**Dechloromonas denitrificans** sp. nov., **Flavobacterium denitrificans** sp. nov., **Paenibacillus anaericanus** sp. nov. and **Paenibacillus terrae** strain MH72, N₂O-producing bacteria isolated from the gut of the earthworm *Aporrectodea caliginosa*

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Earthworms emit nitrous oxide (N₂O) via the activity of bacteria in their gut. Four N₂O-producing facultative aerobes, ED1ᵀ, ED5ᵀ, MH21ᵀ and MH72, were isolated from the gut of the earthworm *Aporrectodea caliginosa*. The isolates produced N₂O under conditions that simulated the microenvironment of the earthworm gut. ED1ᵀ and ED5ᵀ were Gram-negative, motile rods that carried out complete denitrification (i.e. the reduction of nitrate to N₂) and contained membranous c-type cytochromes. ED1ᵀ grew optimally at 30 °C and pH 7. ED1ᵀ oxidized organic acids and reduced (per)chlorate, sulfate, nitrate and nitrite. The closest phylogenetic relative of ED1ᵀ was *Dechloromonas agitata*. ED5ᵀ grew optimally at 25 °C and pH 7. ED5ᵀ grew mainly on sugars, and nitrate and nitrite were used as alternative electron acceptors. The closest phylogenetic relatives of ED5ᵀ were *Flavobacterium johnsoniae* and *Flavobacterium flevense*. MH21ᵀ and MH72 were motile, spore-forming, rod-shaped bacteria with a three-layered cell wall. Sugars supported the growth of MH21ᵀ and MH72. Cells of MH21ᵀ grew in chains, were linked by connecting filaments and contained membranous b-type cytochromes. MH21ᵀ grew optimally at 30–35 °C and pH 7·7, grew by fermentation and reduced low amounts of nitrite to N₂O. The closest phylogenetic relatives of MH21ᵀ were *Paenibacillus borealis* and *Paenibacillus chibensis*. Based on morphological, physiological and phylogenetic characteristics, ED1ᵀ (= DSM 15892ᵀ = ATCC BAA-841ᵀ), ED5ᵀ (= DSM 15936ᵀ = ATCC BAA-842ᵀ) and MH21ᵀ (= DSM 15890ᵀ = ATCC BAA-844ᵀ) are proposed as type strains of the novel species **Dechloromonas denitrificans** sp. nov., **Flavobacterium denitrificans** sp. nov. and **Paenibacillus anaericanus** sp. nov., respectively. MH72 is considered a new strain of **Paenibacillus terrae**.

**INTRODUCTION**

Living earthworms emit the greenhouse gas nitrous oxide (N₂O) and may account for more than 50% of the total N₂O emitted from soils they inhabit (Karsten & Drake, 1997; Matthies *et al*., 1999; Borken *et al*., 2000). The annual global potential of earthworms to produce N₂O has been estimated at 3 × 10⁸ kg N₂O (Drake *et al*., 2005). The earthworm gut is where N₂O originates in the earthworm (Ihssen *et al*., 2003; Horn *et al*., 2003), and the emission of this gas is possibly due to the activation of ingested soil micro-organisms in the special microenvironment of the earthworm gut (Horn *et al*., 2003). The emission of N₂O by earthworms is stimulated by nitrate and nitrite, and denitrifying bacteria...
appear to be involved in this emission (Karsten & Drake, 1997; Matthis et al., 1999). The dissipative reduction of nitrate to nitrite or ammonium can also result in a significant production of N₂O (Anderson & Levine, 1986). Organisms isolated from earthworm casts have a higher probability of reducing nitrate than do soil isolates (Furlong et al., 2002), thus verifying the presence of nitrate-reducers in gut contents of the earthworm and also supporting the probability that soil nitrate-reducers capable of producing N₂O are activated during gut passage (Horn et al., 2003).

Twenty five N₂O-producing isolates were recently obtained from dilution series of earthworm-gut homogenates (Ihssen et al., 2003). At the time of their isolation, four of these isolates, ED¹T, ED⁵T, MH²¹T and MH⁷²T, shared ≤ 97 % 16S rRNA gene sequence similarity with their closest cultured and characterized relatives, and presumably represented novel species in the genera Dechloromonas (ED¹T), Flavobacterium (ED⁵T) and Paenibacillus (MH²¹T and MH⁷²T) (Ihssen et al., 2003). These N₂O-producing isolates were characterized and assessed for their ability to produce N₂O under conditions simulating that of the earthworm gut.

**METHODS**

**Cultivation media.** Anoxic media were prepared using modified techniques of Hungate (1969). Gas phases were 100 % argon for anoxic media and air for aerobic media; the pH was adjusted to 7-9. Solidified media contained 15 g agar per litre. The following media were utilized (unless otherwise indicated, amounts are grams per litre). Medium A: trypic soy broth (TSB) without dextrose (Difco), 2-75. Medium B: Medium A plus 5 mM KNO³ (Karsten & Drake, 1997). Medium C: Medium B plus 50 mM sodium phosphate buffer, pH 7-0. Medium D: tryptic, 2; yeast extract, 2; KNO³, 1 mM; N-acetylg glucosamine, 2 mM. Medium E: KH₂PO₄, 2-68; K₂HPO₄.3H₂O, 0-073; MgCl₂.6H₂O, 0-05; NaCl, 0-4; NH₄Cl, 0-125; CaCl₂.2H₂O, 0-01; trace element solution (Karsten & Drake, 1995), 5 ml; B-vitamin solution (Karsten & Drake, 1995), 5 ml. Medium F: TSB, 27-5; glucose, 10 mM. Medium G: NB, nutrient broth (Difco), 0-8; KNO³, 1 mM; N-acetylglucosamine, 2 mM. Nitrite, glucose, N-acetylg glucosamine and other substrates were added from sterile, anoxic stock solutions after the media were cooled to 55 °C. Media C, A, F and E were used for determining the substrate range of ED¹T, ED⁵T, MH²¹T and MH⁷²T, respectively. The concentrations used for determining the substrate range of ED¹T under denitrifying conditions were: sugars, 2 mM; organic acids, 5-20 mM; alcohols, 10-20 mM; aromatic compounds, 5 mM. The concentrations used for determining the substrate range of ED⁵T, MH²¹T and MH⁷²T under anoxic conditions were 5 mM. Cultures were incubated at 15 °C in the dark.

**Electron microscopy.** Strains were grown under anoxic conditions on liquid or solidified Medium F. For negative staining, cells were harvested from liquid media by centrifugation (2000 g, 10 min), suspended in distilled water, and adsorbed to carbon film (Valentine et al., 1968). Cells were stained with an aqueous uranyl acetate solution (2 %, w/v, pH 4-8). For thin section preparations, cells were fixed in glutaraldehyde/OsO₄ (Traub et al., 1976; Kusel et al., 2000). The fixed specimens were treated with tannic acid (1 %, w/v, 0-1 M cacodylate buffer, pH 7-2) (Bayer & Easterbrook, 1991). Specimens were embedded in Spurr’s resin after dehydration in ethyl alcohol (Spurr, 1969; Traub et al., 1976).

**Membrane preparation and redox difference spectra.** Cell-free extracts, cytoplasmic fractions and membranes were prepared from cells grown under anoxic conditions or anoxic conditions with nitrate (10 mM) as terminal electron acceptor. Oxic preparations (Frostl et al., 1996) of cellular fractions were reduced with sodium dithionite, and reduced-minus-oxidized spectra were measured at room temperature with an Uvikon 930 (Kontron Instruments) double-beam spectrophotometer (Matthis et al., 2001).

**DNA G+C content.** Cells were disrupted by French press. The DNA was purified on hydroxyapatite (Cashion et al., 1977). The hydrolysis of DNA with P1 nuclease, the HPLC analysis of hydrolysate, and the calculation of DNA G+C content were done according to published protocols (Tamaoka & Komagata, 1984; Mesbah et al., 1989). Analysis was obtained commercially at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

**Phylogenetic analysis.** Almost full-length 16S rRNA gene fragments were pre-amplified and sequenced as described previously (Ihssen et al., 2003). Public databases (GenBank, EMBL) were searched for closely related sequences using the program BLAST (Altschul et al., 1997). Alignments of sequences, distance matrix calculations and construction of phylogenetic trees were performed using the program package ARB (Department of Microbiology, Technical University Munich, Germany; http://www.arb-home.de). For calculation of phylogenetic trees, neighbour-joining, parsimony and maximum-likelihood algorithms were applied to 16S rRNA gene sequences; all sequences were more than 1400 nt long. A consensus tree was constructed when consistent branching was obtained with all three methods (Ludwig et al., 1998).

**N₂O production by isolates in sterile soil microcosms under gut-simulated conditions.** Field fresh, autoclaved soil was utilized in soil microcosms. Soil samples (31 g fresh weight) were weighed into 125 ml infusion flasks (Merck ABS); the water content of the soil was adjusted to 55 % with double-distilled water. The flasks were sealed with a screw-cap and butyl-rubber stopper, flushed with 100 % argon, and autoclaved. The physico-chemical environment of the earthworm gut was simulated in soil microcosms as described previously (Horn et al., 2003). Supplements in these microcosms were: NaCl, 130 mM; sodium phosphate buffer, pH 6-8, 10 mM; NH₄Cl, 10 mM; NaNO₃, 3 mM; NaNO₂, 1 mM; glucose, 10 mM; tryptone, 0-2 g l⁻¹; and soytone, 0-2 g l⁻¹. ED¹T-containing microcosms also contained 2 mM acetate. Microcosms of the denitrifiers ED¹T and ED⁵T were inoculated with 3 x 10⁶ cells (g dry weight soil)⁻¹; microcosms of the fermenter MH²¹T and the fermenter/nitrate-dissimilator MH⁷²T were inoculated with 3 x 10⁶ cells (g dry weight soil)⁻¹. These cell numbers were based on most-probable-number counts of denitrifiers and fermenters/nitrate-dissimilators [6 x 10⁵ and 1 x 10⁶ (g dry weight gut section)]⁻¹, respectively) obtained from the gut of earthworms (Ihssen et al., 2003). Rates reported for the production of N₂O by the isolates were corrected by values obtained with uninoculated control microcosms [which approximated 17 pmol N₂O h⁻¹ (g dry weight soil)⁻¹]. Unless otherwise indicated, microcosms were performed in triplicates.

**Analytical methods.** N₂ and high concentrations of N₂O were analyzed with a Hewlett Packard model 5890 series II gas chromatograph equipped with a thermal conductivity detector and either a Molecular Sieve column (Alltech) for N₂ or a Chromosorb 102 column (Alltech) for N₂O (Karsten & Drake, 1995, 1997). Low concentrations of N₂O (< 300 p.p.m.) were analyzed with a Hewlett Packard gas chromatograph equipped with an electron capture detector and a Porapak Q-80/100 column (Supelco) (Karsten & Drake, 1997). Soluble organic compounds were determined by HPLC (Karsten & Drake, 1995); the detection limit for sugars and organic acids was approximately 0-1 mM. Nitrate, nitrite and...
ammonium were determined colorimetrically (Harrigan & Mc Cance, 1966; Cataldo et al., 1975; Gadkari, 1984) with a UVIKON 930 spectrophotometer (Kontron Instruments). Established methods were utilized to determine the classical properties (e.g. enzymological and Gram reactions) of the isolates (Cowen, 1974; Bergey et al., 1990; Smibert & Krieg, 1994). Growth was measured as optical density at 660 nm (OD_{600}). Uninoculated medium served as reference. Values are means of duplicate or triplicate analyses.

RESULTS

Source and isolation of the organisms

ED1^T, ED5^T, MH21^T and MH72 were obtained from the gut homogenates of earthworms (Aporrectodea caliginosa Savigny) collected from garden soil in Bayreuth, Germany, during a screening for micro-organisms that were capable of producing N_2O (Hissen et al., 2003). ED1^T and ED5^T were derived from single colonies obtained by plating 0-1 ml of 10^-6 and 10^-4 dilutions of a gut homogenate, respectively, onto solidified anoxic Medium B with 2 mM glucose. For the isolation of MH21^T and MH72, enrichment cultures that were obtained by inoculating liquid anoxic Medium G and D, respectively, with 10^-6 and 10^-5 dilutions of gut homogenates, respectively, were streaked onto solidified anoxic Medium D. Isolated colonies were screened for the production of N_2O in liquid media and restreaked three times on solidified media to guarantee purity.

Morphological and cytological characteristics

See description of species (below) for information on ED1^T, ED5^T and MH21^T. Cells of ED1^T had polar flagella (Fig. 1a), formed multicellular aggregates and sometimes displayed connecting filaments (Fig. 1b) (Kuhaner et al., 2000; Matthies et al., 2001; Kuesel et al., 2001); outer and cytoplasmic membranes were observed (Fig. 1c). ED5^T formed chains consisting of 3–14 cells that were sometimes tethered to one another by connecting filaments (Supplementary Figure, in IJSEM Online). Cells of MH21^T were also sometimes tethered by connecting filaments (Fig. 1d), had a three-layered cell wall (Fig. 1e) and formed terminal spores (Fig. 1f).

Colonies of MH72 were 1–3 mm in diameter and had a flat, smooth, circular and entire appearance. Cells of MH72 were rods that were 0·7–1·0 by 2·0–4·0 μm. MH72 was Gram-variable, had a three-layered cell wall, no outer membrane and formed terminal to subterminal, ellipsoidal spores (Supplementary Figure). Most of the cells were arranged in pairs. The paired cells sometimes displayed dissimilar morphologies in negatively stained preparations, and some cells appeared to have either plasmolysed or did not have a capsule. In thin sections, cells sometimes appeared empty (ghost cells) or contained degenerated remnants of the protoplast. The protoplasts of intact, paired cells were separated from each other by a septum.

Phylogenetic analysis

The 16S rRNA gene sequence (1487 nt) of strain ED1^T was phylogenetically closely related to the 16S rRNA gene sequences of Ferribacterium limneticum, ‘Dechloromonas aromatica’ and Dechloromonas agitata (97 % sequence similarities to that of ED1^T) (Fig. 2a). ED1^T had a 99 % 16S rRNA gene sequence similarity to that of its closest phylogenetic relative, Dechloromonas sp. SIUL, which is shown in Fig. 2(a) as a representative for several other unclassified strains of Dechloromonas (Coates et al., 1999).

The 16S rRNA gene sequence (1447 nt) of ED5^T was phylogenetically most closely related to the 16S rRNA gene sequences of Flavobacterium flevense and Flavobacterium johnsoniae (95 % sequence similarities to that of ED5^T), and clustered with the 16S rRNA gene sequences of other type strains of the Cytophaga-Flavobacteria group (Fig. 2b). However, different treeing methods yielded inconsistent branchings, indicating that the phylogeny of the genus Flavobacterium is uncertain.

The 16S rRNA gene sequence of MH21^T (1512 bp) was phylogenetically most closely related to the 16S rRNA gene sequences of Paenibacillus borealis and Paenibacillus chibensis (95–96 % sequence similarity to that of MH21^T) and clustered with the 16S rRNA gene sequences of other type strains of the genus Paenibacillus (Fig. 2c).

The 16S rRNA gene sequence (1461 nt) of MH72 was phylogenetically most closely related to the 16S rRNA gene sequences found in the Paenibacillus cluster (Fig. 2c). MH72 had a 99 % 16S rRNA gene sequence similarity to that of its closest phylogenetic relative, Paenibacillus terrae (Yoon et al., 2003).

DNA G+C content

Strains ED1^T, ED5^T, MH21^T and MH72 had a DNA G+C content of 61·2, 34·6, 42·6 and 46·0 mol%, respectively.

Effects of temperature and pH on growth

See description of species (below) for information on ED1^T, ED5^T and MH21^T. MH72 grew optimally at 35 °C; growth occurred at 5–35 °C. Growth occurred at pH 5–2–8·5 and was optimal at pH 7·3–7·7. MH72 had a doubling time of 2·7 h (under optimal conditions).

Substrate range and fermentation product profiles

Of the compounds tested, only organic acids were growth-supportive for ED1^T (Supplementary Table, in IJSEM Online). Vanillate, ferulate, syringate and trimethoxybenzol were not growth-supportive for ED1^T. Many sugars and other compounds, including polymers, were growth-supportive for ED5^T, MH21^T and MH72 (Supplementary Table). Maximum optical densities (OD_{600}) observed for ED1^T, ED5^T, MH21^T and MH72 were approximately 0·4, 0·6, 1·0 and 1·3, respectively.
Fig. 1. Transmission electron micrographs of ED1T (a–c) and MH21T (d–f). (a) Negatively stained cell of ED1T with flagellum. (b) Cell agglomeration with connecting filament. (c) Detailed view of the cell wall. (d) Negatively stained cell of MH21T with connecting filaments. (e) Ultrastructural details of the three-layered cell wall. (f) Longitudinal section of a mother cell containing a forespore. Abbreviations: C, core of a connecting filament; CF, connecting filament; CM, cytoplasmic membrane; CW, cell wall; F, flagellum; Fi, fibril; FSP, forespore; I, inclusion body; IFM, inner forespore membrane; OFM, outer forespore membrane; OM, outer membrane; S, septum; Sh, sheath; SL, surface layer.
MH21<sup>T</sup> and MH72 grew by fermentation when an exogenous terminal electron acceptor was not available. Formate (4.5 mM), acetate (2.0 mM) and ethanol (6.0 mM) were produced when glucose (6.5 mM) was fermented by MH21<sup>T</sup> (the recovery of glucose-derived carbon and reductant in these products was approximately 50 and 62%, respectively; the gas phase was not evaluated). Formate (4.7 mM), acetate (2.2 mM), 2,3-butanediol (2.2 mM), ethanol (6.1 mM), H<sub>2</sub>(4.5 mM) and CO<sub>2</sub>(25 mM) were produced when glucose (9.0 mM) was fermented by MH72 (the recovery of glucose-derived carbon and reductant was approximately 100 and 75%, respectively).

**Alternative electron acceptors and the production of N<sub>2</sub>O**

ED1<sup>T</sup>, ED5<sup>T</sup> and MH72 grew facultatively. ED1<sup>T</sup> and ED5<sup>T</sup> reduced nitrate to N<sub>2</sub>, during which N<sub>2</sub>O was produced as a transient intermediate (Fig. 3a, b). Stationary denitrifying cultures of ED1<sup>T</sup> initially produced N<sub>2</sub>O upon transfer into fresh medium, while exponential cultures initially produced mainly N<sub>2</sub>. A fragment of the nitrous oxide reductase gene <i>nosZ</i> has been sequenced from both ED1<sup>T</sup> and ED5<sup>T</sup> (M. A. Horn, A. Schramm & H. L. Drake, unpublished data), corroborating the ability of these organisms to reduce N<sub>2</sub>O to N<sub>2</sub>. ED1<sup>T</sup> also utilized fumarate, sulfate, chlorate or perchlorate as electron acceptor. ED5<sup>T</sup> did not utilize sulfate or Fe<sup>3+</sup> as electron acceptor. MH72 reduced nitrate to nitrite and produced N<sub>2</sub>O as a side-product under anoxic conditions (Fig. 3c). Sulfate or Fe<sup>3+</sup> was not dissimilated by MH72. MH21<sup>T</sup> reduced Fe<sup>3+</sup> to Fe<sup>2+</sup>, and the recovery of electrons from glucose in Fe<sup>2+</sup> was 0.5–1.0%. Under these conditions, MH21<sup>T</sup> produced formate and acetate, but almost no ethanol. MH21<sup>T</sup> reduced nitrite (1 mM) to N<sub>2</sub>O; nitrite
concentrations above 2 mM inhibited growth. Sulfate or nitrate was not dissimilated by MH21T.

ED1T, ED5T, MH21T and MH72 produced 140 ± 22, 816 ± 33, 66 ± 42 and 58 ± 13 pmol N2O h−1 (g dry weight soil)−1, respectively, during the first 12 h of incubation under conditions designed to simulate the micro-environment of the earthworm gut (see Methods). The isolates produced negligible amounts of N2O in microcosms containing unamended autoclaved soil (data not shown), indicating that the in situ conditions of the gut were important to the ability of the isolates to produce N2O in soil microcosms.

Cytochromes

Redox spectra of membranes from denitrifying and aerobic cultures of ED1T and ED5T were similar. Membranous fractions and cell-free extracts of ED1T and ED5T contained c-type cytochromes with absorption maxima at approximately 425, 523, 552 nm and 425, 525, 554 nm, respectively. Spreading (i.e. the width) of the γ-peak at 425 nm and the shoulders of the α- and β-peaks suggest that membranous b-type cytochromes were also present in ED1T and ED5T.

Membranes from aerobic cultures of MH72 contained a- and b-type cytochromes (Fig. 4a); in contrast, cell-free extracts of MH72 had absorption maxima characteristic of b-type cytochromes (data not shown). Membranes and cell-free extracts of nitrate-dissimilating cells of MH72 only contained b-type cytochromes (Fig. 4b and data not shown). Cell-free extracts and membranes from aerobic cultures of MH21T contained b-type cytochromes with absorption maxima at 426, 534 and 560 nm.

Additional taxonomic properties

See description of species (below) for information on ED1T, ED5T and MH21T. Ammonium served as sole nitrogen source for MH72. MH72 was negative for oxidase and lysine decarboxylase, was positive for catalase, did not deaminate phenylalanine, did not hydrolyse urea, did not form indole and grew in 2 % but not 5 % NaCl.

DISCUSSION

Proposal of novel species

The denitrifier ED1T was phylogenetically placed in the genus Dechloromonas in the β-Proteobacteria. The closest phylogenetic relatives of ED1T with validly published names were Ferribacterium limneticum and D. agitata, both of which have a 97 % 16S rRNA gene sequence similarity to that of ED1T. In contrast to ED1T, Ferribacterium limneticum is an obligate anaerobe that reduces Fe3+ (Cummings et al., 1999). D. agitata is a facultative aerobe that contains c-type cytochrome(s), reduces chlorate and perchlorate, but is not able to grow with Casamino acids or by denitrification (Achenbach et al., 2001). The collective properties of ED1T indicate that it represents a novel species of the genus Dechloromonas, for
which the name *Dechloromonas denitrificans* sp. nov. is proposed.

ED5\textsuperscript{T} is a facultative aerobe that grew by denitrification in mineral medium. The closest phylogenetic relatives of ED5\textsuperscript{T}, *Flavobacterium flevense* and *Flavobacterium johnsoniae* (Bernardet et al., 1996), have 16S rRNA sequence similarities of less than 96% to that of ED5\textsuperscript{T}. In contrast to ED5\textsuperscript{T}, the type strain of *Flavobacterium johnsoniae* is a strict aerobe that has oxidase activity (Reichenbach, 1989; Bernardet et al., 1996). *Flavobacterium flevense* is negative for the flexirubin reaction and cannot grow on gelatin (Van der Meulen et al., 1974; Bernardet et al., 1996). There are conflicting reports on the abilities of *Flavobacterium flevense* and *Flavobacterium johnsoniae* to reduce nitrate (Van der Meulen et al., 1974; Reichenbach, 1989; Bernardet et al., 1996); the reduction of nitrate to N\textsubscript{2} or N\textsubscript{2}O has not been reported for these organisms. ED5\textsuperscript{T} yielded a positive flexirubin reaction, which is typical for non-marine species of *Flavobacterium* (Reichenbach, 1989; Bernardet et al., 1996). The collective properties of ED5\textsuperscript{T} indicate that it represents a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium denitrificans* sp. nov. is proposed.

MH21\textsuperscript{T} was phylogenetically placed in the genus *Paenibacillus* in the *Firmicutes*. The closest phylogenetic relatives of MH21\textsuperscript{T}, *P. borealis* and *P. chibensis*, have a less than 97% 16S rRNA gene sequence similarity to that of MH21\textsuperscript{T}, and have a DNA G+C content that is at least 10% higher than that of MH21\textsuperscript{T} (Shida et al., 1997; Elo et al., 2001) (Table 1). *P. borealis* has a temperature optimum that is 7°C lower than that of MH21\textsuperscript{T} (Elo et al., 2001). In contrast to the anaerobic ability of MH21\textsuperscript{T}, *P. chibensis* is a strict aerobe (Shida et al., 1997). The production of N\textsubscript{2}O and the reduction of Fe\textsuperscript{3+} under anoxic conditions have not been reported for *P. borealis* and *P. chibensis* (Shida et al., 1997; Elo et al., 2001). The collective properties of MH21\textsuperscript{T} indicate it represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus anaeric anus* sp. nov. is proposed.

Upon the initial isolation of MH72, the 16S rRNA gene sequence of its closest phylogenetic neighbour was not greater than 97% similar to that of MH72. However, the 16S rRNA gene sequence of a recently described species of *Paenibacillus, P. terrae* (Yoon et al., 2003), is 99% similar to that of MH72. MH72 produced N\textsubscript{2}O when nitrate was used as a terminal electron acceptor and contained a- and b-type cytochromes. Information on the production of N\textsubscript{2}O, fermentation products, cytochromes and cellular ultrastructure are not provided in the species description of *P. terrae* (Yoon et al., 2003). Nonetheless, given the similarity of the 16S rRNA gene sequences and other properties of MH72 and *P. terrae* (Table 1), it is proposed that MH72 represents a new strain of *P. terrae*.

**Production of N\textsubscript{2}O in the gut of the earthworm**

Earthworms and earthworm gut content produce N\textsubscript{2}O (Karsten & Drake, 1997; Matthies et al., 1999). Depending on the isolate, the initial per cell rates at which the earthworm gut isolates produced N\textsubscript{2}O under gut-simulated conditions were 50–300% (mean 75%) of that obtained in TSB-based liquid media (Ihssen et al., 2003), suggesting that (i) the isolates produce N\textsubscript{2}O under *in situ* conditions and (ii) the nutritional contents of either the gut or TSB foster similar N\textsubscript{2}O-producing activities by the isolates.

The denitrifiers ED1\textsuperscript{T} and ED5\textsuperscript{T} produce N\textsubscript{2}O at cellular rates that are one to two orders of magnitude greater than those of the fermentative MH21\textsuperscript{T} and MH72 (Ihssen et al., 2003), suggesting a predominance of denitrifiers with respect to the production of N\textsubscript{2}O in the earthworm gut. However, the numbers of cultured fermenters in the earthworm gut per gram dry weight of gut content are approximately ten-fold greater than the number of cultured denitrifiers (Ihssen et al., 2003), suggesting that fermentative micro-organisms could be more important to the *in situ* production of N\textsubscript{2}O in the earthworm gut than is suggested.
by the estimated per cell production of N₂O from isolates. Indeed, when microcosms that simulated the gut environment were inoculated with a ten-fold excess of fermentative microbes (i.e. MH21T or MH72) than denitrifiers (i.e. ED1T or ED5T), the microcosms inoculated with the fermentative isolates produced N₂O at rates that were only six-fold lower than those of denitrifier-inoculated microcosms. Thus, the direct contribution of fermenters to the production of N₂O by earthworms may not be negligible.

A major fermentation product of isolates MH21T and MH72 was acetate. Acetate is a common product of fermenters (e.g. Bulthuis et al., 1991; Schlegel & Jannasch, 1992; Kühner et al., 2000), and is a substrate for denitrifiers such as ED1T, suggesting that a mutualistic interaction of certain denitrifiers and fermenters might occur in the gut of earthworms. The production of nitrite via the dissimilatory reduction of nitrate is a characteristic trait of many fermenters (Stouthamer, 1988). Nitrite is a precursor for N₂O during denitrification (Zumft, 1992) and greatly stimulates the production of N₂O by earthworms (Matthies et al., 1999). Thus, the nitrite-dependent production of N₂O by denitrifiers in the earthworm gut might also be enhanced by the nitrite-forming activities of certain fermenters.

**Table 1. Characteristics of earthworm gut isolates Paenibacillus anaericanus and Paenibacillus terrae strain MH72, and closest relatives**

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<td>-</td>
<td>-</td>
<td>NR</td>
</tr>
<tr>
<td>Deamination of phenylalanine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NR</td>
</tr>
<tr>
<td>Hydrolysis of urea</td>
<td>-</td>
<td>-</td>
<td>NR</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>42-6</td>
<td>52-8</td>
<td>53-6</td>
<td>46-0</td>
<td>47-0</td>
</tr>
<tr>
<td>pH optimum (range)</td>
<td>7.7 (5.8–8.5)</td>
<td>7.0 (4.5–9.0)</td>
<td>7.0 (5.6–8.0)</td>
<td>7.3–7.7 (5.2–5.8)</td>
<td>6.5–8.0 (NR)</td>
</tr>
<tr>
<td>Temperature (°C) optimum (range)</td>
<td>30–35 (5–40)</td>
<td>37 (10–50)</td>
<td>28 (5–37)</td>
<td>35 (5–35)</td>
<td>30 (10–40)</td>
</tr>
<tr>
<td>Growth at 2% NaCl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 5% NaCl</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inulin</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pectin</td>
<td>-</td>
<td>NR</td>
<td>+</td>
<td>+</td>
<td>NR</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Sorbitol</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</tr>
</tbody>
</table>

**Description of Dechloromonas denitrificans sp. nov.**

*Dechloromonas denitrificans* (de.ni.tri’fi.canis. L. prep. *de* away from; *L.* *n.* *nitrum* soda; *N.I.* *n.* *nitr* nitrate; *N.I.* *v.* *denitrifico* to denitrify; *N.I.* part. adj. *denitrificans* denitrifying).

Colonies are yellowish and 0.5–1 mm in diameter. Cells are Gram-negative, facultative, short rods, 1·7 × 0·5 μm, motile with a polar flagellum and sometimes form connecting filaments. Membranes contain c-type cytochromes; b-type cytochromes might also occur. Grows from 5 to 36 °C and pH 6·1 to 8·3, with optimal growth at 30 °C and pH 7. Doubling time under optimal conditions is 6·5 h. O₂, NO₃⁻, NO₂⁻, ClO₃⁻, ClO₄⁻, SO₄²⁻ and fumarate are used as electron acceptors. Fe³⁺ is not used as an electron acceptor. N₂O is produced as an intermediate during the reduction of NO₃⁻ to N₂. Utilizes acetate, propionate, butyrate, iso-butyrate, iso-valerate, lactate, pyruvate, succinate, malate, glutamate and Casamino acids as...
electron donors. Formate, H₂, methanol, ethanol, propanol, cellobiose, glucose, fructose, xylose, lactose, galactose, mannose, arabinose, pectin, vanillate, ferulate, syringate and trimethoxybenzol are not growth-supportive. The DNA G+C content is 61.2 mol%. Phylogenetically most closely related to Dechloromonas agitata.

The type strain (EDT²⁵ = DSM 15892²⁵ = ATCC BAA-841²⁵) was isolated from the gut of the earthworm Aporrectodea caliginosa (collected from garden soil in Bayreuth, Germany).

Description of Flavobacterium denitrificans sp. nov.

Flavobacterium denitrificans (de.ni.trি.fi.can{s}. L. prep. de away from; L. n. nitrum soda; N.L. n. nitras nitrate; N.L. v. denitrifico to denitrify; N.L. part. adj. denitrificans denitrifying).

Colonies are flat, circular, entire and yellow. Cells are faculative, rods, 0.8–3.0 × 0.3–0.9 μm, can form chains (3–14 cells), sometimes form connecting filaments, stain Gram-negative, motile and have an outer membrane. Membranes contain c-type cytochromes; b-type cytochromes might also occur. Grows from 10 to 30 °C and pH 5.5 to 8.2, with optimal growth at 25 °C and pH 7. Doubling time under optimal conditions is 7-3 h. Utilizes arabinose, cellobiose, fructose, fumarate, galactose, hexosamines, inositol, isovalerate, lactate, lactic acid, maltose, mannitol, mannose, N-acetylglucosamine, raffinose, saccharose, starch, and xylose as electron donors. 1-Butanol, 1-propanol, acetate, butyrate, citrate, ethanol, ethanolamine, fumarate, galactose, glycolate, i-butyrate, inositol, i-valerate, lactate, oleate, propionate, raffinose, saccharose, sorbitol and tartrate are not growth-supportive. Uses ammonium as nitrogen source. Flexirubin reaction-, arginine dihydrolase- and catalase-positive. Grows at 2 % but not 5 % NaCl. Formate, acetate and ethanol are formed when glucose is fermented. NO₂⁻ and SO₄²⁻ are not dissimilated. Low amounts (1 mM) of NO₂⁻ are reduced to N₂O. The DNA G+C content is 42-6 %. Phylogenetically most closely related to Paenibacillus borcalis.

The type strain (MH21²⁵ = DSM 15890²⁵ = ATCC BAA-844²⁵) was isolated from the gut of the earthworm Aporrectodea caliginosa (collected from garden soil in Bayreuth, Germany).

ACKNOWLEDGEMENTS

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REFERENCES


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Novel N\textsubscript{2}O-producing bacteria of the earthworm gut

