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Isolation and Characterization of Acetate-Utilizing Anaerobes from a Freshwater Sediment

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ABSTRACT

Acetate-degrading anaerobic microorganisms in freshwater sediment were quantified by the most probable number technique. From the highest dilutions a methanogenic, a sulfate-reducing, and a nitrate-reducing microorganism were isolated with acetate as substrate. The methanogen (culture AMPB-Zg) was non-motile and rod-shaped with blunted ends (0.5–1 μm × 3–4 μm long). Doubling times with acetate at 30–35°C were 5.6–8.1 days. The methanogen grew only on acetate. Analysis of the 16S rRNA sequence showed that AMPB-Zg is closely related to *Methanosaeta concilii.* The isolated sulfate-reducing bacterium (strain ASRB-Zg) was rod-shaped with pointed ends (0.5–0.7 μm × 1.5–3.5 μm long), weakly motile, spore forming, and gram positive. At the optimum growth temperature of 30°C the doubling times with acetate were 3.9–5.3 days. The bacterium grew on a range of organic acids, such as acetate, butyrate, fumarate, and benzoate, but did not grow autotrophically with H_2, CO_2, and sulfate. The closest relative of strain ASRB-Zg is *Desulfotomaculum acetoxidans.* The nitrate-reducing bacterium (strain ANRB-Zg) was rod-shaped (0.5–0.7 μm × 0.7–1 μm long), weakly motile, and gram negative. Optimum growth with acetate occurred at 20–25°C. The bacterium grew on a range of organic substrates, such as acetate, butyrate, lactate, and glucose, and did grow autotrophically with H_2, CO_2, and oxygen but not with nitrate. In the presence of acetate and nitrate, thiosulfate was oxidized to sulfate. Phylogenetically, the closest relative of strain ANRB-Zg is *Variovorax paradoxus.*

Introduction

Acetate is the most important intermediate in the degradation of organic matter in anaerobic freshwater environments [12, 20, 25, 26]. Many anaerobic microorganisms capable of growth on acetate as energy source have been described. Methanogens that can grow on acetate are *Methanosarcina* and *Methanosaeta* (formerly *Methanothrix*), and both types can be isolated from different freshwater environments. *Methanosarcina* species are generalists; whereas *Methanosaeta* species are specialists [8]. Different genera of acetate-utilizing sulfate-reducing bacteria are isolated from freshwater environments [34, 36]. *Desulfobacca acetoxidans,* isolated

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from an UASB reactor, can only grow on acetate [16]. Other sulfate reducers such as *Desulforhabdus amnigenus, Desulfobacterium,* and *Desulfotomaculum* species are generalists which use besides acetate a wide variety of substrates such as propionate, hydrogen, and ethanol [3, 14, 35]. Furthermore, some sulfate reducers, e.g., *Desulfovibrio baarsii, Desulfosarcina variablis, Desulfococcus,* and several *Desulfobacterium* species, show very poor growth on acetate, despite the fact that an acetate-degrading pathway is present [34]. They generally prefer other substrates than acetate. Acetate is a common substrate for nitrate-reducing bacteria. The ability to denitrify is widely spread among bacteria and archaea and shows representatives in almost 130 species within more than 50 genera [39]. Most nitrate reducers are regarded as generalists and they are often able to grow with O_2 as an electron acceptor [30]. So far, little is known about the role of nitrate reducers as anaerobic acetate-degraders in natural environments. In most cases the enrichment of nitrate-reducing microorganisms was done with electron donors other than acetate [2, 31, 32]. When tested these microorganisms were found to grow on acetate and nitrate [1, 17, 18].

Recently, we have described studies with freshwater sediment in which labeled acetate was used to examine the influence of sulfate and nitrate on methane production [24]. In the presence of sulfate, addition of acetate stimulated both sulfate reduction and methanogenesis, indicating that both populations were competing for the available acetate. The influence of nitrate on the formation of methane was not clear-cut, as denitrifying bacteria were using other electron donors as well. To understand the impact of inorganic electron acceptors on methanogenesis, we quantified the different groups of bacteria involved in acetate metabolism in freshwater sediment by MPN. In this study we determined the physiologic and phylogenetic characteristics of isolated acetate-utilizing methanogenic, sulfate-reducing, and nitrate-reducing microorganisms obtained from MPN tubes.

Methods

Source of Organisms

An acetate-utilizing methanogen (culture AMPB-Zg), sulfate-reducing bacterium (strain ASRB-Zg), and nitrate-reducing bacterium (strain ANRB-Zg), were isolated from freshwater sediment. Sediment samples were collected September 4, 1995, and April 4, 1996, from ditches next to peat grassland. The grassland area is located near Zegveld in the polder Zegvelderbroek. The polder Zegvelderbroek is a low-lying, deeply drained polder and is located in the major peat area of the western part of the Netherlands (Province of Utrecht: 52°07′N, 4°52′E). In the grassland, which is utilized for agricultural activities, the ditches allow drainage. In the ditches the water level is very shallow with a depth varying from 20 to 40 cm of water. Sediment samples were collected and handled as described previously [25].

Media and Cultivation

A basal bicarbonate buffered medium with a composition as described by Huser et al. was used [7]. To one litre of medium 1 ml of a vitamin solution [37] and 1 ml of an acid and an alkaline trace elements solution was added [27]. The vitamin solution was filter sterilized separately. The gas phase above the medium was 180 kPa N_2/CO_2 (80%/20%) and the pH of the medium was 6.8–6.9. Electron donors and acceptors were added from 1 M sterile, anoxic stock solutions. Except for some heat-labile substrates that were filter sterilized, all substrates were sterilized by heat (20 min, 120°C).

Isolation

All manipulations were done under anaerobic conditions in a glove box. The 0–10 cm layer of the sediment was homogenized, and 15 ml was transferred to a 250-ml serum bottle containing 135 ml of medium. The bottle was closed with a butyl rubber stopper, evacuated, and gassed with N_2/CO_2 (80%/20%). After shaking for 5 min the sediment slurry (15 ml) was serially diluted to the 10^{-10} dilution. A three-tube MPN series was prepared by transferring 5-ml samples to 120-ml serum bottles containing 45 ml of medium. The MPN tests for acetate-utilizing bacteria were performed with 10 mM acetate with or without sulfate or nitrate (10 mM). Incubations were carried out in the dark at 20°C. The highest dilutions that showed growth were used for further isolation. Pure cultures were obtained for the sulfate-reducing bacterium by pasteurizing a full-grown culture for 10 min at 80°C. The spores were transferred to fresh medium and the application of pasteurization was repeated twice. Pure cultures were obtained for the nitrate-reducing bacterium by repeated application of the agar roll-tube-dilution method as described by Hungate [6]. To check purity, isolates were inoculated into medium with 0.2% yeast extract (BBL-Becton Dickinson), lactate, pyruvate, or glucose as substrates. After incubation, the cultures were examined microscopically.

Physiological Tests

Utilization of carbon sources, energy sources, and electron acceptors was tested using concentrations of 10 mM. These tests were performed in 120-ml serum bottles containing 45 ml of medium. The substrates and electron acceptors consumed and the products formed were measured.

Sequence Analysis and Phylogenetic Tree

Nucleic acids from strain ASRB-Zg were isolated by sonification followed by phenol extraction and ethanol precipitation as previ-

ously described [21]. The 16S rRNA gene was amplified by PCR using a set of primers corresponding to positions 8–27 [5′-CACGGATCCAGACTT-TGAT(C/T)(A/C)TGGCTCAG-3′] and 1492–1513 [5′-GTGCTGCAGTACGG(T/C)TACCTTGTTACG-ACTT-3′] of *Escherichia coli*. PCR amplification, purification, and sequencing of the PCR product were performed as previously described [16]. The 16S-rDNA sequencing for culture AMPB-Zg and strain ANRB-Zg was carried out by W. Liesack and H. Lüdemann (Max-Planck-Institute für terrestrische Mikrobiologie, Marburg, Germany) as previously described [5]. The phylogenetic trees for culture AMPB-Zg and strain ANRB-Zg were constructed by H. Lüdemann. The 16S rDNA of the isolates was integrated in an alignment of about 8,000 full and partial primary structures using the respective tools of the ARB software [29]. Only almost complete sequenced 16S rDNA references were used to calculate the dendrogram. The phylogenetic trees were constructed from dissimilarity matrices by the neighbor-joining method implemented in the ARB software package. The phylogenetic tree for strain ASRB-Zg was constructed by S. Stubner (Max-Planck-Institute für terrestrische Mikrobiologie, Marburg, Germany) as previously described [38].

The sequence data of the 16S rDNA of the isolated strains were submitted to the EMBL data bank under the following accession numbers: AJ276397 (AMPB-Zg), AJ276370 (ASRB-Zg), and AJ276398 (ANRB-Zg).

Analytical Techniques

Methane and hydrogen were measured as previously described [23]. Fatty acids were analyzed on a CP9001 gas chromatograph equipped with a FID [25]. Non-volatile organic acids were analysed by high-pressure liquid chromatography (HPLC) on a Merck column (Polyspher OA HY). The mobile phase was 0.01 N H_2SO_4 at a flow of 0.6 ml/min at 60°C. Anions were analyzed by HPLC as previously described [23].

Results

Isolation and Morphological Characterization

The methanogenic culture AMPB-Zg was obtained from 1×10^8-fold diluted, freshwater sediment inoculated with acetate and was obtained by repeated application of dilution method. Formation of methane and consumption of acetate were detected to follow growth. Cells of the isolated methanogen were non-motile and rod-shaped with blunted ends (0.5–1 μm wide and 3–4 μm long). The purity check with medium containing 0.2% yeast extract showed that AMPB-Zg still contained a contaminating bacterium.

The sulfate-reducing strain ASRB-Zg was obtained from 1×10^8-fold diluted sediment inoculated with acetate and sulfate. A pure culture was obtained by pasteurizing a full-grown culture for 10 min at 80°C. The isolated sulfate-reducing bacterium was weakly motile and, rod-shaped with pointed ends (0.5–0.7 μm wide and 1.5–3.5 μm long), occuring singly or in pairs. The formation of bright spores was observed occasionally. Spores were spherical and oriented centrally in the cells. Cells stained gram negative but gram positive cells were observed occasionally.

The nitrate-reducing strain ANRB-Zg was obtained from 1×10^6-fold diluted sediment inoculated with acetate and nitrate. It was isolated by repeated application of the agar roll-tube-dilution method. The isolated nitrate-reducing bacterium was weakly motile and rod-shaped (0.5–0.7 μm wide and 0.7–1 μm long), occurring singly or in pairs. Cells stained gram negative.

Growth and Substrate Utilization

The culture AMPB-Zg grew on acetate at 20 to 40°C. The optimum temperature for the methanogen was around 30–35°C. The isolate did not grow on H_2/CO_2 or formate, nor was methane formed with these substrates. An average growth yield of 0.75 g cell protein was obtained per mole of acetate consumed. With acetate, doubling times of 5.6–8.1 days were measured at 30°C.

The optimum growth temperature for strain ASRB-Zg on acetate and sulfate was around 30°C. No or little growth was observed below 10°C or above 35°C. Strain ASRB-Zg used sodium sulfate (10 mM) or sodium thiosulfate (10 mM) as electron acceptors. However, growth was stimulated when $FeSO_4$ was used as electron acceptor. Sulfur and nitrate could not be used as electron acceptor with acetate as electron donor. The sulfate-reducing bacterium did not grow chemolithoautotrophically with H_2 and sulfate as energy substrate and CO_2 as sole carbon source. It grew chemoorganotrophically with a large number of organic compounds (Table 1). All substrates were oxidized completely to CO_2. The complete oxidation of acetate (19 mM) led to the consumption of 19 mM sulfate. With sulfate, an average growth yield of 2.6 g cell protein was obtained per mol of acetate consumed. With acetate, doubling times of 3.9–5.3 days were measured at 30°C.

Strain ANRB-Zg grew on acetate and nitrate at 4 to 30°C; the optimum temperature for the nitrate-reducing bacterium was around 20–25°C. In the presence of acetate, strain ANRB-Zg used nitrate (10 mM) or oxygen. The nitrate-reducing bacterium grew chemoorganoheterotrophically with a large number of organic compounds (Table 2). All substrates were oxidized completely to CO_2, unless stated otherwise. Strain ANRB-Zg did not grow chemolithoautotrophically with H_2 and nitrate as energy substrate and CO_2

Table 1. Organic compounds tested as electron donors and carbon sources for strain ASRB-Zg in the presence of 10 mM sulfate[a]

Utilized:
H_2–CO_2 (80:20, v/v) plus 1 mM acetate, formate (10), acetate (10), butyrate (10), isobutyrate (10), methanol (10), ethanol (10), succinate (10), fumarate (10), benzoate (5)
Tested, but not utilized:
H_2–CO_2 (80:20, v/v), lactate (10), propanol (10), isopropanol (10), butanol (10), propionate (10), valerate (10), glucose (10), fructose (10), xylose (10)
Tested, but not utilized in the absence of sulfate:
Ethanol (10), lactate (10), pyruvate (10), fumarate (10)
Electron acceptors utilized with acetate:
Thiosulfate (10)
Electron acceptors not utilized with acetate:
Sulfur (5), sulfite (5), ferric iron (10), nitrate (5), oxygen (20% v/v)

[a] Substrate concentrations in parentheses are millimolar unless stated otherwise.

as sole carbon source, but it did with H_2 and oxygen (Table 2). Sulfur sulfite, thiosulfate, and sulfate could not be used as electron acceptor with acetate as electron donor. In the presence of acetate and nitrate, thiosulfate (5 mM) was oxidized to sulfate (5 mM) showing that thiosulfate was a suitable electron donor for the isolate. Hardly any growth was observed in the presence of thiosulphate and nitrate alone.

Phylogenetic Analysis

Comparative 16S rRNA sequence analysis revealed a relationship of culture AMPB-Zg to *Methanosaeta concilii* (sequence similarity 99.8%). The phylogenetic relationships of culture AMPB-Zg derived from 16S rRNA sequence analysis are depicted in Fig. 1. Strain ASRB-Zg shows a relationship with *Desulfotomaculum acetoxidans* (sequence similarity 98.7%). The phylogenetic tree depicted in Fig. 2 reflects the phylogenetic relationship of strain ASRB-Zg to its closest relatives. Comparative 16S rRNA sequence analysis revealed a relationship of strain ANRB-Zg with *Variovorax paradoxus* (Fig. 3). However, the sequence similarity with this strain was only 93.9%.

Discussion

We quantified the different groups of microorganisms involved in the acetate metabolism in a freshwater sediment originating from ditches next to peat grassland [24]. Acetate was shown to be an important intermediate in the degradation of organic matter in freshwater sediment [25]. By using labeled acetate we could show that methanogenesis accounted for more than 70% of the acetate mineralization in the sediment when sulfate concentrations were below 70 µM (*in situ* concentrations in summer). Sulfate reduction was the process dominating the consumption of acetate (>75%) when sulfate concentrations were higher than >500 µM (*in situ* concentrations in winter). Addition of nitrate (150 µM) lowered the acetate consumption rate and inhibited the formation of methane completely. The dominant acetate utilizing microorganisms were methanogens (20×10^8 cells cm^{-3} sediment) and sulfate reducers (2×10^8 cells cm^{-3} sediment). Acetate-utilizing nitrate reducers (5×10^5 cells cm^{-3} sediment) were clearly outnumbered by the methanogens and sulfate reducers [24]. The acetate-utilizing anaerobes obtained from the dilution series and their properties are summarized in Table 3.

The aceticlastic methanogen, culture AMPB-Zg, grew only on acetate, which is the characteristic property for all *Methanosaeta* species [9]. On the basis of the 16S rRNA sequence analysis, it became clear that the isolate is closely related if not identical to *Methanosaeta concilii.* Other acetate-utilizing methanogens are *Methanosarcina* species, but these were only observed in the lower dilutions of the serial dilution ($<10^{-3}$ based on morphology and autofluorescence). The isolation of culture AMPB-Zg from freshwater sediment using the highest positive dilution of a serial dilution on acetate strongly indicated that culture AMPB-Zg is the most abundant acetate-degrading methanogen in the sediment.

Table 2. Organic compounds tested as electron donors and carbon sources for strain ANRB-Z in the presence of 15 mM nitrate or oxygen (20% v/v)

Utilized in the presence of nitrate or O_2:
Formate (10), acetate (10), propionate (10), butyrate (10), lactate (10), pyruvate (10), methanol (10), ethanol (10), propanol (10), glucose (10), fructose (10), benzoate (5), syringate (5)
Utilized in the presence of O_2:
H_2–CO_2 (80:20, v/v), humic acid (2 mg; production of acetate)
Tested, but not utilized in the presence of nitrate:
Xylose (10)
Tested, and utilized in the absence of nitrate:
Glucose (10), fructose (10)
Tested, but not utilized in the absence of nitrate:
Ethanol (10), pyruvate (10)
Electron acceptors utilized with acetate:
Nitrite (5)
Electron acceptors not utilized with acetate:
Sulfur (5), sulfite (5), thiosulfate (10), sulfate (10), ferric iron (10)

[a] Substrate concentrations in parentheses are millimolar unless stated otherwise.

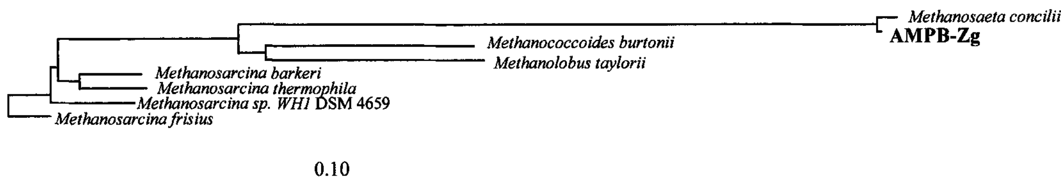

Fig. 1. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationship of the methanogenic culture AMPB-Zg to its closest relatives. Distance matrices were constructed from aligned sequences and corrected for multiple base changes at single positions by the method of Jukes and Cantor [10]. A phylogenetic tree was constructed by the neighbor-joining method of Saitou and Nei [22] by using the ARB software package [29]. The scale bar represents 10 nucleotide substitutions per 100 nucleotides. All nucleotide positions were used for the construction of the phylogenetic tree.

This abundance may reflect the high affinity of *Methanosaeta* species for acetate [8].

Strain ASRB-Zg is a sulfate-reducing bacterium that forms heat-resistant endospores, which is characteristic for *Desulfotomaculum* species [35]. The isolate grew on a variety of organic compounds that are formed during anaerobic degradation of organic matter, such as acetate, butyrate, and ethanol. From analysis of the 16S rRNA sequence it became clear that strain ASRB-Zg is closely related to *Desulfotomaculum acetoxidans.* The nutritional ability of strain ASRB-Zg is comparable to that of *D. acetoxidans* [35], although strain ASRB-Zg has a wider substrate range. The specific growth rate of ASRB-Zg (μ_{max} = 0.13–0.18 day^{-1}) is slightly higher than that of AMPB-Zg (μ_{max} = 0.09–0.12 day^{-1}). Strain ASRB-Zg and AMPB-Zg are the most abundant acetate-degrading microorganisms in the sediment, and both organisms are probably competing for the available acetate when sufficient sulfate is present in the sediment, which is the case in winter and spring [24]. Strain ASRB-Zg is a generalist, and it is possible that acetate degradation is not the only activity of the strain in the sediment. It might prefer other substrates than acetate. This ability to use other substrates besides acetate can give strain ASRB-Zg a competitive advantage over the methanogen under conditions of high sul-

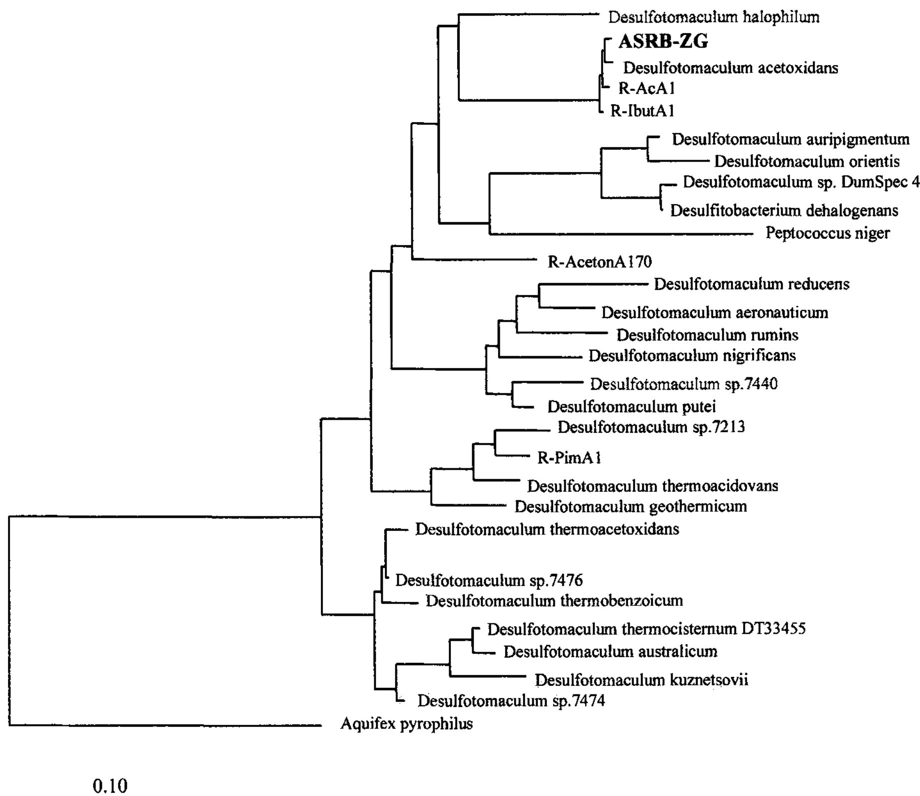

Fig. 2. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationship of the isolated sulfate-reducing bacterium ASRB-Zg to its closest relatives. The tree was constructed with the maximum likelihood method omitting high variable regions of the 16S rDNA with a filter of 50% invariance. Strains R-AcA1 R-IbutAl, R-PimA1, and R-AcetonA170 are gram positive bacteria which are affiliated with the genus *Desulfotomaculum* [38]. The scale bar represents 10 nucleotide substitutions per 100 nucleotides. *Aquifex pyrophilus* was used as an outgroup.

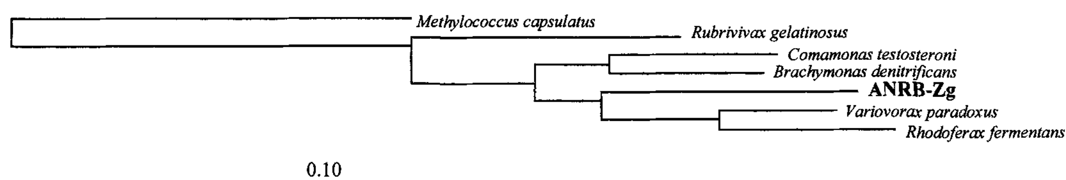

Fig. 3. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationship of the isolated nitrate-reducing bacterium ANRB-Zg and cultured members of the beta subclass of proteobacteria. Distance matrices were constructed from aligned sequences and corrected for multiple base changes at single positions by the method of Jukes and Cantor [10]. A phylogenetic tree was constructed by the neighbor-joining method of Saitou and Nei [22] by using the ARB software package [29]. The scale bar represents 10 nucleotide substitutions per 100 nucleotides. Only nucleotide positions that are in more than 50% of full sequenced beta proteobacteria in the database of the ARB software package were used to calculate the phylogenetic tree. *Methylococcus capsulatus* was used as an outgroup.

fate concentration. However, at low sulfate concentrations versatile acetate-degrading sulfate reducers may prefer other substrates than acetate [15]. Unfortunately, no information is available on how mixed substrate utilization may affect the competition between freshwater methanogens and sulfate reducers for acetate.

Strain ANRB-Zg grew only chemolithoautotrophically with H_2 and oxygen as energy substrate and CO_2 as sole carbon source. Under nitrate-reducing conditions it is a facultative anaerobe that grows only chemoorganotrophically on a variety of electron donors. Although it is not possible to draw a definitive conclusion about the functional role of strain ANRB-Zg in the sediment, some speculations can be made on the basis of its physiological characteristics and 16S ribosomal DNA sequence. The strain was isolated from a freshwater sediment that consists mainly out of organic material (90–95%) [25]. Organic matter is a heterogeneous and complex substance for which no exact chemical and physical structure can be given. It contains compounds such as fulvic acids, humic acids, and humin [28]. The capacity of strain ANRB-Zg to degrade syringate and humic acids may indicate that this organism plays a role in the degradation of naturally occurring aromatic compounds in the sediment. Apparently, strain ANRB-Zg has a metabolic pathway(s) that is effective toward naturally occurring aromatic compounds. It has been suggested that such pathways evolved in to novel metabolic pathways effective toward halogenated aromatics [33]. Intriguingly, Nogales et al. retrieved a 16S rRNA clone library from a highly PCB-polluted moor soil [13]. This culture-independent method based on the RT-PCR amplification of 16S rRNA molecules can be used to obtain an overview of the metabolically active members of the present bacterial community. Most of the sequence types obtained from the PCB-polluted soil belonged to the beta-subclass of Proteabacteria and were limited to two genera, *Burkholderia* and *Variovorax*. The authors argued that the predominance of these sequences might suggest that these organisms play an important role in the microbial activity within the polluted soil. Furthermore, strain ANRB-Zg is closely related to the *Variovorax* strains PLAE6 and HW1 (data not shown). These strains are capable of degrading halogenated aromatics such as 2,4-dichlorophenoxyacetic acid [4, 11]. Although not tested, it could be that strain ANRB-Zg, in addition to growing on acetate, is capable of degrading halogenated aromatics as well.

The physiological property of growth on reduced sulfur compounds such as thiosulfate in the presence of nitrate is a common feature of colorless sulfur bacteria [19]. In an ear-

Table 3. Properties of the isolated acetate-utilizing anaerobes

Species	Morphology	Width (μm)	Length (μm)	Gram staining	Spores	Temperature optimum (°C)	Oxidation of organic substrates
AMPB-Zg	Rod	0.5–1	3–4	ND	NO	30–35	
ASRB-Zg	Rod	0.5–0.7	1.5–3.5	+[a]	+	30	Complete
ANRB-Zg	Rod	0.5–0.7	0.7–1	–	NO	20–25	Complete

ND, not determined; NO, not observed; symbols: +, positive; +[a], positive and negative; –, negative able 3.
Properties of the isolated acetate-utilizing anaerobes

lier study, we noted that the addition of acetate and nitrate to sediment from Zegveld led to the oxidation of reduced sulfur compounds to sulfate [24]. The isolation of an acetate-utilizing nitrate reducer that is capable of oxidizing thiosulfate to sulfate supports these observations. Strain ANRB-Zg might be involved in the oxidation of reduced sulfur compounds to sulfate in the sediment. Analysis of the 16S rRNA sequence revealed that strain ANRB-Zg is related to the genera *Variovorax*. To our knowledge it is not known whether these bacteria are capable of growth on reduced sulfur compounds. At the moment too little information is available to understand the exact role of strain ANRB-Zg in the sulfur cycle of the sediment.

Acknowledgments

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References

1. Azegami K, Nishiyama K, Watanabe Y, Kadota I, Ohuchi A, Fukazawa C (1987) *Pseudomonas plantarii* sp. nov., the causal agent of rice seedling blight. Int J Syst Bacteriol 37:144–152
2. Bazylinski DA, Palome E, Blakemore NA, Blakemore RP (1986) Denitrification by *Chromobacterium violaceum.* Appl Environ Microbiol 52:696–699
3. Brysch K, Schneider C, Fuchs G, Widdel F (1987) Lithoautotrophic growth of sulfate-reducing bacteria, and description of *Desulfobacterium autrophicum* gen. nov., sp. nov. Arch Microbiol 148:264–274
4. Cavalca L, Hartmann A, Rouard N, Soulas G (1999) Diversity of tfdC genes: distribution and polymorphism among 2,4-dichlorophenoxyacetic acid degrading soil bacteria. FEMS Microbiol Ecol 29:45–58
5. Finster K, Coates JD, Liesack W, Pfennig N (1997) *Desulfuromonas thiophila* sp. nov, a new obligately sulfur-reducing bacterium from anoxic freshwater sediment. Int J Syst Bacteriol 47:754–758
6. Hungate RE (1969) A roll tube method for cultivation of strict anaerobes. In: Norris JR, Ribbons DW (eds) Methods in Microbiology, Vol 3b. Academic Press, New York, pp 117–132
7. Huser BA, Wuhrmann K, Zehnder AJB (1982) *Methanothrix soehngenii* gen. nov. sp. nov., a new acetotrophic non-hydrogen-oxidizing methane bacterium. Arch Microbiol 132:1–9
8. Jetten MSM, Stams AJM, Zehnder AJB (1992) Methanogenesis from acetate—a comparison of the acetate metabolism in *Methanothrix soehngenii* and *Methanosarcina* spp. FEMS Microbiol Rev 88:181–197
9. Jones WJ (1991) Diversity and physiology of methanogens. In: Rogers JE, Whitman WB (eds) Microbial Production and Consumption of Greenhouse Gases: Methane, Nitrogen Oxides, and Halomethanes. American Society for Microbiology, Washington, DC, pp 39–55
10. Jukes T, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (ed) Mammalian Protein Metabolism. Academic Press, New York, pp 21–132
11. Kamagata Y, Fulthorpe RR, Tamura K, Takami H, Forney LJ, Tiedje JM (1997) Pristine environments harbor a new group of oligotrophic 2,4-dichlorophenoxyacetic acid–degrading bacteria. Appl Environ Microbiol 63:2266–2272
12. Kiene RP (1991) Production and consumption of methane in aquatic systems. In: Rogers JE, Whitman WB (eds) Microbial Production and Consumption of Greenhouse Gases: Methane, Nitrogen Oxides, and Halomethanes. American Society for Microbiology, Washington, DC, pp 111–146
13. Nogales B, Moore ERB, Abraham W-F, Timmis KN (1999) Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl–polluted moorland soil. Environ Microbiol 1:199–212
14. Oude Elferink SJWH, Maas RN, Harmsen HJM, Stams AJM (1995) *Desulforhabdus amnigenus* gen nov sp nov, a sulfate reducer isolated from anaerobic granular sludge. Arch Microbiol 164:119–124
15. Oude Elferink SJWH, Luppens SBI, Marcelis CLM, Stams AJM (1998) Kinetics of acetate oxidation by two sulfate reducers isolated from anaerobic granular sludge. Appl Environ Microbiol 64:2301–2303
16. Oude Elferink SJWH, Akkermans-van Vliet WM, Bogte JJ, Stams AJM (1999) *Desulfobacca acetoxidans* gen. nov. sp. nov., a novel acetate-degrading sulfate reducer isolated from sulfidogenic granular sludge. Int J Syst Bacteriol 49:345–350
17. Pichinoty F, Mandel M, Garcia J-L (1979) The properties of novel denitrifying *Bacillus* cultures found in tropical soils. J Gen Microbiol 115:419–430
18. Reinhold B, Hurek T, Fendik I, Pot B, Gillis M, Kersters K, Thielemans S, De Ley J (1987) *Azospirrilum halopraeferens* sp. nov., a nitrogen-fixing organism associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth). Int J Syst Bacteriol 37:43–51
19. Robertson LA, Kuenen JG (1992) The colorless sulfur bacteria. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) The Prokaryotes, Vol 1. Springer, Berlin, pp 385–413
20. Rothfuss F, Conrad R (1993) Thermodynamics of methanogenic intermediary metabolism in littoral sediment of lake constance. FEMS Microbiol Ecol 12:265–276
21. Sambrook J, Fritsch EJ, Maniatis T (1989) Moleculair Cloning:

A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
23. Scholten JCM, Stams AJM (1995) The effect of sulfate and nitrate on methane formation in a freshwater sediment. Antonie Van Leeuwenhoek 68:309–315
24. Scholten JCM, van Bodegom PM, Vogelaar J, van Ittersum A, Hordijk K, Roelofsen W, Stams AJM (2000) Effect of sulphate and nitrate on acetate consumption by methanogenic, sulphate- and nitrate-reducing populations in mixed freshwater sediment. Biogeochem, in press.
25. Scholten JCM, Conrad R, Stams AJM (2000) Effect of 2-bromoethane sulfonate, molybdate and chloroform on acetate consumption by methanogenic and sulfate-reducing communities in a freshwater sediment. FEMS Microbiol Ecol 32:35–42
26. Schütz H, Seiler W, Conrad R (1989) Processes involved in formation and emission of methane in rice paddies. Biogeochem 7:33–53
27. Stams AJM, Vandijk JB, Dijkema C, Plugge CM (1993) Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. Appl Environ Microbiol 59:1114–1119
28. Stevenson FJ (1994) Humus Chemistry; Genesis, Composition, Reactions, 2nd ed. John Wiley & Sons, New York
29. Strunk O, Gross O, Reichel B, May M, Hermann S, Stuckman N, Nonhoff B, Lenke M, Finhart A, Vilbig A, Ludwig T, Bode A, Schleifer K-H, Ludwig W (1998) ARB: a software environment for sequence data. http://www.mikro.biologie.tu-muenchen.de. Department of Microbiology, Technische Universität München, Munich, Germany.
30. Thauer RK, Moller-Zinkhan D, Spormann AM (1989) Biochemistry of acetate catabolism in anaerobic chemotrophic bacteria. Ann Rev Microbiol 43:43–67
31. Tomlinson GA, Jahnke LL, Hochstein LI (1986) *Halobacterium denitrificans* sp. nov., an extremely halophilic denitrifying bacterium. Int J Syst Bacteriol 36:66–70
32. Torreblanca M, Rodriguez-Valera F, Juez G, Ventosa A, Kamekura M, Kates M (1986) Classification of non-alkaliphilic Halobacteria based on numerical taxonomy and polar lipid composition, and description *of Haloarcula* gen. nov. and *Haloferax* gen. nov. Syst Appl Microbiol 8:89–99
33. Van der Meer JR (1994) Genetic adaptation of bacteria to chlorinated aromatic compounds. FEMS Microbiol Rev 15:239–249
34. Widdel F (1988) Microbiology and ecology of sulfate- and sulfur-reducing bacteria. In: Zehnder AJB (ed) Biology of Anaerobic Microorganisms. John Wiley & Sons, New York, pp 469–585
35. Widdel F (1992) The genus *Desulfotomaculum.* In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) The Prokaryotes, Vol 2. Springer, Berlin, pp 1792–1799
36. Widdel F, Hansen TA (1992) The dissimilatory sulfate- and sulfur-reducing bacteria. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) The Prokaryotes, Vol 1. Springer, Berlin, pp 583–624
37. Wolin EA, Wolin MJ, Wolfe RS (1963) Formation of methane by bacterial extracts. J Biol Chem 238:2882–2886
38. Wind T, Stubner S, Conrad R (1999) Sulfate-reducing bacteria in rice field soil and on rice roots. Syst Appl Microbiol 22:269–279
39. Zumft WG (1992) The denitrifying prokaryotes. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) The Prokaryotes, Vol 1. Springer, Berlin, pp 554–581