APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1999, p. 3526–3533 Vol. 65, No. 8
0099-2240/99/$04.00+0

Culturable Populations of *Sporomusa* spp. and *Desulfovibrio* spp. in the Anoxic Bulk Soil of Flooded Rice Microcosms

DIRK ROSENCRANTZ,[1] FREDERICK A. RAINEY,[2]† AND PETER H. JANSSEN[1]*

Max-Planck-Institut für terrestrische Mikrobiologie, D-35043 Marburg,[1] and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, D-38124 Braunschweig,[2] Germany

Received 2 February 1999/Accepted 18 May 1999

Most-probable-number (MPN) counts were made of homoacetogenic and other bacteria present in the anoxic flooded bulk soil of laboratory microcosms containing 90- to 95-day-old rice plants. MPN counts with substrates known to be useful for the selective enrichment or the cultivation of homoacetogenic bacteria (betaine, ethylene glycol, 2,3-butanediol, and 3,4,5-trimethoxybenzoate) gave counts of 2.3×10^3 to 2.8×10^5 cells per g of dry soil. Homoacetogens isolated from the terminal positive steps of these dilution cultures belonged to the genus *Sporomusa*. Counts with succinate, ethanol, and lactate gave much higher MPNs of 5.9×10^5 to 3.4×10^7 cells per g of dry soil and led to the isolation of *Desulfovibrio* spp. Counting experiments on lactate and ethanol which included *Methanospirillum hungatei* in the medium gave MPNs of 2.3×10^6 to 7.5×10^8 cells per g of dry soil and led to the isolation of *Sporomusa* spp. The latter strains could grow with betaine, ethylene glycol, 2,3-butanediol, and/or 3,4,5-trimethoxybenzoate, but apparently most cells of *Sporomusa* spp. did not initiate growth in counting experiments with those substrates. Spores apparently accounted for 2.2% or less of the culturable bacteria. It appears that culturable *Desulfovibrio* spp. and *Sporomusa* spp. were present in approximately equal numbers in the bulk soil. Multiple, phylogenetically-distinct, phenotypically-different, strains of each genus were found in the same soil system.

Processes leading to methane emission from rice paddy soils have been quantified (9, 37) and their importance to world climate recognized (14). Since these soils are flooded, they are largely anoxic and typically (but not exclusively) methanogenic. In the oxic zones associated with the plant root system, the chemical reoxidation of reduced inorganic sulfur and iron compounds leads to a significant flow of electrons to sulfate and ferric iron as electron acceptors (19, 42, 51). In the bulk soil, the flow of carbon and electrons is mainly fermentative, with methane being the major reduced end product of organic matter breakdown (4, 42). Plant polymers and root exudates are important sources of carbon and energy for microbial activity, leading to methane formation through a trophic web of largely uncharacterized microbial populations.

Based on ^{14}C-glucose (30) and $^{14}CO_2$ and ^{14}C-acetate (42) turnover studies and mass balance calculations on slurries of rice paddy soil (4), it is known that some 80% of the methane formed is derived from aceticlastic methanogenesis. This is higher than the 67% expected from a normal methanogenic trophic web. To explain this, it has been suggested that homoacetogenic bacteria may play a significant role in acetate production in anoxic rice paddy soil (4, 10, 30, 45). The utilization by homoacetogenic bacteria of intermediates generated by the initial fermentative degradation of organic matter may result in a higher flow of carbon and electrons through acetate to methane. Homoacetogenic bacteria have also been shown to be active in other soils when they become anoxic (32, 47).

Conrad et al. (10) enriched *Acetobacterium* spp. from Italian rice paddy soil using hydrogen as the growth substrate by incubating enrichment cultures at low temperatures to select against hydrogenotrophic methanogens. Enrichment cultures show the presence of microorganisms in the sample material but do not tell us about the sizes of the populations. Molecular techniques have been used to detect homoacetogenic bacteria based on DNA probes targeting the gene for formyltetrahydrofolate synthetase (34), a key enzyme of their central metabolic pathway, but this has not been applied to rice paddy soils. The homoacetogenic bacteria do not represent a phylogenetically coherent group (49), and so 16S rRNA-based approaches are not applicable. We have attempted to enumerate the homoacetogenic bacteria by utilizing substrates favoring their selective enrichment and have developed new approaches which were useful in obtaining high viable counts of homoacetogens. To obtain a better understanding of the microbial community structure of the anoxic bulk soil of the rice paddy system, we have identified and partially characterized numerically significant representative strains isolated by the extinction dilution method.

MATERIALS AND METHODS

Medium preparation. Two sulfide-reduced, bicarbonate-buffered mineral media, supplemented with vitamins, were used in this study, DM and FM (26). Medium DM was used for the enumeration experiments, while medium FM was used for experiments with the pure cultures. Screw-cap bottles were filled, leaving a small gas bubble, or tubes or serum bottles were partially filled (with the headspace gas composed of N_2 plus CO_2 [80:20 {vol/vol}]), and closed with butyl rubber stoppers.

L isomers of organic acids and D isomers of sugars were used. 2,3-Butanediol (Fluka, Buchs, Switzerland) was a mix of racemic and meso forms. Substrates and other supplements were prepared as neutralized (with NaOH or HCl, as required) 200-mM to 2-M stock solutions and sterilized by autoclaving or, in the case of heat-labile compounds and sugars, by sterile filtration (0.2-μm pore size). The substrates were added to sterile media just before inoculation to a final concentration of 10 mM, except for monosaccharides (4 mM), 3,4,5-trimethoxybenzoate (5 mM), formate (20 mM), and succinate (20 mM). Nitrate and sulfate were added as sodium salts to a 20-mM final concentration. Sulfur was prepared as an aqueous slurry (26) and added at approximately 100 mmol/liter. Hydrogen was added to the N_2-CO_2 headspace of partially filled serum bottles or tubes to an overpressure of 0.6 bar, with the addition of 1 mM acetate as a supplementary carbon source unless otherwise noted.

* Corresponding author. Present address: Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3052, Australia. Phone: 61(3) 9344 5706. Fax: 61 (3) 9347 1540. E-mail: p.janssen@microbiology.unimelb.edu.au.

† Present address: Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803.

TABLE 1. MPN counts of bacteria in anoxic soil of a flooded rice paddy soil microcosm

Substrate	Cell count (per g of dry soil)	95% Confidence interval	Strain isolated in pure culture
Lactate[a]	3.7×10^6	8.3×10^5–1.1×10^7	*Desulfovibrio* sp. strain DR10
Ethanol[a]	2.3×10^6	4.4×10^5–8.3×10^6	*Desulfovibrio* sp. strain DR14
Succinate	5.9×10^5	9.8×10^4–2.9×10^6	*Desulfovibrio* sp. strain DR1
Betaine[a]	5.9×10^4	9.8×10^3–2.9×10^5	*Desulfobacterium* sp. strain DR7
Ethylene glycol	1.0×10^4	2.0×10^3–4.4×10^4	*Sporomusa* sp. strain DR6
2,3-Butanediol	1.0×10^4	2.0×10^3–4.4×10^4	*Sporomusa* sp. strain DR16
3,4,5-TMB[a,b]	2.3×10^3	4.4×10^2–8.3×10^3	*Sporomusa* sp. strain DR5

[a] Three further counts with soil from different microcosms were made with these substrates (two incubated at 25°C and one incubated at 15°C); in all cases, the counts were not significantly different ($P < 0.05$) from those obtained in the experiments listed in the table.
[b] 3,4,5-TMB, 3,4,5-trimethoxybenzoate.

Microbial strains. *Desulfovibrio sulfodismutans* ThAc01 (DSM 3696^T), *Acetobacterium woodii* WB1 (DSM 1030^T), *Pelobacter acetylenicus* WoAcy1 (DSM 3246^T), and *Methanospirillum hungatei* JF1 (DSM 864^T) were from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). *A. woodii* and *P. acetylenicus* were cultivated in screw-cap bottles in FM medium with 10 mM ethylene glycol and 10 mM acetoin, respectively. *M. hungatei* was cultivated in FM medium in partially filled serum bottles, closed with butyl rubber stoppers, under a headspace gas composed of N_2 plus CO_2 (80:20 [vol/vol]) and supplemented with 0.6 bar of H_2 and 5 mM acetate.

Enumeration and isolation of bacteria. Rice (*Oryza sativa* var. Roma type japonica) was grown in plastic containers in the laboratory, as described by Frenzel et al. (20), in flooded soil obtained from wetland rice fields of the Italian Rice Research Institute in Vercelli, Italy. Soil cores were taken from laboratory rice cultures in which the plants were 90 to 95 days old by pressing a plastic tube into the soil to a depth of about 15 cm. Only the lower 10 cm of the cores was used. Three-tube most-probable-number (MPN) counts were made as described previously with DM as the growth medium (26). The MPN tubes were incubated in the dark at 25°C (unless otherwise noted) for 13 weeks or at 15°C for 33 weeks. Growth was considered positive if the substrate had been consumed (this could not be assessed in the case of betaine) and products had been formed (organic acids, gallate, CH_4, or sulfide, as appropriate). The MPN was calculated from the dry weight of the soil (by drying it to constant weight at 105°C), the dilution factor, and tables for three parallel dilution series based on a statistical treatment of such counting methods (1). The significance of differences between MPN values was tested as described by Cochran (8).

Some soil samples were pasteurized by heating them in a water bath at 80°C for 15 min prior to making the dilution series. *M. hungatei* was added to some MPN series at 1 ml of a well-grown culture per 9 ml of inoculated medium immediately after the dilution series had been prepared.

Isolation and cultivation of pure cultures. The agar deep method for isolating pure cultures was described by Pfennig (38). The tubes were incubated at 25°C. Culture purity was checked microscopically by growth tests with various growth substrates; by growth in a complex medium consisting of DM or FM medium with (per liter) 0.5 g of yeast extract, 2 mmol of glucose, 5 mmol of fumarate, 5 mmol of pyruvate, and 2 mmol of acetate; and by testing growth on nutrient agar (Difco Laboratories, Detroit, Mich.) plates supplemented with 4 mM glucose.

Pure cultures were normally grown at 25°C in completely filled screw-cap bottles, with the addition of 100 μM (final concentration) sodium dithionite from sterile stocks (48). Defined cocultures with *M. hungatei* and cultures in which gases were to be measured were grown in aliquots of 50 ml of medium in 120-ml serum bottles with N_2 plus CO_2 (80:20 [vol/vol]) gas and closed with butyl rubber stoppers.

Characterization and analytical methods. Genomic DNA was extracted, and the mol% G+C ratio was determined by reversed-phase high-performance liquid chromatography (HPLC) as described by Janssen et al. (25). Phase-contrast photomicrographs were made after immobilizing the cells on an agar-coated microscope slide (39). Gram staining was carried out as described by Süßmuth et al. (44), employing 95% isopropanol as the decolorizing agent and with *A. woodii* and *P. acetylenicus* as controls. A fluorescence test (40) was used to detect the presence of desulfoviridin.

Sporulation was tested by adding 1 ml of sterile soil extract to 50 ml of medium supplemented with 25 mg of thiamine, 25 mg of $CaCl_2 \cdot 2H_2O$, and 15 mg of $MnCl_2 \cdot 4H_2O$. The medium was then inoculated, and spores were looked for in the stationary phase by phase-contrast microscopy. Soil extract was prepared from dried rice paddy soil as described by Cote and Gherna (13).

The concentrations of organic growth substrates and organic end products of metabolism were measured by ion exclusion HPLC (30), except for betaine, which was not measured, and 3,4,5-trimethoxybenzoate and gallate, which were measured by reversed-phase HPLC (28). Hydrogen production was determined by gas chromatography (26). Sulfide production was qualitatively assessed by a copper precipitation test (11) and quantitatively assessed by a colorimetric assay (7). Methane was measured by gas chromatography (18). The increase of ammonium due to nitrate reduction was tested with Merckoquant ammonium test strips (Merck, Darmstadt, Germany).

Enzyme assays. Cultures (50 ml) were grown in serum bottles and harvested by centrifugation at 3,000 × *g* for 30 min at 4°C in the bottles. The supernatants were expelled by inverting the bottles and introducing a stream of N_2 via a hypodermic needle through the stopper while simultaneously allowing the supernatant to be expelled via a second hypodermic needle. The cells were resuspended in 1 ml of anoxic 50 mM potassium phosphate buffer (pH 7.2) and used directly in the enzyme assays. The presence of carbon monoxide dehydrogenase and formate dehydrogenase was assayed spectrophotometrically (24) on whole cells permeabilized in the cuvette by adding 10 μl of a toluene-ethanol mixture (1:9 [vol/vol]) to the 1-ml assay. *A. woodii* and *P. acetylenicus* were used as positive and negative controls, respectively.

The protein content of the cell concentrates was estimated by boiling 200 μl of diluted (in potassium phosphate buffer) cell suspension with 50 μl of 10 M NaOH. Standards of bovine serum albumin were treated the same way. The samples and standards were then centrifuged at 13,000 × *g* for 5 min, and 200 μl of the supernatant was added to 600 μl of phosphate buffer. The protein concentrations in these samples and standards were determined by the method of Bradford (2).

16S rDNA sequence determination and analyses. Genomic DNA extraction, PCR-mediated amplification of the 16S ribosomal DNA (rDNA), and purification of PCR products were carried out by procedures described previously (41). Purified PCR products were sequenced with *Taq* DyeDeoxy terminator cycle sequencing kits (Applied Biosystems, Weiterstadt, Germany) as directed in the manufacturer's protocol. The Applied Biosystems 373A DNA sequencer was used for the electrophoresis of the sequence reaction products. The ae2 editor (36) was used to align the 16S rDNA sequences determined in this study against the 16S rDNA sequences of representatives of the main bacterial lineages available from public databases. Pairwise evolutionary distances were computed by using the correction of Jukes and Cantor (27). Phylogenetic dendrograms were reconstructed from distance matrices by the least squares distance method of De Soete (16).

Nucleotide sequence accession numbers. The accession numbers of the reference strains used in the phylogenetic analyses are as follows: *Acetonema longum*, M61919; *Acidaminococcus fermentans*, X78017; *Clostridium quercicolum*, M59110; *Desulfomicrobium baculatus*, M37311; *Desulfomonas pigra*, M34404; *Desulfovibrio acrylicus*, U32578; *Desulfovibrio africanus*, X99236; *Desulfovibrio desulfuricans* subsp. *desulfuricans*, M34113; *Desulfovibrio gabonensis*, U31080; *Desulfovibrio gigas*, M34400; *Desulfovibrio halophilus*, U48243; *Desulfovibrio longus*, X63623; *Desulfovibrio longreachensis*, Z24450; *Desulfovibrio profundis*, U90726; *Desulfovibrio salexigens*, M34401; *Desulfovibrio termitidis*, X87409; *Desulfovibrio* "*vietnamensis*," X93994; *Desulfovibrio vulgaris* subsp. *vulgaris*, M34399; *Pectinatus cerevisiiphilus*, ARB_2608; *Phascolarctobacterium faecium*, X72865; *Quinella ovalis*, M62701; *Selenomonas lacticifex*, ARB_533F; *Selenomonas ruminantium* subsp. *ruminantium*, ARB_A646; *Sporomusa paucivorans*, M59117; *Sporomusa silvacetica*, Y09976; *Sporomusa termitida*, M61920; *Zymophilus paucivorans*, ARB_B5A2.

The 16S rDNA sequences determined in this study have been deposited in the EMBL database under the following accession numbers: strain DR1, Y17758; strain DR5, Y17761; strain DR6, Y17760; strain DR10, Y17757; strain DR14, Y17756; strain DR15, Y17762; strain DR1/8, Y17763; strain VeLac3, Y17755; and *D. sulfodismutans*, Y17764.

RESULTS

Counts by the MPN method. Serial liquid dilutions were made of anoxic soil from various flooded microcosms in which rice had been grown for 90 to 95 days. The culturable-cell numbers varied, depending on the growth substrate used (Ta-

TABLE 2. MPN counts of bacteria in anoxic soil of two flooded rice paddy soil microcosms[a]

Substrate	Addition	Cell count (per g of dry soil)	95% Confidence interval	Strain isolated in pure culture
Lactate	None	7.5×10^6	1.3×10^6–3.7×10^7	
	M. hungatei	7.5×10^8	1.3×10^8–3.7×10^9	*Sporomusa* sp. strain DR1/8
Lactate	None	5.0×10^6	9.8×10^5–2.1×10^7	
	M. hungatei	1.1×10^7	2.1×10^6–4.0×10^7	
Ethanol	None	2.9×10^6	5.6×10^5–1.1×10^7	
	M. hungatei	2.3×10^6	5.0×10^5–7.8×10^6	*Sporomusa* sp. strain DR15
Ethanol	None	3.4×10^7	1.1×10^7–8.2×10^7	
	M. hungatei	1.3×10^8	3.0×10^7–3.6×10^8	

[a] One soil sample from each microcosm was used for two dilution series on each substrate, one of which contained *M. hungatei* in all tubes.

ble 1), with counts of between about 10^4 and 10^7 cells per g of dry soil. The cell counts obtained with lactate, ethanol, and succinate were not significantly different from each other ($P < 0.05$), nor were the counts obtained with betaine, ethylene glycol, 2,3-butanediol, and 3,4,5-trimethoxybenzoate significantly different from each other. There were, however, significant differences ($P < 0.05$) between the cell counts obtained with lactate, ethanol, and succinate and those obtained with the other substrates used (with the exception that there was no significant difference between the counts on succinate and betaine). To test the variability of the counts with soil from different microcosms, the experiments were repeated with selected substrates (noted in Table 1), but the numbers of culturable bacteria recovered with any one growth substrate did not vary significantly ($P < 0.05$). MPN counts incubated at 15°C (noted in Table 1) were not significantly different ($P < 0.05$) from those obtained at 25°C.

To simulate the low-hydrogen partial pressures occurring in the flooded rice paddy soil (29), counts were made in which all tubes were additionally inoculated with a well-grown culture of *M. hungatei*. This had no significant effect on the cell numbers able to be recovered ($P < 0.05$) except in one experiment where the number of lactate-utilizing bacteria able to be cultivated was 100 times higher than when the methanogen was not added (Table 2). In an independent replicate experiment with soil from a different microcosm, the addition of *M. hungatei* had little effect on the recovery of lactate-utilizing bacteria (Table 2).

Counts of hydrogenotrophic bacteria. The MPN of culturable hydrogenotrophic microorganisms (in the absence of sulfate) was 2.7×10^7 per g of dry soil (95% confidence interval, 5.3×10^6 to 1.0×10^8) when the incubations were carried out at 25°C. The terminal positive tubes of these dilution series contained autofluorescing rod-shaped bacteria, and the culture headspaces contained large amounts (>10% [vol/vol]) of methane. Control tubes containing no added hydrogen produced no methane. Incubation of the counting experiments at 15°C resulted in much lower counts ($P < 0.05$) of methanogenic organisms: 1.3×10^3 cells per g of dry soil (95% confidence interval, 2.5×10^2 to 5.3×10^3), based on the production of methane in the tubes. Based on the appearance of acetate in the tubes incubated at 15°C, a culturable population of 7.1×10^3 hydrogenotrophic homoacetogens per g of dry soil was estimated (95% confidence interval, 1.2×10^3 to 3.4×10^4). MPN counting of hydrogenotrophic sulfate-reducing bacteria revealed a culturable population size of 3.3×10^4 cells per g of dry soil (95% confidence interval, 5.5×10^3 to 1.6×10^5).

Determination of spore numbers. Soil slurries used to inoculate the MPN dilution series were pasteurized to kill cells not present as heat-resistant spores. Dilutions were first made from the soil slurry to determine the total population size, and then the same slurries were incubated at 80°C for 15 min. After this step, the slurries were used to inoculate another series of MPN dilutions. On all four substrates used, the cell counts with the pasteurized soil slurries were significantly lower ($P < 0.05$) than with the untreated slurries. We define the MPN after pasteurization as the number of spores able to initiate growth in our media, and thus estimate the proportion of spores of those culturable bacteria enumerated by comparison to counting experiments without pasteurization. Dormant heat-resistant spores were estimated to represent 2.2% or less of the populations of organisms able to be cultivated on these substrates (Table 3).

Isolation of representative strains. We isolated pure cultures from the terminal positive tubes of the liquid dilution series. Based on the theory of liquid dilution series, the organisms growing in the tubes receiving the most-diluted inoculum should represent numerically significant members of the original community able to grow with that substrate under the conditions used. Ten strains were isolated in pure culture, each from a different MPN series.

Comparative 16S rRNA gene sequence analysis showed that strain DR7 was closely related to members of the genus *Desulfobacterium* (data not shown). Growth was always slow and

TABLE 3. MPN counts of bacteria in anoxic soil of flooded rice paddy soil microcosms[a]

Substrate	Treatment	Cell count (per g of dry soil)	95% Confidence interval	Proportion of spores[b]
Lactate	None	5.0×10^6	9.8×10^5–2.1×10^7	2.2
	Pasteurized	1.1×10^5	2.1×10^4–4.0×10^5	
Ethanol	None	3.4×10^7	1.1×10^7–8.2×10^7	0.03
	Pasteurized	1.1×10^4	2.1×10^3–4.0×10^4	
3,4,5-TMB[c]	None	2.8×10^4	6.4×10^3–8.5×10^4	0.29
	Pasteurized	8.1×10^1	1.6×10^1–3.4×10^2	
Betaine	None	2.8×10^5	6.4×10^4–8.5×10^5	0.64
	Pasteurized	1.8×10^3	3.4×10^2–6.4×10^3	

[a] Soil samples were first diluted as normal (see the text), and then the same sample was heated at 80°C for 15 min and used to carry out a second (pasteurized) dilution.

[b] The ratio of cells and spores culturable after pasteurization to those culturable prior to pasteurization, expressed as a percentage.

[c] 3,4,5-TMB, 3,4,5-trimethoxybenzoate.

TABLE 4. End products of lactate or ethanol metabolism by *Desulfovibrio* sp. strain DR1 and *Sporomusa* sp. strain DR1/8 with the addition of sulfate, co-inoculation of *M. hungatei* JF1, or without addition[a]

Strain	Organic substrate (and electron acceptor)	Amt of organic substrate used (μmol)	Amt of product (μmol)			
			Acetate	Hydrogen	Sulfide	Methane
DR1	Lactate (+ sulfate)	500	488	–[b]	260	–
	Ethanol (+ sulfate)	500	454	–	250	–
DR1	Lactate	48	44	110	–	–
	Ethanol	68	72	120	–	–
DR1 + JF1	Lactate	500	426	–	–	226
	Ethanol	500	451	–	–	245
DR1/8	Lactate	500	664	–	–	–
	Ethanol	500	682	–	–	–
DR1/8 + JF1	Lactate	500	594	–	–	169
	Ethanol	500	453	–	–	214

[a] Experiments were carried out in 50-ml aliquots of FM medium in 120-ml serum bottles incubated at 25°C. Na_2SO_4 was added as noted at 1,000 μmol per 50 ml. Biomass formation was not measured.
[b] –, not determined.

difficult to reproduce, and the strain was not characterized any further.

The remaining nine strains were characterized phenotypically and phylogenetically.

Characterization of strains of the genus *Desulfovibrio*. Strains DR1, DR10, and DR14 were isolated from MPN dilution series on succinate, lactate, and ethanol, respectively, without the addition of *M. hungatei* (Table 1). These strains were isolated on their respective growth substrates in agar deep cultures. Strain VeLac3 was isolated from the MPN dilution series on hydrogen plus sulfate, using 10 mM lactate plus 20 mM sulfate in agar deep cultures. All four strains showed the same morphology as the dominant cell type in the terminal positive tubes of the MPN series from which they were isolated.

Strains DR1, DR10, DR14, and VeLac3 were "vibrio"-shaped, desulfoviridin-containing, non-spore-forming, gram-negative bacteria, with genomic DNA with mol% G+C contents of between 58 and 66. All were motile. The cells were 2 to 8 μm long, depending on the strain, and about 1 μm in diameter. All four strains were able to grow with sulfate as a terminal electron acceptor, with hydrogen, formate, lactate, and ethanol as electron donors but not with acetate or propionate. Acetate was produced from lactate, and sulfate was reduced to sulfide. Strains DR1, DR10, and DR14 were able to ferment, albeit weakly, ethanol and lactate, while strain VeLac3 grew well with lactate without an added electron acceptor but not with ethanol. Strains DR1 and DR14 grew slowly but reproducibly on succinate, producing acetate. We did not investigate this any further. All four strains were unable to grow with hydrogen plus sulfate in the absence of added acetate, although a number of passages in acetate-free medium were required to dilute out acetate introduced with the inoculum. In addition to sulfate, nitrate and elemental sulfur could be used as terminal electron acceptors.

All four strains grew rapidly with lactate or ethanol in coculture with *M. hungatei* in the absence of sulfate. This was investigated in more detail with strain DR1. The balances of substrate and product show that the electron equivalents from the fermentation of lactate and ethanol were recovered as methane. In the absence of both sulfate and *M. hungatei*, strain DR1 fermented lactate and ethanol only poorly, and hydrogen was produced (Table 4).

16S rDNA sequences comprising 1,477 nucleotides were determined for strains DR1, DR10, DR14, and VeLac3, and a 1,479-nucleotide sequence was determined for *D. sulfodismutans* ThAc01. The phylogenetic positions of these strains, based on 16S rDNA sequence comparisons, are shown in Fig. 1. The new strains clearly fall within the radiation of the genus *Desulfovibrio* and cluster with the species *D. sulfodismutans*, to which they have 16S rDNA sequence similarities in the range of 92.9 to 93.3%. The four isolates DR1, DR10, DR14, and VeLac3 have 16S rDNA sequence similarities to each other in the range 98.5 to 99.7%, indicating that they are closely related strains that may represent one or more new species of the genus *Desulfovibrio*.

Characterization of strains of the genus *Sporomusa*. Strains DR1/8 and DR15 were isolated from MPN dilution series on lactate and ethanol, respectively, to which *M. hungatei* had been added (Table 2). Strains DR5, DR6, and DR16 were isolated from MPN dilution series on 3,4,5-trimethoxybenzoate, ethylene glycol, and 2,3-butanediol, respectively (Table 1). All five strains were isolated by using their respective growth substrates in agar deep cultures, without the addition of *M. hungatei*.

Strains DR1/8, DR5, DR6, DR15, and DR16 were all motile curved rods which formed spores. Gram staining was difficult to control, but usually a gram-positive reaction was obtained. The mol% G+C ratios of the genomic DNA were 40.8 to 42.2. The strains varied in cell shape and size, the proportion of spore-containing cells in cultures grown under the same conditions, and the shapes and positions of the spores (Fig. 2). All five strains could grow with hydrogen plus carbon dioxide, ethanol, 2,3-butanediol, betaine, and glycerol, and they varied in their abilities to grow with formate, lactate, methanol, ethylene glycol, 3,4,5-trimethoxybenzoate, succinate, glucose, and fructose (Table 5). Gallate and acetate were formed from 3,4,5-trimethoxybenzoate. Products other than acetate were not determined for betaine. With all other growth substrates, acetate was the sole organic end product. High activities of carbon monoxide dehydrogenase and formate dehydrogenase, key enzymes of the Wood-Ljungdahl acetyl-coenzyme A path-

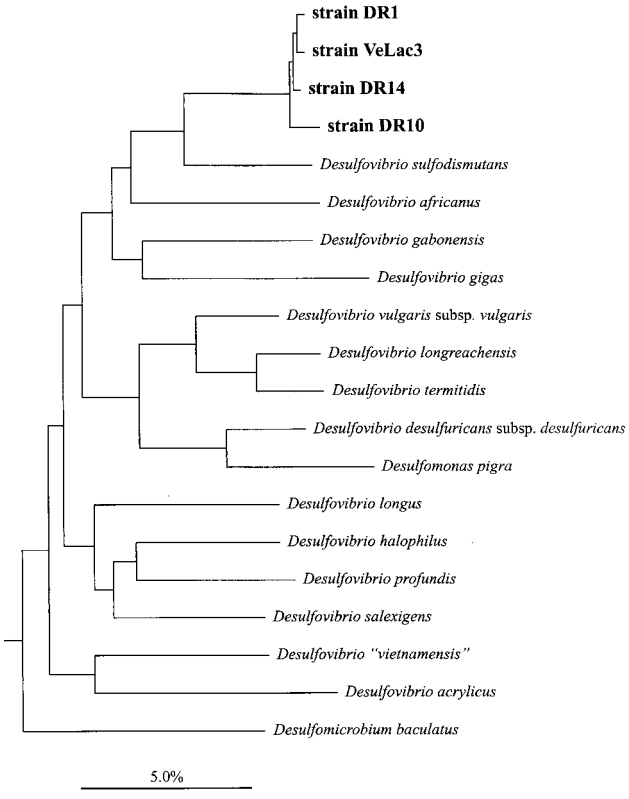

FIG. 1. 16S rDNA-based phylogenetic dendrogram showing the positions of strains DR1, DR10, DR14, and VeLac3 within the radiation of the genus *Desulfovibrio* and related taxa. The scale bar represents 5 inferred nucleotide changes per 100 nucleotides analyzed.

way (52), were detected in pyruvate-grown cells of strains DR1/8 (16.2 and 28.5 μmol · min^{-1} · mg of protein^{-1}, respectively) and DR5 (39.1 and 17.6 μmol · min^{-1} · mg of protein^{-1}, respectively). The balance of substrates and products for strain DR1/8 growing with ethanol and with lactate shows that about 1.5 mol of acetate were formed per mol of substrate (Table 4).

All five strains could grow syntrophically with *M. hungatei*, in which case less acetate was formed and methane was produced. This was investigated in more detail with strain DR1/8. The balance of substrate turnover and product (Table 4) shows that the syntrophic coupling was incomplete and suggests that hydrogen evolution (recovered as methane) and acetate formation by the Wood-Ljungdahl acetyl-coenzyme A pathway occurred simultaneously.

16S rDNA sequences ranging in length from 1,429 to 1,504 nucleotides were determined for strains DR5, DR6, DR15, DR16, and DR1/8. Comparative analyses of these sequences with those available from the public databases showed these strains to be most closely related to members of the genus *Sporomusa* within the low-G+C lineage of the gram-positive bacteria (Fig. 3). The 16S rDNA sequence similarity values between the sequences of the strains DR5, DR6, DR15, and DR1/8 are in the range of 97.2 to 99.8%. Strains DR15 and DR16 have 16S rDNA sequences which are identical over the 1,450 nucleotides determined. Strains DR6 and DR15 are very closely related, showing 99.8% 16S rDNA sequence similarity and between 94.4 and 97.7% similarity to the other *Sporomusa* species, indicating that, together with strain DR16, they could represent a novel species. Strain DR5 has 99.1% 16S rDNA sequence similarity with *S. silvacetica*, a homoacetogen isolated from a forest soil (31). Strain DR1/8 represents a distinct lineage within the *Sporomusa* group, with 95.7 to 97.6% 16S rDNA sequence similarity to the described species and 97.2 to 97.6% similarity to the other *Sporomusa* strains isolated in this study.

DISCUSSION

Viable cell counts. We used the MPN viable count technique to estimate the population sizes of homoacetogenic bacteria in the anoxic bulk soil of microcosms containing 90- to 95-day-old rice plants. This technique is associated with a number of difficulties (33), including the ability to detect only those organisms able to grow on the growth media used. The theory of dilution culture predicts that the organisms growing in the terminal positive tubes of such extinction dilution series were present in higher numbers in the original sample than might be the case for organisms recovered from lower dilutions.

We used a number of approaches to try to maximize the viable counts of homoacetogenic bacteria. We carried out MPN counts with organic growth substrates which support the growth of or favor the enrichment of homoacetogenic bacteria. We attempted to modify the hydrogen partial pressure in the culture medium so that it remained at levels similar to those found in rice paddy soil throughout the incubation period.

Betaine, ethylene glycol, 2,3-butanediol, and 3,4,5-trimethoxybenzoate all gave counts of $\leq 2.8 \times 10^5$ cells per g of dry soil. Except with betaine, the use of these substrates led us to isolate *Sporomusa* spp. (strains DR5, DR6, and DR16) from the terminal positive tubes of these dilution series. These substrates, which are reportedly useful for cultivating and enriching homoacetogens (17), thus favored the growth of homoacetogens, as expected. It appears that homoacetogenic bacteria play a significant role in the carbon and electron flow in rice paddy soil (4, 10, 30, 45). The counts obtained with 2,3-butanediol, ethylene glycol, and 3,4,5-trimethoxybenzoate appear to be much lower than we expected, being maximally some 0.06% of the total microbial cell count (6) in this paddy soil system. These substrates, therefore, did not appear to be useful for assessing the population size of homoacetogens when compared with other results (see below).

Counts with succinate, ethanol, and lactate gave much higher MPNs of 5.9×10^5 to 3.4×10^7 cells per g of dry soil. Counts in soils from different microcosms were not significantly different, suggesting that there was little variation between microcosms. From the terminal positive tubes of these dilution series, we isolated *Desulfovibrio* spp. (strains DR1, DR10, and DR14) rather than homoacetogens. Based on their phenotypic characteristics and the results of comparative 16S rDNA sequence comparisons, strains DR1, DR10, DR14, and VeLac3 are typical members of the genus *Desulfovibrio*. Sulfate-reducing bacteria belonging to the genus *Desulfovibrio* have previously been isolated from the same rice paddy soil from Italy (15, 50).

Counting experiments on lactate and ethanol which included *M. hungatei* in the medium gave counts of 2.3×10^6 to 7.5×10^8 cells per g of dry soil. From the terminal positive tubes of these dilution series, we isolated *Sporomusa* spp. (strains DR1/8 and DR15). The one very high count on lactate in the presence of *M. hungatei* may be the result of the heterogeneity in the soil, although we attempted to produce homogeneous suspensions to inoculate our counting experiments. The general reproducibility of our MPN estimates shows we were mostly successful. Why the addition of *M. hungatei* to counting experiments resulted in the isolation of *Sporomusa* spp. when

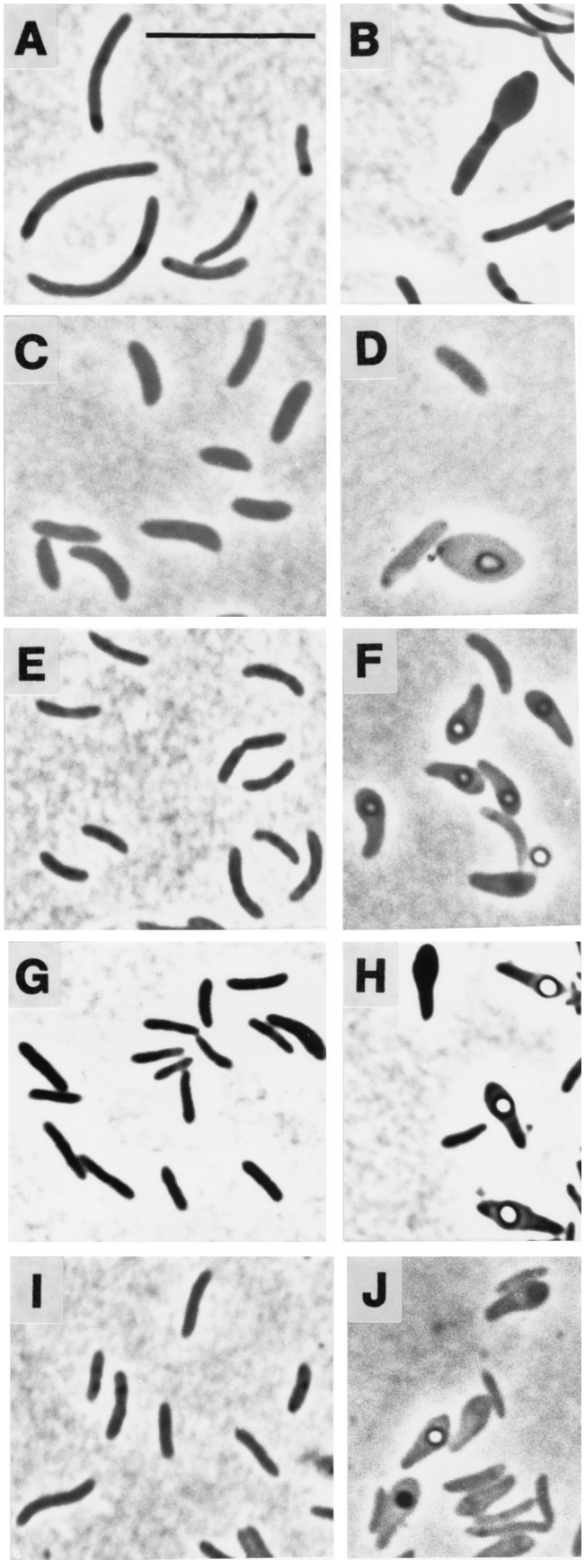

FIG. 2. Phase-contrast photomicrographs of cells of strains of *Sporomusa* spp. Vegetative cells (A) and a sporulating cell (B) of strain DR1/8, vegetative cells (C) and a sporulating cell (D) of strain DR5, vegetative cells (E) and sporulating cells (F) of strain DR6, vegetative cells (G) and sporulating cells (H) of strain DR15, and vegetative cells (I) and a sporulating cell (J) of strain DR16 are shown. Bar = 10 μm (all panels).

the same substrates without the addition of the methanogen led to the isolation of *Desulfovibrio* spp. is not known. The addition of large inocula of *M. hungatei* to cultures of hydrogen-producing bacteria successfully maintains the hydrogen partial pressure at levels similar to those found in rice paddy soil (5). Of course, cultures which were not coinoculated with *M. hungatei* also received methanogens via the soil. The same rice paddy soil contained up to 2.7×10^7 hydrogenotrophic methanogens per g of dry soil, in agreement with earlier findings (21). However, the numbers added to each tube of the MPN dilution series, relative to the number of cells of *Sporomusa* spp., was apparently not high enough to modify the growth conditions to allow the homoacetogens to dominate the cultures on lactate and ethanol in the absence of added *M. hungatei*. Interestingly, the strains of *Sporomusa* spp. isolated from the experiments containing *M. hungatei* could grow with betaine, ethylene glycol, 2,3-butanediol, and/or 3,4,5-trimethoxybenzoate (Table 5), but apparently most cells of *Sporomusa* spp. did not initiate growth in counting experiments with these substrates.

The use of hydrogen with sulfate gave counts of only 3.3×10^4 hydrogenotrophic sulfate-reducing bacteria per g of dry soil, even though the estimate of *Desulfovibrio* spp. counted with other growth substrates, and subsequently shown to be able to grow with hydrogen, was significantly ($P < 0.05$) greater. Similarly, incubation of MPN counting experiments at 15°C with hydrogen in the absence of sulfate did not allow the cultivation of high numbers of hydrogenotrophic homoacetogens, although under these conditions homoacetogens have been shown to outcompete methanogens (10). As with our other attempts to count homoacetogens with organic growth substrates, our present knowledge of the metabolic capabilities of these organisms did not permit us to predict which conditions are best for initiation of growth in microbiological media.

We conclude that *Desulfovibrio* spp. and *Sporomusa* spp. were present in culturable population sizes, each of about 3×10^6 to 3×10^7 cells per g of dry soil. The total microbial community size of this system was estimated at about 5×10^8 4′,6-diamidino-2-phenylindole-stainable cells per g of dry soil (6).

Spore numbers. Homoacetogenic bacteria belonging to the genera *Sporomusa*, *Clostridium*, and *Acetonema* are able to form heat-resistant spores (43). Spores will be counted as active cells, after they germinate, in the viable cell counts. We carried out cell counts on soil samples before and after pasteurization. On four different substrates, the active proportion of the total population was estimated to be 97.8% or greater. This meant that either spores did not germinate under the cultivation conditions used, and therefore were not counted, or only very low numbers of spores were present in the soil. In either case, the population sizes counted in our experiments represent mainly active cells rather than a significant number of spores.

Competition. The nine strains isolated and characterized in more detail belong to two genera able to grow on a range of common substrates. Those substrates which may be significant in the anoxic soil habitat include hydrogen, formate, ethanol, and lactate. This suggests that there may be competition for

TABLE 5. Substrates supporting growth of strains of *Sporomusa* spp.

Substrate	Growth[a]				
	DR1/8	DR5	DR6	DR15	DR16
$H_2 + CO_2$	+	+	+	+	+
Lactate	+	−	+	−	−
Ethanol	+	+	+	+	+
Succinate	−	−	−	−	−
Betaine	+	+	+	+	+
Ethylene glycol	−	+	+	+	+
2,3-Butanediol	+	+	+	+	+
3,4,5-TMB[b]	+	+	−	−	−

[a] +, growth; −, no growth.
[b] 3,4,5-TMB, 3,4,5-trimethoxybenzoate.

growth substrates within and between members of these genera.

At the typical hydrogen concentrations in anoxic rice paddy soil, about 7 to 12 Pa (29), only sulfate-reducing bacteria or methanogenic archaea can be expected to be able to utilize hydrogen, since they have threshold concentrations for hydrogen utilization (12) that are lower than this. *Sporomusa* spp., with threshold concentrations for hydrogen utilization of about 40 to 80 Pa (12), would not be expected to utilize hydrogen at these concentrations. At low sulfate concentrations (<30 μM), sulfate-reducing bacteria cannot compete with methanogens for hydrogen (35). Sulfate is present at concentrations of about 10 μM in the anoxic bulk soil of rice microcosms (51). Thus, neither *Desulfovibrio* spp. nor *Sporomusa* spp. could be expected to utilize hydrogen.

Both *Desulfovibrio* spp. and *Sporomusa* spp. can grow as syntrophs on lactate, ethanol, and other substrates in conjunction with hydrogenotrophic methanogens (3, 22, 23). From thermodynamic considerations, a methanogenic coculture growing on lactate or ethanol should have lower threshold concentrations for organic substrates than a fermenting bacterium alone. This may explain how *Desulfovibrio* spp. and *Sporomusa* spp. can exist in the anoxic bulk soil. Both groups also have alternative strategies for growth, depending on local conditions: either sulfate reduction or homoacetogenesis.

Comparative analysis of 16S rDNA and morphological characteristics suggested that the five strains of *Sporomusa* spp. probably represent three different species. The five strains varied in cell size and shape, shape and position of the spores, and the range of substrates used. The phenotypic differences suggest slight differences which may allow them to occupy different niches, although the differences found in this study may not be those that are significant in nature. Similarly, the four *Desulfovibrio* spp. isolated (three originated from one sample) differ slightly from one another and may occupy slightly different niches within the soil habitat. In a homogeneous system with a single limiting factor, one strain would be expected to outcompete the others. Soil is, however, highly heterogeneous. This high heterogeneity possibly allows a wider range of apparently similar organisms to coexist within one ecosystem (46).

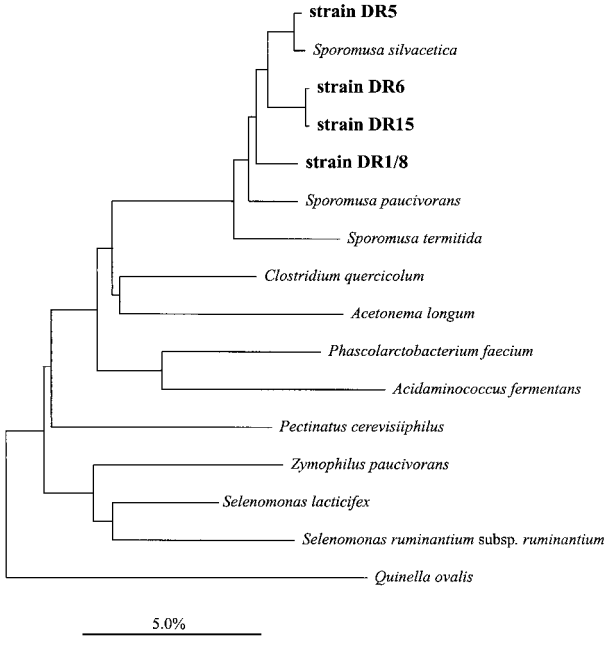

FIG. 3. 16S rDNA-based phylogenetic dendrogram showing the positions of strains DR1/8, DR5, DR6, and DR15 within the radiation of the genus *Sporomusa* and related taxa. The scale bar represents 5 inferred nucleotide changes per 100 nucleotides analyzed.

ACKNOWLEDGMENT

We thank Oliver Kappler for carrying out the analyses of gallate and 3,4,5-trimethoxybenzoate.

REFERENCES

1. **Beliaeff, B., and J. Y. Mary.** 1993. The "most probable number" estimate and its confidence limits. Water Res. **27:**799–805.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Bryant, M. P., L. L. Campbell, C. A. Reddy, and M. R. Crabill.** 1977. Growth of *Desulfovibrio* in lactate or ethanol media low in sulfate in association with H_2-utilizing methanogenic bacteria. Appl. Environ. Microbiol. **33:**1162–1169.
4. **Chin, K.-J., and R. Conrad.** 1995. Intermediary metabolism in methanogenic paddy soil and the influence of temperature. FEMS Microbiol. Ecol. **18:**85–102.
5. **Chin, K.-J., and P. H. Janssen.** Unpublished data.
6. **Chin, K.-J., D. Hahn, U. Hengstmann, W. Liesack, and P. H. Janssen.** Unpublished data.
7. **Cline, J. D.** 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. **14:**454–458.
8. **Cochran, W. G.** 1950. Estimation of bacterial densities by means of the "most probable number." Biometrics **6:**105–116.
9. **Conrad, R.** 1993. Mechanisms controlling methane emission from wetland rice fields, p. 317–355. *In* R. S. Oremland (ed.), Biogeochemistry of global change: radiatively active trace gases. Chapman and Hall, New York, N.Y.
10. **Conrad, R., F. Bak, H. J. Seitz, B. Thebrath, H. P. Mayer, and H. Schütz.** 1989. Hydrogen turnover by psychrotrophic homoacetogenic and mesophilic methanogenic bacteria in anoxic paddy soil and lake sediment. FEMS Microbiol. Ecol. **62:**285–294.
11. **Cord-Ruwisch, R.** 1985. A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate reducing bacteria. J. Microbiol. Methods **4:**33–36.
12. **Cord-Ruwisch, R., H.-J. Seitz, and R. Conrad.** 1988. The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. Arch. Microbiol. **149:**350–357.
13. **Cote, R. J., and R. L. Gherna.** 1994. Nutrition and media, p. 155–178. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular microbiology. American Society for Microbiology, Washington, D.C.
14. **Crutzen, P. J.** 1995. On the role of CH_4 in atmospheric chemistry: sources, sinks and possible reductions in anthropogenic sources. Ambio **24:**52–55.
15. **Dalsgaard, T., and F. Bak.** 1994. Nitrate reduction in a sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, isolated from rice paddy soil: sulfide inhibition, kinetics, and regulation. Appl. Environ. Microbiol. **60:**291–297.
16. **De Soete, G.** 1983. A least squares algorithm for fitting additive trees to proximity data. Psychometrica **48:**621–626.
17. **Diekert, G.** 1992. The acetogenic bacteria, p. 517–533. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer, Berlin, Germany.

18. **Frenzel, P., and U. Bosse.** 1996. Methyl fluoride, an inhibitor of methane oxidation and methane production. FEMS Microbiol. Ecol. **21:**25–36.
19. **Frenzel, P., U. Bosse, and P. H. Janssen.** 1998. Rice roots and methanogenesis in a paddy soil: ferric iron as an alternative electron acceptor in the rooted soil. Soil Biol. Biochem. **31:**421–430.
20. **Frenzel, P., F. Rothfuss, and R. Conrad.** 1992. Oxygen profiles and methane turnover in a flooded rice microcosm. Biol. Fertil. Soils **14:**84–89.
21. **Großkopf, R., P. H. Janssen, and W. Liesack.** 1998. Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl. Environ. Microbiol. **64:**960–969.
22. **Heijthuisen, J. H. F. G., and T. A. Hansen.** 1986. Interspecies hydrogen transfer in co-cultures of methanol-utilizing acidogens and sulfate-reducing or methanogenic bacteria. FEMS Microbiol. Ecol. **38:**57–64.
23. **Hermann, M., M.-R. Popoff, and M. Sebald.** 1987. *Sporomusa paucivorans* sp. nov., a methylotrophic bacterium that forms acetic acid from hydrogen and carbon dioxide. Int. J. Syst. Bacteriol. **37:**93–101.
24. **Janssen, P. H., and B. Schink.** 1995. Catabolic and anabolic enzyme activities and energetics of acetone metabolism of the sulfate-reducing bacterium *Desulfococcus biacutus*. J. Bacteriol. **177:**277–282.
25. **Janssen, P. H., W. Liesack, C. Kluge, S. Seeliger, B. Schink, and C. G. Harfoot.** 1996. Sodium-dependent succinate decarboxylation by a new anaerobic bacterium belonging to the genus *Peptostreptococcus*. Antonie Leeuwenhoek **70:**11–20.
26. **Janssen, P. H., A. Schuhmann, E. Mörschel, and F. A. Rainey.** 1997. Novel anaerobic ultramicrobacteria belonging to the *Verrucomicrobiales* lineage of bacterial descent isolated by dilution culture from anoxic rice paddy soil. Appl. Environ. Microbiol. **63:**1382–1388.
27. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, New York, N.Y.
28. **Kappler, O., P. H. Janssen, J.-U. Kreft, and B. Schink.** 1997. Effects of alternative methyl group acceptors on the growth energetics of the *O*-demethylating anaerobe *Holophaga foetida*. Microbiology **143:**1105–1114.
29. **Krämer, H., and R. Conrad.** 1993. Measurement of dissolved H_2 concentrations in methanogenic environments with a gas diffusion probe. FEMS Microbiol. Ecol. **12:**149–158.
30. **Krumböck, M., and R. Conrad.** 1991. Metabolism of position-labeled glucose in anoxic methanogenic paddy soil and lake sediment. FEMS Microbiol. Ecol. **85:**247–256.
31. **Kuhner, C. H., C. Frank, A. Greißhammer, M. Schmittroth, G. Acker, A. Gößner, and H. L. Drake.** 1997. *Sporomusa silvacetica* sp. nov., an acetogenic bacterium isolated from aggregated forest soil. Int. J. Syst. Bacteriol. **47:**352–358.
32. **Küsel, K., and H. L. Drake.** 1995. Effects of environmental parameters on the formation and turnover of acetate by forest soils. Appl. Environ. Microbiol. **61:**3667–3675.
33. **Liesack, W., P. H. Janssen, F. A. Rainey, N. L. Ward-Rainey, and E. Stackebrandt.** 1997. Microbial diversity in soil: the need for a combined approach using molecular and cultivation techniques, p. 375–439. *In* J. D. van Elsas, J. T. Trevors, and E. M. H. Wellington (ed.), Modern soil microbiology. Marcell Dekker, New York, N.Y.
34. **Lovell, C. R., and Y. Hui.** 1991. Design and testing of a functional group-specific DNA probe for the study of natural populations of acetogenic bacteria. Appl. Environ. Microbiol. **57:**2602–2609.
35. **Lovley, D. R., and M. J. Klug.** 1986. Model for the distribution of sulfate reduction and methanogenesis in freshwater sediments. Geochim. Cosmochim. Acta **50:**11–18.
36. **Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese.** 1994. The Ribosomal Database Project. Nucleic Acids Res. **22:**3485–3487.
37. **Minami, K., and H.-U. Neue.** 1994. Rice paddies as a methane source. Clim. Change **27:**13–26.
38. **Pfennig, N.** 1978. *Rhodocyclus purpureus* gen. nov., sp. nov., a ring-shaped, vitamin B_{12}-requiring member of the family *Rhodospirillaceae*. Int. J. Syst. Bacteriol. **28:**283–288.
39. **Pfennig, N., and S. Wagener.** 1986. An improved method for preparing wet mounts for photomicrographs of microorganisms. J. Microbiol. Methods **4:**303–306.
40. **Postgate, J. R.** 1959. A diagnostic reaction of *Desulphovibrio desulphuricans*. Nature **183:**481–482.
41. **Rainey, F. A., N. Ward-Rainey, R. M. Kroppenstedt, and E. Stackebrandt.** 1996. The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam. nov. Int. J. Syst. Bacteriol. **46:**1088–1092.
42. **Rothfuss, F., and R. Conrad.** 1993. Vertical profiles of CH_4 concentrations, dissolved substrates and processes involved in CH_4 production in a flooded Italian rice field. Biogeochemistry **18:**137–152.
43. **Schink, B.** 1994. Diversity, ecology, and isolation of acetogenic bacteria, p. 197–235. *In* H. L. Drake (ed.), Acetogenesis. Chapman and Hall, New York, N.Y.
44. **Süßmuth, R., J. Eberspächer, R. Haag, and W. Springer.** 1987. Biochemisch-mikrobiologishes Praktikum. Thieme, Stuttgart, Germany.
45. **Thebrath, B., H. P. Mayer, and R. Conrad.** 1992. Bicarbonate dependent production and methanogenic consumption of acetate in anoxic paddy soil. FEMS Microbiol. Ecol. **86:**295–302.
46. **Tilman, D.** 1994. Competition and biodiversity in spatially structured habitats. Ecology **75:**2–16.
47. **Wagner, C., A. Greißhammer, and H. L. Drake.** 1996. Acetogenic capacities and the anaerobic turnover of carbon in a Kansas prairie soil. Appl. Environ. Microbiol. **62:**494–500.
48. **Widdel, F., and F. Bak.** 1992. Gram-negative mesophilic sulfate-reducing bacteria, p. 3352–3378. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer, Berlin, Germany.
49. **Willems, A., and M. D. Collins.** 1996. Phylogenetic relationships of the genera *Acetobacterium* and *Eubacterium* sensu stricto and reclassification of *Eubacterium alactolyticum* as *Pseudoramibacter alactolyticus* gen. nov., comb. nov. Int. J. Syst. Bacteriol. **46:**1083–1087.
50. **Wind, T., and R. Conrad.** 1995. Sulfur compounds, potential turnover of sulfate and thiosulfate, and numbers of sulfate-reducing bacteria in planted and unplanted paddy soil. FEMS Microbiol. Ecol. **18:**257–266.
51. **Wind, T., and R. Conrad.** 1997. Localization of sulfate reduction in planted and unplanted rice field soil. Biogeochemistry **37:**253–278.
52. **Wood, H. G., and L. G. Ljungdahl.** 1991. Autotrophic character of the acetogenic bacteria, p. 201–250. *In* J. M. Shively and L. L. Barton (ed.), Variations in autotrophic life. Academic Press, New York, N.Y.