***Desulfobacca acetoxidans* gen. nov., sp. nov., a novel acetate-degrading sulfate reducer isolated from sulfidogenic granular sludge**

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A mesophilic sulfate reducer, strain ASRB2^T, was isolated with acetate as sole carbon and energy source from granular sludge of a laboratory-scale upflow anaerobic sludge bed reactor fed with acetate and sulfate. The bacterium was oval-shaped, 1.3 × 1.9–2.2 µm, non-motile and Gram-negative. Optimum growth with acetate occurred around 37 °C in freshwater medium (doubling time: 1.7–2.2 d). Enzyme studies indicated that acetate was oxidized via the carbon monoxide dehydrogenase pathway. Growth was not supported by other organic acids, such as propionate, butyrate or lactate, alcohols such as ethanol or propanol, and hydrogen or formate. Sulfite and thiosulfate were also used as electron acceptors, but sulfur and nitrate were not reduced. Phylogenetically, strain ASRB2^T clustered with the delta subclass of the *Proteobacteria*. Its closest relatives were *Desulfosarcina variabilis*, *Desulfacinum infernum* and *Syntrophus buswellii*. Strain ASRB2^T is described as the type strain of *Desulfobacca acetoxidans* gen. nov., sp. nov.

Keywords: *Desulfobacca acetoxidans* gen. nov., sp. nov., acetate, sulfate-reducing bacteria

INTRODUCTION

Sulfate-reducing bacteria play an important role in the degradation of organic matter in anaerobic bioreactors treating sulfate-rich wastewaters, such as those from paper mills, tanneries or food oil industry (Oude Elferink *et al.*, 1994; Colleran *et al.*, 1995). If sufficient sulfate is available, sulfate reducers can easily out-compete hydrogenotrophic methanogens and syntrophic consortia for substrates like hydrogen and propionate (Oude Elferink *et al.*, 1994; Colleran *et al.*, 1995; Visser, 1995). However, the outcome of the competition for acetate between sulfate reducers and methanogens in anaerobic wastewater treatment systems is less clear. In some studies with freshwater or

low-salt systems, acetate conversion via methanogenesis was predominant, even at an excess of sulfate (McCartney & Oleszkiewicz, 1991; Isa *et al.*, 1986; Visser *et al.*, 1993a). Other studies report the predominance of acetate degradation via sulfate reduction (Alphenaar *et al.*, 1993; Visser *et al.*, 1993b; Harada *et al.*, 1994). Factors which could affect the outcome of the competition between methanogens and sulfate reducers are, for example, the kinetic properties of the bacteria involved, the pH and temperature of the reactor, and the chemical oxygen demand (COD)/sulfate ratio of the wastewater (Oude Elferink *et al.*, 1994; Colleran *et al.*, 1995; Visser, 1995). In wastewater with a COD/sulfate ratio below 0.67 (g g⁻¹) there will be excess of sulfate allowing the degradation of all organic material via sulfate reduction.

Acetate is one of the major intermediates in the breakdown of organic matter in anaerobic bioreactors (Gujer & Zehnder, 1983; Smith & Mah, 1966). Therefore, it is important to know which sulfate reducers can compete with the acetoclastic methanogens present in the sludge. Although many mesophilic

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Abbreviations: COD, chemical oxygen demand; UASB, upflow anaerobic sludge bed.

The GenBank accession number for the 16S rRNA gene sequence of strain ASRB2^T is AF002671.

sulfate reducers can grow with acetate as sole electron donor and carbon source (Widdel, 1992; Widdel & Bak, 1992), only a few show good growth with acetate under freshwater conditions. Among them are the Gram-positive *Desulfotomaculum acetoxidans* (Widdel & Pfennig, 1981), the Gram-negative *Desulfobacter* strain AcKo (Widdel, 1987), and the Gram-negative *Desulforhabdus amnigenus*, which was recently isolated from granular sludge of an upflow anaerobic sludge bed (UASB) reactor treating papermill wastewater (Oude Elferink *et al.*, 1995). In this paper we describe the isolation and characterization of a sulfate reducer from granular sludge of a laboratory-scale UASB reactor fed with acetate and an excess of sulfate. In this reactor sulfate reduction had completely superseded methanogenesis after 1 year of reactor operation.

METHODS

Origin of strain ASRB2^T. The sulfate-reducing bacterium, strain ASRB2^T, was isolated from the granular sludge of a pilot-scale UASB reactor (1.7 l) fed with acetate and an excess of sulfate. Initially, the reactor was seeded with sludge from a 10 l UASB reactor that had been fed with acetate and sulfate for more than 2 years. Detailed characteristics of this seed-sludge have been described elsewhere (Visser, 1985). The reactor influent had a COD/sulfate ratio of 0.6 (g g⁻¹) and was treated at a temperature of 30 °C. Sludge samples were taken after 6 months and 1 year of reactor operation. During this period acetate degradation via sulfate reduction increased from approximately 80 to 100%, while degradation via methanogenesis decreased from 20 to 0%.

Media and cultivation. Unless stated otherwise, bacteria were cultured at 37 °C in 120 ml serum vials containing 50 ml of a bicarbonate-buffered medium, and a gas phase of 172.2 kPa N₂/CO₂ (80:20, v/v) as described previously (Oude Elferink *et al.*, 1995). The inoculum size was 1%.

Isolation. Granular sludge samples (10 ml), taken from the reactor after 6 months and after 1 year of operation, were 10-fold diluted and disintegrated immediately after sampling as described previously (Oude Elferink *et al.*, 1995). This crushed granular sludge was used to make 10-fold serial dilutions in liquid media containing acetate and sulfate (20 mM each). For each dilution, 5 ml inoculum was added to 45 ml medium. The cultures were incubated at 30 °C, and the highest dilutions which showed growth were used for further isolation. Pure cultures were obtained by repeated application of the agar roll-tube dilution method as described by Hungate (1969). Purity of the isolates was checked by microscopic observations and by testing anaerobic growth on pyruvate and glucose with 0.1% yeast extract (BBL, Becton Dickinson), and on Wilkins-Chalgren anaerobe broth (Oxoid).

Growth experiments. Utilization of carbon sources, electron donors, and electron acceptors were tested in basal bicarbonate-buffered medium as described before (Oude Elferink *et al.*, 1995). In most cases growth was followed by measuring substrate utilization and sulfide production, and by visual examination of culture turbidity. All tests were performed at a predetermined temperature, pH and salinity, allowing optimal growth of the isolate (Oude Elferink *et al.*, 1995).

Analysis of cell compounds. Desulfovibrin was detected

according to the methods of Postgate (1959). Cytochromes were identified in cell extracts by recording reduced-minus-oxidized difference spectra with a Beckman DU7500 spectrophotometer, and on SDS-PAGE gel according to the methods of Thomas *et al.* (1976). The G+C content of the DNA was determined by HPLC at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Gram staining was done according to standard procedures (Doetsch, 1981). The presence of gas vacuoles was determined by microscopic examination of late-exponential phase cultures before and after a pressure-shock treatment in a hypodermic syringe.

Chemical analysis and enzyme measurements. Substrates were measured by HPLC or GC as described by Oude Elferink *et al.* (1995). Sulfide was determined as described by Trüper & Schlegel (1964), and protein was measured according to the method of Bradford (1976). The enzyme activities of carbon monoxide dehydrogenase, formate dehydrogenase and 2-oxoglutarate dehydrogenase activities were assayed according to Schauder *et al.* (1986), using anoxically prepared cell extracts (Jetten *et al.*, 1990) of cells grown with acetate and sulfate and harvested in the late-exponential phase.

Sequence analysis and phylogenetic tree. The 16S rRNA gene of strain ASRB2^T was selectively amplified as described previously (Harmsen *et al.*, 1993), using a set of universal 16S rRNA-based primers: forward primer [5' CACGGAT-CCAGAGTTTGAT(C/T)-(A/C)TGGCTCAG] corresponded to positions 8–27 of *Escherichia coli* 16S rRNA, and the reverse primer (5' GTGCTGCAGGGTTACCTTGTTACGACT) corresponded to positions 1493 to 1510. Amplification products were cloned in the pGEM®-T vector according to the manufacturers protocol (pGEM®-T Vector Systems, Promega). Plasmids of the clones were isolated by Wizard Plus Minipreps DNA Purification System according to the manufacturer's instruction (Promega). The inserts were amplified using primers T7 (5' AATACGACTCACTATAG) and Sp6 (5' ATTTAGGTGACACTATA). The PCR products were sequenced with a LICOR 4000L sequencer, by using Thermo Sequenase fluorescent-labelled primer cycle sequencing with 7-deaza-dGTP according to the manufacturer's protocols (Amersham). The total 16S rRNA gene sequence was determined and aligned to those of other bacterial sequences, taking into account sequence similarity and higher order structure, using the alignment tool of the ARB program package (Ludwig & Strunk, 1996).

RESULTS

Isolation and morphological characterization

Strain ASRB2^T was the dominant acetoclastic sulfate reducer in sludge samples taken from the reactor after 6 months and 1 year of operation. The highest sludge dilutions (1 × 10⁸ and 1 × 10⁹) showing growth on acetate and sulfate were used for the isolation of strain ASRB2^T by a repeated application of the agar roll-tube dilution method. In agar the strain grew in greyish colonies with an irregular shape. Cells of the isolate were non-motile oval to rod-shaped (1.3 µm wide × 1.9–2.2 µm long), and appeared singly or in pairs (Fig. 1). Cells stained Gram-negative. Spores were never observed. Late-exponential phase or stationary phase cells often contained light-reflecting inclusions that

Fig. 1. Phase-contrast photomicrograph of strain ASRB2^T. Arrowhead indicates a cell with a gas vacuole. Bar, 10 µm.

could be destroyed by pressure-shock treatment, indicating that the inclusions were gas vacuoles.

Growth conditions and substrate utilization

The optimum growth temperature for strain ASRB2^T on acetate and sulfate was between 36 and 40 °C. Little growth was observed below 27 °C or above 47 °C. The

optimum pH for growth was 7.1–7.5; growth was possible between pH 6.5 and 8.3. The shortest doubling time on acetate was 1.7–2.2 d. Growth in brackish medium was slow (the doubling time increased 4–8-fold), and no growth was observed in marine medium. When vitamins were omitted from the media, cultures could be transferred (1% inoculum size) at least four times without any growth retardation.

In the presence of acetate, strain ASRB2^T could use (mM) sulfate (20), thiosulfate (20) or sulfite (5) as electron acceptor; sulfur (5), nitrate (5) and fumarate (10) were not used. Strain ASRB2^T was specialized in the degradation of acetate, and complete oxidation of 10 mM acetate led to a concomitant formation of 9.6 mM sulfide. The threshold for acetate was below 15 µM, the detection threshold of our gas chromatograph. Compounds tested but not utilized as electron donors by strain ASRB2^T were (mM): propionate (20), butyrate (20), lactate (20), H₂/CO₂ (80:20, v/v) with or without acetate (2), formate (10) with or without acetate (2), ethanol (20), propanol (10), butanol (10), pyruvate (20), fumarate (20), glucose (20), crotonate (5), benzoate (1), phenol (0.5), aspartate (5) and glutamate (5).

The pathway of acetate oxidation was studied by enzyme measurements of key enzymes in cell-free extracts. The specific activities of carbon monoxide

Fig. 2. Distance matrix tree reflecting the phylogenetic relationships of *Desulfobacca acetoxidans* strain ASRB2^T with other sulfate reducers and relatives belonging to the delta subclass of the *Proteobacteria*. Bar, 0.10 K_{nuc} .

dehydrogenase and formate dehydrogenase were 0.63 and 0.84 mol min⁻¹ (mg protein)⁻¹, respectively. 2-Oxoglutarate dehydrogenase activity could not be detected.

Pigments and other cell compounds

Dithionite-reduced versus air-oxidized spectra of cell extracts of ASRB2^T revealed absorption maxima at 422, 527 and 557 nm, indicating the presence of c-type cytochromes (Mahler & Cordes, 1969). The presence of c-type cytochromes was confirmed with the staining procedure on SDS gel (Thomas *et al.*, 1976) (results not shown). Desulfoviridin could not be detected. The G + C content of the DNA was 51.1 ± 0.2 mol %.

Phylogenetic analysis

The phylogenetic relationships of strain ASRB2^T derived from 16S rRNA sequence analysis are depicted in Fig. 2. The 16S rRNA sequence shows that strain ASRB2^T is a member of the delta subclass of the *Proteobacteria*. A 16S rRNA sequence highly similar to that of ASRB2^T was not available in the database. *Desulfosarcina variabilis*, *Desulfacinum infernum* and *Syntrophus buswellii* were the closest relatives of ASRB2^T; the level of sequence similarity was 86.9, 85.6 and 85.5%, respectively. The acetate-degrading sulfate reducer *Desulforhabdus amnigenus* was only moderately related to strain ASRB2^T (sequence similarity 85.1%).

DISCUSSION

Physiology, ecology and taxonomy of strain ASRB2^T

Strain ASRB2^T was isolated from granular sludge of a laboratory-scale UASB reactor fed with acetate and an excess of sulfate. Cells resembling those of strain ASRB2^T were isolated from the sludge before and after sulfate reduction had superseded methanogenesis, by using the highest positive dilutions of two serial dilution ranges on acetate and sulfate. This strongly indicates that strain ASRB2^T is the most abundant acetate-degrading sulfate reducer in this sludge and is able to outcompete acetate-degrading methanogens. The only two genera of acetate-degrading methanogenic archaea known are *Methanosarcina* and *Methanosaeta* ('*Methanotherix*') (Whitman *et al.*, 1992). *Methanosaeta* species generally are the most important methanogenic acetate degraders in anaerobic bioreactors, because of their high affinity and low threshold (7–69 µM) for acetate (Oude Elferink *et al.*, 1994; Jetten *et al.*, 1992). The threshold of strain ASRB2^T (<15 µM) is in the same range as that of *Methanosaeta* sp. However, strain ASRB2^T has a higher specific growth rate ($\mu_{\max} = 0.32\text{--}0.41\text{ d}^{-1}$) than *Methanosaeta* sp. ($\mu_{\max} = 0.08\text{--}0.29\text{ d}^{-1}$). This could be one of the reasons why strain ASRB2^T is able to outcompete the acetate-degrading methanogens in

the reactor. Strain ASRB2^T seems to be specialized in acetate consumption, this in contrast to the nutritionally versatile acetate-degrading *Desulforhabdus amnigenus*, which was recently isolated from a pilot-scale UASB reactor treating papermill wastewater using the same isolation procedures as described for strain ASRB2^T (Oude Elferink *et al.*, 1995). Apparently, the acetate-degrading sulfate-reducing population in the laboratory-scale reactor differs significantly from the population in the pilot-scale reactor. This is probably due to the different conditions in the pilot-scale reactor, such as the limiting sulfate concentration (COD/sulfate = 1.1 g g⁻¹) and the more complex wastewater. The nutritional specialization of strain ASRB2^T is comparable to that of *Desulfobacter* sp. (Widdel, 1987), although some *Desulfobacter* species can use hydrogen and ethanol as well. However, the mean specific growth rate of *Desulfobacter* sp. ($\mu_{\max} = 0.8\text{--}1.1\text{ d}^{-1}$) (Oude Elferink *et al.*, 1994) is approximately twice as high as that of strain ASRB2^T. The oxidation of acetate in *Desulfobacter* sp. and in strain ASRB2^T occurs via different pathways. *Desulfobacter* sp. use the citric acid cycle (Widdel, 1987), while strain ASRB2^T degrades acetate via the CO-dehydrogenase pathway. This is indicated by the high activity in cell-free extracts of strain ASRB2^T, of carbon monoxide dehydrogenase and formate dehydrogenase, two key enzymes of the CO-dehydrogenase pathway, together with the absence of 2-oxoglutarate dehydrogenase activity, a key enzyme of the citric acid cycle (Schauder *et al.*, 1986).

Phylogenetically, strain ASRB2^T clusters with the delta subclass of the *Proteobacteria*. *Desulfobacter* sp. is only distantly related to strain ASRB2^T. The closest relatives are *Desulfosarcina variabilis* (86.9% similarity), *Desulfacinum infernum* (85.6% similarity) and *Syntrophus buswellii* (85.5% similarity).

Physiologically and phylogenetically strain ASRB2^T differs significantly from the syntrophically benzoate-oxidizing *S. buswellii* (Wallrabenstein *et al.*, 1995), the thermophilic *Desulfacinum infernum* (Rees *et al.*, 1995), and the nutritionally versatile *Desulfosarcina variabilis* (Widdel & Bak, 1992). Therefore, we propose that strain ASRB2^T represents a new species of a new genus. We propose the name *Desulfobacca acetoxidans* gen. nov., sp. nov. for this organism.

Description of *Desulfobacca* gen. nov.

Desulfobacca (de.sul.fo.bac'ca. L. pref. *de* from; L. n. *sulfur* sulfur; M.L. pref. *Desulfo-* desulfuricating, used to characterize a dissimilatory sulfate-reducing prokaryote; L. fem. n. *baca* or *bacca* berry, especially olive; M.L. fem. n. *Desulfobacca* a sulfate-reducing, olive-shaped bacterium).

Non-motile, oval to rod-shaped cells. Sulfate or other inorganic sulfur compounds, but not elemental sulfur, serve as terminal electron acceptor and are reduced to H₂S. Acetate is the common electron donor and carbon

source, and is completely oxidized to CO₂ via the CO-dehydrogenase pathway. *Desulfobacca* belongs to the delta subclass of the *Proteobacteria*; the closest relatives are *Desulfosarcina variabilis*, *Desulfacinum infernum* and *Syntrophus buswellii*.

Description of *Desulfobacca acetoxidans* sp. nov.

Desulfobacca acetoxidans (a.cet.o'xi.dans. L. n. acetum vinegar; M.L. part. pres. oxidans oxidizing, M.L. part. adj. acetoxidans acetate-oxidizing).

Cells are oval to rod-shaped, 1.3 × 1.9–2.2 µm, singly or in pairs. Cells do not form spores and are Gram-negative. Acetate is the only electron donor and carbon source used. Sulfate, sulfite and thiosulfate can serve as electron acceptors. The optimum pH is 7.7–7.5, the optimum temperature is 37 °C. Growth is optimal in freshwater medium. The G + C content of the DNA is 51.1 ± 0.2 mol%. Habitat is granular sludge from an upflow anaerobic sludge bed (UASB) reactor fed with acetate and sulfate. The type strain is ASRB2^T (= DSM 11109^T).

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