12H16 (FN659160) - 1 - - 1 - - -
freshwater bacterium (DQ990029)
Exiguobacterium sibiricum (CP001022)
"Paenibacillaceae"
t51_13L62 (FN659644) - - - - - - 2 -
arthropod gut bacterium (AJ302026)
Paenibacillus graminis (FJ544322)
goose feces bacterium (FJ390556)
t51_13L111 (FN659677) - - - 1 - - 1 -
Bacillus circulans (Y13061)
freshwater bacterium (AY345548)
t51_12H84 (FN659560) - - 3 2 - - - -
"Paenibacillus caespitis" (AM745263)
rhizosphere bacterium (FJ175072)
t51_12L53 (FN659450) - - 1 - - - - -
"Paenibacillus riograndensis" (EU257201)
freshwater bacterium (AY345554)
Paenibacillus amylolyticus (AM237378)
t51_13L117 (FN659682) - - - - - - 1 -
Paenibacillus anaericanus (AJ318909)
t51_12L92 (FN659482) - - 2 1 - - 1 -
glacier bacterium (AY315170)
"Paenibacillus kukuduoahitejonii" (AB360547)
t0_13L1 (FN659239) - - - - 2 - - -
"Paenibacillus pocheonensis" (AB245386)
soil bacterium (AJ863229)
Clostridiaceae
t0_12H64 (FN659196) 1 1 3 4 - - 3 1
rhizosphere bacterium (EF019973)
Clostridium bowmanii (AJ506120)
Clostridium thiosulfatireducens (EU937735)
t51_13H13 (FN659696) - - 1 1 - - - 1
human intestine bacterium (EF434326)
t51_13H67 (FN659734) - - 2 - - - 1 1
Clostridium sartagoforme (Y18175)
swine manure bacterium (AY542483)
sheep feces bacterium (EU773345)
t51_12H34 (FN659536) - - - 1 - - - -
Clostridium frigidicarnis (AF069742)
t51_12L50 (FN659448) - - 2 1 - - - 2
freshwater bacterium (AF418957)
Clostridium paraputrificum (X73445)
t51_13H23 (FN659705) - - 4 1 - - 3 3
Clostridium gasigenes (AF092548)
river sediment bacterium (EF590059)
t51_13H52 (FN659729) ★ - - 4 4 - - - 21
"Clostridium chromoreductans" (AY228334)
soil bacterium (EU134677)
t51_12H32 (FN659534) - - - 2 - - - -
soil bacterium (AM084895)
Clostridium saccharobutylicum (AM998793)
t51_13H105 (FN659762) ★ - - 3 - - - 1 7
biofilm bacterium (AY168741)
Clostridium beijerinckii (CP000721)
t51_13H95 (FN659756) ★ - - 5 7 - - 1 9
freshwater bacterium (FJ535169)
Clostridium puniceum (X71857)
"Clostridium corinoforum" (X76742)
"Peptostrepto-coccaceae"
polar bear feces bacterium (EU460527)
Clostridium glycolicum (AY007244)
t51_12H118 (FN659587) - - 1 1 - - - -
polar bear feces bacterium (EU777676)
Clostridium bifermentans (DQ978211)
t51_12L112 (FN659495) - - 1 - - - - -
"Clostridium maritimum" (EU089965)
t51_13L6 (FN659603) - - 6 7 1 - 14 1
soil bacterium (FM956963)
t51_13L38 (FN659627) - - 7 8 - - 31 3
"Clostridium ruminantium" (EU089964)
sheep feces bacterium (EU773375)
"Lachno-spiraceae"
t51_12H96 (FN659571) - - 1 2 - - - 1
Clostridium phytofermentans (CP000885)
swine manure bacterium (EU358714)
Clostridium lentocellum (X71851)
t51_13H73 (FN659740) - - - - - - - 1
hedgehog feces bacterium (EU459269)
0.01

These glucose-assimilating taxa occur in soils (Hamberger *et al.*, 2008; Degelmann *et al.*, 2009; Schellenberger *et al.*, 2010) and have robust metabolic capacities and doubling times that can range between 0.5 and 3 h (Francis *et al.*, 2000; Wiegel *et al.*, 2006), factors that likely contributed to their competitiveness.

Many fermentative or facultative microorganisms can reduce nitrate or nitrite (Cole, 1990; Umarov, 1990). Active genera included *Aeromonas*, *Bacillus*, *Buttiauxella*, *Clostridium*, *Enterobacter*, *Flavobacterium*, *Paenibacillus* and *Pseudomonas* (Table 2, Figures 4a and b), and earthworm gut isolates affiliated with these genera have the ability to reduce either nitrate or nitrite and produce N_2O (Ihssen *et al.*, 2003). Different regions of the alimentary canal yield different organic acid, H_2 and N_2O signatures (Wüst *et al.*, 2009a), reflecting potential trophic links that might occur between fermentation and denitrification during gut passage (for example, when denitrifiers use fermentation products as a source of reductant).

Clostridiaceae

Most sequences derived from 'heavy' fractions after [^{13}C]glucose incubation were affiliated with the genus *Clostridium sensu stricto* (Collins *et al.*, 1994; Figure 4a). Species of this genus are considered to be obligate anaerobes and can dissimilate glucose to acetate, butyrate, lactate, ethanol, H_2 and CO_2 (Rainey *et al.*, 2009). The formation of these products was enhanced by glucose (Figure2 and Table 1). The closest related cultured species of labeled clostridial sequences, *C. beijerinckii* and *C. puniceum,* can produce substantial amounts of H_2 in addition to butyrate, acetate, formate and butanol (Rainey *et al.*, 2009). Although H_2 is a trophic link to methanogenesis and acetogenesis (Drake *et al.*, 2006, 2008; Hedderich and Whitman, 2006; Liu and Whitman, 2008; McInerney *et al.*, 2008), methanogens and acetogens are not metabolically significant in the earthworm gut (Karsten and Drake, 1995).

Most electrons derived from supplemental glucose were recovered in lactate, but classic lactate-producing genera such as *Lactococcus*, *Lactobacillus* and *Streptococcus* were not detected, which is not surprising, as most species in these taxa are not considered to be common to soil (Teuber *et al.*, 1992; Hammes and Hertel, 2009; Whiley and Hardie, 2009). Similarly, *Lactovum*, a lactate-forming genus isolated from acidic soil (Matthies *et al.*, 2004; Drake, 2010), was not detected. Lactate can also be produced by saccharolytic clostridia and can be the main fermentation product of these organisms under certain conditions (Wiegel *et al.*, 2006). For example, *C. perfringens* produces mainly lactate under carbon-rich conditions (Macfarlane and Macfarlane, 2003). Some sequences were closely affiliated with *C. gasigenes* and *C. frigidicarnis* (Figure 4a), species that can grow at low temperatures and secret hydrolytic exoenzymes (Broda *et al.*, 1999, 2000; Akila and Chandra, 2003), indicating that psychrotolerant clostridia might be involved in the degradation of organic matter in the earthworm alimentary canal. '*Peptostreptococcaceae*' are closely related to *Clostridiaceae* (Ludwig *et al.*, 2009 and Figure 4a) and were enriched during incubation (Table2 and Figure 4a). However, the high relative abundance of '*Peptostreptococcaceae*' -related sequences in the 'light' fractions of the [^{13}C]glucose treatment indicated that '*Peptostreptococcaceae*' used endogenous sources of organic carbon.

Enterobacteriaceae

Species of the family *Enterobacteriaceae* are facultative aerobes having both a respiratory and a fermentative metabolism (Brenner, 1992). Characteristic fermentation products of *Enterobacteriaceae* include formate, succinate, lactate, acetate, ethanol, H_2 and CO_2 (Brenner and Farmer, 2005), products consistent with those detected in glucose treatments (Figure2 and Table 1) and also with the apparent fermentation products detected along the earthworm alimentary canal (Wüst *et al.*, 2009a). Most *Enterobacteriaceae* are able to reduce nitrate to nitrite (Brenner, 1992) and might therefore be at least partly responsible for the low nitrate concentrations in the alimentary canal compared with those of pre-ingested soil (Horn *et al.*, 2003; Wüst *et al.*, 2009a). Several genera within the *Enterobacteriaceae* (*Buttiauxella*, *Kluyvera*, *Pantoea*, *Raoultella* and *Serratia*) were labeled with [^{13}C]glucose-derived carbon (Figure 4b). Species of these genera are able

Figure 4 Phylogenetic tree of 16S rRNA complementaryDNA sequences (bold) retrieved from the earthworm gut and reference sequences of the *Firmicutes* (**a**) and *Gammaproteobacteria* (**b**). Only representative sequences are shown. The values next to the branches represent the percentages of replicate trees (>50%) in which the associated taxa clustered together in the bootstrap test (10 000 replicates). Dots at nodes indicate confirmation of topology by AxML using the same data set. Labeled taxa are marked with stars. *Methanosarcina barkeri* (AF028692) was used as outgroup. Accession numbers are in parentheses. Quotation marks indicate nonvalidated taxa (Euzéby, 2010). Abbreviations: t_0, at the start of incubation; t_{51}, at 51 h of incubation; L, 'light' fractions; H, 'heavy' fractions. (**a**): The family *Veillonellaceae* was excluded because the sequence that affiliated with this family (t51_12H90) was shorter than 700 bp. The phylogenetic tree was calculated using the neighbor-joining method (50% minimum similarity filter; 651 valid positions between 104 and 815 of the 16S rRNA gene of *E. coli*). Bar indicates 0.01 estimated change per nucleotide. (**b**): The labeled sequence t51_13H112 (next cultivated species *Serratia fonticola*, AY236502, 99% 16S rRNA gene similarity) was excluded from tree calculation because it was shorter than 700 bp. The phylogenetic tree was calculated using the neighbor-joining method (50% minimum similarity filter; 698 valid positions between 101 and 816 of the 16S rRNA gene of *E. coli*). Bar indicates 0.05 estimated change per nucleotide.

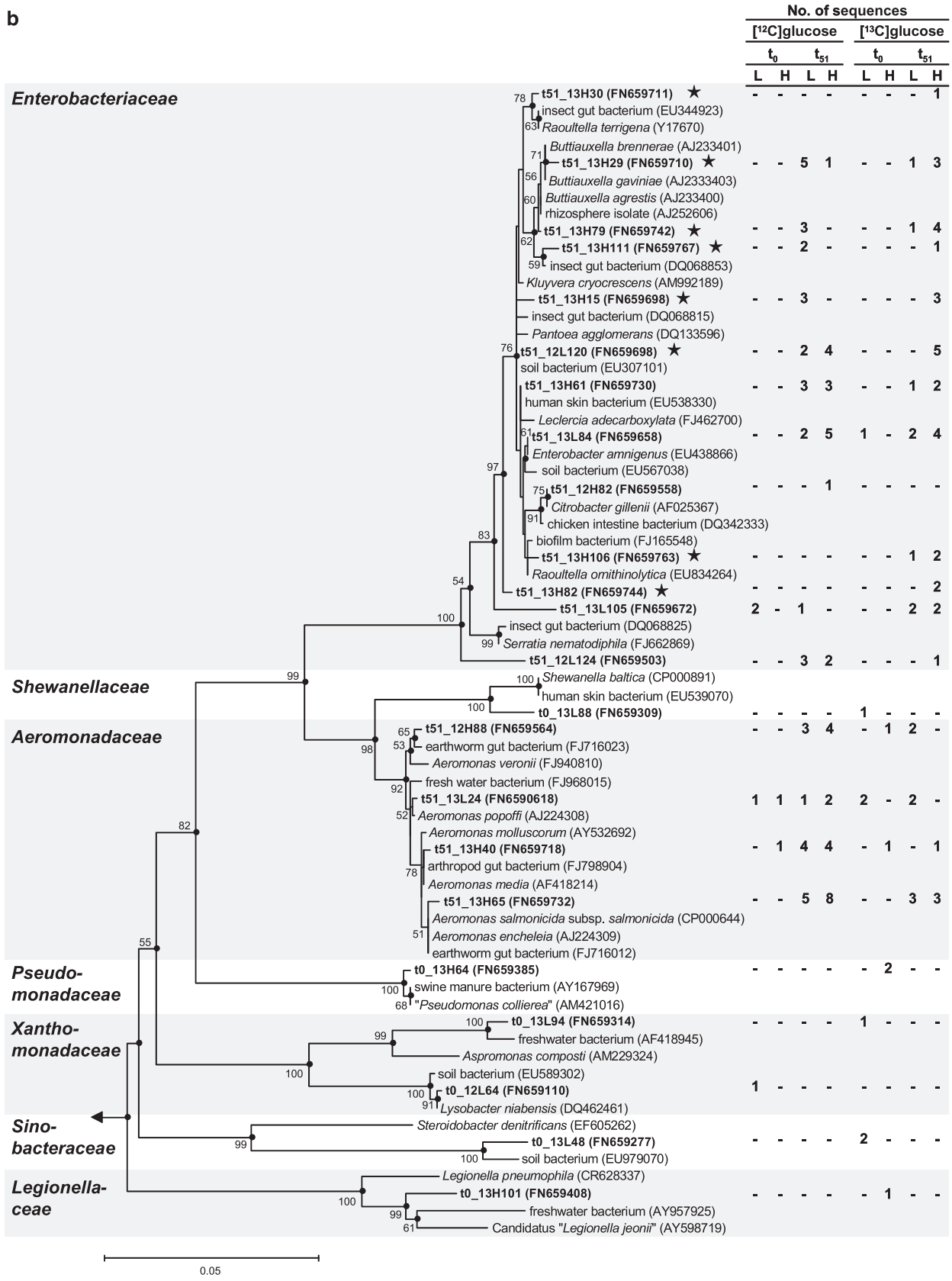


Figure 4 Continued.

to ferment glucose (Müller *et al.*, 1996; Ihssen *et al.*, 2003; Brenner and Farmer, 2005; Grimont and Grimont, 2005). Some labeled enterobacterial sequences were affiliated with *Buttiauxella agrestis* (Figure 4b), strains of which can reduce N_2O to N_2 (Kaldorf *et al.*, 1993). Many labeled enterobacterial sequences were related to *P. agglomerans* (Figure 4b). *P. agglomerans* produces exoenzymes chitinase, pectinase and protease (Mahmoud *et al.*, 2008), and might therefore contribute to the degradation of complex organic matter in the earthworm gut. The numerous anaerobic capabilities of *P. agglomerans* (Grimont and Grimont, 2005) might confer a selective advantage in O_2-limited habitats similar to the earthworm gut. In addition to growing by fermentation, this organism is capable of dissimilating metals such as Fe(III) by using lactate, acetate and H_2 as electron donors (Francis *et al.*, 2000). The large amounts of Fe(II) in the earthworm alimentary canal suggest that Fe(III) reduction occurs *in situ* and could be driven by such fermentation products (Wüst *et al.*, 2009a).

Limitations and conclusions

Microcosm experiments do not fully duplicate *in situ* conditions. Furthermore, a major constraint in interpreting stable isotope probing experiments is the potential labeling that can occur because of cross-feeding (Neufeld *et al.*, 2007). Thus, the limitations of this study should be examined. The 1–10 dilution of gut contents necessitated for obtaining samples for chemical and molecular analyses diluted both taxa and endogenous nutrients, and cross-feeding could have resulted in labeling of taxa that did not directly assimilate glucose. For example, the apparent consumption of small amounts of formate and lactate in unsupplemented microcosms indicated that a limited amount of [^{13}C]glucose-derived formate and lactate might have been consumed and thus assimilated. Also, only one of several saccharides potentially available in the gut (Wüst *et al.*, 2009a) was evaluated. The above interpretations must therefore be qualified within the constraints of these limitations. Nevertheless, anoxia, 15 °C, 10 mM glucose, neutral pH, and the relatively short incubation period approximated important parameters likely to be of importance to fermentation in the earthworm gut. In addition, if cross-feeding occurred in the microcosms, it could and likely does occur *in situ*. For example, acetate was a major fermentation product of earthworm gut content (Table 1) and might constitute an *in situ* trophic link between fermenters and denitrifiers, as it is a substrate for many denitrifiers (Tiedje, 1988). Thus, the labeling observed might theoretically reflect the type of interconnected events that occur during gut passage. However, midgut and hindgut contents contain only marginal amounts of nitrate and nitrite (Wüst *et al.*, 2009a), and no N_2O was produced in the stable isotope probing microcosms (data not shown), suggesting that cross-feeding of denitrifiers was very unlikely. Indeed, although denitrifying taxa were detected at the beginning of incubation, they were neither labeled nor detected at the end of 51 h of incubation (Table 2). Although we cannot fully exclude the possibility that cross-feeding occurred, it was likely minimal because of the relatively short incubation period and limited availability of alternative electron acceptors. Furthermore, the majority of ^{13}C from [^{13}C]glucose was recovered in fermentation products that accumulated with time, suggesting that subsequent assimilation of these products was minimal. Similarly, selective death and selective predation seem unlikely causes of the marked enrichment of *Clostridiaceae* and *Enterobacteriaceae*.

Within these constraints and considerations, the findings of this study suggest that *Clostridiaceae* and *Enterobacteriaceae* are subsets of diverse active taxa in the alimentary canal of the earthworm that are capable of consuming mucus- and plant-derived saccharides during gut passage, and illustrate the effect that gut passage might have on selectively stimulating fermentative taxa of ingested soil. Although a minimal cross-feeding might have occurred, the substantial labeling of *Clostridiaceae* and *Enterobacteriaceae* indicates that certain ingested obligate anaerobes and facultative aerobes from soil can concomitantly metabolize the same source of carbon. This possibility is consistent with the formation of products considered indicative of clostridial (for example, butyrate) and enterobacterial (for example, succinate) fermentations (Brenner and Farmer, 2005; Rainey *et al.*, 2009) in the midgut and hindgut of the alimentary canal (Wüst *et al.*, 2009a).

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