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ORIGINAL ARTICLE

Methanogenic food web in the gut contents of methane-emitting earthworm *Eudrilus eugeniae* from Brazil

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The anoxic saccharide-rich conditions of the earthworm gut provide an ideal transient habitat for ingested microbes capable of anaerobiosis. It was recently discovered that the earthworm *Eudrilus eugeniae* from Brazil can emit methane (CH_4) and that ingested methanogens might be associated with this emission. The objective of this study was to resolve trophic interactions of bacteria and methanogens in the methanogenic food web in the gut contents of *E. eugeniae*. RNA-based stable isotope probing of bacterial 16S rRNA as well as *mcrA* and *mrtA* (the alpha subunit of methyl-CoM reductase and its isoenzyme, respectively) of methanogens was performed with [^{13}C]-glucose as a model saccharide in the gut contents. Concomitant fermentations were augmented by the rapid consumption of glucose, yielding numerous products, including molecular hydrogen (H_2), carbon dioxide (CO_2), formate, acetate, ethanol, lactate, succinate and propionate. *Aeromonadaceae*-affiliated facultative aerobes, and obligate anaerobes affiliated to *Lachnospiraceae*, *Veillonellaceae* and *Ruminococcaceae* were associated with the diverse fermentations. Methanogenesis was ongoing during incubations, and ^{13}C-labeling of CH_4 verified that supplemental [^{13}C]-glucose derived carbon was dissimilated to CH_4. Hydrogenotrophic methanogens affiliated with *Methanobacteriaceae* and *Methanoregulaceae* were linked to methanogenesis, and acetogens related to *Peptostreptoccocaceae* were likewise found to be participants in the methanogenic food web. H_2 rather than acetate stimulated methanogenesis in the methanogenic gut content enrichments, and acetogens appeared to dissimilate supplemental H_2 to acetate in methanogenic enrichments. These findings provide insight on the processes and associated taxa potentially linked to methanogenesis and the turnover of organic carbon in the alimentary canal of methane-emitting *E. eugeniae*.
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Introduction

Earthworms go mostly unnoticed because of their subsurface lifestyle. However, the propensity of earthworms to consume and transform their habitat has significant impact on terrestrial ecosystems and soil processes (Darwin, 1881; Lee, 1985; Edwards and Bohlen, 1996; Makeschin, 1997; Brown and James, 2006; Nechitaylo *et al.*, 2010). For example, *Lumbricus terrestris* can consume the entire annual litter fall ($300\,g\,m^{-2}$) of forests it inhabits (Satchell, 1967). Earthworms can likewise be a dominant macrofauna of soil and up to 2000 individuals per square meter of soil have been recorded (Edwards and Bohlen, 1996). Assuming an average gut volume of approximately $250\,mm^3$ ($80\,mm$ length $\times$ $1\,mm^2$ radius $\times\,\pi$), this number of earthworms would theoretically yield approximately 500 ml of gut contents per square meter of soil, illustrating that the alimentary canal of the earthworm can be an important component of soil.

The alimentary canal of the earthworm constitutes a mobile anoxic micro-compartment in aerated soils (Drake and Horn, 2007). The *in situ* conditions of the earthworm gut include anoxia, near neutral pH, relatively low redox potentials and millimolar concentrations of monosaccharide equivalents in the aqueous phase (Horn *et al.*, 2003; Drake and Horn, 2007; Wüst *et al.*, 2009a). The high concentration of saccharides in the gut appears to be derived from the saccharide-rich mucus that is secreted in the alimentary canal for facilitating gut passage and digestion of the ingested matter (Lavelle, 1986; Barois, 1987, Edwards and Bohlen, 1996; Trigo *et al.*, 1999;

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Brown *et al*., 2000). The *in situ* conditions of the gut stimulate ingested obligate anaerobes and facultative aerobes that promote diverse fermentations along the alimentary canal, and fermentation-derived molecular hydrogen (H_2) is emitted *in vivo* by earthworms (Karsten and Drake, 1995; Schmidt *et al*., 2001; Wüst *et al*., 2009a, 2011). Fermentation products in the gut may serve as substrates for soil-derived denitrifiers and be trophically linked to the *in vivo* emission of nitrous oxide (N_2O) and dinitrogen (N_2) (Karsten and Drake, 1997; Matthies *et al*., 1999; Depkat-Jakob *et al*., 2013). In this regard, earthworms can contribute to the capacity of soils to emit nitrogenous gases (Karsten and Drake, 1997; Matthies *et al*., 1999; Rizhiya *et al*., 2007; Lubbers *et al*., 2011), and it has been postulated that the emission of H_2 by earthworms might drive energy-dependent processes in soil such as the fixation of N_2 or carbon dioxide (CO_2) (Wüst *et al*., 2009a). Soil iron is likewise subject to anaerobic redox transformations during gut passage (Wüst *et al*., 2009a). These findings illustrate that the alimentary canal of earthworms augments diverse anaerobic microbial activities that impact on the cycling of elements in soils.

Several studies have indicated that earthworms do not emit methane (CH_4) (Hornor and Mitchell, 1981; Karsten and Drake, 1995; Drake and Horn, 2007; Šustr and Šimek, 2009). It was therefore surprising that certain earthworms in Brazil, in particular *Eudrilus eugeniae*, were recently discovered to emit CH_4 *in vivo* (Depkat-Jakob *et al*., 2012). *E. eugeniae* is an epigeic species that is native to certain African soils but is commonly used in vermicomposting in other countries, including Brazil (Martinez, 1998; Domínguez, 2004; Oboh *et al*., 2007). The emission of CH_4 by *E. eugeniae* appeared to be linked to the ingested methanogens of the families *Methanosarcinaceae*, *Methanobacteriaceae* and *Methanomicrobiaceae* (Depkat-Jakob *et al*., 2012). We hypothesized that the capacity of *E. eugeniae* to emit CH_4 is linked to diverse fermentations in the alimentary canal, and the main objective of this study was to resolve anaerobic processes and associated taxa that can potentially drive methanogenesis in the gut of *E. eugeniae*.

Material and methods

Earthworms
Adult earthworms of the species *E. eugeniae* (*Eudrilidae*) were obtained in May 2012 from the distributer Minhobox (Juiz de Fora, Minas Gerais, Brazil). Earthworms were 2.3 ± 0.2 g and 11–20 cm in length. The gut passage time of adult specimens of *E. eugeniae* is approximately 6 h (Mba, 1988).

In vivo emission of CH_4
Individual specimens of *E. eugeniae* were washed with sterile deionized water, dried by blotting with tissue paper, weighed and placed into sterile 160-ml serum vials (one earthworm per replicate). Serum vials were sealed with butyl rubber septum stoppers and incubated for 5 h at room temperature in the dark. The gas phase was air.

Gut content microcosms
Earthworms were washed and exposed to ice-cold, sparkling mineral water. Gut content (approximately 25 g) was squeezed out from approximately 100 earthworms while gassing with 100% argon to minimize exposure of the gut content to air (Depkat-Jakob *et al*., 2012). Gut content was kept under an anoxic atmosphere of 100% argon in sterile serum bottles and mixed to a 1:10 (w/v) dilution with sterile anoxic sodium phosphate buffer (Wüst *et al*., 2011). The gut content homogenate was distributed into nine sterile anoxic 160-ml serum bottles that were crimp sealed with sterile butyl rubber stoppers and wrapped with tinfoil to minimize exposure to light; the gas phase was 100% argon. Each serum bottle contained 18-ml gut content homogenate (corresponding to approximately 1.8-g gut content) and was preincubated overnight in the dark at 25 °C to ensure that residual molecular oxygen (O_2) was consumed. Replicates of three serum bottles were treated with either 2 ml of 100 mM [^{12}C]-glucose (AppliChem GmbH, Darmstadt, Germany) or 2 ml of 100 mM [^{13}C]-glucose (Sigma-Aldrich Chemie GmbH, Munich, Germany; 99 atom% ^{13}C). Two milliliters of sterile anoxic water was added to three control bottles. Bottles were incubated in the dark at 25 °C. Samples were taken at different intervals after the addition of substrate with sterile syringes that had been flushed with sterile argon. Samples (1 ml) for molecular analyses were preserved in 9-ml RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany) and stored at −20 °C. Gas samples (3 ml) were collected in Exetainers (Labco Limited, High Wycombe, UK). Aqueous samples (1 ml) for chemical analyses were filter sterilized through autoclaved nylon filters (0.2-μm pore size; Infochroma, Zug, Switzerland) and stored at −20 °C in sterile safe-lock tubes (Eppendorf, Hamburg, Germany).

Methanogenic-enrichment culture
The enrichment medium contained (in mg l^{-1}) mineral salts (KH_2PO_4 10; NH_4Cl 4.6; $MgCl_2 \bullet 6H_2O$ 10; $CaCl_2 \bullet 2H_2O$ 10, (modified from Wüst *et al*., 2009b)), trace metals ($MnSO_4 \bullet H_2O$ 2.5; $FeCl_2 \bullet 4H_2O$ 0.7; $CoCl_2 \bullet 2H_2O$ 1; $CaCl_2 \bullet 2H_2O$ 1; $ZnCl_2$ 0.5; $AlK(SO_4)_4 \bullet 12H_2O$ 0.2; H_3BO_3 0.1; $Na_2MoO_4 \bullet 2H_2O$ 0.1; $CuSO_4 \bullet 5H_2O$ 0.1; $Na_2WO_4 \bullet 2H_2O$ 0.05; $NiCl_2 \bullet 2H_2O$ 0.2; H_2SeO_3 0.5 (modified Balch *et al*., 1979)), 10 ml vitamin solution (Balch *et al*., 1979), 0.5 g yeast extract, 0.5 g tryptone, 15 g $NaHCO_3$, 0.1 mg resazurin, 0.03 g cysteine and 0.03 g NaS. The pH was 7. Enrichments were incubated at 25 °C

with a CO_2 head space and supplemented with either 22 mM H_2 or 5 mM acetate for 14 days. Unsupplemented microcosms served as controls. Enrichments were prepared in triplicate.

Analytical methods

The pressure in the incubation bottles was measured with a pressure transducer (DMG 2120, Ballmoos Elektronik AG, Horgen, Switzerland). The pH of liquid samples was measured with a pH-Meter (WTW pH 330, Wissenschaftliche Werkstätten, Weilheim, Germany) and was used for calculating CO_2 production. The fresh weight (FW) of gut content at each sampling point was calculated by subtracting the amount of gut content removed at previous samplings. Gas concentrations of CO_2, H_2 and CH_4 were determined with a SRI8610C gas chromatograph (SRI Instruments, Torrance, CA, USA). CH_4 and H_2 were separated with a Molecular Sieve Column (13 ×, 2 m × 1/8 in; Restek, Bellefonte, PA, USA). CO_2 was separated with a HayeSep-D column (2 m × 1/8 in; SRI instruments). The carrier gas was helium at a flow rate of 40 ml min^{-1} for CO_2 and 20 ml min^{-1} for CH_4 and H_2. The oven temperature was 60 °C. The temperature of the detector (thermal conductivity detector) for CO_2 was 175 °C. The temperature of the detector (helium ionization detector) for CH_4 and H_2 was 150 °C. Gas concentrations were calculated as previously described (Küsel and Drake, 1995). Organic compounds in the aqueous phase were analyzed by high performance liquid chromatography as previously described (Wüst *et al.*, 2009a). The ^{13}C content of CH_4 and CO_2 was determined by gas chromatograph combustion isotoperatio mass spectrometry as previously described (Conrad *et al.*, 2007). The CH_4 emitted by living earthworms was measured with a Hewlett-Packard Co. 5980 series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) (Küsel and Drake, 1995).

Extraction of nucleic acids

Nucleic acids were extracted as previously described (Depkat-Jakob *et al.*, 2012), and the extracts of each replicate were pooled per treatment. DNA was degraded by treatment with 1 U DNAse I per µl (Fermentas GmbH, St Leon-Rot, Germany), and RNA was purified by precipitation with 0.7 volume isopropyl alcohol and 0.1 volume of 5 mM sodium chloride (Merck KGaA, Darmstadt, Germany). The removal of DNA was confirmed by the inability to amplify 16S rRNA gene fragments by PCR without reverse transcription (see below).

RNA stable isotope probing (RNA SIP)

RNA SIP was performed per published protocol (Whiteley *et al.*, 2007). A gradient solution (buoyant density of 1.793 ± 0.002 g ml^{-1}) was prepared by mixing 4.61 ml caesiumtrifluoroacetate solution (buoyant density: 2.0 ± 0.05 g ml^{-1}; GE Healthcare, Buckinghamshire, UK), 0.175 ml formamide and a variable amount of gradient buffer (100 mM KCl, 100 mM Tris, 1 mM EDTA, pH 8). The gradient solution was added to 68–506 ng RNA and placed in 4.9-ml OptiSeal Polyallomer Tubes (13 × 48 mm; Beckmann, Palo Alto, CA, USA). A centrifugation tube filled with gradient solution and 20 µl diethylpyrocarbonate (DEPC)–H_2O served as blank for determining the densities of fractions after centrifugation. All gradients were set up with the same gradient solution. The separation of [^{12}C]-RNA and [^{13}C]-RNA was achieved by isopycnic centrifugation at 130 000 *g* (37 800 r.p.m.) at 20 °C for 67 h in a LE-70 ultracentrifuge (Beckman Coulter, Fullerton, CA, USA). The rotor stopped without braking. Ten fractions (0.45 ml) of each gradient were collected manually (Manefield *et al.*, 2002) using a suction pump (Econo Pump1, Bio-Rad, Hercules, CA, USA). The densities of the fractions were determined by weighting at 25 °C. RNA in each fraction was precipitated according to Lottspeich and Engels (2006). In all, 200 µl of RNA was mixed with 130 µl of 3 M RNAse-free sodium acetate buffer (pH 5.2), 13.6 µl of a sterilized solution of 10 mg glycogen per ml and 1020 µl of ice-cold 96% ethanol. RNA was precipitated overnight at − 20 °C and centrifuged for 20 min at 14 000 r.p.m. and 4 °C. The supernatant was removed, and the RNA-pellet was washed with 500 µl of ice-cold RNAse-free 70% ethanol. The purified RNA was eluted in DEPC–H_2O. The concentration of RNA in each fraction was determined with a Quant-iT RiboGreen RNA Kit (Invitrogen, Eugene, OR, USA). RNA was stored at − 80 °C.

Reverse transcription and PCR amplification

Extracted RNA was transformed into complementary DNA (cDNA) by reverse transcription with SuperScript III reverse transcriptase as previously described (Depkat-Jakob *et al.*, 2012). PCR amplification of bacterial 16S rRNA genes from cDNA was performed with the primers 27F and 907RM (5′-AGAGTTTGATCMTGGCTC-3′; 5′-CCGTCAATTC MTTTGAGTTT-3′; Lane, 1991). The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 5 cycles at 95 °C for 60 s, at 40 °C for 60 s, and at 72 °C for 90 s, and 35 subsequent cycles at 95 °C for 60 s, at 50 °C for 30 s, and at 72 °C for 90 s. The final elongation was at 72 °C for 5 min. Final concentrations of PCR reagents were 1 × 5Prime Master Mix (5Prime, Hamburg, Germany), 1 mM magnesium chloride and 0.6 µM of each primer. cDNA of *mcrA* and *mrtA* transcripts of the RNA SIP analysis and *mcrA* and *mrtA* of the methanogenic H_2–CO_2 enrichment culture were amplified as previously described (Depkat-Jakob *et al.*, 2012).

Sequence analyses

Cloning of PCR products for *mcrA*, *mrtA* and 16S rRNA cDNA retrieved from pooled fractions

2 and 3 (representing the 'heavy' [labeled] RNA; buoyant density: 1.803 ± 0.001–$1.794 \pm 0.003\,g\,ml^{-1}$; Supplementary Figure S1) and from pooled fractions 9 and 10 (representing the 'light' [unlabeled] RNA; buoyant density: 1.743 ± 0.003–$1.735 \pm 0.004\,g\,ml^{-1}$; Supplementary Figure S1), and also of PCR products obtained from the enrichment culture, was performed as previously described (Schmidt *et al.*, 2014). PCR products of clones with correct inserts were selected for sequencing at Macrogen Europe (Amsterdam, the Netherlands). Sequences were analyzed with MEGA 5.1 (Tamura *et al.*, 2011), ARB (Version 2005; Ludwig *et al.*, 2004) and BLASTn (Altschul *et al.*, 1990). *McrA* and *mrtA* clone sequences and reference sequences were translated *in silico* into amino-acid sequences and aligned with ARB, resulting in a final alignment of 130 aligned amino-acid positions. SINA Webaligner was applied to align 16S rRNA cDNA sequences, which were then merged with the 16S rRNA gene database from SILVA homepage (http://www.arb-silva.de/; last visit: 15/01/13; Pruesse *et al.*, 2007). The resulting alignment contained 880 aligned nucleotide positions. Chimeric 16S rRNA cDNA gene sequences were identified as described (Schmidt *et al.*, 2014). Potential chimeric sequences were blasted (BLASTn) and corrected by removing the shorter part of the sequence at the connection point of the different fragments. Retrieved amino-acid sequences were assigned to different Operational Taxonomic Units (OTUs) with DOTUR (Schloss and Handelsman, 2005). A conservative threshold value of 87.5% was used for determining family-level OTUs of 16S rRNA gene sequences (Yarza *et al.*, 2008). A conservative threshold value of 85.7% was used for creating species-level OTUs of *mcrA* and *mrtA* sequences (Hunger *et al.*, 2011); a maximal identity of $\leqslant$85.7% is indicated by 'spp.' after the genus name. The classification of 16S rRNA gene sequences was accomplished with the RDP classifier (Wang *et al.*, 2007), by DOTUR analysis and via BLASTn. A sequence was assigned to a novel family when the maximum identity to a known sequence in the NCBI database was $<$87.5%. The coverage of the gene libraries was calculated according to Schloss *et al.* (2004), and rarefaction curves were constructed with aRarefact (http://www.uga.edu/~strata/software; last accessed: 8 January 2013) (Hurlbert, 1971; Heck *et al.*, 1975).

Phylogenetic analyses

Phylogenetic trees of *mcrA* and *mrtA* were constructed with ARB by applying the algorithms neighbor-joining (Kimura correction (Saitou and Nei, 1987); 10 000 boostraps), maximum-likelihood (Dayhoff PAM modell, Phylip PROML; 100 bootstraps) and maximum-parsimony (Phylip PROTPARS; 500 bootstraps). Phylogenetic trees of *mcrA* and *mrtA* were based on a 100% similarity filter and 130 valid amino acids between positions 327 and 457 of *mcrA* of *Methanocella paludicola* SANAE. Phylogenetic trees of 16S rRNA cDNA sequences were constructed using neighbor-joining (Felsenstein correction (Felsenstein, 1985; Saitou and Nei, 1987); 10 000 bootstraps), AxML and maximum-parsimony methods and applying a 100% similarity filter of 880 valid nucleotides between positions 26 and 906 of the 16S rRNA gene sequence of *Escherichia coli* ATCC 11775.

Nucleotide sequence accession numbers

Sequences were submitted to the European Nucleotide Archive (accession numbers: HG964568–HG964633 (16S rRNA), HG964544–HG964567 (*mcrA* and *mrtA* of RNA SIP), and LK936462–LK936502 (mcrA and *mrtA* of methanogenic enrichment)).

Results

The experiments outlined below were designed to first demonstrate that *E. eugeniae* emitted CH_4 *in vivo* and to subsequently examine the capacity of gut contents from *E. eugeniae* to dissimilate glucose, selected as a model saccharide found in the gut. [^{13}C]-glucose was utilized as substrate (a) so that glucose-derived carbon could be traced to CH_4 and (b) for the RNA SIP-based assessment of taxa involved in the methanogenic food web.

In vivo emission of CH_4

E. eugeniae emitted CH_4, with the average *in vivo* emission approximating 40 nmol CH_4 per gram FW in 5 h (Figure 1).

Dissimilation of glucose

Approximately 90 μmol supplemental glucose per gram FW gut content was consumed within 18 h in the [^{12}C]- and [^{13}C]-glucose treatments (Figure 2a). Glucose consumption occurred without apparent delay, indicating that microbes in the gut contents were poised to respond rapidly to nutrient input under anoxic conditions. Approximately 1 μmol CH_4 per gram FW and 4 μmol CO_2 per gram FW accumulated during the preincubation period.

Gaseous (Figure 2) and soluble (Figure 3) products were similar for [^{12}C]- and [^{13}C]-glucose treatments. Up to approximately 88 and 74 μmol H_2 per gram FW accumulated during the [^{12}C]- and [^{13}C]-glucose treatments, respectively, whereas only approximately 5 μmol H_2 per gram FW was detected in unsupplemented controls (values are the difference between t_0 and t_{24} time points) (Figure 2d). CO_2 accumulation was rapid and relatively linear after the addition of glucose and yielded approximately 135 and 119 μmol CO_2 per gram FW during the [^{12}C]- and [^{13}C]-glucose treatments, respectively (values are the difference between t_0 and t_{24} time points) (Figure 2c). The accumulated CO_2 in the [^{13}C]-glucose treatment was strongly enriched in ^{13}C,

reaching 70 atom%. Approximately 14 μmol CO_2 per gram FW accumulated in the unsupplemented controls (Figure 2c) (value is the difference between t_0 and t_{24} time points).

Approximately 6 μmol CH_4 per gram FW accumulated in glucose treatments, whereas approximately half this much CH_4 accumulated in unsupplemented controls (Figure 2b) (values are the difference between t_0 and t_{24} time points). The accumulated CH_4 in the [^{13}C]-glucose treatment was strongly enriched in ^{13}C, reaching 50 atom%. Based on a linear *in vivo* emission of CH_4 (Depkat-Jakob *et al.*, 2012) and an hourly *in vivo* emission rate of approximately 8 nmol CH_4 per g FW earthworm (calculated from Figure 1), approximately 0.2 μmol CH_4 per g FW earthworm would be emitted *in vivo* in 24 h. In contrast, approximately 3 μmol CH_4 per g FW gut content was produced during the 24-h incubation without supplemental glucose (this value is the difference between the t_0 and t_{24} time points). In addition, approximately 1 μmol CH_4 per g FW gut content was produced during the overnight preincubation. Thus, on a FW basis, the gut content produced substantially more CH_4 than did living earthworms, a result consistent with the fact that the gut content represents a very small amount of the total FW of the earthworm but is nonetheless the source of the CH_4 that is emitted *in vivo*.

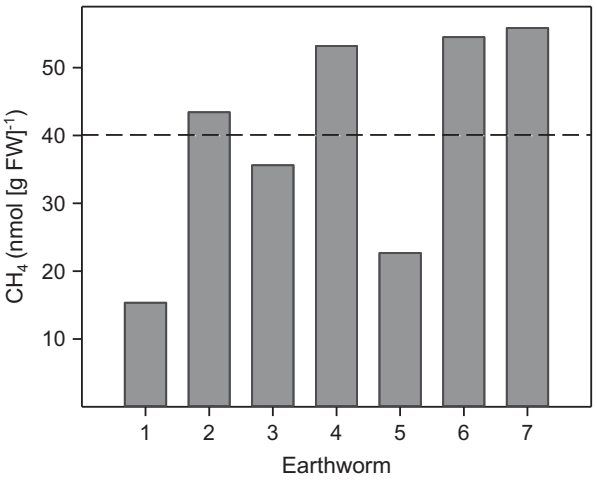

Figure 1 *In vivo* emission of CH_4 by seven individual specimens of *E. eugeniae*. Values represent the amount of CH_4 emitted during a 5-h incubation period under an atmosphere of air. The dashed line indicates the mean value.

Diverse fermentations were substantially more stimulated by supplemental glucose than was methanogenesis (Figures 2 and 3). Formate, ethanol, lactate and succinate were produced and subject to subsequent consumption in glucose treatments; in contrast, acetate and propionate accumulated as end products in glucose treatments (Figure 3). Trace amounts of butyrate and isobutyrate were detected in controls and glucose treatments. At the end of the 24 h of incubation, approximately 69% and 61% of supplemented carbon and approximately 63% and 53% of supplemented electrons were recovered in the detected products of the [^{13}C]- and [^{12}C]-glucose treatments, respectively (Table 1). Unrecovered carbon and electrons may have been due in part to non-detected processes (for example, poor recovery of CO_2 because of undetected carbonates, non-detected fermentation products or incomplete recovery of those detected, formation of storage polymers and assimilation of carbon).

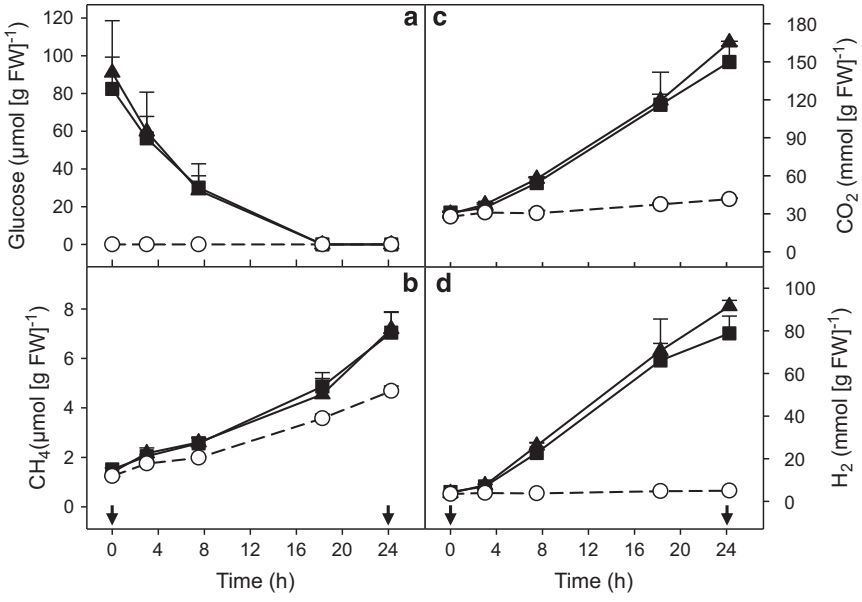

Figure 2 Effect of supplemental glucose (**a**) on the production of CH_4 (**b**), CO_2 (**c**) and H_2 (**d**) by the gut contents of *E. eugeniae* incubated under anoxic conditions. Symbols represent the mean of triplicate incubations, and error bars represent the s.d. Arrows indicate the time point at which the samples were taken for RNA-SIP analysis. Symbols: empty circles, control (no supplemental glucose); filled squares, [^{13}C]-glucose treatments; filled triangles, [^{12}C]-glucose treatments.

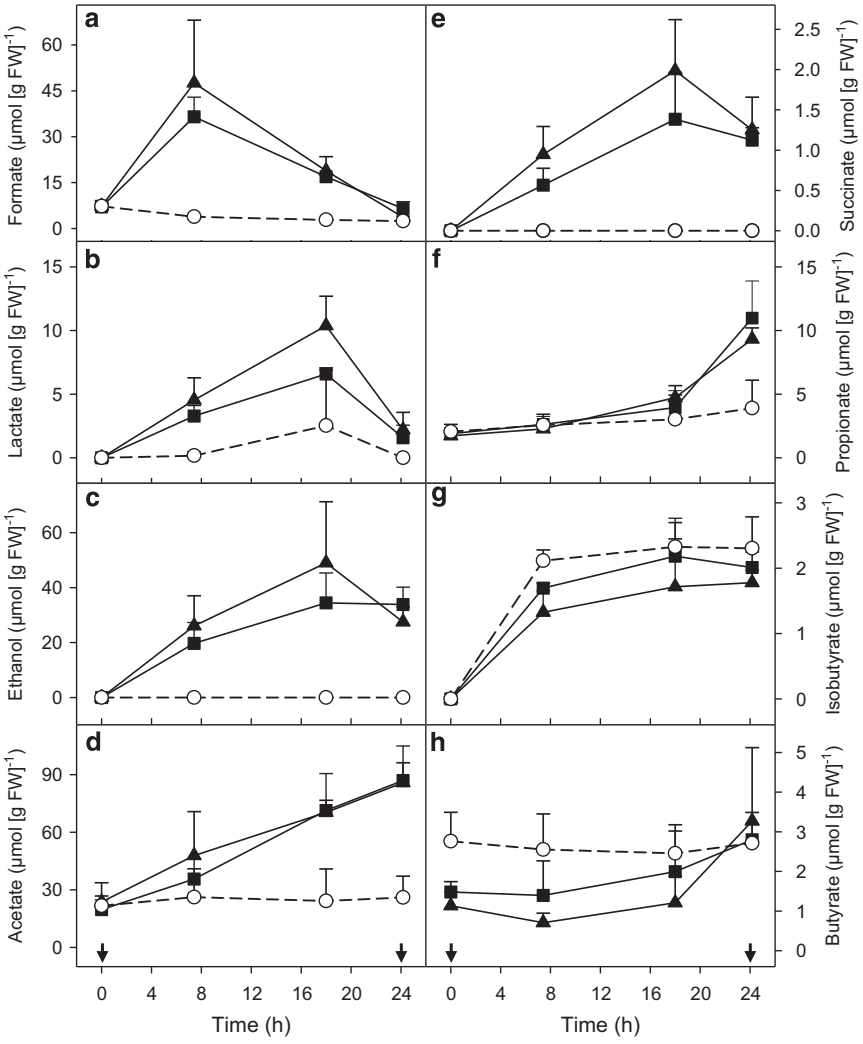

Figure 3 Effect of supplemental glucose on the production of formate (**a**), lactate (**b**), ethanol (**c**), acetate (**d**), succinate (**e**), propionate (**f**), isobutyrate (**g**) and butyrate (**h**) by the gut contents of *E. eugeniae* incubated under anoxic conditions. Symbols represent the mean of triplicate incubations, and error bars indicate the s.d. Arrows indicate the time point at which the samples were taken for RNA-SIP analysis. Symbols: empty circles, control (no supplemental glucose); filled squares, [^{13}C]-glucose treatments; filled triangles, [^{12}C]-glucose treatments.

Detected bacteria

A total of 528 bacterial 16S rRNA cDNA sequences were analyzed. The family-level coverage per clone library ranged between 85% and 94% (Supplementary Table S1), and rarefaction analyses confirmed that sequencing was sufficient for family-level coverage (Supplementary Figure S2A). Sequences from all clone libraries were affiliated with 46 different families and sub-orders; four families were defined as novel (Table 2). Families (and sub-orders) were assigned to the phyla *Firmicutes* (46.2%), *Proteobacteria* (19.5%, dominated by *Gammaproteobacteria* (11.4%)), *Actinobacteria* (16.5%), *Fusobacteria* (5.5%), *Planctomycetes* (5.5%), *Tenericutes* (4.5%), *Spirochaetes* (0.8%), *Verrucomicrobia* (0.8%), *Bacterioidetes* (0.6%) and *Chloroflexi* (0.2%) (Figure 4a and Table 2). Sequences obtained from cDNA at the start of incubation were related to 28 different families and sub-orders (Table 2). Those sequences were mostly related to the phyla *Actinobacteria* (21.7%), *Fusobacteria* (15.0%) and *Firmicutes* (28.3%), of which 39.2% were related to the family *Peptostreptococcaceae* (Figure 4a).

The bacterial 16S rRNA cDNA sequences obtained from the glucose treatments at the end of the 24-h incubation were affiliated with 41 different families and sub-orders (Table 2). Those sequences were mostly related to the phyla *Firmicutes* (55.2%), Proteobacteria (20.4%, dominated by *Gammaproteobacteria* (12.9%)) and Actinobacteria (13.8%) (Figure 4a). *Lachnospiraceae I* (18.1%), *Peptostreptococcaceae* (14.1%) and *Aeromonadeaceae* (9.8%) were the most abundant families.

Bacterial community composition

The shift of the buoyant densities of extracted RNA from [^{13}C]-glucose treatments toward fractions with higher densities (Supplementary Figure S1)

Table 1 Recoveries of carbon (C-mol balance) and electrons (E-mol balance) in the detected products at the end of the 24-h incubation

Products	*Concentration of the detected products (μmol (g FW)$^{-1}$)*		*C-mol balance (%)*		*E-mol balance (%)*	
	[^{12}C]-Glc	*[^{13}C]-Glc*	*[^{12}C]-Glc*	*[^{13}C]-Glc*	*[^{12}C]-Glc*	*[^{13}C]-Glc*
Carbon dioxide	121.4	105.1	22.2	21.3	NA	NA
Methane	2.3	2.1	0.4	0.4	0.9	0.8
Hydrogen	86.1	72.9	NA	NA	7.9	7.4
Formate	1.3	4.3	0.2	0.9	0.1	0.4
Lactate	2.2	1.6	1.2	1.0	1.2	1.0
Ethanol	27.5	33.9	10.1	13.7	15.1	20.6
Acetate	57.8	62.9	21.2	25.5	21.2	25.5
Succinate	1.3	1.1	0.9	0.9	0.8	0.8
Propionate	5.8	7.2	3.2	4.4	3.7	5.1
Butyrate	2.2	1.4	1.6	1.1	2.0	1.4
Total	307.9	292.1	61.0	69.1	52.8	62.9

Abbreviations: FW, fresh weight; NA, not applicable. The concentrations of the detected products represent means of triplicates and are based on the differences between the t_0 and t_{24} time points, corrected by the concentration of the detected products of the unsupplemented controls. Balances are based on the amount of carbon or electrons recovered in the products versus the amount of carbon or electrons available in the glucose that was consumed.

reinforces the likelihood that some of the glucose-derived carbon was assimilated by microorganisms in the *E. eugeniae* gut content. The bacterial community structure of 'heavy' fractions of the [^{13}C]-treatment was distinct from that of the 'light' fractions of the [^{13}C]-treatment and 'heavy' fractions of the [^{12}C]-glucose treatment (Figure 4a). 16S rRNA cDNA sequences obtained from 'heavy' fractions of the [^{13}C]-glucose treatment at the end of the 24-h incubation were affiliated with 12 different families and sub-orders, most of which were related to *Aeromonadaceae* (27.8%) and *Lachnospiraceae I* (27.8%) (Table 2). Sequences assigned to those families were closely related to *Aeromonas hydrophila* strain ANSE1 (GU296671, 99% similarity, Supplementary Table S3) and *Clostridium propionicum* strain JCM 1430 (AB649276, 97–99% similarity, Supplementary Table S3). *Veillonellaceae*- and *Ruminococcaceae*-affiliated sequences were also mainly abundant in the 'heavy' fractions of the [^{13}C]-glucose treatment (Table 2). Sequences assigned to *Veillonellaceae* and *Ruminococcaceae* were closely related to *Succinispira mobilis* strain 19gly1 (NR_028868, 99% similarity) and *Clostridium viride* strain DSM 6836 (NR_026204, 99% similarity, Supplementary Table S3), respectively. Approximately 19% of the sequences retrieved from 'heavy' fractions of the [^{13}C]-glucose treatment were affiliated to *Peptostreptococcaceae* (Table 2), with the closest known relatives (98–99% similarity, Supplementary Table S3) being *Clostridium mayombei* (FR733682) and *Clostridium glycolicum* strain CIN5 (AY007244).

Detected methanogens

A total of 497 *mcrA* and *mrtA* sequences were assigned to 14 species-level OTUs. The coverage for each clone library was >97% (Supplementary Table S2), and rarefaction analyses confirmed that sequencing was sufficient for species-level coverage (Supplementary Figures S2B and C). Sequences retrieved at the start of incubation were affiliated with *Methanosarcinaceae* (37.2%), *Methanocellaceae* (25.6%), *Methanosaetaceae* (26.1%) and *Methanobacteriaceae* (11.1%) (Figure 4b). The active *mcrA* community structure changed after 24 h of incubation; sequences affiliated with *Methanoregulaceae* were detected, and the relative abundance of *Methanosaetaceae* and *Methanocellaceae* was decreased. *McrA* and *mrtA* sequences of the 'heavy' fraction of [^{13}C]-glucose treatments were assigned to the species-level OTUs 10, 11 and 13. Those OTUs were affiliated with *Methanoregula* spp. and *Methanobacterium formicicum* (Figure 5). Sequences related to those species were not detected or were not abundant in 'light' fractions of [^{13}C]-glucose treatments and 'heavy' fractions of [^{12}C]-glucose treatments.

Methanogenic-enrichment culture

The results above suggested that H_2 rather than acetate was an important driver of methanogenesis. To evaluate this possibility in more detail, enrichment cultures prepared from the gut content of *E. eugeniae* were supplemented with H_2–CO_2 or acetate and incubated for 14 days. Methanogenesis was only stimulated by H_2–CO_2 (0.8 mM CH_4 compared with 0.1 mM CH_4 in controls lacking H_2). Two species-level *mrtA* and four species-level *mcrA* phylotypes affiliated with *Methanobacteriaceae* were detected in the H_2–CO_2 enrichments. The relative abundance of phylotypes and affiliated species were: 39% *Methanobacterium ivanovii* (*mrtA*), 34% *M. ivanovii* (*mcrA*), 15% *Methanobacterium* sp. (*mcrA*), 5% *Methanobacterium formicicum* (*mrtA*), 5% *M. formicicum* (*mcrA*), and 2% *Methanobacterium kanagiense* (*mcrA*). It is noteworthy that acetate production was more

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

Phylogenetic affiliation[a]		*Relative abundance of sequences (%)*					
Phylum and classes	*Order, sub-order (*) and family*	*[^{13}C]-glucose*				*[^{12}C]-glucose*	
		t_0, *L*	t_0, *H*	t_{24}, *L*	t_{24}, *H*	t_{24}, *L*	t_{24}, *H*
Actinobacteria (16.5)	*Acidimicrobiales* (2.2)						
	Acidimicrobiaceae	—	3.3	1.2	—	1.1	—
	Unclassified Acidimicrobineae	3.4	1.1	1.2	—	2.2	—
	Actinomycetales (13.3)						
	Corynebacterineae (Mycobacteriaceae/Dietziaceae/ Nocardiaceae)*	1.1	8.7	1.2	1.1	3.3	8.1
	Micrococcineae (Microbacteriaceae/Micrococcaceae)*	3.4	7.6	3.7	—	4.4	3.5
	Propionibacterineae (Propionibacteriaceae/Nocardioidaceae)	8.0	2.2	3.7	3.3	8.8	7.0
	Coriobacteriales (0.2)						
	Coriobacteriaceae	—	1.1	—	—	—	—
	Rubrobacterales (0.8)						
	Rubrobacteraceae	1.1	2.2	—	—	1.1	—
Bacterioidetes (0.6)	*Bacteroidales* (0.4)						
	Porphyromonadaceae	1.1	—	—	1.1	—	—
	Cytophagales (0.2)						
	Incertae sedis	—	—	—	—	1.1	—
Chloroflexi (0.2)	*Anaerolineales* (0.2)						
	Anaerolineaceae	—	1.1	—	—	—	—
Firmicutes (46.2)	*Bacillales* (2.1)						
	Bacillaceae	1.1	1.1	4.9	—	1.1	3.5
	Planococcaceae	1.1	—	—	—	—	—
	Clostridiales (42.0)						
	Clostridiaceae 1	5.7	5.4	3.7	1.1	6.6	8.1
	Lachnospiraceae I(C. propionicum)	—	—	3.7	27.8	16.5	23.3
	Lachnospiraceae II	4.6	4.4	8.6	3.3	4.4	2.3
	Lachnospiraceae III (C. lentocellum)	2.3	1.1	5.0	1.1	2.2	1.2
	Peptostreptococcaceae	10.2	12.0	12.4	18.9	14.3	10.5
	Peptococcaceae	—	1.1	—	—	—	—
	Ruminococcaceae	1.1	—	4.9	8.9	3.3	5.8
	Veillonellaceae	—	—	—	4.4	3.3	—
	Erysipelotrichales (1.1)						
	Erysipelotrichaceae	2.3	1.1	2.5	—	—	1.2
	Lactobacillales (0.8)						
	Aerococcaceae	2.3	—	1.2	—	—	—
	Carnobacteriaceae	—	—	1.2	—	—	—
Fusobacteria (5.5)	*Fusobacteriales* (5.5)						
	Fusobacteriaceae	20.5	9.8	—	—	1.1	1.2
Planctomycetes (5.5)	Planctomycetales (5.5)						
	Planctomycetaceae I (Gemmata)	2.3	8.7	1.2	—	1.1	—
	Planctomycetaceae II (Rhodospirellula)	—	1.1	—	—	—	2.3
	Planctomycetaceae III (Pirellula)	1.1	1.1	2.5	—	—	—
	Planctomycetaceae IV (Isospaera/Sangulispaera)	—	1.1	2.5	—	—	1.2
	Planctomycetaceae V	1.1	1.1	1.2	—	—	1.2
	Novel family[b]	1.1	—	—	—	1.1	—
Proteobacteria (19.5)							
Alphaproteobacteria (3.2)	*Rhizobiales* (1.7)						
	Hyphomicrobiaceae	—	3.3	5.0	—	—	—
	Phyllobacteriaceae	—	—	2.5	—	—	—
	Rhodobacterales (0.2)						
	Rhodobacteraceae	—	—	1.2	—	—	—
	Rhodospirillales (1.4)						
	Acetobacteraceae	—	1.1	1.2	—	1.1	1.2
	Rhodospirillaceae	—	1.1	2.5	—	—	—
Betaproteobacteria (0.8)	*Burkholderiales* (0.6)						
	Oxalobacteraceae	—	3.3	—	—	—	—
	Neisseriales(0.2)						
	Neisseriaceae	—	—	1.2	—	—	—

Table 2 (Continued)

Phylogenetic affiliation[a]		*Relative abundance of sequences (%)*					
Phylum and classes	*Order, sub-order (*) and family*	*[13C]-glucose*				*[12C]-glucose*	
		t_0, *L*	t_0, *H*	t_{24}, *L*	t_{24}, *H*	t_{24}, *L*	t_{24}, *H*
Deltaproteobacteria (4.2)	*Myxococcales* (4.2)						
	Novel family[b]	—	—	1.2	—	—	—
	Novel family[b]	—	—	—	—	—	1.2
	Polyangiaceae	8.0	2.2	6.2	—	4.4	2.3
Gammaproteobacteria (11.4)	*Aeromonadales* (9.3)						
	Aeromonadaceae	8.0	8.7	—	27.8	7.7	2.3
	Enterobacteriales (1.3)						
	Enterobacteriaceae	—	—	—	1.1	3.3	3.5
	Methylococcales (0.8)						
	Methylococcaceae	—	—	2.5	—	2.2	—
Spirochaetes (0.8)	*Spirochaetales* (0.8)						
	Leptospiraceae	—	—	—	—	3.3	1.2
Tenericutes (4.5)	*Mycoplasmatales* (4.5)						
	Mycoplasmataceae	8.0	4.4	9.9	—	1.1	4.7
Verrucomicrobia (0.8)	Novel family[b](0.8)	1.1	—	—	—	—	3.5

Abbreviations: H, 'heavy' fraction; L, 'light' fractions; t_0, sampling point at the start of incubation; t_{24} sampling point after 24 h of incubation.
[a]Sequences were assigned to bacterial families by using the RDP Classifier, DOTUR analysis and BLASTn search. The number of sequences retrieved from cDNA of 'heavy' and 'light' fractions ranged from 81 to 92 (Supplementary Table S1). The percentage of relative abundances for phyla and orders are shown in parentheses.
[b]Considered as a novel family based on gene sequence similarities $<87.5\%$.

significantly stimulated by H_2–CO_2 than was methanogenesis (13 mM acetate compared with 6 mM acetate in controls lacking H_2), suggesting that H_2 enriched not only methanogens from the gut contents of *E. eugeniae* but acetogens as well.

Discussion

The diverse anaerobic activities that occur in the alimentary canal of earthworms appear to be dominated by ingested microbes that become activated by the unique *in situ* conditions of the alimentary canal (Karsten and Drake, 1995; Matthies *et al.*, 1999; Furlong *et al.*, 2002; Horn *et al.*, 2003; Drake and Horn, 2007; Knapp *et al.*, 2009; Wüst *et al.*, 2009a; Depkat-Jakob *et al.*, 2012). This generalization for the earthworm gut is in contrast to other invertebrates whose guts are heavily colonized by novel endemic microbes (for example, termites; Brune, 2006; Dietrich *et al.*, 2014). The methanogenic food webs of classic methane-emitting ecosystems such as ruminants and wetlands are primarily linked to the initial hydrolysis of autotrophically produced polymers (for example, the hydrolysis of plant-synthesized lignocelluloses; Morrison *et al.*, 2009; Bridgham *et al.*, 2013). In contrast, the methanogenic food web of *E. eugeniae* is likely linked to the hydrolysis of heterotrophically produced polymers, that is, the hydrolysis of earthworm-derived polysaccharide-rich mucus (Wüst *et al.*, 2009a). Indeed, 100 mM monosaccharide equivalents, including >10 mM glucose can occur in the aqueous phase of gut contents (Horn *et al.*, 2003; Wüst *et al.*, 2009a). Glucose was therefore selected as a model saccharide to experimentally evaluate potential trophic interactions of prokaryotes that yield CH_4 in the digestive system of *E. eugeniae*. The rapid and nearly linear production of H_2 and CO_2 by the gut contents of *E. eugeniae* in response to glucose (Figure 2) corroborate previous studies that demonstrated the capacity of microbes in the alimentary canal of various earthworms to augment anaerobic processes (Karsten and Drake, 1995; Matthies *et al.*, 1999; Wüst *et al.*, 2009a, 2011).

Fermentations and fermenters in E. eugeniae gut contents

Concomitant as well as successive fermentations were stimulated by supplemental glucose (Figure 3). The production of formate, acetate, succinate, lactate, ethanol, CO_2 and H_2 is indicative of mixed acid fermentation by facultative aerobes (Gottschalk, 1986). Most sequences retrieved from 'heavy' fractions of [^{13}C]-glucose treatments were affiliated with *Aeromonadaceae*, members of which are facultative aerobes (Martin-Carnahan and Joseph, 2005b). *A. hydrophila* was the closest cultured species to retrieved *Aeromonadaceae*-related sequences and is a facultative aerobe that can ferment sugars (including glucose) to ethanol, formate, acetate, succinate, CO_2 and H_2 (Martin-Carnahan and Joseph, 2005a). Certain sequences of 'heavy fractions' from [^{13}C]-glucose treatments were closely affiliated with *C. viride* (*Ruminococcaceae*), an obligate anaerobe that can ferment glucose to acetate, formate, succinate, lactate and ethanol (Ezaki, 1984; Buckel *et al.*, 1994). *C. viride* is

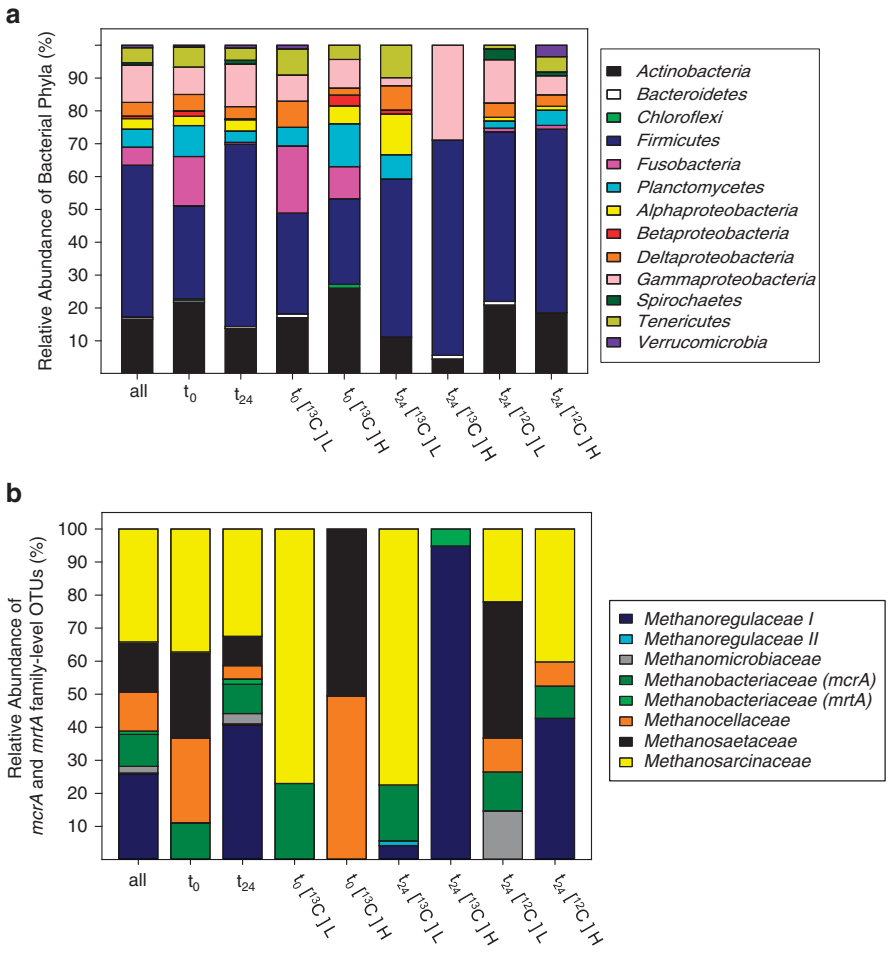

Figure 4 Phylogenetic distribution of bacterial 16S rRNA cDNA (**a**) and *mcrA* and *mrtA* (**b**) sequences of [^{13}C]- and [^{12}C]-glucose treatments. Sequences were assigned to bacterial phyla with the RDP Classifier. Assignment of sequences to methanogenic families was based on ⩽75.4% gene sequence similarities (Hunger ***et al.***, 2011). As indicated in the lower axes, sequence analyses were performed for the total number of sequences (lane designated all), for all sequences at the start (t_0) or at 24 h (t_{24}) of incubation and for each fraction per time point and treatment. H, 'heavy' fractions; L, 'light' fraction; t_0, sampling point at the start of incubation; t_{24}, sampling point at 24 h of incubation; 13 and 12, [^{13}C]- and [^{12}C]-glucose treatments, respectively.

likewise able to ferment amino acids (Buckel *et al.*, 1994), suggesting that bacteria related to *C. viride* might have additionally used endogenous amino acids in the earthworm gut contents (Drake and Horn, 2007). The formation of butyrate, isobutyrate and propionate (Figure 3) is indicative of the fermentation of amino acids (Nanninga and Gottschal, 1985; Gottschalk, 1986), and the high relative abundance of *Peptostreptococcaceae*-affiliated sequences suggests that members of this amino-acid-fermenting taxon (for example, *Clostridium lituseburense*) may have utilized amino acids (Hippe *et al.*, 1992; Ezaki, 2009).

The production of propionate subsequent to the consumption of lactate and succinate (Figure 3) is indicative of *Propionibacteria* (Stackebrandt *et al.*, 2006). However, propionate production could have also been linked to members of the detected family *Lachnospiraceae I* that form propionate when utilizing lactate (for example, *C. propionicum*) (Leaver *et al.*, 1955). Members of *Veillonellaceae* can also produce propionate as well as various volatile fatty acids, CO_2 and H_2 (Morrison, 1984). Certain sequences were closely related to the *Veillonellaceae*-affiliated species *S. mobilis*, a strict anaerobe that can ferment organic and amino acids (but not carbohydrates such as glucose) and produce formate, acetate, propionate, CO_2 and H_2 (Janssen, 1984), compounds detected in the incubations (Figures 2 and 3).

Trophic links of methanogens in the gut contents of E. eugeniae

CH_4 production was not as significantly stimulated by supplemental glucose as was fermentation (Figures 2 and 3). However, ^{13}C-labeling of CH_4 verified that supplemental [^{13}C]-glucose-derived carbon was dissimilated to CH_4, reinforcing the likelihood that glucose-derived carbon was also assimilated by methanogens. High amounts of the methanogenic substrates H_2 and acetate (Zinder, 1993) accumulated in glucose treatments (Figures 2 and 3). These combined findings indicate that methanogens in the

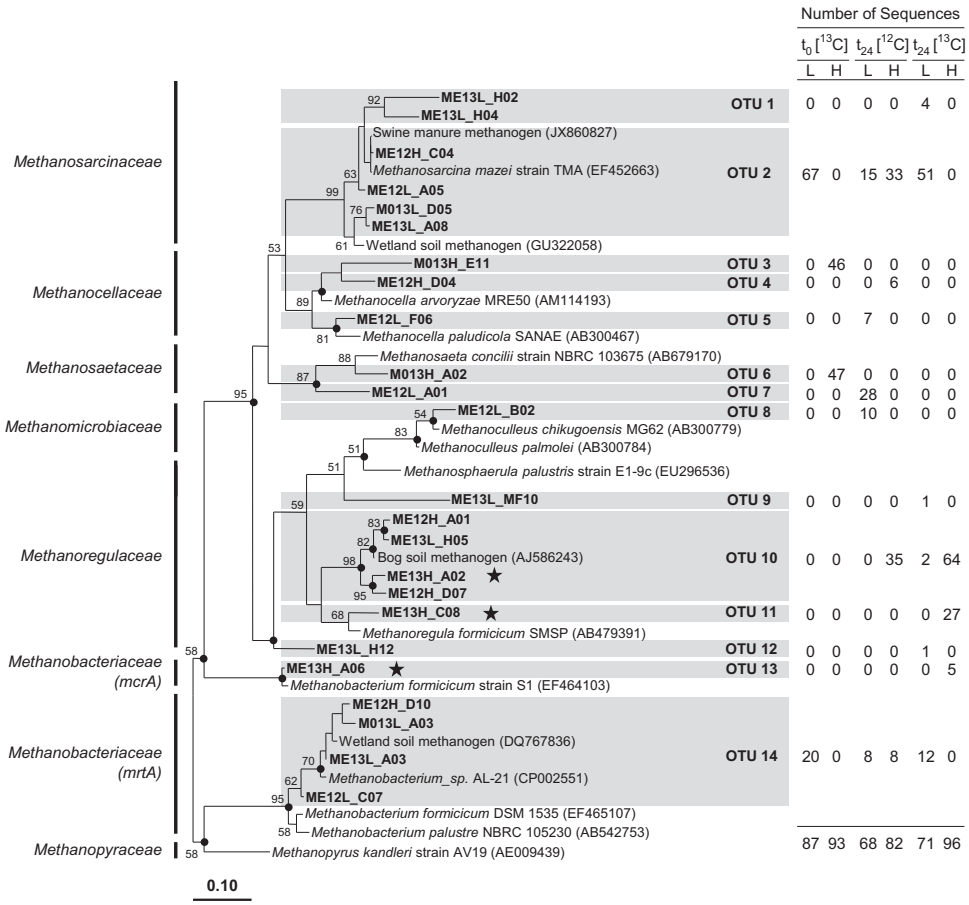

Figure 5 Phylogenetic neighbor-joining tree of representative *mcrA* and *mrtA* amino-acid sequences of [^{13}C]- and [^{12}C]-glucose treatments. Numbers next to the branches represent the percentages of replicate trees (>50%) in which the associated taxa clustered together in the bootstrap test (10 000 bootstraps). Dots at nodes indicate the confirmation of the tree topology by maximum-parsimony and maximum-likelihood calculations with the same data set. Bar represents a 0.1 estimated change per amino acid. Accession numbers of reference sequences are in parentheses. Stars identify labeled *mcrA* and *mrtA* phylotypes. The table outlines the origin of sequences per species-level OTU (based on 85.7% gene sequence similarity; Hunger ***et al.***, 2011). Abbreviations in sequence identifier code: H, 'heavy' fractions; L, 'light' fractions; M0, methanogen sequence from sampling point t_0 (that is, at the start of incubation); ME, methanogen sequence from sampling point t_{24} (that is, at 24 h of incubation); 13 and 12, [^{13}C]- and [^{12}C]-glucose treatments, respectively.

alimentary canal of *E. eugeniae* are closer to substrate saturation than are fermentative taxa.

Methanogens of the families *Methanosarcinaceae*, *Methanobacteriaceae* and *Methanomicrobiaceae* were previously detected in the *E. eugeniae* gut contents (Depkat-Jakob *et al.*, 2012), and the detection of *mcrA* sequences related to these taxa (Figure 4b) suggest that these taxa are linked to methanogenesis in the alimentary canal. Members of *Methanosarcinaceae* are acetoclastic, that is, can convert acetate to CO_2 and CH_4 (Boone *et al.*, 1993; Hedderich and Whitman, 2006). However, *mcrA* sequences related to the genus *Methanosarcina* were not detected in 'heavy' fractions of [^{13}C]-glucose treatments in which acetate accumulated (Figure 3). Thus acetate derived from supplemental glucose did not appear to be a dominant substrate for methanogenesis.

Analysis of *mcrA* sequences from 'heavy' fractions of [^{13}C]-glucose treatments indicated that methanogens closely related to *M. formicicum* and *Methanoregula* species were likely involved in the consumption of glucose-derived fermentation products. *M. formicicum* and species of *Methanoregula* utilize H_2–CO_2 and formate (Schauer and Ferry, 1980; Bräuer *et al.*, 2011; Yashiro *et al.*, 2011). However, which source of reductant (H_2 or formate) was used for methanogenesis remains unclear. Four molecules of H_2 or four molecules of formate are needed to produce one molecule of CH_4 (Hedderich and Whitman, 2006). Approximately 80 µmol H_2 per gram FW accumulated, and approximately 34 µmol formate per gram FW transiently accumulated and were subsequently consumed in the [^{13}C]-glucose treatments (Figures 2 and 3), values that could theoretically yield approximately 20 µmol CH_4 per gram FW and 8 µmol CH_4 per gram FW, respectively. That only an additional 2 µmol CH_4 per gram FW accumulated in glucose treatments compared with the amount of CH_4 that accumulated in unsupplemented controls indicates that relatively little of the glucose-derived H_2 and formate was linked to methanogenesis.

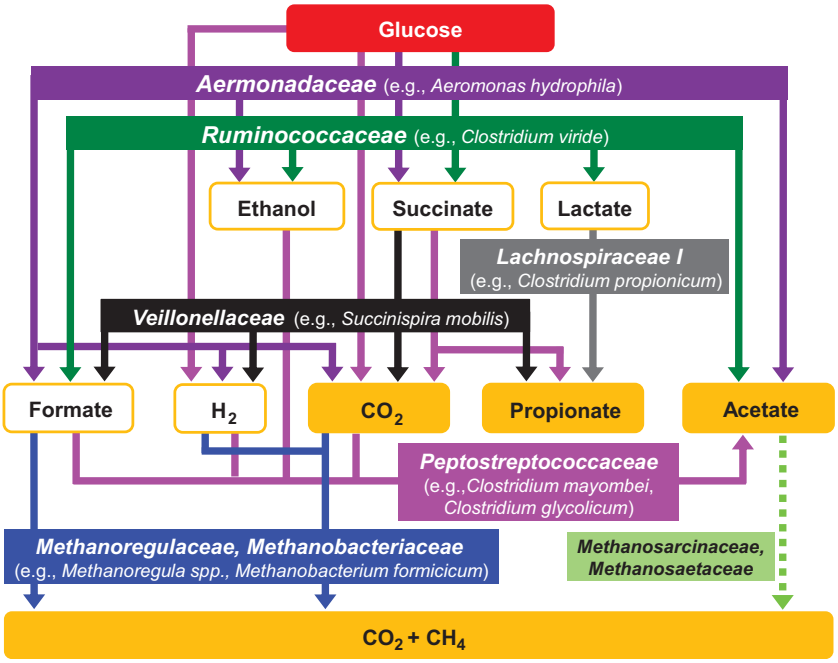

Figure 6 Hypothetical model of the methanogenic food web of the glucose-supplemented gut contents of the CH_4-emitting earthworm *E. eugeniae*. The model is based on detected processes and known functions of the detected taxa. Compounds that accumulated as end products are shown in orange boxes. Species names in brackets represent the closest cultured relatives to retrieved sequences. The dashed light green arrow identifies a potential reaction that was not clearly resolved.

Four molecules of formate are required to synthesize one molecule of acetate via acetogenesis (Drake *et al.*, 2008). Thus approximately 9 µmol acetate per gram FW could have been formed from the approximate 34 µmol formate per gram FW transiently formed from glucose in [^{13}C]-glucose treatments. However, because acetate synthesis was continuous during the entire incubation period and far exceeded this value (Figure 3d), it is not possible to accurately correlate the synthesis of acetate via acetogenesis with the consumption of glucose-derived formate. Nonetheless, the occurrence of acetogens in the gut contents was indicated by (a) the detection of sequences in 'heavy' fractions of [^{13}C]-glucose treatments that were related to the acetogens *C. glycolicum* (Küsel *et al.*, 2001) and *C. mayombei* (Kane *et al.*, 1991) and (b) the H_2-dependent stimulation of acetate synthesis in methanogenic enrichments. Thus acetate production may have been linked to both fermentation and acetogenesis. *C. glycolicum* and *C. mayombei* can utilize diverse substrates for acetogenesis, including H_2–CO_2, carbohydrates (for example, glucose), alcohols and organic acids (for example, formate) (Kane *et al.*, 1991; Küsel *et al.*, 2001), illustrating the potential of acetogens to facilitate multiple trophic links during the flow of carbon in the gut contents. *C. mayombei* can also convert succinate to propionate and CO_2 (Kane *et al.*, 1991). The capacity of acetogens to utilize a variety of electron donors and electron acceptors (Drake *et al.*, 2008) reinforce the likelihood that acetogens could be metabolically active in the complex milieu of the gut contents of *E. eugeniae*.

Conclusions

The collective findings of this study provide the basis for a hypothetical model of the processes and microbial taxa linked to the production of CH_4 (Figure 6). Facultative aerobes of the family *Aeromonadaceae* and obligate anaerobes of the families *Lachnospiraceae I*, *Veillonellaceae* and *Ruminococcaceae* were stimulated by supplemental glucose and are conceived to be representative of taxa involved in the consumption of saccharides in the gut of *E. eugeniae*. Fermentation-derived fatty acids may serve as nutrients for the earthworm under *in situ* conditions (Wüst *et al.*, 2009a).

Different H_2- and fatty acid-producing fermentations can be spatially distributed along the alimentary canal (Wüst *et al.*, 2009a). Thus methanogenesis might be favored in parts of the alimentary canal where specific physico-chemical conditions favor methanogenesis. Fermentation-derived intermediates such as formate and H_2 were likely utilized for methanogenesis by members of the families *Methanobacteriaceae* and *Methanoregulaceae* (Figure 6). Although acetoclastic methanogens (for example, *Methanosarcinaceae* and *Methanosaetaceae*) were detected, assimilation of glucose-derived acetate by such methanogens was not apparent, and acetate did not appear to stimulate acetoclastic methanogens in the SIP experiment or enrichments. However, it cannot be excluded that acetoclastic methanogens are active in the alimentary canal under *in situ* conditions.

Glucose was utilized in the present study as a representative saccharide found in the gut contents.

However, the occurrence of other diverse saccharides such as maltose, mannose, galactose, arabinose and rhamnose in the alimentary canal of earthworms (Wüst *et al*., 2009a) suggests that the *in situ* methanogenic food web in the alimentary canal is more complex than that resolved in the present study. In addition, a more in-depth sequencing will be required to gain a more complete understanding of the prokaryotic species-level diversities of the microbial community associated with the methanogenic food web in the alimentary canal. Current studies are focused on the potential effects that different saccharides might have on trophic interactions that drive methanogenesis and on the *in situ* spatial orientation and regulation of methanogenesis in the alimentary canal of *E. eugeniae*.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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