Dechloromonas denitrificans sp. nov., *Flavobacterium denitrificans* sp. nov., *Paenibacillus anaericanus* sp. nov. and *Paenibacillus terrae* strain MH72, N_2O-producing bacteria isolated from the gut of the earthworm *Aporrectodea caliginosa*

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Earthworms emit nitrous oxide (N_2O) via the activity of bacteria in their gut. Four N_2O-producing facultative aerobes, ED1^T, ED5^T, MH21^T and MH72, were isolated from the gut of the earthworm *Aporrectodea caliginosa*. The isolates produced N_2O under conditions that simulated the microenvironment of the earthworm gut. ED1^T and ED5^T were Gram-negative, motile rods that carried out complete denitrification (i.e. the reduction of nitrate to N_2) and contained membranous *c*-type cytochromes. ED1^T grew optimally at 30 °C and pH 7. ED1^T oxidized organic acids and reduced (per)chlorate, sulfate, nitrate and nitrite. The closest phylogenetic relative of ED1^T was *Dechloromonas agitata*. ED5^T grew optimally at 25 °C and pH 7. ED5^T grew mainly on sugars, and nitrate and nitrite were used as alternative electron acceptors. The closest phylogenetic relatives of ED5^T were *Flavobacterium johnsoniae* and *Flavobacterium flevense*. MH21^T and MH72 were motile, spore-forming, rod-shaped bacteria with a three-layered cell wall. Sugars supported the growth of MH21^T and MH72. Cells of MH21^T grew in chains, were linked by connecting filaments and contained membranous *b*-type cytochromes. MH21^T grew optimally at 30–35 °C and pH 7·7, grew by fermentation and reduced low amounts of nitrite to N_2O. The closest phylogenetic relatives of MH21^T were *Paenibacillus borealis* and *Paenibacillus chibensis*. Based on morphological, physiological and phylogenetic characteristics, ED1^T (=DSM 15892^T=ATCC BAA-841^T), ED5^T (=DSM 15936^T=ATCC BAA-842^T) and MH21^T (=DSM 15890^T=ATCC BAA-844^T) 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*.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Dechloromonas denitrificans* ED1^T, *Flavobacterium denitrificans* ED5^T and *Paenibacillus terrae* MH72 are AJ318917, AJ318907 and AJ318910, respectively.

A table of the substrate utilization profiles of earthworm-gut isolates and transmission electron micrographs of strains ED5^T and MH72 are available as supplementary material in IJSEM Online.

INTRODUCTION

Living earthworms emit the greenhouse gas nitrous oxide (N_2O) and may account for more than 50 % of the total N_2O emitted from soils they inhabit (Karsten & Drake, 1997; Matthies *et al.*, 1999; Borken *et al.*, 2000). The annual global potential of earthworms to produce N_2O has been estimated at 3×10^8 kg N_2O (Drake *et al.*, 2005). The earthworm gut is where N_2O 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 N_2O by earthworms is stimulated by nitrate and nitrite, and denitrifying bacteria

appear to be involved in this emission (Karsten & Drake, 1997; Matthies *et al.*, 1999). The dissimilative reduction of nitrate to nitrite or ammonium can also result in a significant production of N_2O (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 N_2O are activated during gut passage (Horn *et al.*, 2003).

Twenty five N_2O-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, ED1^T, ED5^T, MH21^T and MH72, shared $\leqslant$97 % 16S rRNA gene sequence similarity with their closest cultured and characterized relatives, and presumably represented novel species in the genera *Dechloromonas* (ED1^T), *Flavobacterium* (ED5^T) and *Paenibacillus* (MH21^T and MH72) (Ihssen *et al.*, 2003). These N_2O-producing isolates were characterized and assessed for their ability to produce N_2O 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 oxic media; the pH was adjusted to 7·0. Solidified media contained 15 g agar per litre. The following media were utilized (unless otherwise indicated, amounts are grams per litre). Medium A: tryptic soy broth (TSB) without dextrose (Difco), 2·75. Medium B: Medium A plus 5 mM KNO_3 (Karsten & Drake, 1997). Medium C: Medium B plus 50 mM sodium phosphate buffer, pH 7·0. Medium D: tryptone, 2; yeast extract, 2; KNO_2, 1 mM; *N*-acetylglucosamine, 2 mM. Medium E: KH_2PO_4, 2·68; $K_2HPO_4.3H_2O$, 0·073; $MgCl_2.6H_2O$, 0·05; NaCl, 0·4; NH_4Cl, 0·125; $CaCl_2.2H_2O$, 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; KNO_2, 1 mM; *N*-acetylglucosamine, 2 mM. Nitrite, glucose, *N*-acetylglucosamine 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 ED1^T, ED5^T, MH21^T and MH72, respectively. The concentrations used for determining the substrate range of ED1^T 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 ED5^T, MH21^T and MH72 under oxic conditions were 5 mM. Cultures were incubated at 15 °C in the dark.

Electron microscopy. Strains were grown under oxic 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/OsO_4 (Traub *et al.*, 1976; Küsel *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 oxic conditions or anoxic conditions with nitrate (10 mM) as terminal electron acceptor. Oxic preparations (Fröstl *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 (Matthies *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 established 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).

N_2O 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; NH_4Cl, 10 mM; $NaNO_3$, 3 mM; $NaNO_2$, 1 mM; glucose, 10 mM; tryptone, 0·2 g l^{-1}; and soytone, 0·2 g l^{-1}. ED1^T-containing microcosms also contained 2 mM acetate. Microcosms of the denitrifiers ED1^T and ED5^T were inoculated with 3×10^6 cells (g dry weight soil)$^{-1}$; microcosms of the fermenter MH21^T and the fermenter/nitrate-dissimilator MH72 were inoculated with 3×10^7 cells (g dry weight soil)$^{-1}$. These cell numbers were based on most-probable-number counts of denitrifiers and fermenters/nitrate-dissimilators [6×10^6 and 1×10^7 (g dry weight gut section)$^{-1}$, respectively] obtained from the gut of earthworms (Ihssen *et al.*, 2003). Rates reported for the production of N_2O by the isolates were corrected by values obtained with uninoculated control microcosms [which approximated 17 pmol N_2O h^{-1} (g dry weight soil)$^{-1}$]. Unless otherwise indicated, microcosms were performed in triplicates.

Analytical methods. N_2 and high concentrations of N_2O were analysed with a Hewlett Packard model 5890 series II gas chromatograph equipped with a thermal conductivity detector and either a Molecular Sieve column (Alltech) for N_2 or a Chromosorb 102 column (Alltech) for N_2O (Karsten & Drake, 1995, 1997). Low concentrations of N_2O ($<$300 p.p.m.) were analysed 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 (Cowan, 1974; Bergey *et al.*, 1990; Smibert & Krieg, 1994). Growth was measured as optical density at 660 nm (OD_{660}). 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 (Ihssen *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) (Kuhner *et al.*, 2000; Matthies *et al.*, 2001; Küsel *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_{660}) observed for ED1^T, ED5^T, MH21^T and MH72 were approximately 0·4, 0·6, 1·0 and 1·3, respectively.

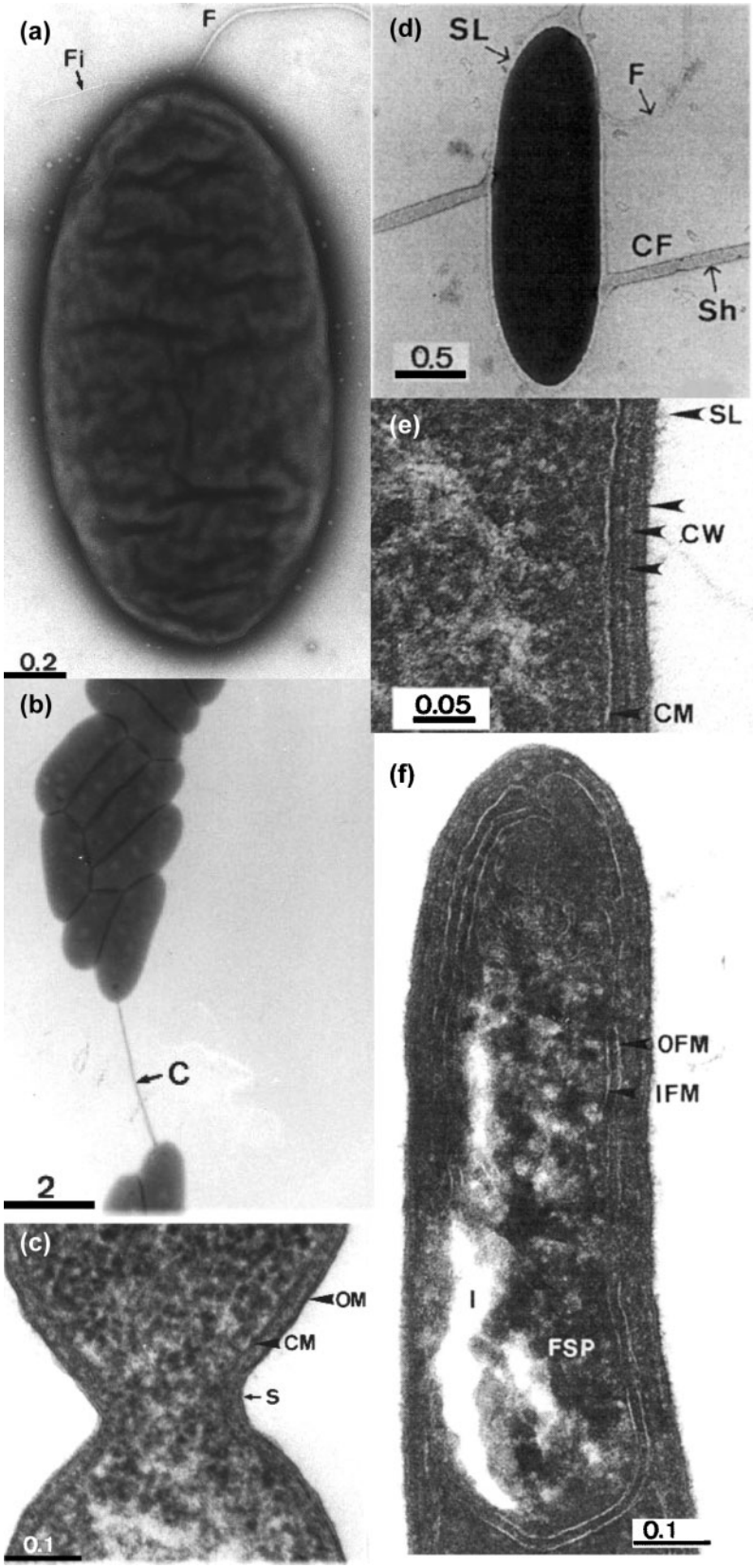

Fig. 1. Transmission electron micrographs of ED1^T (a–c) and MH21^T (d–f). (a) Negatively stained cell of ED1^T with flagellum. (b) Cell agglomeration with connecting filament. (c) Detailed view of the cell wall. (d) Negatively stained cell of MH21^T with connecting filaments. (e) Ultrastructural details 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.

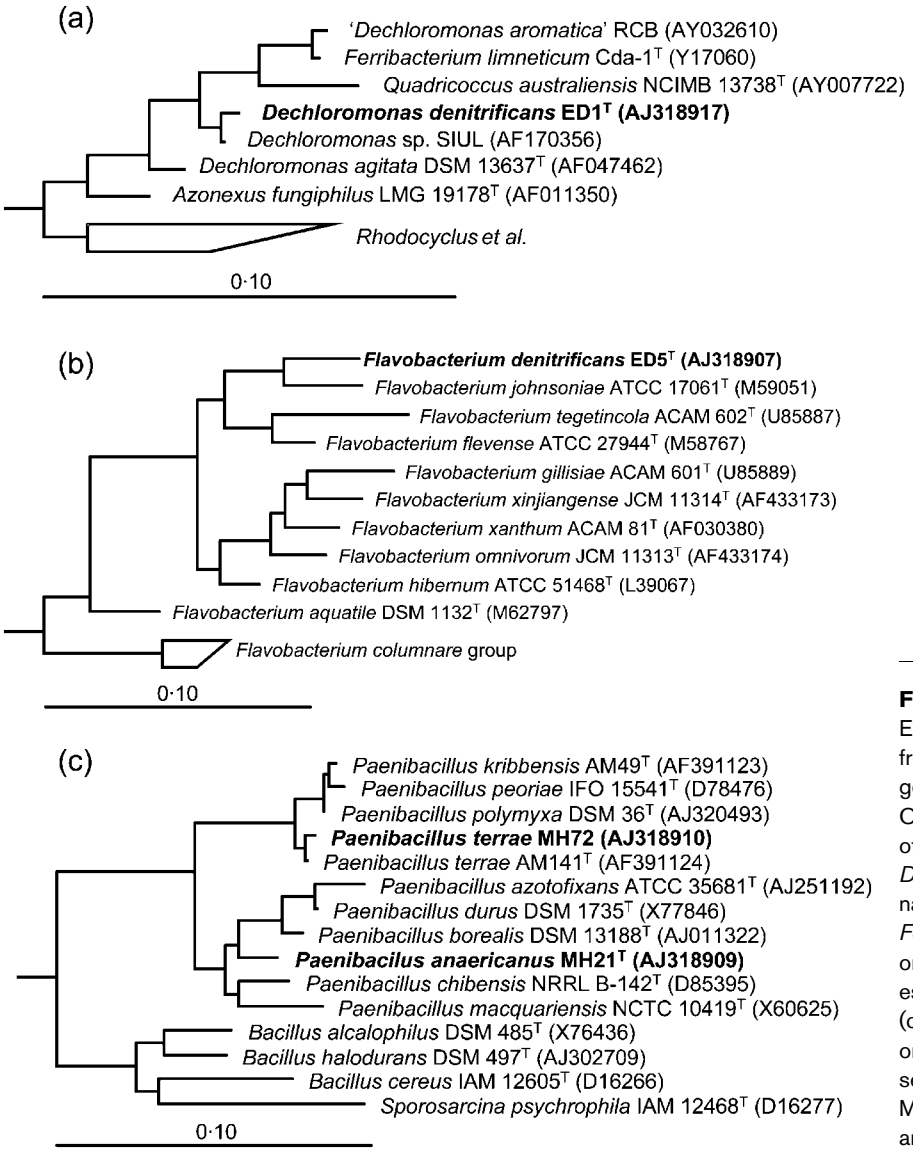

Fig. 2. Phylogenetic positions of ED1^T (a), ED5^T (b), MH21^T and MH72 (c) as inferred from comparative analysis of 16S rRNA gene sequence data (approx. 1400 nt). Outgroups for (a), (b) and (c) were species of the genera *Azovibrio*, *Psychroflexus* and *Dechloromonas*, respectively. A strain designation is not provided for the type strain of *Ferribacterium limneticum* because the organism is no longer available. Bar, a 0·1 estimated change per nucleotide. (a) And (c) are consensus trees. (b) Is based solely on the parsimony algorithm; a stable consensus tree could not be constructed (see Methods). Sequence accession numbers are in parentheses.

MH21^T 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^T (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_2 (4·5 mM) and CO_2 (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_2O

ED1^T, ED5^T and MH72 grew facultatively. ED1^T and ED5^T reduced nitrate to N_2, during which N_2O was produced as a transient intermediate (Fig. 3a, b). Stationary denitrifying cultures of ED1^T initially produced N_2O upon transfer into fresh medium, while exponential cultures initially produced mainly N_2. A fragment of the nitrous oxide reductase gene *nosZ* has been sequenced from both ED1^T and ED5^T (M. A. Horn, A. Schramm & H. L. Drake, unpublished data), corroborating the ability of these organisms to reduce N_2O to N_2. ED1^T also utilized fumarate, sulfate, chlorate or perchlorate as electron acceptor. ED5^T did not utilize sulfate or Fe^{3+} as electron acceptor. MH72 reduced nitrate to nitrite and produced N_2O as a side-product under anoxic conditions (Fig. 3c). Sulfate or Fe^{3+} was not dissimilated by MH72. MH21^T reduced Fe^{3+} to Fe^{2+}, and the recovery of electrons from glucose in Fe^{2+} was 0·5–1 %. Under these conditions, MH21^T produced formate and acetate, but almost no ethanol. MH21^T reduced nitrite (1 mM) to N_2O; nitrite

concentrations above 2 mM inhibited growth. Sulfate or nitrate was not dissimilated by MH21^T.

ED1^T, ED5^T, MH21^T and MH72 produced 140 ± 22, 816 ± 33, 66 ± 42 and 58 ± 13 pmol N_2O h^{-1} (g dry weight soil)$^{-1}$, respectively, during the first 12 h of incubation under conditions designed to simulate the microenvironment of the earthworm gut (see Methods). The isolates produced negligible amounts of N_2O 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 N_2O in soil microcosms.

Cytochromes

Redox spectra of membranes from denitrifying and aerobic cultures of ED1^T and ED5^T were similar. Membranous fractions and cell-free extracts of ED1^T and ED5^T 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 ED1^T and ED5^T.

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 MH21^T contained *b*-type cytochromes with absorption maxima at 426, 534 and 560 nm.

Additional taxonomic properties

See description of species (below) for information on ED1^T, ED5^T and MH21^T. 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 ED1^T was phylogenetically placed in the genus *Dechloromonas* in the *β-Proteobacteria*. The closest phylogenetic relatives of ED1^T with validly published names were *Ferribacterium limneticum* and *D. agitata*, both of which have a 97 % 16S rRNA gene sequence similarity to that of ED1^T. In contrast to ED1^T, *Ferribacterium limneticum* is an obligate anaerobe that reduces Fe^{3+} (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 ED1^T indicate that it represents a novel species of the genus *Dechloromonas*, for

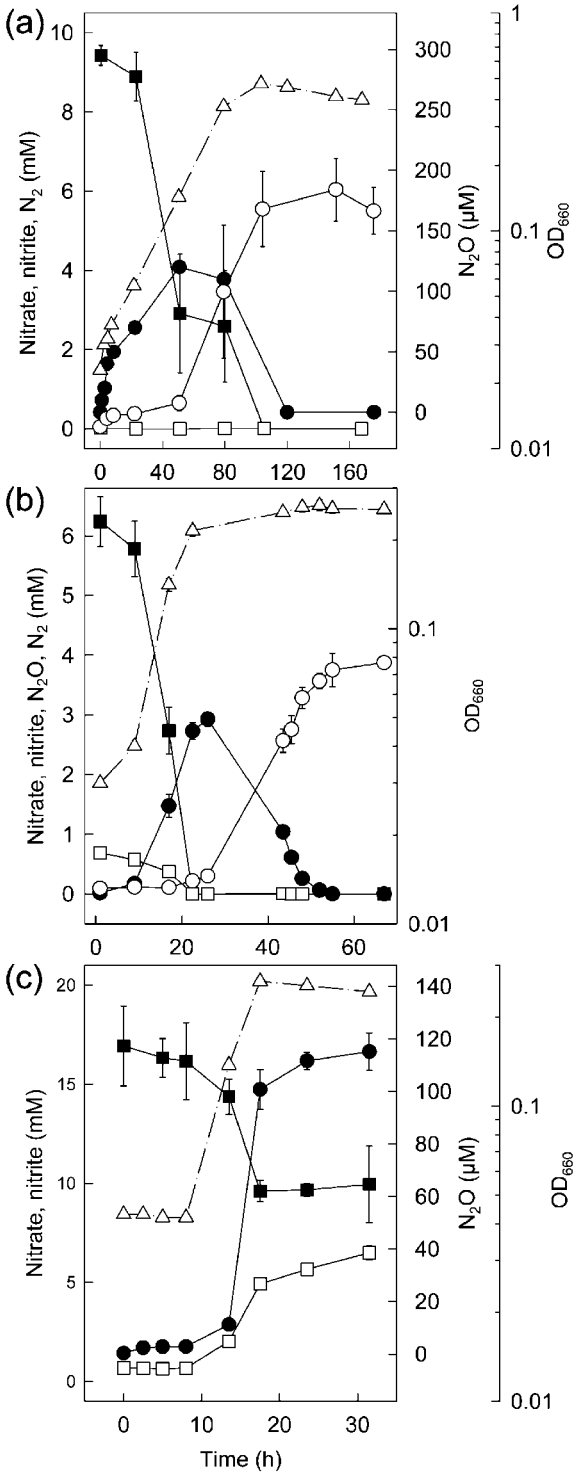

Fig. 3. Reduction of nitrate and production of N_2O during growth of ED5^T (a), ED1^T (b) and MH72 (c) under anoxic conditions. Symbols: ■, nitrate; □, nitrite; ●, N_2O; ○, N_2; △, optical density (OD_{660}).

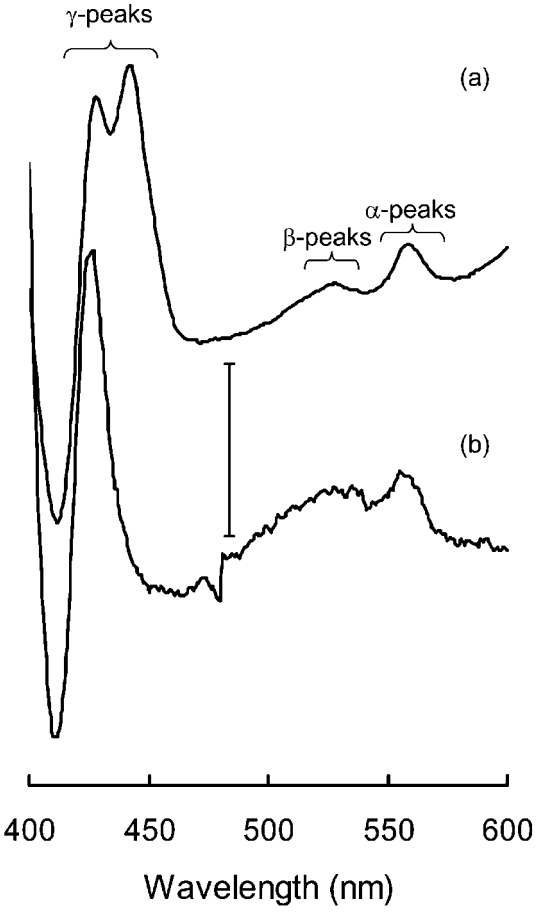

Fig. 4. Sodium dithionite-reduced minus air-oxidized membrane difference spectra derived from aerobic (a) and nitrate-dissimilating (b) cultures of MH72. The bar represents a relative absorbance of 0·10 (a) and 0·02 (b).

which the name *Dechloromonas denitrificans* sp. nov. is proposed.

ED5^T is a facultative aerobe that grew by denitrification in mineral medium. The closest phylogenetic relatives of ED5^T, *Flavobacterium flevense* and *Flavobacterium johnsoniae* (Bernardet *et al.*, 1996), have 16S rRNA sequence similarities of less than 96 % to that of ED5^T. In contrast to ED5^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_2 or N_2O has not been reported for these organisms. ED5^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^T indicate that it represents a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium denitrificans* sp. nov. is proposed.

MH21^T was phylogenetically placed in the genus *Paenibacillus* in the *Firmicutes*. The closest phylogenetic relatives of MH21^T, *P. borealis* and *P. chibensis*, have a less than 97 % 16S rRNA gene sequence similarity to that of MH21^T, and have a DNA G+C content that is at least 10 % higher than that of MH21^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^T (Elo *et al.*, 2001). In contrast to the anaerobic ability of MH21^T, *P. chibensis* is a strict aerobe (Shida *et al.*, 1997). The production of N_2O and the reduction of Fe^{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^T indicate it represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus anaericanus* 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_2O when nitrate was used as a terminal electron acceptor and contained *a*- and *b*-type cytochromes. Information on the production of N_2O, 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_2O in the gut of the earthworm

Earthworms and earthworm gut content produce N_2O (Karsten & Drake, 1997; Matthies *et al.*, 1999). Depending on the isolate, the initial per cell rates at which the earthworm gut isolates produced N_2O 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_2O under *in situ* conditions and (ii) the nutritional contents of either the gut or TSB foster similar N_2O-producing activities by the isolates.

The denitrifiers ED1^T and ED5^T produce N_2O at cellular rates that are one to two orders of magnitude greater than those of the fermentative MH21^T and MH72 (Ihssen *et al.*, 2003), suggesting a predominance of denitrifiers with respect to the production of N_2O 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_2O in the earthworm gut than is suggested

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

1, *P. anaericanus* MH21^T; 2, *P. chibensis* HSCC 442^T; 3, *P. borealis* KK19^T; 4, *P. terrae* strain MH72; 5, *P. terrae* AM141^T. Data of other *Paenibacillus* species are adopted from Shida *et al.* (1997), Yoon *et al.* (2003) and Elo *et al.* (2001). +, Reaction positive; −, reaction negative; NR, not reported.

Characteristic	1	2	3	4	5
Cytochrome *c* oxidase	+	−	−	−	−
Catalase	+	+	+	+	+
Anaerobic growth	+	−	+	+	+
Nitrate reduction	−	+	−	+	+
Fe^{3+} reduction	+	NR	NR	−	NR
Voges–Proskauer reaction	−	−	−	−	NR
Arginine dihydrolase	−	NR	NR	−	NR
Lysine decarboxylase	−	NR	NR	−	NR
Formation of indole	−	NR	−	−	NR
Deamination of phenylalanine	−	−	−	−	NR
Hydrolysis of urea	−	−	NR	−	−
DNA G+C content (mol%)	42·6	52·8	53·6	46·0	47·0
pH optimum (range)	7·7 (5·8–8·5)	7·0 (4·5–9·0)	7·0 (5·6–8·0)	7·3–7·7 (5·2–5·8)	6·5–8·0 (NR)
Temperature (°C) optimum (range)	30–35 (5–40)	37 (10–50)	28 (5–37)	35 (5–35)	30 (10–40)
Growth at 2 % NaCl	+	+	+	+	+
Growth at 5 % NaCl	−	−	−	−	−
Hydrolysis of:					
Inulin	−	+	+	+	+
Starch	+	+	−	+	+
Gelatin	−	−	−	−	+
Pectin	−	NR	+	+	NR
Utilization of:					
N-Acetylglucosamine	+	+	+	−	+
Glycerol	+	−	+	+	+
Sorbitol	−	−	+	−	−

by the estimated per cell production of N_2O from isolates. Indeed, when microcosms that simulated the gut environment were inoculated with a ten-fold excess of fermentative microbes (i.e. MH21^T or MH72) than denitrifiers (i.e. ED1^T or ED5^T), the microcosms inoculated with the fermentative isolates produced N_2O at rates that were only six-fold lower than those of denitrifier-inoculated microcosms. Thus, the direct contribution of fermenters to the production of N_2O by earthworms may not be negligible.

A major fermentation product of isolates MH21^T and MH72 was acetate. Acetate is a common product of fermenters (e.g. Bulthuis *et al.*, 1991; Schlegel & Jannasch, 1992; Kuhner *et al.*, 2000), and is a substrate for denitrifiers such as ED1^T, 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 N_2O during denitrification (Zumft, 1992) and greatly stimulates the production of N_2O by earthworms (Matthies *et al.*, 1999). Thus, the nitrite-dependent production of N_2O by denitrifiers in the earthworm gut might also be enhanced by the nitrite-forming activities of certain fermenters.

Description of *Dechloromonas denitrificans* sp. nov.

Dechloromonas denitrificans (de.ni.tri′fi.cans. 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 yellowish and 0·5–1 mm in diameter. Cells are Gram-negative, facultative, short rods, $1{\cdot}7 \times 0{\cdot}5$ 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. O_2, NO_3^-, NO_2^-, ClO_3^-, ClO_4^-, SO_4^- and fumarate are used as electron acceptors. Fe^{3+} is not used as an electron acceptor. N_2O is produced as an intermediate during the reduction of NO_3^- to N_2. Utilizes acetate, propionate, butyrate, iso-butyrate, iso-valerate, lactate, pyruvate, succinate, malate, glutamate and Casamino acids as

electron donors. Formate, H_2, 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 (ED1^T = DSM 15892^T = ATCC BAA-841^T) 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.tri′fi.cans. 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 facultative, 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, gelatin, glucose, glutamate, inulin, lactose, maltose, mannitol, mannose, *N*-acetylglucosamine, pectin, starch, succinate and xylose as electron donors. 1-Butanol, 1-propanol, acetate, butyrate, chitin, citrate, ethanol, ethanolamine, glycerol, glycolate, i-butyrate, inositol, i-valerate, lactate, oxalate, propionate, raffinose, saccharose, sorbitol and tartrate are not growth-supportive. Uses ammonium as nitrogen source. Flexirubin reaction-, arginine dihydrolase- and catalase-positive. Oxidase- and lysine decarboxylase-negative. Does not deaminate phenylalanine, hydrolyse urea or form indole. Grows at 2 % but not 5 % NaCl. O_2, NO_3^- and NO_2^- are used as electron acceptors. N_2O is produced as an intermediate during the reduction of NO_3^- to N_2. SO_4^{2-} and Fe^{3+} are not used as electron acceptors. Does not grow by fermentation. The DNA G+C content is 34·6 mol%. Phylogenetically most closely related to *Flavobacterium johnsoniae*.

The type strain (ED5^T = DSM 15936^T = ATCC BAA-842^T) was isolated from the gut of the earthworm *Aporrectodea caliginosa* (collected from garden soil in Bayreuth, Germany).

Description of *Paenibacillus anaericanus* sp. nov.

Paenibacillus anaericanus (an.ae.ri.ca′nus. Gr. pref. *an* no/not; Gr. n. *aer* air; Gr. adj. *ikanos* capable; N.L. masc. adj. *anaericanus* capable of anaerobic growth).

Colonies are flat, smooth, circular and entire. Cells are facultative, motile rods with flagella, 2·0–5·0 × 0·5–1·0 μm, grow in chains, are linked by connecting filaments, form terminal to subterminal, ellipsoidal spores, stain Gram-negative and have a three-layered cell wall with no outer membrane. Cells contain *b*-type cytochromes. Grows at 5–40 °C, with an optimum at 30–35 °C. Grows at pH 5·8–8·6, with an optimum at pH 7·7. Doubling time under optimal conditions is 5 h. Grows aerobically on arabinose, cellobiose, chitin, fructose, glucose, glycerol, lactose, maltose, mannitol, mannose, *N*-acetylglucosamine, raffinose, saccharose, starch and xylose. 1-Butanol, 1-propanol, acetate, butyrate, citrate, ethanol, ethanolamine, fumarate, gelatin, glutamate, glycolate, i-butyrate, inositol, inulin, i-valerate, lactate, oxalate, pectin, propionate, sorbitol, succinate and tartrate are not growth-supportive. Catalase- and oxidase-positive. Grows at 2 % but not 5 % NaCl. Formate, acetate and ethanol are formed when glucose is fermented. NO_3^- and SO_4^{2-} are not disssimilated. Low amounts (1 mM) of NO_2^- are reduced to N_2O. The DNA G+C content is 42·6 %. Phylogenetically most closely related to *Paenibacillus borealis*.

The type strain (MH21^T = DSM 15890^T = ATCC BAA-844^T) was isolated from the gut of the earthworm *Aporrectodea caliginosa* (collected from garden soil in Bayreuth, Germany).

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REFERENCES

Achenbach, L. A., Michaelidou, U., Bruce, R. A., Fryman, J. & Coates, J. D. (2001). *Dechloromonas agitata* gen. nov., sp. nov. and *Dechlorosoma suillum* gen. nov., sp. nov., two novel environmentally dominant (per)chlorate-reducing bacteria and their phylogenetic position. *Int J Syst Evol Microbiol* **51**, 527–533.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Anderson, I. C. & Levine, J. S. (1986). Relative rates of nitric oxide and nitrous oxide production by nitrifiers, denitrifiers, and nitrate respirers. *Appl Environ Microbiol* **51**, 938–945.

Bayer, M. E. & Easterbrook, K. (1991). Tubular spinae are long-distance connectors between bacteria. *J Gen Microbiol* **137**, 1081–1086.

Bergey, D. H., Krieg, N. R. & Holt, J. G. (1990). *Bergey's Manual of Systematic Bacteriology*. Baltimore: Williams & Wilkins.

Bernardet, J.-F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K. & Vandamme, P. (1996). Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *Int J Syst Bacteriol* **46**, 128–148.

Borken, W., Grundel, S. & Beese, F. (2000). Potential contribution of *Lumbricus terrestris* L. to carbon dioxide, methane and nitrous oxide fluxes from a forest soil. *Biol Fertil Soils* **32**, 142–148.

Bulthuis, B. A., Rommens, C., Koningstein, G. M., Stouthamer, A. H. & van Verseveld, H. W. (1991). Formation of fermentation products and extracellular protease during anaerobic growth of *Bacillus licheniformis* in chemostat and batch-culture. *Antonie van Leeuwenhoek* **60**, 355–371.

Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). Rapid method for base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

Cataldo, D. A., Haroon, M., Schrader, L. E. & Young, V. L. (1975). Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun in Soil Sci Plant Anal* **6**, 71–80.

Coates, J. D., Michaelidou, U., Bruce, R. A., O'Connor, S. M., Crespi, J. N. & Achenbach, L. A. (1999). Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria. *Appl Environ Microbiol* **65**, 5234–5241.

Cowan, S. T. (1974). *Cowan & Steel's Manual for the Identification of Medical Bacteria*, 2nd edn. New York: Cambridge University Press.

Cummings, D. E., Caccavo, F., Spring, S. & Rosenzweig, R. F. (1999). *Ferribacterium limneticum*, gen. nov., sp. nov., an Fe(III)-reducing microorganism isolated from mining-impacted freshwater lake sediments. *Arch Microbiol* **171**, 183–188.

Drake, H. L., Schramm, A. & Horn, M. (2005). Earthworm gut microbial biomes: their importance to soil microorganisms, denitrification, and the terrestrial production of the greenhouse gas N_2O. In *Intestinal Microorganisms of Termites and Other Invertebrates*. Edited by H. König & A. Varma. New York: Springer (in press).

Elo, S., Suominen, I., Kämpfer, P., Juhanoja, J., Salkinoja-Salonen, M. & Haahtela, K. (2001). *Paenibacillus borealis* sp. nov., a nitrogen-fixing species isolated from spruce forest humus in finland. *Int J Syst Evol Microbiol* **51**, 535–545.

Fröstl, J. M., Seifritz, C. & Drake, H. L. (1996). Effect of nitrate on the autotrophic metabolism of the acetogens *Clostridium thermoautotrophicum* and *Clostridium thermoaceticum*. *J Bacteriol* **178**, 4597–4603.

Furlong, M. A., Singleton, D. R., Coleman, D. C. & Whitman, W. B. (2002). Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm *Lumbricus rubellus*. *Appl Environ Microbiol* **68**, 1265–1279.

Gadkari, D. (1984). Influence of herbicides Goltix and Sencor on nitrification. *Zentralbl Mikrobiol* **139**, 623–631.

Harrigan, W. F. & McCance, M. E. (1966). *Laboratory Methods in Microbiology*. London: Academic Press.

Horn, M. A., Schramm, A. & Drake, H. L. (2003). The earthworm gut: an ideal habitat for ingested N_2O-producing microorganisms. *Appl Environ Microbiol* **69**, 1662–1669.

Hungate, R. E. (1969). A roll tube method for cultivation of strict anaerobes. *Methods Microbiol* **3B**, 117–132.

Ihssen, J., Horn, M. A., Matthies, C., Gößner, A., Schramm, A. & Drake, H. L. (2003). N_2O-producing microrganisms in the gut of the earthworm *Aporrectodea caliginosa* are indicative of ingested soil bacteria. *Appl Environ Microbiol* **69**, 1655–1661.

Karsten, G. R. & Drake, H. L. (1995). Comparative assessment of the aerobic and anaerobic microfloras of earthworm guts and forest soils. *Appl Environ Microbiol* **61**, 1039–1044.

Karsten, G. R. & Drake, H. L. (1997). Denitrifying bacteria in the earthworm gastrointestinal tract and in vivo emission of nitrous oxide (N_2O) by earthworms. *Appl Environ Microbiol* **63**, 1878–1882.

Kuhner, C. H., Matthies, C., Acker, G., Schmittroth, M., Gößner, A. & Drake, H. L. (2000). *Clostridium akagii* sp. nov. and *Clostridium acidisoli* sp. nov.: acid-tolerant, N_2-fixing clostridia isolated from acidic forest soil and litter. *Int J Syst Evol Microbiol* **50**, 873–881.

Küsel, K., Dorsch, T., Acker, G., Stackebrandt, E. & Drake, H. L. (2000). *Clostridium scatologenes* strain SL1 isolated as an acetogenic bacterium from acidic sediments. *Int J Syst Evol Microbiol* **50**, 537–546.

Küsel, K., Karnholz, A., Trinkwalter, T., Devereux, R., Acker, G. & Drake, H. L. (2001). Physiological ecology of *Clostridium glycolicum* RD-1, an aerotolerant acetogen isolated from sea grass roots. *Appl Environ Microbiol* **67**, 4734–4741.

Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. & Schleifer, K.-H. (1998). Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**, 554–568.

Matthies, C., Griesshammer, A., Schmittroth, M. & Drake, H. L. (1999). Evidence for involvement of gut-associated denitrifying bacteria in emission of nitrous oxide (N_2O) by earthworms obtained from garden and forest soils. *Appl Environ Microbiol* **65**, 3599–3604.

Matthies, C., Kuhner, C. H., Acker, G. & Drake, H. L. (2001). *Clostridium uliginosum* sp. nov., a novel acid-tolerant, anaerobic bacterium with connecting filaments. *Int J Syst Evol Microbiol* **51**, 1119–1125.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Microbiol* **39**, 159–167.

Reichenbach, H. (1989). Order I. Cytophagales Leadbetter 1974, 99^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 3, pp. 2011–2082. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore: Williams & Wilkins.

Schlegel, H. G. & Jannasch, H. W. (1992). Prokaryotes and their habitats. In *The Prokaryotes*, vol. 1, pp. 75–125. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.

Shida, O., Takagi, H., Kadowaki, K., Nakamura, L. K. & Komagata, K. (1997). Emended description of *Paenibacillus amylolyticus* and description of *Paenibacillus illinoisensis* sp. nov. and *Paenibacillus chibensis* sp. nov. *Int J Syst Bacteriol* **47**, 299–306.

Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–657. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Spurr, A. R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. *J Ultrastruct Res* **26**, 31–43.

Stouthamer, A. H. (1988). Dissimilatory reduction of oxidized nitrogen compounds. In *Biology of Anaerobic Microorganisms*, pp. 245–301. Edited by E. J. B. Zehnder. New York: Wiley.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reverse-phased high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Traub, W. H., Acker, G. & Kleber, I. (1976). Ultrastructural surface alterations of *Serratia marcescens* after exposure to polymyxin B and/or fresh human serum. *Chemotherapy* **22**, 104–113.

Valentine, R. C., Shapiro, B. M. & Stadtman, E. R. (1968). Regulation of glutamine synthetase. XII. Electron microscopy of the enzyme from *Escherichia coli*. *Biochemistry* **7**, 2143–2152.

Van der Meulen, H. J., Harder, W. & Veldkamp, H. (1974). Isolation and characterization of *Cytophaga flevensis* sp. nov., a new agarolytic flexibacterium. *Antonie van Leeuwenhoek* **40**, 329–346.

Yoon, J.-H., Oh, H.-M., Yoon, B.-D., Kang, K. H. & Park, Y.-H. (2003). *Paenibacillus kribbensis* sp. nov. and *Paenibacillus terrae* sp. nov., bioflocculants for efficient harvesting of algal cells. *Int J Syst Evol Microbiol* **53**, 295–301.

Zumft, W. G. (1992). The denitrifying prokaryotes. In *The Prokaryotes*, 2nd edn, pp. 554–582. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.