APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1999, p. 3319–3324 Vol. 65, No. 8
0099-2240/99/$04.00+0

Anaerobic Mineralization of Quaternary Carbon Atoms: Isolation of Denitrifying Bacteria on Dimethylmalonate

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Received 19 January 1999/Accepted 18 May 1999

The microbial capacity to degrade simple organic compounds with quaternary carbon atoms was demonstrated by enrichment and isolation of five denitrifying strains on dimethylmalonate as the sole electron donor and carbon source. Quantitative growth experiments showed a complete mineralization of dimethylmalonate. According to phylogenetic analysis of the complete 16S rRNA genes, two strains isolated from activated sewage sludge were related to the genus *Paracoccus* within the α-*Proteobacteria* (98.0 and 98.2% 16S rRNA gene similarity to *Paracoccus denitrificans*^{T}), and three strains isolated from freshwater ditches were affiliated with the β-*Proteobacteria* (97.4 and 98.3% 16S rRNA gene similarity to *Herbaspirillum seropedicae*^{T} and *Acidovorax facilis*^{T}, respectively). Most-probable-number determinations for denitrifying populations in sewage sludge yielded 4.6×10^4 dimethylmalonate-utilizing cells ml^{-1}, representing up to 0.4% of the total culturable nitrate-reducing population.

Quaternary carbon atoms bind with all four single σ bonds to carbon atoms. This structural motif is found in many isoprenoic compounds, e.g., the monoterpenes pinene and carene, cholesterol, and carotene. The biosynthetic pathway involves an irreversible cationic polymerization of alkene bonds to newly formed carbon-carbon single bonds. As a result, quaternary carbon atoms are formed from tertiary carbon atoms (9).

The microbial degradation of compounds with quaternary C atoms has not been studied thoroughly. Already-discovered mineralization pathways in aerobic microorganisms often involve molecular oxygen as a cosubstrate. Camphor and eucalyptol feature a ketone in an α position with respect to a quaternary C-atom. This functional group enables a biological Baeyer-Villiger oxygenation to a lactone that yields, after hydrolysis, a tertiary alcohol (31). A second pathway is provided by *Candida albicans* sterol 14α-demethylase, a cytochrome P450 monooxygenase. This enzyme catalyzes the oxidation of a methyl group adjacent to a quaternary C atom to an aldehyde that is oxidized in a third monooxygenation reaction and is eliminated as formate. Thus, the quaternary C atom is changed via radical intermediates into a tertiary C atom with an alkene bond (27). This aldehyde lyase reaction was first described for aromatase, which catalyzes the C-19 removal of androgens, leading to the formation of estrogens (2, 32). Another pathway present in aerobic bacteria involves an oxygenation of the C-9 atom of cholesterol. The 9α-hydroxy-androsta-1,4-dien-2,17-dione formed is labile and reacts nonenzymatically to produce 9,10-seco-androsta-1,3,5(10)-trien-3-ol-9,17-dione. The spontaneous carbon-carbon cleavage obliterates a quaternary C atom and a tertiary alcohol (16).

One example of an oxygen-independent pathway is the cleavage of α-pinene oxide by a lyase. Three rings are opened in an isomerization reaction, and at the same time a quaternary C atom is transformed into a tertiary C atom (11). This rather unusual intramolecular depolymerization reaction can be perceived as the reverse of the biosynthetic pathway.

Research on the fate of quaternary carbon atoms in anoxic habitats advanced with the recent report of denitrifying β-*Proteobacterium* strain 72Chol, that anaerobically mineralizes cholesterol (14). In a theoretical analysis, we developed a plausible pathway for the degradation of cholesterol via carboxy-methylmalonates as possible intermediates (17). Hence, dimethylmalonate was chosen for enrichment and isolation of denitrifying bacteria to verify the existence of the physiological capacity to mineralize a small compound with a quaternary carbon atom in the absence of molecular oxygen.

MATERIALS AND METHODS

Sources of organisms. Enrichment cultures were inoculated with activated sludge obtained from a local wastewater treatment plant (Lintel, Osterholz-Scharmbeck, Germany) or with a water-mud mixture obtained from freshwater ditches located in Bremen, Germany. Most-probable-number (MPN) counts were performed with the activated sludge. *Alcaligenes defragrans* strains (8), *Azoarcus* sp. strain 22Lin (12), and *Thauera linaloolentis* and *Thauera terpenica* strains (7) were maintained in our laboratory since their isolation.

Media and culture conditions. Anoxic media and cultivation techniques were used in this study (33). The basic medium contained (per liter of distilled water) 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NH_4Cl, 0.5 g of KH_2PO_4, 0.1 g of $CaCl_2$, and 0.85 g of $NaNO_3$ (10 mM). After autoclaving, 2 ml of a chelated trace element mixture (1 liter of distilled water contained 2,100 mg of $FeSO_4 \cdot 7H_2O$, 30 mg of H_3BO_3, 100 mg of $MnCl_2 \cdot 4H_2O$, 190 mg of $CoCl_2 \cdot 6H_2O$, 24 mg of $NiCl_2 \cdot 6H_2O$, 29 mg of $CuSO_4 \cdot 5H_2O$, 144 mg of $ZnSO_4 \cdot 7H_2O$, 36 mg of $NaMoO_4 \cdot 7H_2O$, and 5.2 g of EDTA, pH 6.5), 2 ml of selenite-tungstate solution (0.4 g of NaOH $liter^{-1}$, 8 mg of $Na_2WO_4 \cdot 2H_2O$ $liter^{-1}$, and 6 mg of $Na2SeO_3 \cdot 5H_2O$ $liter^{-1}$) (33), 1 ml of vitamin solution (4 mg of 4-aminobenzoic acid, 2 mg of D-(+)-biotin, 10 mg of nicotinic acid, 5 mg of calcium D-(+)-pantothenate, 15 mg of pyridoxin hydrochloride, 4 mg of folic acid, and 1 mg of lipoic acid in 100 ml of 10 mM NaH_2PO_4, pH 7.1), 1 ml of cyanocobalamin solution (50 mg $liter^{-1}$), 1 ml of thiamine solution (10 mg of thiamine hydrochloride in 100 ml of 25 mM NaH_2PO_4, pH 3.4), 1 ml of riboflavin solution (2.5 mg in 100 ml of 25 mM NaH_2PO_4, pH 3.2) (1), and 50 ml of $NaHCO_3$ solution (1 M) were added, and the pH was adjusted to 7.2. N_2-CO_2 (90:10, vol/vol) was used as the gas phase for all anoxic cultures. Electron donors were added from sterile stock solutions prior to inoculation. All incubations were performed at 28°C in the dark.

Enrichment and isolation of denitrifying bacteria on dimethylmalonate. Enrichment cultures contained 330 ml of medium with 5 mM dimethylmalonate and 20 ml of sewage sludge or 250 ml of medium and 100 ml of a water-mud mixture in 0.5-liter bottles that were sealed with thick butyl rubber stoppers. Control enrichments were prepared in parallel without dimethylmalonate to account for endogeneous carbon sources of the inoculate. Overpressure due to gas formation and the amounts of nitrate and nitrite in the culture were determined regularly,

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and 10 mM nitrate was added when the electron acceptor was depleted. Transfer of the enrichment cultures involved an anaerobically performed serial dilution prior to inoculation to obtain an inoculation size of 1.0×10^{-6} (vol/vol) for a medium volume of 150 ml. After three passages, bacteria were isolated via repeated dilution in agar with 5 mM dimethylmalonate and 10 mM nitrate (33). Isolated colonies were pure strains according to microscopic observations of cultures grown on AC broth (Difco, Detroit, Mich.), yeast extract (0.5 g liter^{-1}), glucose (5 mM), or pyruvate (10 mM) under fermenting or denitrifying conditions and according to the formation of homogenous colonies on oxic agar plates containing either AC broth or dimethylmalonate.

Strain maintenance and growth tests. Isolated strains were kept in culture tubes (21 ml) with 15 ml of anoxic medium under selective growth conditions (5 mM dimethylmalonate and 10 mM nitrate). Transfer of an inoculum of 5% (vol/vol) into freshly prepared culture medium was done every second month. Grown cultures were transferred to a refrigerator and kept at 8°C until the next transfer. Growth experiments were performed in duplicate with culture tubes with an inoculum of 2% (vol/vol). Growth was monitored by turbidimetry at 660 nm, and nitrate and nitrite were analyzed in the late stationary phase. Control experiments demonstrated that the carbon sources present in the vitamin solutions and the inoculate did not support observable microbial growth.

The quantification of dimethylmalonate degradation was done by inoculating a 1- or 5-ml portion of a recently grown culture into 200 ml of medium in a 250-ml Erlenmeyer flask with a depressed sidearm for turbidimetric determinations and a threaded neck to hold the butyl rubber stopper with a screw cap against the overpressure that is build up by denitrification. The culture was regularly sampled with sterile, nitrogen-flushed syringes for analyses of organic acids, nitrite, and nitrate. Nitrogen formation was measured in cultures containing an He-CO_2 (90:10, vol/vol) atmosphere. Formation of carbon dioxide was assayed in cultures that were made without bicarbonate and carbon dioxide and were buffered by potassium phosphate (20 mM, pH 7.0).

Denitrifying growth on dimethylmalonate occurred not only in oxygen-free media but also in chemically reduced media. Anoxic media were prereduced in control experiments with 4 mM ascorbate. The strains did not utilize ascorbate as a carbon and energy source. Thus, ascorbate could routinely be added to ensure anoxic conditions in the culture.

Enumeration of dimethylmalonate-utilizing denitrifying bacteria. The size of the denitrifying population in sewage sludge was estimated with MPN dilutions in liquid medium (3). MPN counts were performed with the medium described above and either 5 mM dimethylmalonate or a mixture of fatty acids (acetate, propionate, and butyrate [2 mM each compound] and succinate, valerate, isovalerate, α-methylbutyrate, and isobutyrate [50 μM each compound]) as the sole electron donor. A 10-fold dilution was used, with three portions per dilution. The MPN culture tubes were incubated in the dark at 20°C for 18 weeks.

Chemical analyses. Biomass formation was measured turbidimetrically at 660 nm. Cell dry weight determinations were performed as described previously (12). Qualitative measurements of nitrate consumption were performed with an indicator strip (Merck, Darmstadt, Germany). Nitrate and nitrite were determined quantitatively by high-pressure liquid chromatography (13), and gases (dinitrogen oxide, dinitrogen, and carbon dioxide) were determined by packed-column gas chromatography as described previously (14). Ammonium was measured photometrically by the indophenol method (14). Organic acids were quantified by ion exclusion chromatography on a WA1 column (7.8 by 300 mm; Sarasep, San Jose, Calif.) with a high-pressure liquid chromatography system (Sykam, Garching, Germany) equipped with a UV detector (Linear Instruments, Fremont, Calif.). Culture samples of 950 μl were acidified with 50 μl of 1 M H_2SO_4 and clarified by centrifugation. Subsamples of 50 μl were separated with 5 mM H_2SO_4 as the liquid phase at a flow rate of 0.6 ml min^{-1} at 35°C. Organic acids were detected at 210 nm. Net retention times for dimethylmalonate, acetate, and propionate were 5.09, 9.23, and 11.97 min, respectively.

Cells were observed with a standard phase-contrast microscope (Zeiss, Oberkochen, Germany) with an oil immersion objective (100/1.6). Dense cultures were wet mounted on glass slides coated with 2% washed agar (26) and photographed by using Ortho 25 film (Agfa-Gevaert, Leverkusen, Germany).

16S rRNA gene sequence and data analysis. In vitro amplification of the 16S rRNA gene and direct sequencing were performed as previously described (29). Sequencing reactions were performed with a Taq Dyedeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) and were run on an Applied Biosystems 373S DNA sequencer. The obtained sequences were added to an alignment of about 5,300 homologous bacterial 16S rRNA primary structures (22), applying the aligning tool of the ARB program package (30). Phylogenetic trees were constructed by using subsets of data that included sequences from outgroup reference organisms as well as representative and genealogically related sequences of members of the alpha and beta subclasses of *Proteobacteria* (22). Topologies were evaluated by using distance matrix, maximum-parsimony, and maximum-likelihood methods as implemented in the ARB software to elaborate a consensus tree (21).

Whole-cell in situ hybridization. Microbes that were grown in highly diluted MPN tubes were analyzed by whole-cell in situ hybridization for the presence of α- and β-*Proteobacteria* as described by Manz et al. (23). Probes EUB338 (23) and ALF968 (24) were used to detect *Eubacteria* and α-*Proteobacteria*, respectively. For β-*Proteobacteria*, CY3-labelled probe BET42a was used with a non-labelled competitor probe, GAM42a (23).

Nucleotide sequences accession numbers. The sequences determined in this work have been deposited under EMBL accession numbers AJ012067 to AJ012071.

RESULTS

Enrichment and isolation. The capacity of nitrate-reducing microorganisms to degrade dimethylmalonate was investigated with activated sludge and a water-mud mixture from freshwater ditches. A small inoculum of the former was used to reduce the amount of endogenous electron donors. The enrichment containing 5 mM dimethylmalonate and 10 mM nitrate consumed all nitrate within 9 days and formed 30 ml of gas. After nitrate addition, the gas formation continued to a total volume of 47 ml on day 16. A control experiment without dimethylmalonate produced only 8 ml of gas in this time. The freshwater enrichment contained a significant amount of endogenous electron donors for nitrate reduction. The control consumed nearly 30 mM nitrate in 20 days and formed 89 ml of gas. Gas formation in the enrichment culture started to exceed that in the control on day 14 and accumulated to 116 ml on day 20, when 40 mM nitrate was consumed. Small inocula of these enrichment cultures (150 nl obtained by serial dilution) were transferred, resulting in a dilution of 1 ppm to select for bacteria that were probably present in larger amounts in the enrichment. Two additional passages with small inocula (1 ppm) ensured a selection for bacteria that grew well on dimethylmalonate. Isolation by agar dilution series was then successfully attempted. Two strains, B8B1 and B8B2, originated from activated sludge, and three strains, G7A1, G8A1, and G8B1, originated from the freshwater ditches.

Characterization of the isolated strains. Strains B8B1 and B8B2 were nonmotile spherical cells or short rods with dimensions of 0.7 to 1.0 by 1.0 to 2.0 μm, resembling *Paracoccus*. Strains G7A1 and G8B1 were *Pseudomonas*-like motile straight rods with sizes of 0.5 to 0.7 by 1.5 to 2.5 μm. The motile strain G8A1 was a small spirillum, 0.3 to 0.5 by 1.0 to 2.0 μm in size (Fig. 1). The microorganisms grew on several organic acids, including isobutyrate. Strains G7A1 and G8B1 did not utilize methylsuccinate. Sugars were utilized only by strains B8B1 and B8B2 (Table 1).

Carbon limitation was achieved with 2 mM dimethylmalonate in the presence of 10 mM nitrate. Grown cultures of all strains contained nitrate and dinitrogen oxide but no nitrite. Nitrate-limited cultures were obtained with 5 mM dimethylmalonate and 10 mM nitrate. Nitrate and nitrite were depleted under these conditions. Nitrogen formation was observed in cultures with a helium atmosphere. Ammonium present in the medium was assimilated during denitrifying growth, excluding ammonification as a catabolic process.

Different biomass yields were observed for the strains in carbon-limited cultures and in nitrate-limited cultures; i.e., strains B8B1 and B8B2 produced an optical density (OD) at 660 nm of 0.6 on 10 mM nitrate, whereas strains G7A1, G8A1, and G8B1 produced an OD of only 0.3. Koch showed that, in the absence of pigments, cells with a volume range of 0.4 to 2 μm^3 had an average dry weight/OD ratio of 371 mg (dry weight) liter^{-1} OD at 660 nm^{-1} (18). To account for the smaller size of strain G8A1 cells, we determined the dry weight formed in nitrate-limited denitrifying cultures: strain B8B2 formed 21.4 g (dry weight) mol of nitrate^{-1}, whereas strains G8A1 and G8B1 formed only 10.3 and 10.8 g (dry weight) mol of nitrate^{-1}, respectively.

All strains grew aerobically on nitrate-free agar plates with dimethylmalonate.

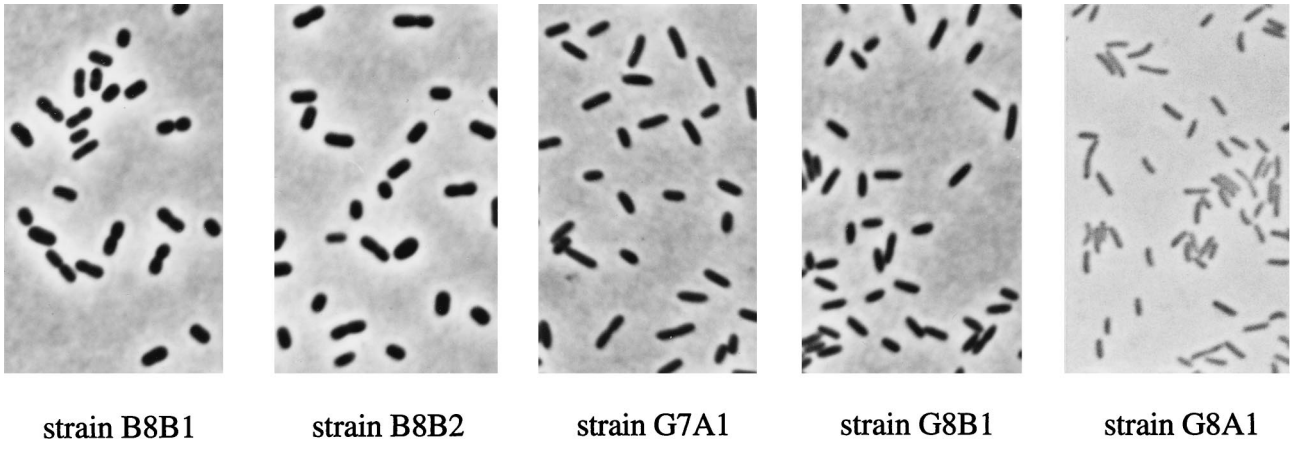

FIG. 1. Phase-contrast photomicrographs of new denitrifying isolates grown with dimethylmalonate (5 mM) and nitrate (10 mM). Bar, 10 μm.

Quantification of denitrifying growth on dimethylmalonate. Strain B8B2 was used to determine quantitatively dimethylmalonate and nitrate consumption (Fig. 2). A biomass of 32.6 mg was formed based on a measured correlation of 427.6 mg (dry mass) liter^{-1} to 1 liter of culture with an OD of 1.0. According to the assimilation equation $17C_5H_8O_4 + 2H_2O \rightarrow 20C_4H_7O_3 + 5CO_2$, this corresponds to a consumption of 269 μmol of dimethylmalonate. Since 870 μmol of dimethylmalonate disappeared during growth, complete mineralization of the calculated amount of dissimilated dimethylmalonate (601 μmol) would provide a total of 12 mmol of electrons for denitrification. Ten millimoles of electrons is required to account for a reduction of nitrate (2 mmol consumed) to dinitrogen. In separate experiments, the nitrogen and carbon balances were analyzed. In nitrate-limited cultures grown under a helium atmosphere, nitrate consumption of 2.56 mmol of nitrogen atoms occurred with the synthesis of 160 μmol of dinitrogen oxide N and 2.13 mmol of nitrogen N. Carbon dioxide formation correlated with the amount of dimethylmalonate supplied. In grown carbon-limited cultures (2 mmol of C as dimethylmalonate), we detected 1.35 mmol of carbon dioxide; the increase in biomass represented, according to the aforementioned equation, an assimilation of 776 μmol of C. Thus, the electron, nitrogen, and carbon balances support within exper-

TABLE 1. Substrates for denitrifying growth of the isolated strains

Electron donor (concn, mM)	Growth[a] of:				
	Paracoccus sp. strain B8B1	*Paracoccus* sp. strain B8B2	*Acidovorax* sp. strain G7A1	*Acidovorax* sp. strain G8B1	*Herbaspirillum* sp. strain G8A1
Acetate (10)	+++	+++	++	++	++
Propionate (6)	+++	+++	++	++	++
Butyrate (4)	+++	+++	++	++	++
Isobutyrate (4)	+++	+++	++	++	++
2,2-Dimethylpropionate (5)	−	−	−	−	−
Dimethylmalonate (5)	+++	+++	++	++	+
Pyruvate (10)	+	+	+	+	++
Succinate (6)	+++	+++	++	++	++
Methylsuccinate (5)	+++	+++	−	−	++
Benzoate (2)	−	−	−	−	−
L-Ascorbate (10)	−	−	−	−	−
D-Gluconate (5)	+++	+++	−	−	−
D-Glucose (5)	+++	+++	−	−	−
D-Fructose (5)	+++	+++	−	−	−
D-Galactose (5)	+++	+++	−	−	−
D-Ribose (5)	+	+	−	−	−
Inositol (5)	+++	+++	−	−	−
Methanol (10)	−	−	−	−	−
L-Glutamate (5)	+++	+++	++	++	+
L-Arginine (5)	−	−	−	−	−
L-Valine (5)	++	++	−	−	−
L-Cysteine (5)	++	++	+++	+++	++
L-Phenylalanine (5)	−	−	−	−	−
L-Isoleucine (5)	+++	+++	+	++	+

[a] Bacterial growth was determined as the increase in OD at 660 nm, as follows: −, <0.030; +, >0.070; ++, >0.200, +++, >0.400.

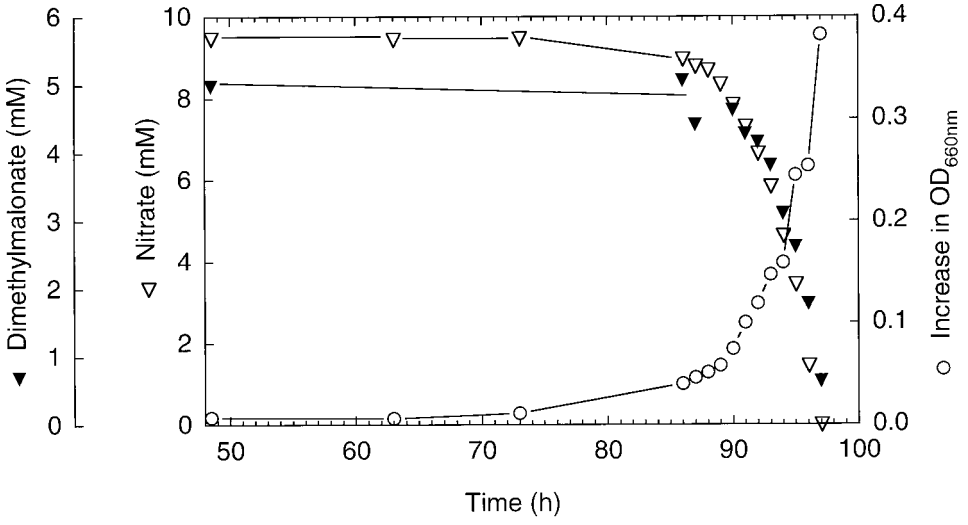

FIG. 2. Growth of strain B8B2 on dimethylmalonate and nitrate.

imental uncertainties the complete mineralization of dimethylmalonate according to the catabolic reaction $C_5H_8O_4 + 4H^+ + 4NO_3^- \rightarrow 5CO_2 + 2N_2 + 6H_2O$.

Phylogenetic affiliations of the isolated strains. Almost-complete 16S rRNA gene sequences from the isolates were obtained by in vitro amplification and direct sequencing. The sequences of strains B8B1 and B8B2 were nearly identical, with a similarity of 99.8%, and strains G7A1 and G8B1 had identical sequences. Sequence analysis showed that both activated-sludge strains B8B1 and B8B2 were affiliated with the genus *Paracoccus* of the α-*Proteobacteria* (Fig. 3). Their closest relative was *P. denitrificans*, with a sequence similarity of 98.0%. Similarities of this strains to one group of *Paracoccus* species (*P. alcaliphilus*, *P. aminophilus*, *P. aminovorans*, *P. thiocyanatus*, and *P. versutus*) ranged from 96.8 to 97.5%, and those to a second group of *Paracoccus* species (*P. alkenifer*, *P. marcusii*, *P. solventivorans*, and *P. kocurii*) ranged from 94.9 to 95.4%. In contrast, all three ditch isolates were affiliated with the β-*Proteobacteria*. Strains G7A1 and G8B1 were affiliated with the genus *Acidovorax*, having 98.3% sequence similarity with the type strains of *Acidovorax facilis*. The closest relative to both ditch isolates, with a similarity of 99.3%, was the activated-sludge clone sequence T20 (28). Strain G8A1 was affiliated with the same branch as *Herbaspirillum seropedicae* (97.4% similarity) and a soil ultramicrobacterium, strain MY14, (97.0% similarity) within the *Oxalobacter-Telluria-Duganella* lineage. Based on these phylogenetic affiliations and the consistent cell morphologies, three isolated strains were selected for deposition at the German Collection of Microorganisms and Cell Cultures, Braunschweig (8a), as *Acidovorax* sp. strain G8B1 (DSM 12578), *Herbaspirillum* sp. strain G8A1 (DSM 12579), and *Paracoccus* sp. strain B8B2 (DSM 12584).

Population sizes of denitrifying bacteria. Volatile fatty acids are major primary fermentation products and may as such be important carbon sources and electron donors for denitrifying bacteria in sewage plants. The population size of nitrate-reducing bacteria was determined by MPN counts with either a defined mixture of fatty acids or AC broth as an electron donor. Both measurements yielded the same population size (data not shown). Thus, we now routinely use a defined mixture of fatty acids as a carbon source for MPN counts of nitrate-reducing bacteria in order to avoid the growth of fermentative bacteria.

Clarification of the wastewater at the plant in Lintel involved a limited aeration to introduce early a sequential nitrification-denitrification. MPN counts on samples from the first and third basins indicated a population of 4.6×10^4 bacteria ml^{-1} that were capable of denitrifying growth on dimethylmalonate. The total denitrifying population decreased from the first basin (1.1×10^8 bacteria ml^{-1}) to the third basin (1.1×10^7 bacteria ml^{-1}). In situ hybridization of cells was performed on the highest-diluted MPN tubes exhibiting growth. The *Bacteria*-specific probe EUB338 visualized 75% ± 24% of DAPI (4′,6-diamidino-2-phenylindole)-stained cells from the fatty acid-utilizing, nitrate-reducing microorganisms with a statistical population size of greater than 10^5 bacteria ml^{-1}. None of the cells hybridized with the α-*Proteobacteria*-specific probe, but the probe for the beta subclass detected 52% ± 18% of DAPI-stained cells. Major contributions to the dimethylmalonate-degrading denitrifying subpopulation within the sludge were made by β-*Proteobacteria*. In MPN tubes representing greater than 10^3 cells ml^{-1}, the EUB338 and BET42a probes indicated a relative abundance of 71% ± 24% of *Bacteria* and of 52% ± 18% of members of the beta subclass. A small contribution of α-*Proteobacteria* (3% of DAPI-stained cells) was found in a single MPN tube that represented a statistical population density of greater than 10^3 cells ml^{-1}.

Some β-*Proteobacteria* that were recently isolated anaerobically on a comparable minimal medium with a single carbon and electron source and nitrate as an acceptor were tested for denitrifying growth on dimethylmalonate. *Azoarcus* sp. strain 22Lin and *Alcaligenes defragrans* 51Men and 65Phen mineralized dimethylmalonate. No utilization was observed in cultures of *A. defragrans* 54Pin^T and 62Car, *Thauera linaloolentis* 47Lol^T, and *Thauera terpenica* 21Mol and 58Eu^T.

DISCUSSION

In this study we isolated, for the first time to our knowledge, bacteria that are able to grow on dimethylmalonate as a carbon source and electron donor. The anoxic culture conditions were chosen to select for an oxygen-independent degradation pathway. In addition, aerobic growth of the isolated microorganisms on dimethylmalonate demonstrated the presence of dimethylmalonate-degrading capacities in oxygen-respiring bacteria. The observation of quantitative dimethylmalonate

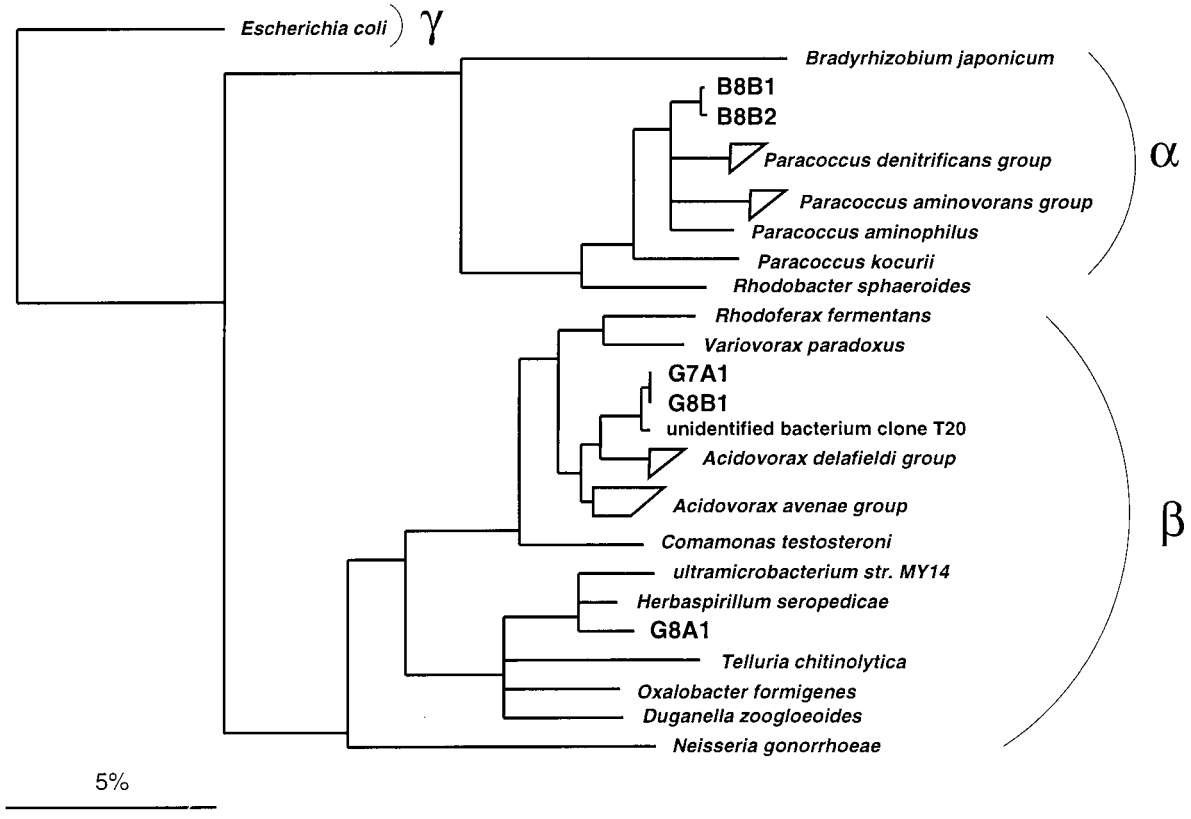

FIG. 3. 16S rRNA-based tree reflecting the phylogenetic relationships of (i) strains B8B1 and B8B2 and a selection of *Proteobacteria* from the alpha subclass and (ii) strains G7A1, G8B1, and G8A1 and a selection of *Proteobacteria* of the beta subclass. The tree is based on the results of a distance matrix analysis including complete or almost-complete 16S rRNA sequences from representative bacteria of the α, β, and γ subclasses (22). The topology of the tree was evaluated and corrected according to the results of distance matrix, maximum-parsimony, and maximum-likelihood analyses of various data sets. The phylogenetic positions of the analyzed strains did not differ in any of the treeing approaches. Multifurcations indicate topologies that could not be unambiguously resolved. The bar indicates 5% estimated sequence divergence. The *P. denitrificans* group comprises the species *P. denitrificans* (GenBank accession no. X69159) and *P. versutus* (D32243 and D32244) and *Paracoccus* sp. strains KL1, KS1, and KS2 (U58017, U58016, and U58015). The *P. aminovorans* group comprises the species *P. aminovorans* (D32240), *P. alcaliphilus* (D32238), and *P. thiocyanatus* (D32242). The *A. delafieldii* group comprises the species *A. delafieldii* (AF078764), *A temperans* (AF078766), and *A. facilis* (AF078765) and *Acidovorax* sp. strain 7078 (AF078767). The *A. avenae* group comprises *A. anthurii* (AJ007013), *A. konjaci* (AF078760), *A. avenae* subsp. *avena* (AF078759), *A. avenae* subsp. *citrulli* (AF078761), *A. avenae* subsp. *cattleyae* (AF078762), and *Acidovorax* sp. strain IMI 357678 (AF078763). Accession numbers of other bacterial 16S rRNA gene sequences are U00006 (*Escherichia coli*), D13429 (*Bradyrhizobium japonicum*), D32239 (*P. aminophilus*), D32241 (*P. kocurii*), X53855 (*Rhodobacter sphaeroides*), D16211 (*Rhodoferax fermentans*), D30793 (*Variovorax paradoxus*), Z93964 (unidentified bacterium clone T20), M11224 (*Comamonas testosteroni*), AB008503 (ultramicrobacterium strain MY14), Y10146 (*H. seropedicae*), X65590 (*Telluria chitinolytica*), U49757 (*Oxalobacter formigenes*), X74914 (*Duganella zoogloeoides*), and X07714 (*Neisseria gonorrhoeae*).

oxidation attests to the capacity of microorganisms to mineralize simple compounds with quaternary carbon atoms. The presence of the dimethylmalonate-degrading capacity in a medium-sized population of bacteria in sewage sludge suggests that dimethylmalonate and related disubstituted malonates may be intermediates of the microbial degradation of organic matter.

The enrichment procedure, with inocula sizes of 10^{-6} (vol/vol), allows the isolation of a probably abundant bacterium with efficient growth on dimethylmalonate and nitrate. The isolation of related strains B8B1 and B8B2 from sewage sludge and G7A1 and G8B1 from freshwater ditches reflects the high selection pressure during enrichment. The isolation of *Paracoccus* sp. strains B8B1 and B8B2 from sewage sludge corresponds to the general methylotrophic physiology of members of the genus *Paracoccus* (20); e.g., *Paracoccus* cells constituted 3.5% of the total population in a denitrifying sand filter fed with methanol (25). Denitrification is also widespread among *Paracoccus* species (20). However, analysis of the dimethylmalonate-degrading bacteria grown in highly diluted MPN tubes with oligodeoxynucleotide probes indicated a predominance of β-*Proteobacteria*. Hence, the *Paracoccus* strains B8B1 and B8B2 had successfully competed with the β-*Proteobacteria* during the enrichment. The key advantage of the nitrate-limited enrichment was probably the higher biomass yield of the *Paracoccus* strains. The partition of β-*Proteobacteria* in dimethylmalonate degradation was confirmed by the freshwater isolates and by denitrifying growth of *Azoarcus* sp. strain 22Lin and *A. defragrans* 51Men and 65Phen on dimethylmalonate.

The isolates from freshwater ditches are β-*Proteobacteria* related to the genera *Acidovorax* and *Herbaspirillum*. The presence of β-*Proteobacteria* in highly diluted MPN tubes is in agreement with in situ investigations of activated sludge that found a dominance of β-*Proteobacteria* (28). Curiously, a cloned 16S rRNA gene sequence obtained in that study (28), clone T20, has the highest 16S rRNA gene sequence similarity (98.3%) to the isolated strains G7A1 and G8B1. Members of *Acidovorax* can denitrify (34, 35), but the described *H. rubrisubalbicans* and *H. seropedicae* are only able to reduce nitrate to nitrite (4). Dinitrogen formation did not occur. Phylogenetically related ultramicrobacteria were not tested for the capacity to denitrify (15). Isolate G8A1 is the first isolate in the

phylogenetic group that reduces nitrate to dinitrogen oxide and further to dinitrogen.

Two pathways for dimethylmalonate degradation can be considered. In model studies for the mechanism of the coenzyme B_{12}-dependent methylmalonyl-coenzyme A (CoA) mutase, dimethylmalonate derivatives of organocobalamin were found to decompose spontaneously in neutral aqueous solutions. The products formed included methylsuccinate derivatives (10). A similar enzymatic rearrangement based on radical intermediates seems feasible, especially because of the utilization of methylsuccinate by strains G8A1, B8B1, and B8B2. The alternative is a decarboxylation yielding isobutyrate derivatives. During incubation with $^{14}CO_2$, Mn^{2+}, ATP, and isobutyryl-CoA, enzyme fractions of *Mycobacterium* sp. strain IBS-M formed two labelled acids that were identified as succinate and dimethylmalonate (19). Besides this observation, a decarboxylase entity is imaginable based on the knowledge of methylmalonyl-CoA and malonyl-CoA decarboxylases (5, 6). We will investigate these possibilities in future research.

ACKNOWLEDGMENT

This study was supported by the Max-Planck-Gesellschaft.

ADDENDUM IN PROOF

Acidovorax sp. strains G7A1 and G8B1 belong to the recently established species *Acidovorax defluvii* (26a) on the basis of 16S rRNA sequences.

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