RESEARCH ARTICLE

Microbial sulfide oxidation in the oxic–anoxic transition zone of freshwater sediment: involvement of lithoautotrophic *Magnetospirillum* strain J10

Jeanine S. Geelhoed[1,2], Dimitry Y. Sorokin[1,3], Eric Epping[4], Tatjana P. Tourova[3], Horia L. Banciu[5], Gerard Muyzer[1], Alfons J.M. Stams[2] & Mark C.M. van Loosdrecht[1]

[1]Department of Biotechnology, Environmental Biotechnology, Delft University of Technology, Delft, The Netherlands; [2]Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands; [3]Winogradsky Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia; [4]Royal Netherlands Institute for Sea Research, Texel, The Netherlands; and [5]Faculty of Biology and Geology, Babes-Bolyai University, Cluj-Napoca, Romania

Correspondence: Jeanine S. Geelhoed, Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands. Tel.: +31 317 484 099; fax: +31 317 483 829; e-mail: jeanine.geelhoed@wur.nl

Received 2 April 2009; revised 19 June 2009; accepted 19 June 2009.
Final version published online 29 July 2009.

DOI:10.1111/j.1574-6941.2009.00739.x

Editor: Michael Wagner

Keywords
lithoautotrophic growth; sulfur oxidation; *Magnetospirillum*; isolation; oxic–anoxic transition zone.

Abstract

The oxic–anoxic transition zone (OATZ) of freshwater sediments, where opposing gradients exist of reduced iron and sulfide with oxygen, creates a suitable environment for microorganisms that derive energy from the oxidation of iron or sulfide. Gradient microcosms incubated with freshwater sediment showed rapid microbial turnover of sulfide and oxygen compared with sterile systems. Microcosms with FeS as a substrate also showed growth at the OATZ and subsequent dilution series resulted in the isolation of three novel strains, of which strain J10 grows chemolithoautotrophically with reduced sulfur compounds under microaerobic conditions. All three strains are motile spirilla with bipolar flagella, related to the genera *Magnetospirillum* and *Dechlorospirillum* within the *Alphaproteobacteria*. Strain J10 is closely related to *Magnetospirillum gryphiswaldense* and is the first strain in this genus found to be capable of autotrophic growth. Thiosulfate was oxidized completely to sulfate, with a yield of 4 g protein mol^{-1} thiosulfate, and autotrophic growth was evidenced by incorporation of ^{13}C derived from bicarbonate into biomass. A putative gene encoding ribulose 1,5-bisphosphate carboxylase/oxygenase type II was identified in strain J10, suggesting that the Calvin–Benson–Bassham cycle is used for autotrophic growth. Analogous genes are also present in other magnetospirilla, and in the autotrophically growing alphaproteobacterium magnetic vibrio MV-1.

Introduction

The oxic–anoxic transition zone (OATZ) in sediments and water columns is characterized by opposing gradients of oxygen and reduced iron and sulfur compounds. Microorganisms that are capable of lithoautotrophic growth utilize the energy stored in these chemical gradients by the oxidation of reduced sulfur and/or iron. Gradient microcosms have been used to cultivate and enrich neutrophilic microaerophilic Fe(II) and sulfide-oxidizing microorganisms in the laboratory. These gradient microcosms provide a combination of microaerobic conditions and a low iron or sulfide concentration, and mimic natural conditions in which chemical oxidation of iron(II) and sulfide by oxygen is limited due to their low concentrations (Nelson *et al.*, 1986; Emerson & Moyer, 1997).

Magnetotactic bacteria are ubiquitously present at the OATZ of freshwater sediments and marine waters (Spring *et al.*, 1993; Frankel *et al.*, 1997; Simmons *et al.*, 2004; Flies *et al.*, 2005a). They produce high-purity crystals of magnetite, a mixed Fe(II)/Fe(III) oxide inside the cell, and are assumed to play an important role in the geochemical cycling of iron in natural sediments (for reviews, see Bazylinski & Frankel, 2004b; Schüler, 2008). The abundance of magnetotactic bacteria at the OATZ might indicate that these bacteria are able to derive energy from the conversion of sulfide and/or iron. Indeed, it was shown that marine magnetotactic bacteria MV-1, MV-2 and MC-1 can grow autotrophically with

reduced sulfur compounds as electron donors (Bazylinski *et al.*, 2004a; Williams *et al.*, 2006). However, attempts to enrich or cultivate freshwater magnetotactic bacteria under autotrophic conditions have so far been unsuccessful (Schüler *et al.*, 1999; Bazylinski *et al.*, 2004a; Flies *et al.*, 2005b).

Most freshwater magnetotactic bacteria are affiliated with the *Alpha* subclass of the *Proteobacteria* (Spring *et al.*, 1992; Flies *et al.*, 2005b), with the majority of cultured freshwater isolates belonging to the genus *Magnetospirillum* (Maratea & Blakemore, 1981; Matsunaga *et al.*, 1991; Schleifer *et al.*, 1991; Schüler *et al.*, 1999; Flies *et al.*, 2005b). In addition, freshwater magnetotactic bacteria affiliated with the *Deltaproteobacteria* [*Desulfovibrio magneticus* (Sakaguchi *et al.*, 2002), uncultured multicellular prokaryote (DeLong *et al.*, 1993)] and the *Nitrospira* phylum [uncultured large rods '*Magnetobacterium bavaricum*' (Spring *et al.*, 1993) and MHB-1 (Flies *et al.*, 2005b)] have been described. For marine magnetotactic bacteria, the phylogenetic diversity appears to be more widespread within the *Proteobacteria* (Simmons *et al.*, 2004, 2007).

In the genomes of the freshwater species *Magnetospirillum magneticum* AMB-1 (Matsunaga *et al.*, 2005), *Magnetospirillum gryphiswaldense* (Richter *et al.*, 2007) and *Magnetospirillum magnetotacticum* MS-1 (Bazylinski *et al.*, 2004a; http://img.jgi.doe.gov), putative genes encoding ribulose-1,5-bisphosphate carboxylase/oxygenase type II (Rubisco)(*cbbM*) have been identified, showing 77–72% identity with *cbbM* of *Rhodobacter sphaeroides* (Wagner *et al.*, 1988). Rubisco is a key enzyme of the Calvin–Benson–Bassham (CBB) cycle for CO_2 fixation and inorganic carbon assimilation. The presence of this gene in magnetospirilla may indicate the ability for autotrophic growth; however, autotrophic growth of *Magnetospirillum* species has not been demonstrated (Schüler *et al.*, 1999; Bazylinski *et al.*, 2004a).

In this study, we investigated the potential for lithoautotrophic growth of microorganisms populating the OATZ. Gradient microcosms with opposing gradients of reduced iron and sulfide, derived from solid FeS, and oxygen were inoculated with freshwater sediment and monitored for bacterial growth. Microcosms with gradients of sulfide (from Na_2S) and oxygen were used to quantify the importance of biological sulfide oxidation. From the FeS microcosms, three novel bacterial strains were isolated. One of these strains, J10, showed lithoautotrophic growth supported by oxidation of reduced sulfur compounds under microaerobic conditions and was studied in more detail.

Materials and methods

Source of sediment and gradient microcosms

Sediment was taken from the top layer of the sediment of a freshwater ditch in Delft (the Netherlands). The malodorous smell indicated the release of sulfide from the water. Gradient microcosms were set up with opposing gradients of oxygen and ferrous iron and sulfide derived from FeS in an agarose-stabilized medium and 50–100 μL of sediment (Emerson & Moyer, 1997). Gradient systems were also prepared with 10 mM $FeCl_2$ or 10 mM Na_2S, buffered with $NaHCO_3/CO_2$. The substrate was added to 2.5 mL bottom plug (1% agarose), which was overlaid with 10 mL mineral medium [concentration (mM): NH_4Cl 5.6, $MgSO_4$ 1.5, $CaCl_2$ 0.7 and KH_2PO_4 0.2] containing 0.15% agarose and supplemented with trace elements ($1\,mL\,L^{-1}$; Pfennig & Lippert, 1966), vitamins ($1\,mL\,L^{-1}$; Wolin *et al.*, 1963) and resazurin ($0.5\,mg\,L^{-1}$). Sterilized substrates, medium and gradient tubes were prepared anoxically in closed bottles/tubes. After preparation of the gradient tubes, the stoppers were replaced with cotton wool plugs and the tubes were incubated for 24–48 h before inoculation, to allow the development of gradients of ferrous iron, sulfide and oxygen.

Sulfide oxidation potential in the top layer of the sediment was assessed by diluting this sediment in sulfide–oxygen gradient microcosms and measuring the sulfide and oxygen gradients that develop. Ten milliliters of medium with 10 mM sulfide and 2% agar was overlaid with 30 mL of medium stabilized with 0.2% agar. These agar-stabilized microcosms were 27 mm in diameter and 70 mm in height. After pouring the media, the columns were incubated at 30 °C in air for 42 h to allow the development of the oxygen and sulfide gradients before inoculation with 0.5 mL of sediment. Blank columns were measured after 54 and 170 h, and inoculated columns after 170, 219 and 412 h of total incubation time. Microprofiles of H_2S, oxygen and pH were determined following the methods detailed by Pringault *et al.* (1998, 1999). The total sulfide concentration in the profile was calculated from the measured concentration of H_2S and pH using the pK_1 of sulfide. Diffusion coefficients of oxygen and sulfide in water at 30 °C (O_2 $2.7 \times 10^{-9}\,m^2\,s^{-1}$, HS^- $1.9 \times 10^{-9}\,m^2\,s^{-1}$; Broecker & Peng, 1974; Li & Gregory, 1974) were used to calculate the diffusive fluxes of oxygen and sulfide in the column.

Batch culture

Batch culture incubations with thiosulfate (25 mM) were performed statically using medium with 25 mM $NaHCO_3$, 10 mM phosphate buffer (pH 7) and N_2/CO_2 (80% : 20%) headspace. Dilution series in liquid medium were performed in closed 120-mL bottles with 20 mL of liquid. For the growth curve experiment, 35 mL of medium was used in 260-mL bottles, inoculated with 0.5 mL of a thiosulfate-grown culture. Oxygen was injected periodically to reach an O_2 concentration of 1–2% in the headspace. The pH was maintained at 6.6–7.1 by the addition of aliquots of 1 M $NaHCO_3$ to prevent decomposition of thiosulfate at low pH.

Other batch incubations were performed with acetate or lactate as an electron donor and hydrous ferric oxide, fumarate or oxygen (1–2% in the headspace) as an electron acceptor. Pure cultures were grown in a medium with acetate supplemented with 100 μM ferric citrate under microaerobic conditions and examined for magnetotactic behavior. All incubations were carried out at 30 °C.

Microorganisms

Strains J10, L70 and LD2 were isolated from freshwater sediment. *Magnetospirillum gryphiswaldense* (DSM 6361^T) and *M. magnetotacticum* (DSM 3856^T) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

Analysis of incorporation of ^{13}C bicarbonate into biomass

Triplicate batch cultures of strain J10 with 25 mM thiosulfate were grown in P-buffered (20 mM, pH 7) medium (35 mL in 260-mL bottles) with N_2 headspace. The medium was supplemented with 5 mM $NaH^{13}CO_3$ (Isotec, Sigma-Aldrich, ^{13}C 99.3%) and inoculated with 0.5-mL thiosulfate-grown culture. Another set of triplicate batch cultures was grown in exactly the same way, but supplied with 5 mM unlabeled $NaHCO_3$. Oxygen was added to a concentration of 1–2% in the headspace and resupplied as required. Appropriate amounts of 1 M NaOH were added to maintain a pH between 6.6 and 7.1. After 35 days of growth, cells were collected by centrifugation and washed with 0.1 M NaCl+10 mM P-buffer, pH 7. The cell pellet was resuspended in 1 mL of the same buffer and stored at −20 °C until analysis.

For analysis, suspended cell material was transferred to a small vial and the vial was closed with a viton stopper. Subsequent additions were made to the closed vials using syringes and needles. The biomass was hydrolyzed in 7 M H_2SO_4 and oxidized with chromic acid (0.25 M final concentration). The vials were incubated at 55 °C overnight. The concentrations of $^{12}CO_2$ and $^{13}CO_2$ in the headspace were analyzed by GC with mass-spectrometric detection, using a DSQ-Trace GC Ultra system (Thermo Electron Corporation) with a QPlot column operated at 50 °C.

Other analytical methods

Cell pellets originating from the growth curve experiment were washed with 0.1 M NaCl and extracted with acetone to remove elemental sulfur. The pelleted cells were lysed in 1 M NaOH at 46 °C for 30 min and the protein concentration was measured using the Lowry method (Lowry *et al.*, 1951). Thiosulfate and sulfate concentrations were analyzed by HPLC using an Ionpac AS9-SC column and an ED40 electrochemical detector (Dionex, Sunnyvale). The acetone extracts were assayed for elemental sulfur (Sörbo, 1957). The O_2 concentration was analyzed using a gas chromatograph (GC 14B, Shimadzu) equipped with a molsieve column and a thermal conductivity detector.

Bacterial suspensions for transmission electron microscopy (TEM) were adsorbed on a carbon-coated 400-mesh copper grid and fixed for 1 min in 3% glutaraldehyde (prepared in 0.15 M phosphate buffer, pH 7.2) at room temperature. Negative staining was performed according to Bradley (1962) by adding one drop of 1% uranyl acetate (aqueous solution) to the fixed sample and incubated for 30 s. The sample was subsequently vacuum dried and examined under a 100-kV transmission electron microscope (JEM-1010, JEOL Ltd, Tokyo, Japan).

Amplification and phylogenetic analysis of 16S rRNA genes and putative genes involved in CO_2 fixation

Total DNA from strains J10, L70 and LD2 was extracted using the UltraClean Soil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA). The nearly complete 16S rRNA gene was amplified using primers 63F and 1387R, and the PCR product was purified using the QIAquick PCR purification kit (Qiagen, Germany). Sequencing was performed by BaseClear (Leiden, the Netherlands) using 63F, 341F, 907R and 1387R primers (Table 1).

Described degenerate oligonucleotide primers for the amplification of fragments of genes encoding 2-oxoglutarate : ferredoxin oxidoreductase (OorA) of the reductive tricarboxylic acid (TCA) cycle and Rubisco type I (CbbL) and type II (CbbM) of the CBB cycle were used to test for the presence of these genes in the genome of strain J10, *M. gryphiswaldense* and *M. magnetotacticum*. Oligonucleotide primers for *oorA* were 67F and 177R (Campbell *et al.*, 2003). Primer combinations for *cbbL* were 379F–634R (Takai *et al.*, 2005), and for *cbbM* 519F–918R (Campbell & Cary, 2004) and 328F–1124R (Spiridonova *et al.*, 2004) were used (Table 1). Positive controls were included using genomic DNA of microorganisms known to possess the listed genes: *Thermotoga maritima*, *Thermoanaerobacter tengcongensis* and *Desulfovibrio vulgaris* were used as positive controls for *oorA*, *Acidithiobacillus ferrooxidans* for *cbbL* and *Thiobacillus denitrificans* for *cbbM*. Negative controls with DNA of microorganisms lacking the genes, as well as controls without a template, were included.

PCR conditions were as follows: denaturation at 94 °C for 2–3 min, followed by 35 cycles of 94 °C for 30 s, the appropriate annealing temperature for 40 s and extension at 72 °C with a duration suitable for the size of the expected product. The cycles were followed by a final extension period of 5–7 min at 72 °C. For different primer combinations, the

Table 1. Oligonucleotide primers used in this study

Target	Primers	Sequence 5′ → 3′	References
16S rRNA gene	63F	CAGGCCTAACACATGCAAGTC	Marchesi *et al*. (1998)
	1387R	GGGCGGWGTGTACAAGGC	Marchesi *et al*. (1998)
	341F	CCTACGGGAGGCAGCAG	Muyzer *et al*. (1993)
	907R	CCGTCAATTCCTTTRAGTTT	Lane (1991)
rTCA cycle (*oorA*)	67F	TTCTTCGCTGGGTAYCCNATHACNCC	Campbell *et al*. (2003)
	177R	CATACCAGCTATYTCRTCYTCCATYTG	Campbell *et al*. (2003)
Rubisco type I (*cbbL*)	379F	GACCAGTCGGYAAYGTNTTYGGNTTYAA	Takai *et al*. (2005)
	634R	CTGACCAGTCTNAYRTTYTCRTCRTCYTT	Takai *et al*. (2005)
Rubisco type II (*cbbM*)	519F	TTCTGGCTGGGBGGHGAYTTYATYAARAAYGACGA	Campbell & Cary (2004)
	918R	CCGTGRCCRGCVCGRTGGTARTG	Campbell & Cary (2004)
	328F	GGHAACAACCARGGYATGGGYGA	Spiridonova *et al*. (2004)
	1124R	CGHAGIGCGTTCATGCCRCC	Spiridonova *et al*. (2004)

Designations for degenerated positions are as follows: R = A, G; Y = C, T; W = A, T; B = C, G, T; H = A, C, T; V = A, C, G; N = A, C, G, T; I = inosine.

annealing temperatures applied were 52 °C for oorA 67F–177R, 54 °C for cbbL 379F–634R, 56 °C for cbbM 519F–918R and 60 °C for cbbM 328F–1124R. The cbbM 328F–1124R amplicons obtained were sequenced using standard Sanger technology and the Big Dye Terminator v3.1 sequencing reaction kit using an ABI 3730 DNA automatic sequencer (Applied Biosystems).

Partial sequences of 16S rRNA and of Rubisco type II genes were assembled using the CAP3 sequence assembly tool (http://pbil.univ-lyon1.fr/cap3.php). Comparative analysis of the assembled gene sequence with sequences in the NCBI database was carried out using BLAST (http://www.ncbi.nlm.nih.gov). CbbM protein sequences were retrieved from the UniProt database (http://www.uniprot.org). The 16S rRNA gene sequences and CbbM protein sequences were aligned in CLUSTALX and phylogenetic trees were generated using the neighbor-joining method. Bootstrap values were calculated for 1000 replicates (Thompson *et al*., 1997). The partial 16S rRNA gene sequences of strains LD2, L70 and J10 have been deposited in GenBank under accession numbers FJ860935–FJ860937. The CbbM sequence of strain J10 is available in GenBank under number FJ657517.

Results

Microprofiles of sulfide and oxygen

Gradients of oxygen and total sulfide were measured in inoculated gradient microcosms and in sterile systems. In the sterile systems, the OATZ shifted from 22 to 26 mm depth below the surface after 2.2 days of incubation to 6–12 mm depth after 7.1 days. For the gradient microcosms (time of sediment addition at $t = 1.75$ days), the zone in which oxygen and sulfide gradient overlap was much more narrow and positioned at a lower depth in the column (5–6 mm after 7.1 days of total incubation time). Over time, the OATZ moved further upwards to 3.5–4 mm after 17.2 days. Measured profiles for $t = 7.1$ days are shown in Fig. 1. The total flux of sulfide in the OATZ was 2.3 pmol cm^{-2} s^{-1} for the control system and 7.2 for the gradient microcosm. Over time, the flux in the OATZ of the gradient microcosm increased to 10.1 at 9.1 days and 13.6 pmol cm^{-2} s^{-1} at 17.2 days of total incubation time. Using the diffusive flux that was estimated from the near-linear concentration gradients of sulfide and oxygen outside the OATZ and the average amount of sulfide and oxygen that is present in the OATZ, the turnover time of sulfide and oxygen was calculated. For the control columns in which only chemical oxidation is taking place ($t = 2.2$ and 7.1 days), the turnover time of both sulfide and oxygen was about equal, with an average value of 0.71 ± 0.04 h. In contrast, in the gradient microcosms turnover times were much faster, with a 30–33-s turnover time for both sulfide and oxygen determined at $t = 7.1$ days of total incubation time. A plate of bacterial cells was present at the OATZ of the gradient microcosms. Microscopic examination showed the dominant presence of highly motile spirillum-shaped cells.

FeS gradient microcosms and isolation

Gradient microcosms with FeS as the substrate showed the development of a thin plate of cells after *c*. 2 weeks. The tubes contained opposing gradients of Fe(II) and sulfide, derived from FeS present in the bottom of the tube, and oxygen from air at the top of the tube. The cells were present at the oxic–anoxic interface, as was indicated by the color of the redox indicator resazurin in the medium. From the thin plate of cells, subsamples were cultured in FeS gradient microcosms a few times, and subsequently transferred to a dilution series in gradient systems with either sulfide (10 mM Na_2S) or ferrous iron (10 mM $Fe(II)Cl_2$) in the bottom plug.

In the sulfide dilution series, growth occurred up to a dilution of 10^7 cells taken from the OATZ, and a culture strongly dominated by motile spirilla was obtained. Following a second dilution series in sulfide–oxygen gradients, the

culture was purified using a dilution series in a liquid culture with thiosulfate as the electron donor under microaerophilic conditions. The isolate was designated J10.

Gradient enrichment cultures with $Fe(II)Cl_2$ showed a band of Fe(III)-hydroxide, which may be the product of chemical or biological oxidation of ferrous iron with oxygen. Just below this band, in the anaerobic part of the gradient, a very thin plate of bacteria was visible. This suggested that the bacteria present in this plate were utilizing freshly precipitated Fe(III) hydroxide as the electron acceptor, while utilizing organic compounds from the agarose as the electron donor. Cells from the bacterial plate of two different gradient systems with $Fe(II)Cl_2$ were transferred to two dilution series of anaerobic batch cultures with freshly prepared amorphous Fe(III) hydroxide as the electron acceptor and lactate as the electron donor. This resulted in the isolation of strains L70 and LD2, both of which are also motile spirilla.

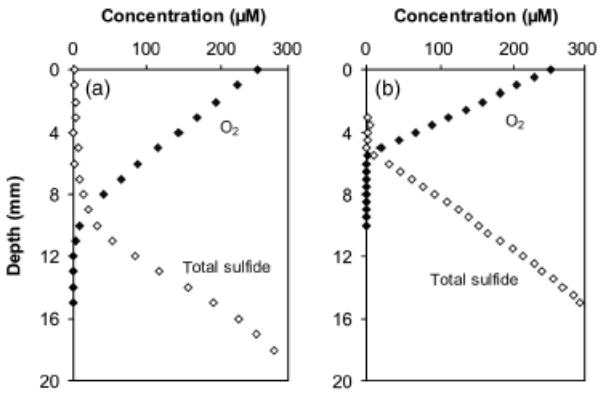

Fig. 1. Oxygen and total sulfide profiles in gradient microcosms, (a) control and (b) with 0.5 mL of sediment added at $t = 1.75$ days. Measurements were taken after 7.1 days of total incubation time. Zero depth is the air/agar-stabilized medium interface. The microcosms initially contained 10 mL of 10 mM sulfide with 2% agar and 30 mL of medium with 0.2% agar; the initial sulfide/nonsulfide interface was at a depth of 53 mm.

Phylogenetic analysis based on the 16S rRNA gene sequence

Phylogenetic analysis of nearly complete 16S rRNA gene sequences showed that the three isolated strains are most closely related to the genera *Magnetospirillum* and *Dechlorospirillum* within the *Alphaproteobacteria* (Fig. 2). The 16S rRNA gene of strain J10 has 99.6% sequence identity with the 16S rRNA gene of the magnetotactic bacterium *M. gryphiswaldense* (Schleifer *et al.*, 1991). These strains are separate from a cluster that contains *M. magnetotacticum* (Blakemore *et al.*, 1979) and *Magnetospirillum* species capable of degradation of aromatic compounds, for example, strains CC-26 and TS-6 (Shinoda *et al.*, 2000; Kawaguchi *et al.*, 2006). The 16S rRNA gene sequence identity of strain J10 with *Magnetospirillum* species in the latter cluster is around 95%. The genus *Phaeospirillum* (formerly *Rhodospirillum*), which contains phototrophic bacteria, forms another branch in this cluster.

Strain L70 is more closely related to *Dechlorospirillum* strains, which are capable of using (per)chlorate as an electron acceptor (Coates *et al.*, 1999; Bender *et al.*, 2004). The sequence identity of the 16S rRNA gene of strain L70 with *Dechlorospirillum* strains WD, DB and VDY is 99.2–99.4%. Strain LD2 is approximately equally related to *Dechlorospirillum* strains WD, DB and VDY (97.6–97.8% 16S rRNA gene sequence identity) and *Magnetospirillum* strains MSM-3 and MSM-4 (97.4–97.5%). Based on these

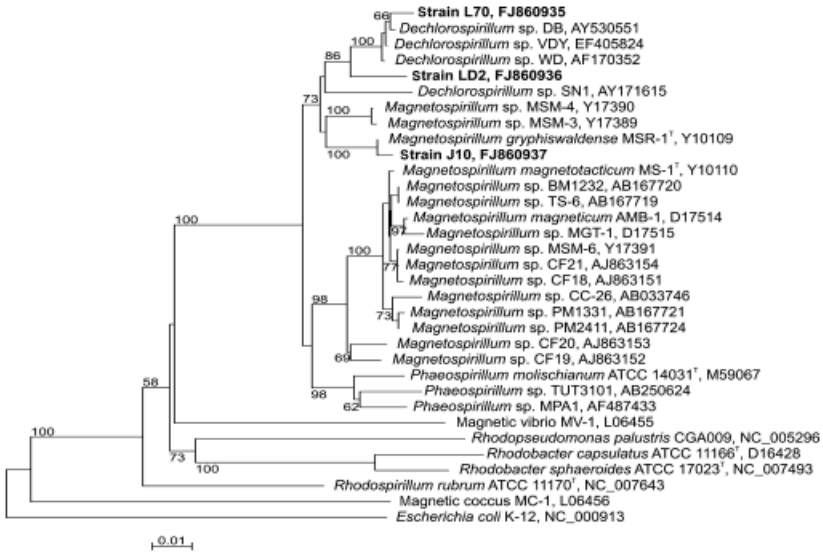

Fig. 2. A phylogenetic tree based on the 16S rRNA gene sequence comparisons of strains J10, L70 and LD2 (in bold), cultured species of the genera *Magnetospirillum*, *Phaeospirillum*, *Dechlorospirillum*, magnetotactic strains MV-1 and MC-1, and selected phototrophic purple nonsulfur bacteria, all of which belong to the *Alphaproteobacteria*. The tree was constructed using the neighbor-joining method with *Escherichia coli* as the outgroup. Numbers indicate percentage bootstrap sampling using 1000 replicates.

levels of sequence identity, strain LD2 likely represents a separate species (Stackebrandt & Ebers, 2006).

Growth characteristics of the novel strains

Autotrophic growth of the novel isolated strains with sulfide or ferrous iron as an electron donor was tested in agarose-stabilized gradient systems. Strain J10 grew with sulfide, but not with ferrous iron. Strains L70 and LD2 did not grow in gradient systems with sulfide and did not oxidize ferrous iron. Similar to the enrichments, the growth of strains L70 and LD2 was observed in gradient systems with ferrous iron; however, a plate of cells developed below the oxic–anoxic interface where ferric hydroxide derived from chemical oxidation of Fe(II) with oxygen was deposited. Strains L70 and LD2 were able to grow with short organic acids such as acetate or lactate as an electron donor. As an electron acceptor, low concentrations of oxygen ($< 2\%$ in the headspace) and nitrate could be used, but not perchlorate, chlorate, sulfate and fumarate.

Cultivation of the three isolates under microaerobic conditions using a medium supplemented with Fe-citrate did not result in magnetotactic behavior. For all three isolates, cells were extremely motile with a forward-and-backward corkscrew motion. TEM showed the presence of a single polar flagellum at each pole (Fig. 3). Cells of strain J10 differed strongly in size depending on the growth conditions. Cells grown with sulfide were longer, with more turns of the helical coil compared with cells grown with acetate (Fig. 3a and b). The TEM images showed areas differing in electron density, but the presence of magnetite crystals, which should appear as very electron-dense particles, was not observed. Cells of strains L70 and LD2 (Fig. 3d and e) also contained round areas with lighter electron density, which may indicate the presence of storage polymers. In addition, linear structures could be seen on the surface of cells of strain LD2 (Fig. 3f) that might be involved in anchoring of the flagella.

Liquid batch culture experiments showed that strain J10 is able to gain energy for growth from the oxidation of thiosulfate with low concentrations of oxygen in media with bicarbonate/CO_2 as the sole carbon source (Fig. 4). No organic compounds were added to the medium, except vitamins (1 mL of the vitamin mixture per liter of the medium). Thiosulfate was oxidized completely to sulfate, which resulted in the production of *c.* 4 mg of protein per millimole of thiosulfate converted (Fig. 4a). Assuming a protein concentration of 60% in the dry weight, and hence a yield of 6.7 g dry weight mol^{-1} thiosulfate, the amount of biomass C produced is *c.* 87 mg C L^{-1}. The organic C concentration in the medium derived from the vitamin solution is only about 25 μg C L^{-1}, which strongly suggests that strain J10 grows autotrophically. The maximum observed conversion rate (Fig. 4a) was *c.* 12 mmol thiosulfate g^{-1} protein h^{-1} (calculated for days 19–23), resulting in a maximum growth rate of 0.05 h^{-1}. The growth values are well within the range observed for other (facultative) autotrophic thiosulfate-oxidizing microorganisms, for which growth rates ranged from 0.04 to 0.6 h^{-1}, and yields were in the range of 2.5–20.6 g dry weight mol^{-1} thiosulfate (data collected by Banciu *et al.* 2004).

Oxygen was supplied to statically incubated cultures in a fed-batch manner, ensuring that the oxygen concentration in the headspace was a maximum of 2% (Fig. 4b). The total amount of oxygen consumed agreed well with the amount of thiosulfate converted. The observed stoichiometry was 1.9 : 1 $O_2 : S_2O_3$, whereas the theoretical ratio was 1.7 : 1 based on the conversion of thiosulfate to sulfate and the generation of reducing equivalents from thiosulfate for the

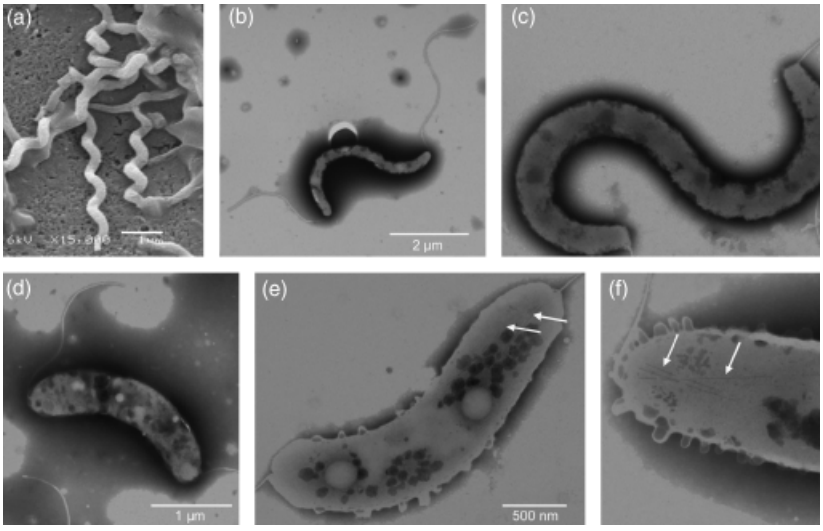

Fig. 3. Scanning and transmission electron micrographs of the three novel strains. (a) SEM of strain J10 grown autotrophically with thiosulfate, (b–f) TEM of negatively stained cells of strain J10 (b, c), L70 (d) and LD2 (e, f) grown with acetate. Arrows indicate linear structures observed in strain LD2.

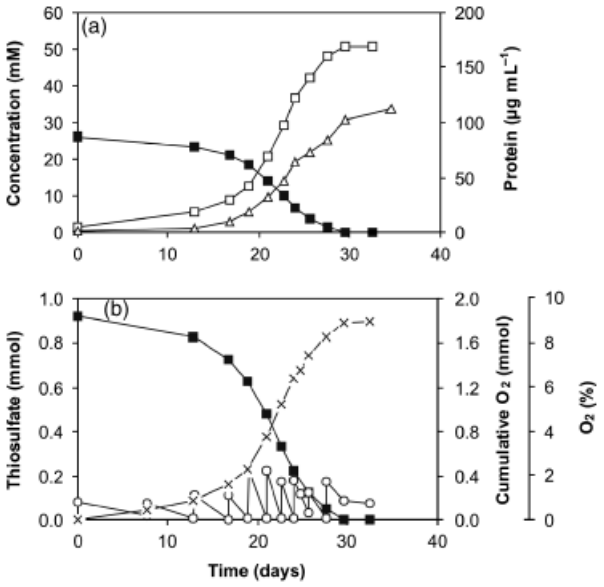

Fig. 4. Growth of strain J10 on thiosulfate. (a) Conversion of thiosulfate (■) to sulfate (□) coupled to growth, measured as protein (Δ). (b) Thiosulfate (■) is oxidized with oxygen (×) under microaerophilic conditions indicated by the oxygen concentration in the headspace (○). Data shown are a representative example of three replicates.

production of biomass. In batch cultures where oxygen was depleted or too much oxygen was present, elemental sulfur was formed. Subsequent prolonged incubation at an adequate oxygen concentration resulted in the oxidation of the produced sulfur to sulfate (data not shown).

Cultures of strain J10 grown with thiosulfate under microaerobic conditions, which were supplied with ^{13}C bicarbonate as the carbon source, showed a ^{13}C signature of the biomass of $81.2 \pm 1.1\%$ ($n = 3$). The ^{13}C content of the added bicarbonate was 99.3%, and some ^{12}C carbon was present from the inoculum (both in the cell material and bicarbonate in the liquid) and the vitamin solution. In identically grown cultures with unlabeled bicarbonate, the fraction of ^{13}C in the assimilated carbon was $1.24 \pm 0.05\%$ ($n = 3$), which is close to the natural abundance of the ^{13}C heavy isotope of 1.11%. The observed incorporation of ^{13}C from bicarbonate into biomass confirms the autotrophic growth of strain J10.

Besides thiosulfate and sulfur, strain J10 also seems to use sulfide as an electron donor. Experiments with sulfide were carried out in agarose-stabilized gradient systems, and therefore the utilization of organic carbon derived from agarose cannot be ruled out. Type strains *M. gryphiswaldense* and *M. magnetotacticum* were also tested for growth in agarose-stabilized sulfide–oxygen gradient systems. In tubes inoculated with *M. gryphiswaldense*, a plate of cells was visible at the oxic–anoxic interface. Dilution series in gradient systems with cells from the cell plate showed growth up to a dilution of 10^{10}. In gradient systems inoculated with *M. magnetotacticum*, a plate of cells did not develop. Transfers of acetate-grown cells of *M. gryphiswaldense* and *M. magnetotacticum* into liquid batch cultures with thiosulfate under microaerobic conditions did not grow.

Detection and phylogenetic analysis of *cbbM*

The presence of putative genes involved in CO_2 fixation was studied using degenerate oligonucleotide primers that target genes encoding Rubisco in the CBB cycle, and the 2-oxoglutarate:ferredoxin oxidoreductase (oor) step in the reductive TCA (rTCA) cycle. Selective amplification resulted in amplicons of the correct size for all positive controls. For strain J10, *M. gryphiswaldense* and *M. magnetotacticum*, amplification products were obtained for the gene encoding Rubisco type II (*cbbM*). Two different primer sets were used for *cbbM*, which both resulted in products of the correct size. Using specific primers for genes encoding Rubisco type I (*cbbL*) and the *oor* step in the rTCA cycle, no amplification products were obtained. Another indication for the absence of an rTCA cycle is the inability of strain J10 to reduce fumarate, which was tested in batch cultures with acetate as the electron donor and fumarate as the electron acceptor. The capacity of strain J10 to use acetate as the electron donor was ascertained in batch experiments with 1–2% oxygen in the headspace, in which growth occurred.

The partial *cbbM* gene sequence obtained for strain J10 (717 bp) has 91% sequence identity with *M. gryphiswaldense* (Richter *et al.*, 2007) and *c.* 78% identity with *M. magnetotacticum* (Bazylinski *et al.*, 2004a) and *M. magneticum* (Matsunaga *et al.*, 2005). CbbM of strain J10 clusters together with analogous sequences detected in the *Alphaproteobacteria*, except for the CbbM sequence of magnetic vibrio MV-1 (Fig. 5). With respect to other CbbM sequences in the database, CbbM of *Rhodospirillum rubrum* is the closest branch to the magnetospirilla. This differs from the 16S rRNA gene relatedness, where MV-1 and also *Rhodospeudomonas* and *Rhodobacter* species are more closely related to the magnetospirilla than *R. rubrum* (Fig. 2). At the amino acid level, the identity of CbbM of strain J10 is 96% with *M. gryphiswaldense* and 75–82% with analogous sequences of other *Magnetospirillum* strains and phototrophic purple nonsulfur bacteria (Anderson & Fuller, 1969; Wagner *et al.*, 1988). The CbbM sequence of the alphaproteobacterium magnetic vibrio MV-1 (Bazylinski *et al.*, 2004a) clusters with sequences found in the *Beta-* and the *Gammaproteobacteria*. These include several autotrophic sulfide-oxidizing species, some of which occur as symbionts in tubeworms and clams.

Discussion

Microbial sulfide oxidation in the OATZ

Microprofiles of sulfide and oxygen in gradient microcosms and in control columns (Fig. 1) showed clear differences

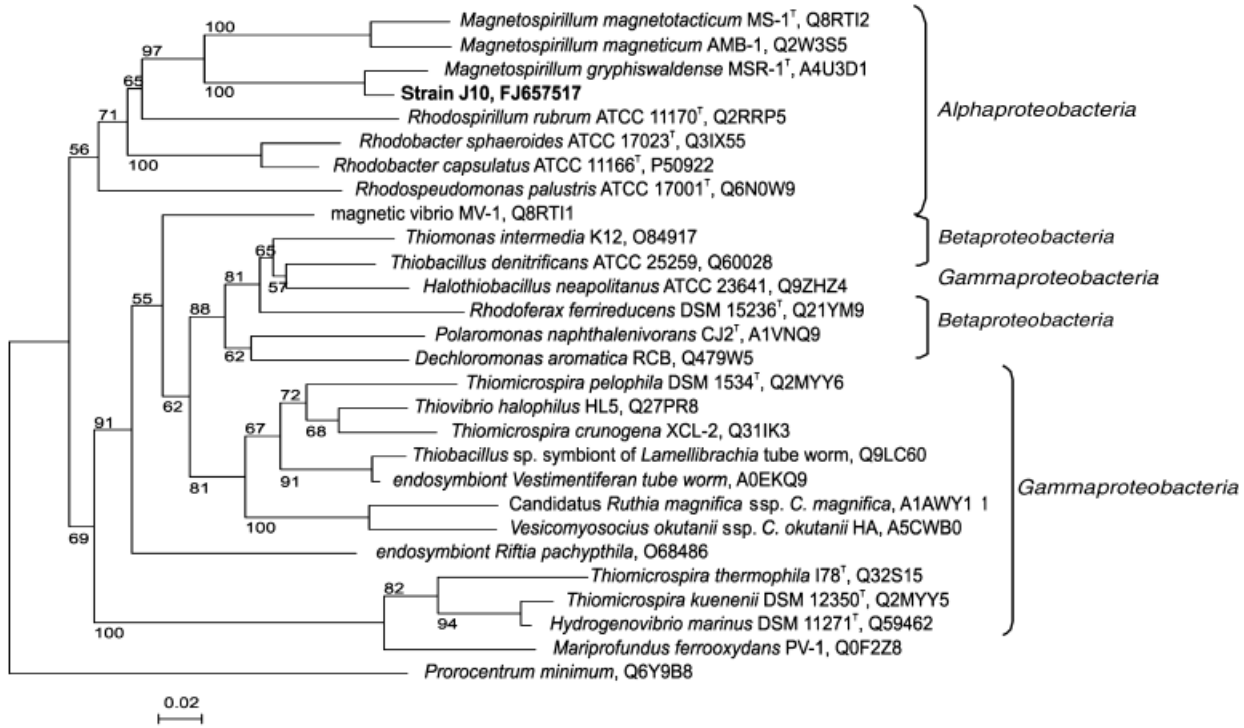

Fig. 5. Phylogenetic tree constructed for amino acid sequences of bacterial Rubisco type II (CbbM; strain J10 in bold) with dinoflagellate species *Prorocentrum minimum* as the outgroup. The scale bar indicates the evolutionary distance corresponding to 2 substitutions per 100 amino acids. Numbers at the branches indicate percentage bootstrap values calculated for 1000 replicates. Affiliation to the *Alpha-*, *Beta-* or *Gamma-* subclasses of the *Proteobacteria* is also indicated.

indicative of active microbial oxidation of sulfide. In the microcosms, there was less overlap of sulfide and oxygen gradients resulting from higher fluxes in the OATZ. The total flux is comprised of the diffusive flux and the utilization of sulfide and oxygen for chemical and biological oxidation processes. Five days after addition of sediment to the microcosms, the sulfide flux was more than three times larger compared with the control, and calculated turnover times were 0.5 min for the sediment microcosms and 42 min for the control. Turnover values for microbially mediated sulfide oxidation are in good agreement with observations by Nelson *et al.* (1986) for sulfide–oxygen gradients inoculated with a pure culture of a *Beggiatoa* sp. In contrast, turnover values for chemical oxidation are about half compared with the latter study, which may be caused by the presence of particulate metals in the sediment that catalyze sulfide oxidation (Zhang & Millero, 1994; Yao & Millero, 1996). The observed straight concentration gradient of oxygen above the OATZ suggests that aerobic microbial growth utilizing compounds from the inoculum or the agar is of minor importance.

The observation of active biological oxidation of sulfide at the OATZ and the dominant presence of spirillum-shaped bacteria at this position suggests that at least part of this microbial population is involved in sulfide oxidation. The population probably also comprises sulfur-oxidizing strain J10, which was isolated from the same sediment. When we assume that sulfide and thiosulfate are metabolized at the same rate (Timmer-ten Hoor, 1981; Stefess *et al.*, 1996) and use the observed conversion rate of strain J10 as a guideline for sulfur-oxidizing metabolism, the biomass density of the sulfur-oxidizing population can be estimated. From the observed flux in the overlap zone and the conversion rate, we estimate a biomass concentration of 15 μg protein mL^{-1} or *c.* 2×10^8 cells mL^{-1}. This high cell density confirms that sulfide-oxidizing bacteria constitute an important part of the bacterial population in the OATZ.

Isolation and phylogenetic affiliation of novel strains

Gradient microcosms with sediment from the OATZ of a freshwater ditch resulted in microbial populations that were present at the very narrow interface of oxic–anoxic conditions. In FeS microcosms both ferrous iron and sulfide could act as electron donors and therefore transfers were made to gradient systems with either sulfide or ferrous iron. Only in the case of sulfide did this result in the enrichment and

isolation of a bacterial strain (J10) that oxidizes the supplied electron donor. Autotrophic growth of strain J10 was confirmed by assimilation of heavy isotopic ^{13}C bicarbonate. From gradient systems with ferrous iron, two isolates (strains L70 and LD2) capable of using low concentrations of oxygen and nitrate as an electron acceptor were obtained.

The three isolated strains are motile spirilla using bipolar flagella and are phylogenetically relatively closely related, despite their different metabolic capacities. The 16S rRNA gene sequences of the three strains are $>$ 95.8% identical, clustering with the genera *Magnetospirillum* and *Dechlorospirillum* in the *Alphaproteobacteria*. The closest relative of strain J10 is *M. gryphiswaldense*, with 99.6% sequence identity.

Strain J10 is a novel facultative lithoautotrophic *Magnetospirillum* sp.

Species of the genus *Magnetospirillum* are best known for the ability to produce high-purity magnetite crystals in the cell; however, not all isolated strains in this genus are magnetotactic (e.g. strains CC-26 and TS-6; Shinoda *et al.*, 2000, 2005). None of the three novel isolated strains was found to align in the magnetic field of a bar magnet or to contain magnetite crystals inside the cell. The property of magnetosome formation may be easily lost upon repeated transfer (Schübbe *et al.*, 2003; Flies *et al.*, 2005b; Ullrich *et al.*, 2005), and therefore it is possible that this capacity was initially present in strain J10, but was lost during the isolation process. Many genes involved in the magnetosome synthesis and assembly are clustered within a magnetosome island. Phylogenetic analyses and the composition of magnetosome islands in the genomes of magnetospirilla and strains MC-1 and MV-1 suggest that magnetotaxis evolved by horizontal gene transfer (Schübbe *et al.*, 2003; Ullrich *et al.*, 2005; Jogler *et al.*, 2009).

The novel *Magnetospirillum* strain J10 is able to derive energy from the oxidation of thiosulfate, sulfur and, probably, sulfide under microaerobic conditions. Like the other described magnetospirilla, strain J10 is also able to use organic compounds such as acetate for growth at microaerobic levels of oxygen (Maratea & Blakemore, 1981; Schleifer *et al.*, 1991). In the enrichment and isolation process of strain J10, gradient systems were applied to provide a combination of microaerobic conditions and a low-sulfide concentration. Using this approach, several strains of microaerophilic magnetotactic spirilla have been isolated previously, but none of these was able to grow without an organic substrate (Schüler *et al.*, 1999; Flies *et al.*, 2005b). Therefore, while sulfide/oxygen gradients are instrumental for maintaining microaerobic conditions, growth in these agarose-stabilized gradients does not necessarily implicate utilization of sulfide as an electron donor; a situation that may also be the case for our growth experiment with *M. gryphiswaldense* in sulfide–oxygen gradients. We intend to study the possible utilization of sulfide for the growth of *M. gryphiswaldense* further in a chemically defined medium and under controlled oxygen conditions.

Indications for use of reduced sulfur compounds by *Magnetospirillum* species are present in the (draft) genome sequences. The reduced sulfur-oxidizing Sox pathway is one of the enzyme systems that may be used by chemolithotrophic sulfur-oxidizing bacteria and many *Alphaproteobacteria* contain genes that encode this pathway. A number of sox genes have also been detected in the genomes of *M. gryphiswaldense* and other magnetospirilla (Friedrich *et al.*, 2005; Meyer *et al.*, 2007; Ogawa *et al.*, 2008). Reverse dissimilatory sulfite reductase (Dsr) catalyzes the oxidation of sulfur to sulfite, and in the genomes of *Magnetospirillum* species putative *dsr* operons have been identified (Dahl *et al.*, 2008).

Marine magnetotactic vibrios MV-1 and MV-2, and coccus MC-1, all *Alphaproteobacteria*, also grow chemolithoautotrophically with sulfide or thiosulfate as electron donors under microaerobic conditions. Strains MV-1 and MV-2 use the CBB pathway for autotrophic growth, while strain MC-1 appeared to use the rTCA cycle for autotrophy (Bazylinski *et al.*, 2004a; Williams *et al.*, 2006). Strain J10 possesses a Rubisco type II gene (*cbbM*) with 91–78% sequence identity to *cbbM* of *M. gryphiswaldense*, *M. magnetotacticum* and *M. magneticum*. Type II Rubisco is mostly found in anaerobic and microaerophilic prokaryotes owing to their poor affinity to CO_2 and low capacity to discriminate between CO_2 and O_2, in contrast to the high-affinity type I that is present in aerobic prokaryotes (Hernandez *et al.*, 1996; Tabita, 1999). The presence of Rubisco type II is therefore consistent with the obligate microaerobic characteristic of magnetospirilla. An rTCA cycle is probably absent in strain J10. Together, these results suggest that strain J10 uses the CBB pathway for CO_2 fixation and autotrophic growth.

Ecological niche

Magnetospirilla are involved in the geochemical cycles of iron, nitrogen and sulfur. Iron is incorporated into magnetite that may be deposited in sediments and preserved as magnetofossils (Kopp & Kirschvink, 2008). In addition, both oxidized and reduced iron may be used in respiration (Guerin & Blakemore, 1992). Dinitrogen gas may be assimilated through nitrogenase activity (Bazylinski *et al.*, 2000) and nitrate may be used as an electron acceptor in respiration (Escalante-Semerena *et al.*, 1980; Bazylinski & Blakemore, 1983; Shinoda *et al.*, 2000, 2005). Interaction with the sulfur cycle has been implicated previously for several other sulfur-oxidizing magnetotactic bacteria (Bazylinski *et al.*,

2004a; Williams *et al*., 2006) and for magnetotactic bacteria that produce greigite [mixed Fe(II)/Fe(III) sulfide] rather than magnetite (DeLong *et al*., 1993; Simmons *et al*., 2004). The present study shows the involvement of magnetospirilla with the sulfur cycle through the utilization of reduced sulfur compounds as an energy source for growth.

Incubations of sediment in FeS gradient microcosms resulted in 10^7 cells per 100 μL present at the OATZ that were involved in sulfide oxidation. Transfer to dilution series yielded the dominant sulfide-oxidizing species, which was identified as a *Magnetospirillum* sp. (strain J10). The utilization of sulfide as the electron donor, combined with the obligate microaerophilic growth by strain J10, fits the natural conditions at the oxic–anoxic interface where *Magnetospirillum* species thrive and opposing gradients of oxygen and sulfide exist. In the natural environment, strain J10 would likely benefit from a facultative autotrophic or mixotrophic life style (Gottschal & Kuenen, 1980; Kelly, 1999). Energy available in reduced sulfur compounds can be captured, while at the same time, small organic acids may be used as an electron donor and/or a carbon source.

Acknowledgements

We thank Paul Roosken and Darko Korosic (Department of Biotechnology, Delft University of Technology) for technical assistance and Astrid Paulitsch (Wetsus, Leeuwarden, the Netherlands) for the SEM image of strain J10. We are also grateful to Dr Lucian Barbu-Tudoran and Professor Dr Constantin Craciun (Electron Microscopy Center, Babes-Bolyai University of Cluj-Napoca, Romania) for TEM images. Financial support by DIOC Water of Delft University of Technology is gratefully acknowledged.

References

Anderson RE & Fuller RC (1969) Photosynthesis in *Rhodospirillum rubrum*. IV Isolation and characterization of ribulose 1,5-diphosphate carboxylase. *J Biol Chem* **244**: 3105–3109.

Banciu H, Sorokin DY, Kleerebezem R, Muyzer G, Galinski EA & Kuenen JG (2004) Growth kinetics of haloalkaliphilic, sulfur-oxidizing bacterium *Thioalkalivibrio versutus* strain ALJ 15 in continuous culture. *Extremophiles* **8**: 185–192.

Bazylinski DA & Blakemore RP (1983) Denitrification and assimilatory nitrate reduction in *Aquaspirillum magnetotacticum*. *Appl Environ Microb* **46**: 1118–1124.

Bazylinski DA & Frankel RB (2004b) Magnetosome formation in prokaryotes. *Nat Rev Microbiol* **2**: 217–230.

Bazylinski DA, Dean AJ, Schüler D, Phillips EJP & Lovley DR (2000) N_2-dependent growth and nitrogenase activity in the metal-metabolizing bacteria, *Geobacter* and *Magnetospirillum* species. *Environ Microbiol* **2**: 266–273.

Bazylinski DA, Dean AJ, Williams TJ, Kimble Long L, Middleton SL & Dubbels BL (2004a) Chemolithoautotrophy in the marine, magnetotactic bacterial strains MV-1 and MV-2. *Arch Microbiol* **182**: 373–387.

Bender KS, Rice MR, Fugate WH, Coates JD & Achenbach LA (2004) Metabolic primers for detection of (per)chlorate-reducing bacteria in the environment and phylogenetic analysis of cld gene sequences. *Appl Environ Microb* **70**: 5651–5658.

Blakemore RP, Maratea D & Wolfe RS (1979) Isolation and pure culture of a freshwater magnetic spirillum in chemically defined medium. *J Bacteriol* **140**: 720–729.

Bradley DE (1962) A study of the negative staining process. *J Gen Microbiol* **29**: 503–516.

Broecker WS & Peng TH (1974) Gas-exchange rates between air and sea. *Tellus* **26**: 21–35.

Campbell BJ & Cary SC (2004) Abundance of reverse tricarboxylic acid cycle genes in free-living microorganisms at deep-sea hydrothermal vents. *Appl Environ Microb* **70**: 6282–6289.

Campbell BJ, Stein JL & Cary SC (2003) Evidence of chemolithoautotrophy in the bacterial community associated with *Alvinella pompejana*, a hydrothermal vent polychaete. *Appl Microbiol Biot* **69**: 5070–5078.

Coates JD, Michaelidou U, Bruce RA, O'Connor SM, Crespi JN & Achenbach LA (1999) Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria. *Appl Environ Microb* **65**: 5234–5241.

Dahl C, Schulte A, Stockdreher Y, Hong C, Grimm F, Sander J, Kim R, Kim SH & Shin DH (2008) Structural and molecular genetic insight into a widespread sulfur oxidation pathway. *J Mol Biol* **384**: 1287–1300.

DeLong EF, Frankel RB & Bazylinski DA (1993) Multiple evolutionary origins of magnetotaxis in bacteria. *Science* **259**: 803–806.

Emerson D & Moyer C (1997) Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. *Appl Environ Microb* **63**: 4784–4792.

Escalante-Semerena JC, Blakemore RP & Wolfe RS (1980) Nitrate dissimilation under microaerophilic conditions by a magnetic spirillum. *Appl Environ Microb* **40**: 429–430.

Flies CB, Jonkers HM, De Beer D, Bosselmann K, Böttcher ME & Schüler D (2005a) Diversity and vertical distribution of magnetotactic bacteria along chemical gradients in freshwater microcosms. *FEMS Microbiol Ecol* **52**: 185–195.

Flies CB, Peplies J & Schüler D (2005b) Combined approach for characterization of uncultivated magnetotactic bacteria from various aquatic environments. *Appl Environ Microb* **71**: 2723–2731.

Frankel RB, Bazylinski DA, Johnson MS & Taylor BL (1997) Magneto-aerotaxis in marine coccoid bacteria. *Biophys J* **73**: 994–1000.

Friedrich CG, Bardischewsky F, Rother D, Quentmeier A & Fischer J (2005) Prokaryotic sulfur oxidation. *Curr Opin Microbiol* **8**: 253–259.

Gottschal JC & Kuenen JG (1980) Mixotrophic growth of *Thiobacillus* A2 on acetate and thiosulfate as growth limiting substrates in the chemostat. *Arch Microbiol* **126**: 33–42.

Guerin WF & Blakemore RP (1992) Redox cycling of iron supports growth and magnetite synthesis by *Aquaspirillum magnetotacticum*. *Appl Environ Microb* **58**: 1102–1109.

Hernandez JM, Baker SH, Lorbach SC, Shively JM & Tabita FR (1996) Deduced amino acid sequence, functional expression, and unique enzymatic properties of the form I and form II ribulose bisphosphate carboxylase/oxygenase from the chemoautotrophic bacterium *Thiobacillus denitrificans*. *J Bacteriol* **178**: 347–356.

Jogler C, Kube M, Schubbe S, Ullrich S, Teeling H, Bazylinski DA, Reinhardt R & Schuler D (2009) Comparative analysis of magnetosome gene clusters in magnetotactic bacteria provides further evidence for horizontal gene transfer. *Environ Microbiol* **11**: 1267–1277.

Kawaguchi K, Shinoda Y, Yurimoto H, Sakai Y & Kato N (2006) Purification and characterization of benzoate-CoA ligase from *Magnetospirillum* sp. strain TS-6 capable of aerobic and anaerobic degradation of aromatic compounds. *FEMS Microbiol Lett* **257**: 208–213.

Kelly DP (1999) Thermodynamic aspects of energy conservation by chemolithotrophic sulfur bacteria in relation to the sulfur oxidation pathways. *Arch Microbiol* **171**: 219–229.

Kopp RE & Kirschvink JL (2008) The identification and biogeochemical interpretation of fossil magnetotactic bacteria. *Earth-Sci Rev* **86**: 42–61.

Lane DJ (1991) 16S/23S rRNA sequencing. *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds), pp. 115–175. John Wiley & Sons, Chichester, UK.

Li Y & Gregory S (1974) Diffusion of ions in sea water and in deep-sea sediments. *Geochim Cosmochim Acta* **38**: 703–714.

Lowry OH, Rosebrough NJ, Farr AL & Randall RJ (1951) Protein measurement with the Folin-phenol reagents. *J Biol Chem* **193**: 265–275.

Maratea D & Blakemore RP (1981) *Aquaspirillum magnetotacticum* sp. nov., a magnetic spirillum. *Int J Syst Bacteriol* **31**: 452–455.

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ & Wade WG (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microb* **64**: 795–799.

Matsunaga T, Sakaguchi T & Tadokoro F (1991) Magnetite formation by a magnetic bacterium capable of growing aerobically. *Appl Microbiol Biot* **35**: 651–655.

Matsunaga T, Okamura Y, Fukumori Y, Wahyudi AT, Murase Y & Takeyama H (2005) Complete genome sequence of the facultative anaerobic magnetotactic bacterium *Magnetospirillum* sp. strain AMB-1. *DNA Res* **12**: 157–166.

Meyer B, Imhoff JF & Kuever J (2007) Molecular analysis of the distribution and phylogeny of the *soxB* gene among sulfur-oxidizing bacteria – evolution of the Sox sulfur oxidation enzyme system. *Environ Microbiol* **9**: 2957–2977.

Muyzer G, de Waal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microb* **59**: 695–700.

Nelson DC, Jørgensen BB & Revsbech NP (1986) Growth pattern and yield of a chemoautotrophic *Beggiatoa* sp. in oxygen-sulfide microgradients. *Appl Environ Microb* **52**: 225–233.

Ogawa T, Furusawa T, Nomura R, Seo D, Hosoya-Matsuda N, Sakurai H & Inoue K (2008) SoxAX binding protein, a novel component of the thiosulfate-oxidizing multienzyme system in the green sulfur bacterium *Chlorobium tepidum*. *J Bacteriol* **190**: 6097–6110.

Pfennig N & Lippert KD (1966) Über das Vitamin B12-bedürfnis phototropher Schwefel bacterien. *Arch Microbiol* **55**: 245–256.

Pringault O, Kuhl M, de Wit R & Caumette P (1998) Growth of green sulphur bacteria in experimental benthic oxygen, sulphide, pH and light gradients. *Microbiol-UK* **144**: 1051–1061.

Pringault O, Epping E, Guyoneaud R, Khalili A & Kuhl M (1999) Dynamics of anoxygenic photosynthesis in an experimental green sulphur bacteria biofilm. *Environ Microbiol* **1**: 295–305.

Richter M, Kube M, Bazylinski DA, Lombardot T, Glockner FO, Reinhardt R & Schuler D (2007) Comparative genome analysis of four magnetotactic bacteria reveals a complex set of group-specific genes implicated in magnetosome biomineralization and function. *J Bacteriol* **189**: 4899–4910.

Sakaguchi T, Arakaki A & Matsunaga T (2002) *Desulfovibrio magneticus* sp. nov., a novel sulfate-reducing bacterium that produces intracellular single-domain-sized magnetite particles. *Int J Syst Evol Micr* **52**: 215–221.

Schleifer KH, Schüler D, Spring S, Weizenegger M, Amann R, Ludwig W & Köhler M (1991) The genus *Magnetospirillum* gen. nov. Description of *Magnetospirillum gryphiswaldense* sp. nov. and transfer of *Aquaspirillum magnetotacticum* to *Magnetospirillum magnetotacticum* comb. nov. *Syst Appl Microbiol* **14**: 379–385.

Schübbe S, Kube M, Scheffel A, Waver C, Heyen U, Meyerdieks A, Madkour M, Mayer F, Reinhardt R & Schüler D (2003) Characterization of a spontaneous nonmagnetic mutant of *Magnetospirilum gryphiswaldense* reveals a large deletion comprising a putative magnetosome island. *J Bacteriol* **185**: 5779–5790.

Schüler D (2008) Genetics and cell biology of magnetosome formation in magnetotactic bacteria. *FEMS Microbiol Rev* **32**: 654–672.

Schüler D, Spring S & Bazylinski DA (1999) Improved technique for the isolation of magnetotactic spirilla from a freshwater sediment and their phylogenetic characterization. *Syst Appl Microbiol* **22**: 466–471.

Shinoda Y, Sakai Y, Ue M, Hiraishi A & Kato N (2000) Isolation and characterization of a new denitrifying spirillum capable of anaerobic degradation of phenol. *Appl Environ Microb* **66**: 1286–1291.

Shinoda Y, Akagi J, Uchihashi Y, Hiraishi A, Yukawa H, Yurimoto H, Sakai Y & Kato N (2005) Anaerobic degradation of aromatic compounds by *Magnetospirillum* strains: isolation and degradation genes. *Biosci Biotech Bioch* **69**: 1483–1491.

Simmons SL, Sievert SM, Frankel RB, Bazylinski DA & Edwards KJ (2004) Spatiotemporal distribution of marine magnetotactic bacteria in a seasonally stratified coastal salt pond. *Appl Environ Microb* **70**: 6230–6239.

Simmons SL, Bazylinski DA & Edwards KJ (2007) Population dynamics of marine magnetotactic bacteria in a meromictic salt pond described with qPCR. *Environ Microbiol* **9**: 2162–2174.

Sörbo B (1957) A colorimetric method for the determination of thiosulfate. *Biochim Biophys Acta* **23**: 412–416.

Spiridonova EM, Berg IA, Kolganova TV, Ivanovsky RN, Kuznetsov BB & Tourova TP (2004) An oligonucleotide primer system for amplification of the ribulose-1,5-bisphosphate carboxylase/oxygenase genes of bacteria of various taxonomic groups. *Microbiology* **73**: 316–325.

Spring S, Amann R, Ludwig W, Schleifer KH & Petersen N (1992) Phylogenetic diversity and identification of nonculturable magnetotactic bacteria. *Syst Appl Microbiol* **15**: 116–122.

Spring S, Amann R, Ludwig W, Schleifer KH, Van Gemerden H & Petersen N (1993) Dominating role of an unusual magnetotactic bacterium in the microaerobic zone of a freshwater sediment. *Appl Environ Microb* **59**: 2397–2403.

Stackebrandt E & Ebers J (2006) Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* **Nov**: 152–155.

Stefess GC, Torremans RAM, deSchrijver R, Robertson LA & Kuenen JG (1996) Quantitative measurement of sulphur formation by steady state and transient state continuous cultures of autotrophic *Thiobacillus* species. *Appl Microbiol Biot* **45**: 169–175.

Tabita FR (1999) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: a different perspective. *Photosynth Res* **60**: 1–28.

Takai K, Campbell BJ, Cary SC *et al.* (2005) Enzymatic and genetic characterization of carbon and energy metabolisms by deep-sea hydrothermal chemolithoautotrophic isolates of *Epsiloproteobacteria*. *Appl Environ Microb* **71**: 7310–7320.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **24**: 4876–4882.

Timmer-ten Hoor A (1981) Cell yield and bioenergetics of *Thiomicrospira denitrificans* compared with *Thiobacillus denitrificans*. *A van Leeuw J Microb* **47**: 231–243.

Ullrich S, Kube M, Schübbe S, Reinhardt R & Schüler D (2005) A hypervariable 130-kilobase genomic region of *Magnetospirillum gryphiswaldense* comprises a magnetosome island which undergoes frequent rearrangements during stationary growth. *J Bacteriol* **187**: 7176–7184.

Wagner SJ, Stevens SEJ, Nixon BT, Lambert DH, Quivey RG & Tabita FR (1988) Nucleotide and deduced amino acid sequence of the *Rhodobacter sphaeroides* gene encoding form II ribulose-1,5-bisphosphate carboxylase/oxygenase and comparison with other deduced form I and II sequences. *FEMS Microbiol Lett* **55**: 217–222.

Williams TJ, Zhang CL, Scott JH & Bazylinski DA (2006) Evidence for autotrophy via the reverse tricarboxylic acid cycle in the marine magnetotactic coccus strain MC-1. *Appl Environ Microb* **72**: 1322–1329.

Wolin EA, Wolin MJ & Wolfe RS (1963) Formation of methane by bacterial extracts. *J Biol Chem* **238**: 2882–2886.

Yao WS & Millero FJ (1996) Oxidation of hydrogen sulfide by hydrous Fe(III) oxides in seawater. *Mar Chem* **52**: 1–16.

Zhang JZ & Millero FJ (1994) Kinetics of oxidation of hydrogen sulfide in natural waters. *Environmental Geochemistry of Sulfide Oxidation, ACS Symposium Series 550* (Alpers CN & Blowes DW, eds), pp. 393–409. American Chemical Society, Washington, DC.