APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2003, p. 1662–1669 Vol. 69, No. 3
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.3.1662–1669.2003

The Earthworm Gut: an Ideal Habitat for Ingested N_2O-Producing Microorganisms

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Received 5 August 2002/Accepted 26 November 2002

The in vivo production of nitrous oxide (N_2O) by earthworms is due to their gut microbiota, and it is hypothesized that the microenvironment of the gut activates ingested N_2O-producing soil bacteria. In situ measurement of N_2O and O_2 with microsensors demonstrated that the earthworm gut is anoxic and the site of N_2O production. The gut had a pH of 6.9 and an average water content of approximately 50%. The water content within the gut decreased from the anterior end to the posterior end. In contrast, the concentration of N_2O increased from the anterior end to the mid-gut region and then decreased along the posterior part of the gut. Compared to the soil in which worms lived and fed, the gut of the earthworm was highly enriched in total carbon, organic carbon, and total nitrogen and had a C/N ratio of 7 (compared to a C/N ratio of 12 in soil). The aqueous phase of gut contents contained up to 80 mM glucose and numerous compounds that were indicative of anaerobic metabolism, including up to 9 mM formate, 8 mM acetate, 3 mM lactate, and 2 mM succinate. Compared to the soil contents, nitrite and ammonium were enriched in the gut up to 10- and 100-fold, respectively. The production of N_2O by soil was induced when the gut environment was simulated in anoxic microcosms for 24 h (the approximate time for passage of soil through the earthworm). Anoxia, high osmolarity, nitrite, and nitrate were the dominant factors that stimulated the production of N_2O. Supplemental organic carbon had a very minimal stimulatory effect on the production of N_2O, and addition of buffer or ammonium had essentially no effect on the initial N_2O production rates. However, a combination of supplements yielded rates greater than that obtained mathematically for single supplements, suggesting that the maximum rates observed were due to synergistic effects of supplements. Collectively, these results indicate that the special microenvironment of the earthworm gut is ideally suited for N_2O-producing bacteria and support the hypothesis that the in situ conditions of the earthworm gut activate ingested N_2O-producing soil bacteria during gut passage.

Denitrification in the earthworm gut is involved in the in vivo emission of N_2O by earthworms (23), cultured denitrifiers occur in high numbers in the earthworm gut (17), and denitrification can occur in earthworm casts (9, 35). Most denitrifiers possess the capacity to both produce and consume N_2O (6), and the net release of N_2O during denitrification is regulated by various parameters, including pH (29), the phase of growth (3), and the concentrations of nitrate and electron donors (19). High numbers of other organisms that are capable of producing N_2O (i.e., nitrate-dissimilating and nitrifying bacteria) are also present in the earthworm gut (14). Production of N_2O by nitrate-dissimilating bacteria is favored in systems that contain high levels of organic carbon, like the rumen or the gastrointestinal tracts of higher animals (18, 36). Some nitrifiers are able to use nitrate or nitrite as electron acceptors and by using this nitrifier denitrification system can produce N_2O and/or N_2 under oxygen-limited conditions (12, 26).

In the companion paper, enumeration and isolation of N_2O-producing bacteria of the earthworm gut are described, and the following activation hypothesis is proposed (14): inactive or dormant soil bacteria that are ingested into the favorable physicochemical environment of the earthworm gut are activated and produce N_2O during passage through the gut. The goal of this study was to address this hypothesis, and our two main objectives were (i) to characterize the physicochemical parameters of the earthworm gut environment and (ii) to simulate gut conditions in soil microcosms and identify the parameters that could be responsible for activating the N_2O-producing soil microorganisms that are ingested into the microenvironment of the earthworm gut.

MATERIALS AND METHODS

Field sites and sampling. Earthworms ($n = 210$) were identified by standard protocols (4) as *Apporectoedea caliginosa* (Savigny), *Allolobophora chlorotica* (Savigny), *Lumbricus terrestris* L., and *Lumbricus rubellus* (Hoffmeister). Earthworms and soil samples from the uppermost 10 to 40 cm of soil were obtained from seven different sites near Bayreuth, Germany (meadow, garden, and field) (Table 1); each site contained all four of the earthworm species mentioned above. Earthworms and soil samples were transported in aseptic beakers, and they were used immediately or were stored at 2°C in the dark until they were processed. Several randomly chosen worms from all sites tested positive for in vivo emission of N_2O (the protocol used was the protocol described previously [23]).

Microsensor measurements. Earthworms that were washed with sterile double-distilled water and sedated with ethanol (40%) were embedded in a horizontal position in 1.5% agarose prior to in situ microsensor measurements; the upper half of each body was left exposed to air and was not embedded in agar. Clark-type microsensors for O_2 (27) and N_2O (1) with internal references and guard cathodes were purchased from Unisense (Aarhus, Denmark). The stirring sensitivities were <2%, and the tip diameters were <10 μm (O_2 sensor) and <25 μm (N_2O sensor). The 90% response times were <3 and <20 s for the O_2 and N_2O sensors, respectively. The O_2 sensors were calibrated with N_2 and air-saturated water. The N_2O sensors were calibrated with N_2O concentrations ranging from 0 to 19 μM. Liquid ion-exchange membrane pH microsensors were manufactured as described previously (7). The microsensors were mounted on a

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TABLE 1. Study sites

Site	Location	Use	Cultivation	Soil texture	Soil pH (H_2O)
H	Heinersreuth	Garden	Organic[a]	Sandy clay loam	7.1
U	Unternschreez	Garden	Organic[a]	Silty loam	6.8
UD	Upper Dappert	Field	Organic[a]	Sandy loam	6.9
LD	Lower Dappert	Field	Organic[a]	Sandy loam	6.9
TW	Trafo Wiese	Meadow	Organic[a]	Sandy clay loam	6.8
B	Brunnacker	Field	Conventional[b]	Loamy sand	6.6
L	Leite	Field	Conventional[b]	Sandy loam	6.6
HW	Hofmanns Wiese	Meadow	Conventional[b]	Silty loam	7.1

[a] Mineral fertilizers and pesticides were not added.
[b] Mineral fertilizers and pesticides were added.

micromanipulator (Märtzhäuser, Wetzlar, Germany), and radial concentration profiles were measured through whole earthworms. Alternatively, single-point measurements were obtained with a microsensor tip positioned at approximately the center of the gut.

Extraction of gut contents and soil. Worms were washed three times with sterile double-distilled H_2O and sedated with ethanol (40%); the gut contents were released by squeezing intact worms from the anterior end to the posterior end. Alternatively, the worms were dissected, and the gut behind the gizzard was divided into the following four parts: anterior region, midgut region A, midgut region B, and posterior region. Gut contents from worms belonging to the same species were pooled to obtain samples that weighed approximately 0.5 g (fresh weight). Pooled gut contents (fresh weight, 0.5 g) and soil (fresh weight, 20 g) were extracted with 2 and 20 ml of double-distilled H_2O, respectively, by vortexing at the maximum speed for approximately 1 min and subsequent extraction for 16 h at 2°C (similar results were obtained with an extraction time of 1 h). Solid matter was separated by centrifugation (10,000 × *g*), and the supernatant fluids (extracts) were filtered (pore size, 0.2 μm) and stored at −20°C until they were analyzed.

Microbial production of N_2O in gut simulations with soil microcosms. Soil samples (fresh weight, 5 to 10 g) were aseptically placed into serum vials (40 ml). Anoxic soil microcosms were flushed with argon. Supplements were added from sterile, anoxic stock solutions. The final concentrations of additives in the aqueous phase of soil in the microcosms were as follows: saline (NaCl), 130 mM; sodium phosphate buffer (pH 6.8), 10 mM; NH_4Cl, 10 mM; $NaNO_3$, 1 mM; $NaNO_2$, 1 mM; glucose, 10 mM; tryptone, 0.2 g liter^{-1}; and soytone, 0.2 g liter^{-1}. The water content was adjusted to the levels indicated below by addition of sterile, anoxic, double-distilled water. The gas phases in oxic and anoxic microcosms were sterile air and argon, respectively. Gaseous samples were withdrawn by using aseptic techniques and were analyzed immediately. Microcosms were prepared in triplicate and incubated vertically at 20°C in the dark for 24 h.

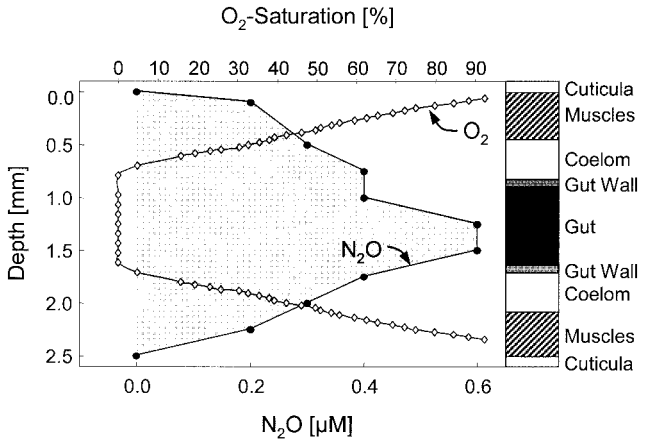

FIG. 1. N_2O (●) and O_2 (◇) profiles for the midgut region of an ethanol-sedated *L. rubellus*. The right axis identifies the anatomical regions of a cross section of a worm.

Analytical methods. N_2O was analyzed with a gas chromatograph equipped with an electron capture detector and a Porapak Q-80/100 column (Supelco, Bellefonte, Pa.) (17). The N_2O concentrations are expressed below as the amount of N_2O in the gas phase plus the amount of N_2O in the aqueous phase (calculated from the water content of a sample). Inorganic anion and cation contents were measured by ion chromatography by using a 733 IC separation center with a 753 supressor module and a 732 IC detector and a 690 ion chromatograph (MetrOhm, Herisau, Switzerland). In certain cases, nitrate, nitrite, and ammonium were also quantified by flow injection analysis (QuickChem AE; Lachat Instruments, Milwaukee, Wis.). The contents of soluble organic compounds were determined by high-performance liquid chromatography (16, 24, 28); the detection limit for sugars and organic acids was approximately 0.1 mM. Glucose concentrations were verified by using a glucose oxidase assay kit according to the manufacturer's protocol (Sigma, St. Louis, Mo.). Amino sugar concentrations were determined colorimetrically at 510 nm after acetylation and reaction with dimethylaminobenzaldehyde (Ehrlich's reagent [30]). The concentrations of soluble compounds in soil or gut contents are expressed below as millimolar concentrations and reflect the number of millimoles per liter of aqueous phase (calculated from the water content of each sample). Oven-dried (60°C), homogenized gut contents and soil were analyzed to determine total nitrogen and total carbon contents with an element analyzer (CHN-O rapid; Foss-Heraeus, Hanau, Germany). The water contents of soil and gut contents were determined by weighing samples before and after drying at 60°C for 48 h. An Ingold U457-S7/110 combination pH electrode was used to measure pH values in gut extracts.

RESULTS

In situ concentrations of N_2O and O_2 in earthworms. N_2O concentrations increased from the cuticle towards the gut in a radial transect of *L. rubellus* (Fig. 1). The highest concentrations of N_2O were found in the gut, a finding that is consistent with the hypothesis that the gut is where N_2O is produced in the earthworm. In a longitudinal transect of *L. rubellus* (Fig. 2), the in situ N_2O concentrations in the gut were 2.7 μM behind the gizzard, 5.6 μM in the midgut region, and 0.2 μM near the anus. The concentrations of N_2O in the gut of *A. caliginosa*

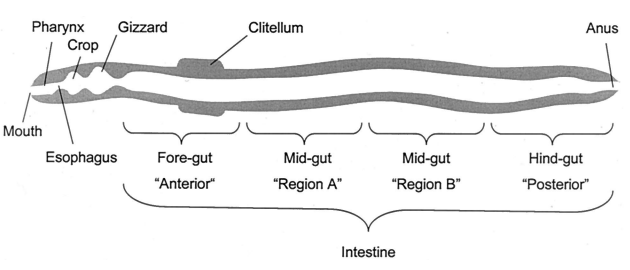

FIG. 2. Diagram of the digestive system of an earthworm (based on information obtained from references 8 and 21).

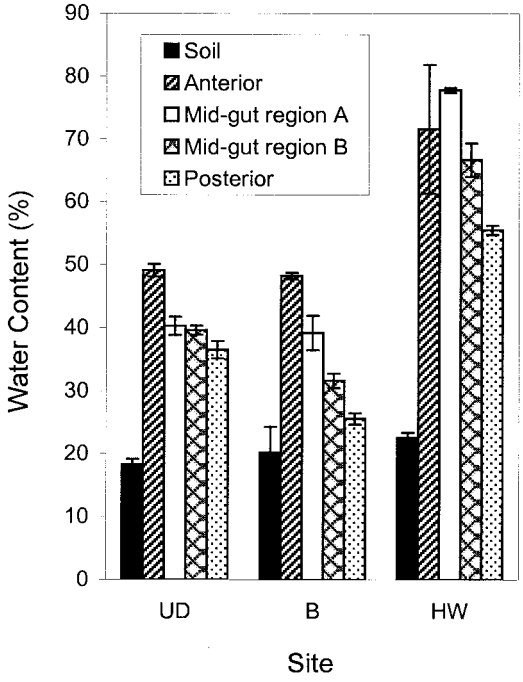

FIG. 3. Water contents of soil and gut contents of different regions in the earthworm gut (Fig. 2). Similar patterns were obtained for earthworms from sites LD, TW, and L (Table 1). The error bars indicate standard errors ($n = 3$).

ranged from 2 to 17.6 μM ($n = 5$). Sedated worms displayed a steep gradient of O_2 from the cuticle to the coelom; the gut was anoxic (Fig. 1). O_2 was never detected in the gut or gizzard of *A. caliginosa* ($n = 15$), *L. rubellus* ($n = 15$), or *L. terrestris* ($n = 1$).

Species variability of gut contents. Although the water content, pH, and concentrations of chemical compounds varied between individual sets of pooled gut contents, no major differences or trends were apparent between different species of earthworms from the same site. Therefore, for the analysis described below, data from all worms from each site were combined.

Water content and pH in gut contents and soil. The water contents of gut contents ranged from 40 to 65% and were approximately twofold greater than the water contents of the soils from which the worms were obtained (Fig. 3). In general, the water content of gut contents decreased from the anterior end to the posterior end along the gastrointestinal tract, but it was always higher than that of soil. In longitudinal transects (seven data points each), the pH was near neutral in the guts of *L. rubellus* ($n = 3$) and *A. caliginosa* ($n = 1$), and in each case there was a slight decrease in the posterior part of the gut (data not shown). The pH increased slightly from the gizzard to the anterior part of the gut, remained fairly constant in the middle parts, and dropped slightly again in the posterior part. The pH values of gut extracts from 47 individual worms collected from six different sites ranged from 6.8 to 7.1, and the values were slightly higher than the pH values of the corresponding soils (data not shown). The pH (H_2O) of the soil was more variable than that of the gut, indicating that there is a

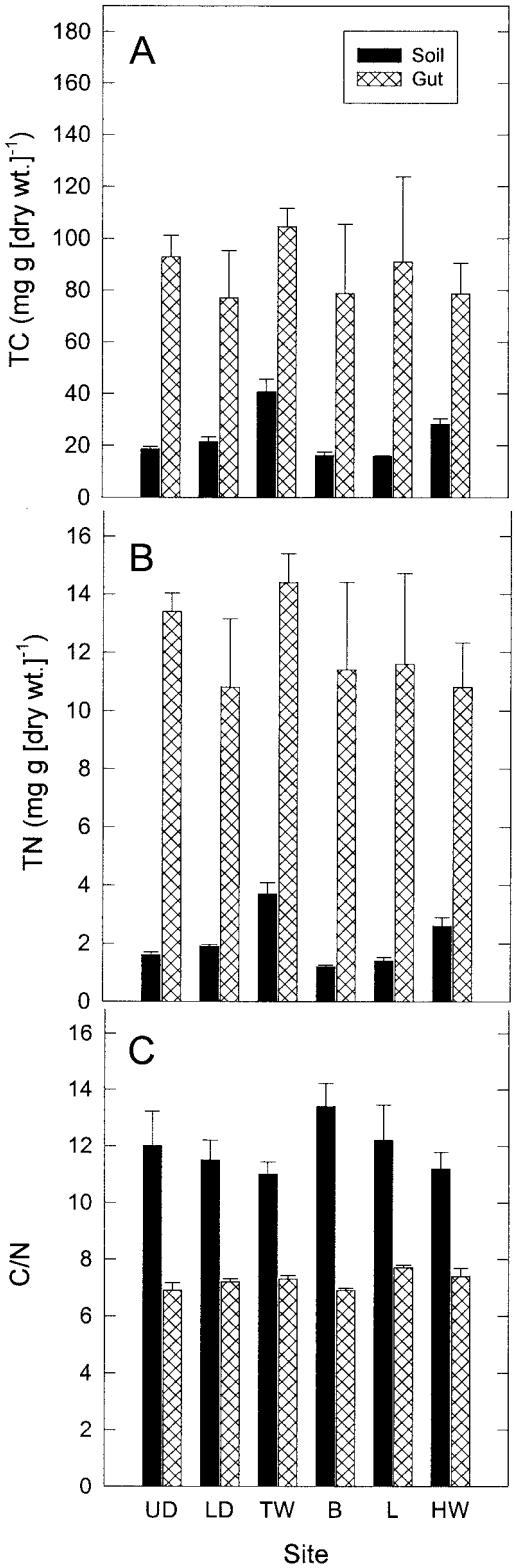

FIG. 4. Total carbon contents (TC) (A), total nitrogen contents (TN) (B), and C-to-N ratios (C/N) (C) of soil ($n = 5$) and earthworm gut contents ($n = 3$ to 5). The error bars indicate standard errors. The sites are described in Table 1.

TABLE 2. Organic compounds in soil and earthworm gut contents

Site[b]	Material	Concn (mmol liter [water content]$^{-1}$)[a]					
		Glucose	Maltose	Formate	Acetate	Lactate	Succinate
H	Soil	0.0	0.0	0.0	0.0	0.0	0.0
	Gut	25.3	1.6	0.0	2.3	1.1	2.2
U	Soil	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Gut	22.8 ± 3.0	7.5 ± 3.0	0 ± 0	1.7 ± 0.1	1.0 ± 0.0	1.3 ± 0.4
UD	Soil	0.1 ± 0.1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Gut	13.5 ± 14.4	0.3 ± 0.4	3.8 ± 3.8	2.2 ± 2.2	0 ± 0	0.6 ± 0.9
LD	Soil	0.1 ± 0.2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Gut	14.8 ± 11.6	0.3 ± 0.6	2.6 ± 3.6	5.3 ± 8.4	2.9 ± 1.9	1.1 ± 0.8
TW	Soil	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Gut	46.7 ± 39.7	4.9 ± 6.9	8.8 ± 8.8	1.1 ± 1.5	2.6 ± 1.9	0.6 ± 0.9
B	Soil	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Gut	43.0 ± 27.5	1.0 ± 1.4	5.2 ± 7.3	0 ± 0	1.1 ± 0.8	0.4 ± 0.6
L	Soil	0.1 ± 0.1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Gut	69.0 ± 49.2	5.6 ± 6.5	4.9 ± 4.9	0.3 ± 0.4	0.5 ± 0.7	1.0 ± 1.4
HW	Soil	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Gut	16.6 ± 16.4	0.3 ± 0.5	3.6 ± 3.6	8.1 ± 11.4	0.5 ± 0.4	0.4 ± 0.3

[a] The values are means ± standard deviations for three or four replicates. When no standard deviation is given, two replicates were analyzed, and the value is the average for the replicates.
[b] The sites are described in Table 1.

homeostasis near neutral pH in the earthworm gut environment.

Quantities of carbon and nitrogen in gut contents and soil. The mean values for the total carbon content in gut contents were three- to fivefold higher than those in soil (Fig. 4A). The mean values for total nitrogen and organic carbon contents were likewise higher in gut contents than in soil (Fig. 4B and data not shown). The C/N ratio for gut contents was smaller than the C/N ratio for soil (Fig. 4C).

Quality of carbon and nitrogen in gut and soil. Gut contents contained high concentrations of easily degradable organic compounds (Table 2). The average concentrations of glucose and maltose in the aqueous phase of the earthworm gut were approximately 32 and 3 mM, respectively. Although >10 mM maltose was detected in certain cases, the occurrence of maltose in the gut was more variable than the occurrence of glucose. Acetate, formate, lactate, and succinate were also detected in the aqueous phase of the gut (Table 2), indicating that fermentative organisms were active in the earthworm gut. Amino sugars (1 to 40 mM) were also found in some gut samples and, at lower concentrations, in soil (data not shown). Other sugars (except for trace amounts of glucose in three soils) and organic acids were not detected in soil (Table 2).

With the exception of the two meadow sites (sites TW and HW), the concentration of nitrate in the aqueous phase of soil was significantly greater than the concentration of nitrate in the aqueous phase of the gut (Fig. 5A). In contrast, the concentrations of nitrite and ammonium were greater in the gut than in the soil (Fig. 5B and C). In particular, the concentrations of ammonium in earthworm guts were markedly greater than the concentrations of ammonium in soils (Fig. 5C). Based on data obtained by ion chromatography, the average osmolarity of the aqueous phase of the gut was approximately 130 mosmol liter^{-1}, a value that was equivalent to the osmolarity of saline. The concentrations of amino acids were approximately 40-fold greater in the gut than in soil (Table 3). The mean concentration of free alanine in the aqueous phase of the gut was 50 ± 25 μM.

Microbial production of N_2O in gut simulations. Soil was subjected to some of the physicochemical conditions of the gut of the earthworm, and the effect of these conditions on the production of N_2O was monitored for 24 h. N_2O was not detected when unamended soil was incubated under oxic conditions; in contrast, anoxia stimulated the production of N_2O in unamended soil (Table 4). The production of N_2O by anoxic, unamended soil decreased with increasing water content (Table 4). This observation might have been due to dilution of nitrate and nitrite (likely precursors of N_2O) in the aqueous phase of the soil.

Under anoxic conditions, soil that was adjusted to a water content of 50% (which was approximately the water content of the gut [Fig. 3]) and was amended with glucose, proteins, saline, buffer, nitrite, and ammonium produced N_2O at an initial rate that was 24-fold greater than that of unamended soil (Table 5, condition 2). In the absence of supplemental inorganic nitrogenous compounds, the production of N_2O by anoxic soil amended with glucose, proteins, saline, and buffer was stimulated 11-fold (Table 5, condition 3). Addition of small amounts of nitrite and nitrate increased the rates of N_2O production by anoxic soil (Table 5, conditions 4 and 6, respectively); the duration of net N_2O production was extended in these nitrite- and nitrate-supplemented microcosms (data not shown), indicating that N_2O production was linked to the consumption of these supplements. Saline (NaCl) also increased the production of N_2O (Table 5, condition 5), demonstrating that an increase in osmolarity can stimulate the capacity of the microbiota of soil to produce N_2O. When added as single supplements, glucose, proteins, buffer, and ammonium had negligible effects on the capacity of soil to produce N_2O. However, the fact that a combination of these supplements (Table 5, conditions 2 and 3) yielded rates that were greater than the rate obtained mathematically for single supplements indicates

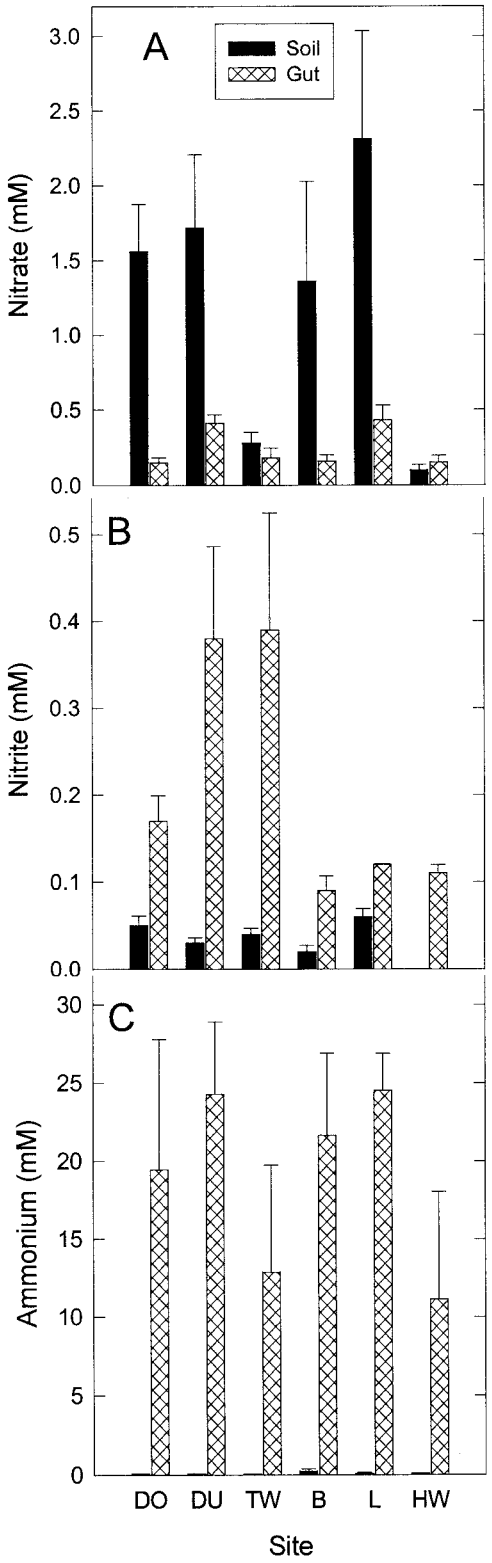

FIG. 5. Nitrate contents (A), nitrite contents (B), and ammonium contents (C) of the aqueous phase of soil ($n = 3$) and earthworm gut contents ($n = 3$ or 4). The error bars indicate standard errors. The sites are described in Table 1.

TABLE 3. Free and total amino acids in soil and earthworm gut contents

Site	Material	Concn (μmol liter [water content]$^{-1}$)[a]	
		Free amino acids	Total amino acids
B	Soil	10 ± 0	10 ± 0
	Gut	630	1,680 ± 350
L	Soil	10 ± 0	20 ± 0
	Gut	210	730 ± 620
HW	Soil	10 ± 0	20 ± 0
	Gut	380 ± 70	1,420 ± 130

[a] The values are means ± standard deviations for three replicates. When no standard deviation is given, two replicates were analyzed, and the value is the average for the replicates.

that the maximum rates observed were due to synergistic effects of supplements.

DISCUSSION

Microenvironment of the earthworm gut. The in vivo production of N_2O by earthworms is associated with the microbiota of their gut contents (14), and the highest in situ concentrations of N_2O in the earthworms occurred in the gut lumen (Fig. 1). Denitrification and the dissimilatory reduction of nitrate are most likely the main microbial processes responsible for the production of N_2O by earthworms (14, 17, 23). This conclusion is supported by the finding that the concentration of nitrate in gut contents is less than that in the soil that is ingested by the earthworms. Denitrification and the reductive dissimilation of nitrate in soil are repressed when oxygen is readily available (36). Thus, the anoxia of the earthworm gut (Fig. 1) should favor the reductive dissimilation of nitrate (2, 17, 23, 40).

The availability of reductant is important to the reductive dissimilation of nitrate (36). Thus, another factor that favors the reduction of nitrate in the gut is the availability of high-quality electron donors, such as sugars, organic acids, and amino acids (Tables 2 and 3). The high concentrations of organic carbon in the gut might be derived from (i) ingested plant- and soil-derived materials, including fungal hyphae and large bacterial cells (31, 32), that are partially degraded by digestive enzymes (e.g., proteases, chitinases, *N*-acetylglucosaminases, and maltases [37, 38, 42]) and (ii) the intestinal mucus that is secreted by the earthworm (39). The gut contents may contain up to 80% proteinaceous and polysaccharide-like

TABLE 4. Effects of oxygen and water content on the initial production of N_2O by soil[a]

Conditions	Water content (%)	N_2O production (nmol h^{-1} g [dry wt]$^{-1}$)[b]
Oxic	18[c]	0.00 ± 0.00
Anoxic	18[c]	0.90 ± 0.19
Anoxic	50	0.21 ± 0.10
Anoxic	70	0.18 ± 0.01

[a] Microcosms were prepared with soil from site H (see Table 1). The rates of production are based on the linear production of N_2O ($0.90 \leq r^2 \leq 0.99$, minimum of four data points) during the first 10 to 24 h of incubation.
[b] The values are means ± standard deviations for triplicate microcosms.
[c] Water content of fresh soil.

TABLE 5. Effects of chemical parameters on the initial production of N_2O by soil in anoxic microcosms[a]

Condition	Supplement(s)[b]	N_2O production	
		nmol h^{-1} g (dry wt)$^{-1}$[c]	% of control
1	None (control)	0.2 ± 0.1	NA[d]
2	Glucose, tryptone/soytone, saline, phosphate buffer, $NaNH_4$, KNO_2[e]	4.8 ± 0.4	2,400
3	Glucose, tryptone/soytone, saline, phosphate buffer	2.3 ± 0.1	1,150
4	KNO_2	1.1 ± 0.0	550
5	Saline	0.7 ± 0.1	350
6	$NaNO_3$	0.6 ± 0.1	300
7	Glucose	0.4 ± 0.0	200
8	Tryptone/soytone	0.3 ± 0.0	150
9	Phosphate buffer	0.2 ± 0.0	100
10	NH_4Cl	0.1 ± 0.0	50[f]

[a] Microcosms were prepared with soil from site H (see Table 1), and the rates of production are based on the linear production of N_2O ($0.90 \leq r^2 \leq 0.99$, minimum of four data points) during the first 10 to 24 h of incubation.
[b] The concentrations used are described in Materials and Methods. The water content was adjusted to 50% (which was approximately the water content of gut contents).
[c] The values are means ± standard deviations for triplicate microcosms.
[d] NA, not applicable.
[e] Autoclaved soil did not produce N_2O under condition 2.
[f] The amount of N_2O produced in oxic soil supplemented with NH_4Cl was 0.03 ± 0.00 nmol h^{-1} g (dry weight)$^{-1}$.

mucus (22, 39), and it has been postulated that this mucus might stimulate ingested soil microorganisms in a mutualistic digestion system (20).

The availability of electron donors, the high concentrations of ammonium, a water content of approximately 50%, a near-neutral pH, and anoxia are factors that should greatly enhance anaerobic activities in the gut of the earthworm. The occurrence of succinate, lactate, and acetate in gut contents indicates that fermentative microorganisms are active in the gut of the earthworm and is consistent with the distribution of fermentation products detected in anoxic, high most-probable-number dilutions of gut contents (14). Many known fermentative microorganisms (e.g., species of *Clostridium*) or facultative microorganisms (e.g., species of *Bacillus*) can also reduce nitrate or nitrite and thereby produce N_2O (5, 41). Indeed, such microorganisms are abundant in the guts of earthworms (14).

The in situ conditions of the gut are ideal for activation of dormant (or inactive) bacteria and bacterial spores that might be present in soil. Many endospore-forming bacilli are facultative anaerobes (34), are abundant in soil (10, 25), have been detected in the guts of earthworms (13, 14), and can reduce nitrate or nitrite to N_2O (14, 33). Glucose and L-alanine stimulate the germination of endospores (15), and high concentrations of these compounds were present in the gut environment (Table 2). The increased detectability of endospores by fluorescence in situ hybridization during gut passage in *L. terrestris* was attributed to the onset of germination of endospores (11). Activation of dormant soil bacteria during passage through the gastrointestinal tract of the earthworm could theoretically account for the high numbers of cultured microorganisms detected in gut contents of the earthworm (14, 16, 17).

Gut simulations. Additional evidence that there is activation of N_2O-producing soil microorganisms in the earthworm gut was obtained from the gut simulations. Aerated, oxic soil produced N_2O under conditions that simulated the conditions found in the gut (Table 5, condition 2), and endogenous sources of nitrogen in the soil were sufficient for significant

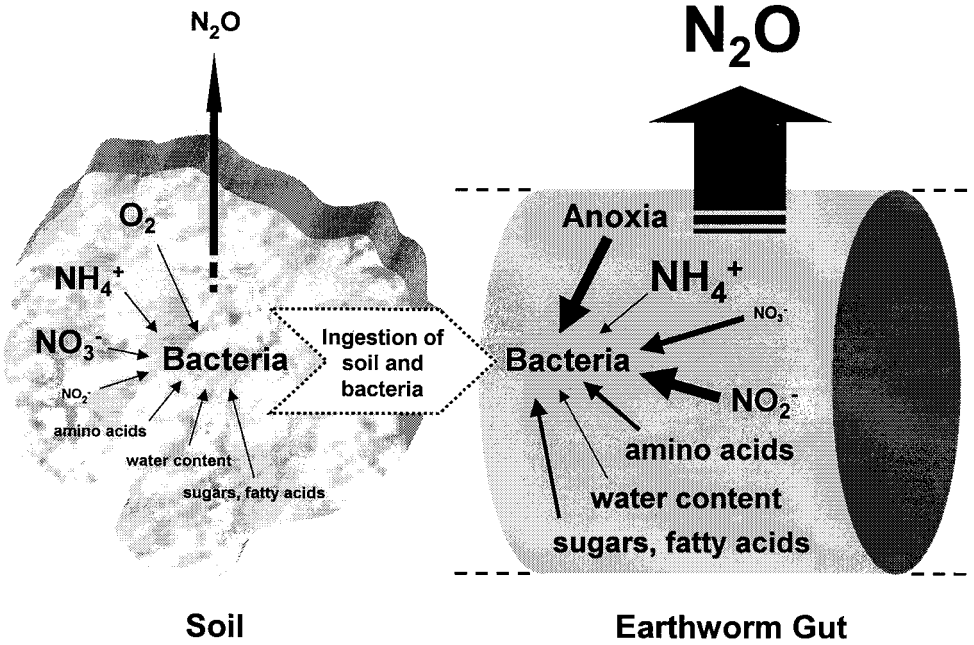

FIG. 6. Hypothetical model illustrating which factors stimulate the production of N_2O by bacteria ingested into the gut of the earthworm. The relative concentrations of compounds are indicated by the font sizes, and the relative effect of each compound on the production of N_2O in the gut is indicated by the thickness of the arrow.

production of N_2O under anoxic conditions (Table 5, condition 3). However, amendment of anoxic soil with low concentrations of nitrite or nitrate was also stimulatory, which is in accordance with results obtained with gut microcosms (14) and whole worms (23). Increased osmolarity stimulated the production of N_2O, whereas buffer or an increased water content was not stimulatory, indicating that the osmotic conditions in the gut of the earthworm enhance the activity of the N_2O-producing microorganisms of the soil.

Although the gut simulations oversimplify the conditions found in the gut of a living earthworm (e.g., excretion, absorption, and the kinetics of trophically linked processes were not taken into consideration), the data collected in this study and the data described in the accompanying paper (14) support the model illustrated in Fig. 6. It is proposed that bacteria from the soil and rhizosphere are ingested by earthworms as part of their diet. The change from the relatively dry and oxygen-rich but substrate-poor conditions in the soil to the moist, high-osmolarity, anoxic, substrate-rich conditions in the gut leads to activation of ingested bacteria and to the onset of the reductive processes that lead to the production of N_2O. Delayed synthesis of N_2O reductase and high concentrations of nitrite could theoretically enhance the production of N_2O during passage through the gut. Although evidence collected to date suggests that denitrifying bacteria are primarily responsible for the production of N_2O in the gut of the earthworm, other bacteria capable of reductive dissimilation of nitrate and nitrite might also be involved under certain in situ conditions. Current studies are aimed at further resolving specific structure-function relationships of the N_2O-producing microbiota of the earthworm gut.

ACKNOWLEDGMENTS

Support for this study was provided by the Deutsche Forschungsgemeinschaft (grant DFG DR310/2-1) and the German Ministry of Education, Research and Technology (grant PT BEO 51-0339476C).

We thank Hans-Peter Grossart (University of Oldenburg) for analysis of amino acids, Unisense (Aarhus, Denmark) for assistance with the microsensor analyses, and Peter Rothenhöfer for assistance with the ion chromatography.

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