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Desulfovibrio magneticus sp. nov., a novel sulfate-reducing bacterium that produces intracellular single-domain-sized magnetite particles

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A novel type of dissimilatory sulfate-reducing bacterium, designated strain RS-1^T, capable of producing intracellular magnetite particles (magnetosomes) was isolated from freshwater sulfide-rich sediments. Phylogenetic analysis based on 16S rDNA sequences revealed that RS-1^T is a member of the genus *Desulfovibrio*. Its closest known relative is *Desulfovibrio burkinensis* (sequence similarity of 98·7%). Strain RS-1^T contains desulfoviridin, *c*-type cytochromes and, unlike other *Desulfovibrio* spp., it possesses menaquinone MK-7(H_2) instead of MK-6 or MK-6(H_2). Strain RS-1^T is also unique compared with other members of *Desulfovibrio* in its ability to synthesize intracellular magnetite particles. A novel species, *Desulfovibrio magneticus* sp. nov., is proposed for RS-1^T (= ATCC 700980^T = DSM 13731^T), a sulfate-reducing magnetotactic bacterium.

Keywords: *Desulfovibrio magneticus* sp. nov., iron biomineralization, magnetotactic bacteria, sulfate-reducing bacteria, magnetite

INTRODUCTION

The abundant occurrence of magnetotactic bacteria has been observed in various aquatic environments. Most magnetotactic bacteria currently available in pure culture belong to the *α-Proteobacteria* (Eden *et al*., 1991; Schleifer *et al*., 1991; Burgess *et al*., 1993; DeLong *et al*., 1993). Discovery of multicellular magnetotactic prokaryotes containing magnetic iron sulfur crystals (Farina *et al*., 1990; Mann *et al*., 1990) suggests a role for magnetic biomineralization, even in sulfidic environments. Phylogenetic analysis has shown that magnetic iron sulfur-containing micro-organisms are related to the dissimilatory sulfate-reducing bacteria within the *δ-Proteobacteria* (DeLong *et al*., 1993). However, accurate physiological and biochemical characteristics of these micro-organisms are unknown.

Fe(III)-reducing bacteria are also involved in magnetite formation in aquatic environments owing to the dissimilatory reduction of ferric iron by anaerobic respiration. These organisms differ from magnetotactic bacteria in that they produce magnetite extracellularly and need ferric oxyhydroxide [poorly crystalline Fe(III) oxide] for formation of magnetite. Fe(III)-reducing bacteria occur in several genera of the *δ-Proteobacteria*, known species being, for instance, *Geobacter metallireducens* (Lovley *et al*., 1993), *Geobacter sulfurreducens* (Caccavo *et al*., 1994), *Pelobacter carbinolicus* (Lovley *et al*., 1995), *Desulfuromonas acetoxidans* (Roden & Lovley, 1993) and *Desulfuromonas palmitatis* (Coates *et al*., 1995). This group of organisms can be grown in pure culture and has been well-characterized taxonomically.

Strain RS-1^T is a dissimilatory sulfate-reducing obligate anaerobe capable of producing intracellular magnetite that responds to an external magnetic field. This bacterium is also capable of producing extracellular magnetic iron sulfide. Strain RS-1^T represents a bacterium with novel metabolic features within the magnetotactic bacteria, suggesting the presence of a hitherto unknown mechanism to produce magnetite crystals. Partial characterization (Sakaguchi *et al*., 1993) and phylogenetic analysis based on the partial 16S rDNA sequence (Kawaguchi *et al*., 1995) have

The DDBJ accession number for the 16S rDNA sequence of strain RS-1^T is D43944.

been carried out for strain RS-1^T. Results show that this magnetotactic bacterium belongs to the dissimilatory sulfate-reducing bacteria within the *δ-Proteobacteria* in the genus *Desulfovibrio*.

In this study, the characteristics and phylogenetic position of RS-1^T are described and the name *Desulfovibrio magneticus* sp. nov. is proposed.

METHODS

Isolation and culture conditions. Strain RS-1^T was isolated by non-magnetic enrichment from the aquatic phase of a sample from freshwater sediment of Kameno River waterway (Wakayama, Japan) (Sakaguchi *et al*., 1993, 1996). The isolation procedure involved a novel method without magnetic collection (Sakaguchi *et al*., 1996). The method consisted of incubation of sediments, enrichment of bacteria in the medium, isolation of enriched bacteria via colonies and observation of magnetite formation.

Cells of strain RS-1^T were anaerobically cultured and maintained in the growth medium as described previously (Sakaguchi *et al*., 1993). The medium contained 32 μM ferric quinate, 0·93 mM sulfate and 0·3 g yeast extract l^{-1}. The growth medium was sterilized by autoclaving for 10 min (2 atm, 120 °C) and cooled under oxygen-free argon gas. After cooling, the medium was distributed into 25 ml vials (10 ml medium) under anaerobic conditions. The vials were sealed with rubber and aluminium crimp caps and filled with argon gas. A volume of 100 μl sterile 28·5 mM L-cysteine solution, which was made oxygen-free by flushing with argon, was added to the glass vials using a disposable syringe. The vial was sparged with pure argon gas for 5 min before inoculation. The procedure for generating anaerobic conditions was also applied to the large-scale cultures for physiological and biochemical tests.

Using a haemocytometer, growth was measured by counting the number of cells using a phase-contrast microscope (Olympus BH-2). Magneto-sensitivity was determined by the response to a magnet placed on the microscope platform which was rotated by hand. *Desulfovibrio magneticus* was negatively stained with 1 % phosphotungstic acid and cells were placed on the surface of carbon-coated, collodion-covered copper mesh grids for observation with a transmission electron microscopy (Hitachi H-700H). The number of magnetite particles in an individual cell was counted. Values were obtained by averaging magnetite particles from 100 individual cells.

Utilization of electron donors and acceptors. Electron donors and carbon sources, as well as electron acceptors, were tested in 25 ml glass vials containing 10 ml growth medium. Electron donors, carbon sources and electron acceptors were added from sterile stock solutions. Growth on different organic compounds was tested by adding test substrates (5 mM final concentration) to lactate-free growth medium. Fumarate, nitrate, sulfite, thiosulfate, elemental sulfur and iron (III) citrate (5 mM final concentration) were tested with lactate as the carbon source and electron donor in sulfate-free medium. Growing cultures were transferred three times into fresh media with the same electron donor/acceptor combination to ensure that growth was not due to residual lactate and/or sulfate from the original stock. Growth on H_2/CO_2 was examined in liquid medium containing 5 mM sodium acetate and 5 mM sulfate. Growth was determined after 7 d cultivation and compared that with the control vial in medium without electron donors or acceptors. Growth at various temperatures (20–38 °C), pH values (pH 5–10) and NaCl concentrations (0–1 %, w/v) was determined after 7 d. All assays used an initial inoculum of strain RS-1^T of 1×10^6 cells ml^{-1}.

Pigments. For pigment determination, cells were cultivated in medium containing sulfate as the electron acceptor. During late exponential growth, cells were harvested, washed, resuspended in phosphate buffer (0·6 g NaH_2PO_4 l^{-1} and 0·7 g Na_2HPO_4 l^{-1}, pH 7·0) and broken by sonication. The lysate was then centrifuged at 190000 ***g*** for 30 min and the supernatant was scanned with a spectrophotometer (UV1600; Shimadzu). The presence of desulfoviridin was determined by its characteristic absorption at 630 nm. *Desulfovibrio vulgaris* ATCC 29579^T was used as a positive control in the desulfoviridin test. Cytochromes were identified by recording the redox difference spectrum (dithionite-reduced minus air-oxidized) using spectrophotometry. Menaquinones were extracted as described previously (Hiraishi, 1988) and identified using HPLC with a UV detector and compared against standards.

16S rDNA gene sequence analysis and DNA–DNA hybridization. DNA was extracted by standard procedures (Sambrook *et al*., 1989) and 16S rDNA gene fragments were amplified by PCR as described previously (Kawaguchi *et al*., 1995). The PCR forward primer was 5′-AGAGTTTGATCCTGGCTCAG-3′ (*Escherichia coli* positions 8–25) and the reverse primer was 5′-TAAGGAGGTGATCCAACCGC-3′ (*E. coli* positions 1523–1542). Amplified PCR products were ligated into plasmid pCR2.1 with the original TA cloning kit (Invitrogen) and used to transform *E. coli* DH5α. Plasmids containing 16S rDNA from strain RS-1^T were purified and sequenced with an automated DSQ-2000L DNA sequencer (Shimadzu) using the thermosequenase fluorescent-labelled primer cycle sequencing kit (Amersham Pharmacia Biotech). Sequences similar to the 16S rDNA sequence from strain RS-1^T were found using GenBank and DDBJ databases and FASTA software (Wilbur & Lipman, 1983). 16S rDNA sequences were aligned for phylogenetic analysis with the CLUSTAL W algorithm program (Thompson *et al*., 1994). The tree was constructed using the neighbour-joining algorithm (Saitou & Nei, 1987). Tree topology was re-examined using 1000 bootstrapped datasets.

DNA was prepared by the hydroxyapatite method as described by Suzuki *et al*. (2001). DNA–DNA hybridization was performed using the microplate method as described previously (Ezaki *et al*., 1989) at 50 °C in hybridization mixture (2 × SSC, 5 × Denhardt's solution, 3 % dextran sulfate, 50 % formamide, 50 μg denatured salmon DNA ml^{-1}) containing photobiotin-labelled DNA probes. A microplate reader (FLUO star Galaxy; BMG Labtechnologies) was used for the fluorescence measurements.

Cellular fatty acid analysis. For analysis of total cellular fatty acids, cells were cultivated in medium containing sulfate as an electron acceptor. Cells were harvested during late exponential growth by centrifugation and washed with phosphate buffer. The pellet was dried and 20 mg was subjected to HCl methanolysis (Katayama & Kuraishi, 1978). Cells were added to 2 ml 5 % (w/v) HCl/methanol. The mixture was shaken vigorously and incubated at 90 °C for 45 min. Fatty acid methyl esters were extracted from rapidly cooled mixtures by washing tube contents three times with 1·5 ml *n*-hexane. Anhydrous sodium sulfate

powder was added to the combined hexane mixture and the mixture was evaporated. Samples were analysed by GC (GC-14B; Shimadzu) utilizing a capillary column (HR-SS-10; Shinwa); the column temperature was held at 180 °C for 1 min, ramping to 220 °C at a rate of 2 °C min^{-1} and finally held for 10 min; the injector temperature was 250 °C and helium flow rate was 4 ml min^{-1}. Methyl esters were identified by comparing retention times of peaks from samples to those of a standard fatty acid mixture.

RESULTS AND DISCUSSION

Physiological and biochemical characteristics

Although strain RS-1^T is catalase-positive, it could not be cultured in the presence of oxygen and therefore is a strict anaerobe. Oxidase tests were negative. Results of growth tests with various electron acceptors are shown in Table 1. In the presence of sulfate or thiosulfate, cells produced H_2S causing precipitation of iron sulfide in medium containing iron salts. Precipitation of iron sulfide was prevented when fumarate was used as the terminal electron acceptor, because H_2S is not produced in the absence of sulfate and thiosulfate. Fumarate was used as the preferential electron acceptor in the presence of both fumarate and sulfate, and sulfide accumulated only after fumarate had been consumed, presumably because the redox potential of fumarate/succinate is more positive than that of sulfate/sulfide. Fe_3S_4 was not formed intracellularly.

RS-1^T produced only a small amount of magnetite when sulfide was formed in the presence of sulfate. It was observed by transmission electron microscopy that individual cells contained a mean of six magnetite particles when pyruvate and fumarate were used as the carbon source and electron acceptor, respectively (Fig. 1). The majority of cells did not contain magnetite particles when pyruvate and sulfate were used. The number of magnetotactic cells was higher when fumarate was used as the electron acceptor compared with sulfate. When pyruvate and fumarate were used, approximately 70% cells responded to an artificial magnetic field. When sulfate was used as the electron acceptor, the majority of cells did not exhibit magnetotaxis and less than 1% were magnetotactic. Magnetotaxis in *Desulfovibrio magneticus* is distinctly different from *Magnetospirillum* spp. or magnetic cocci which swim along magnetic fields (Matsunaga *et al*., 1991; Frankel *et al*., 1997). *Desulfovibrio magneticus* cells swam or migrated randomly in different directions. Strain RS-1^T and *Magnetospirillum* also differed with respect to their magnetite particles. *Magnetospirillum* produces crystalline magnetite particles (Matsunaga & Sakaguchi, 1992; Bazylinski *et al*., 1994), whereas strain RS-1^T produced irregular bullet-shaped particles (Sakaguchi *et al*., 1993). It is therefore assumed that the biosynthetic mechanism in strain RS-1^T is different or that this strain lacks control factors for crystallization. Intracellular magnetite produced by strain RS-1^T is covered with an organic membrane (Gorby *et al*.,

Table 1. Comparison of the properties of strain RS-1^T and three *Desulfovibrio* strains

Strains: 1, RS-1^T; 2, *Desulfovibrio burkinensis*; 3, *Desulfovibrio alcoholivorans*; 4, *Desulfovibrio fructosivorans*. Features such as cell shape, optimum temperature, pH and salinity range of the four strains were similar. All four strains utilized typical substrates for *Desulfovibrio* sp., such as lactate, malate and ethanol as electron donors, and sulfate and thiosulfite as electron acceptors. Other substrates tested in the presence of sulfate and fumarate (5 mM) but not utilized by strain RS-1^T were as follows: acetate, formate, maleate, citrate, *n*-butyrate, propionate, tartrate, glycolate, alanine, glycine, fructose, glucose, lactose, saccharose, inositol, 1-propanol, 2-propanol, toluene, benzene and naphthalene. All substrates were added at 5 mM final concentration. ND, Not determined; +, growth; −, no growth; w, weak.

Characteristic	1	2*	3†	4‡
Major menaquinone	MK-7(H_2)	ND	MK-6	MK-6
G+C content (mol%)	65·8	67·0	64·5	64·1
Electron acceptors (with lactate):				
Sulfur	−	+	+	+
Fumarate	+	+	−	+
Electron donors (with sulfate):				
Pyruvate	+	+	+	+
H_2+CO_2 (10^5 Pa)+acetate	−	+	+	+
1,2-Propanediol	−	+	+	−
Succinate	−	w	+	−

* From Ouattara *et al*. (1999).

† From Qatibi *et al*. (1991).

‡ From Ollivier *et al*. (1988).

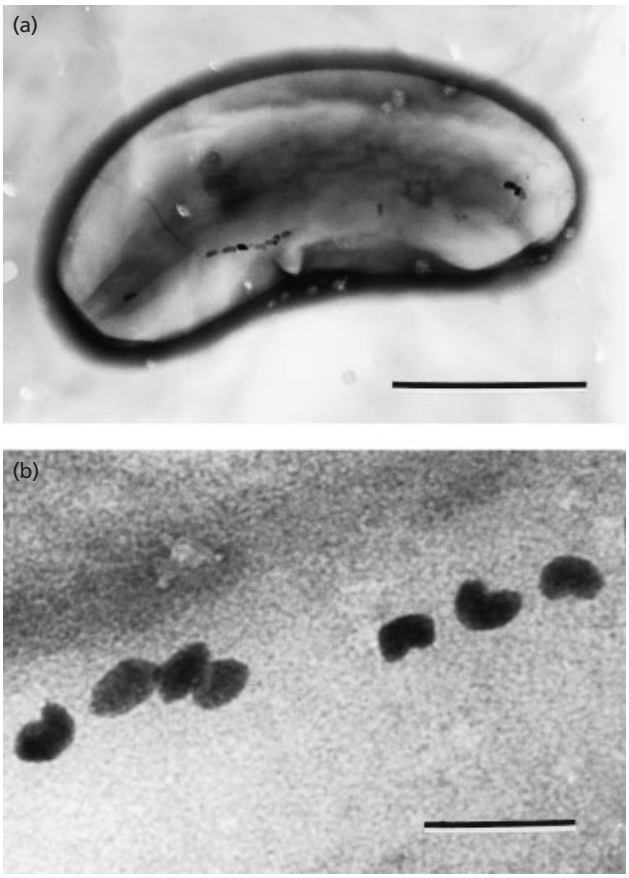

Fig. 1. Transmission electron micrographs of sulfate-reducing magnetotactic bacterium RS-1^T grown under fumarate-reducing conditions. (a) Whole cell (bar, 1 μm); (b) presumed magnetite particles (bar, 100 nm).

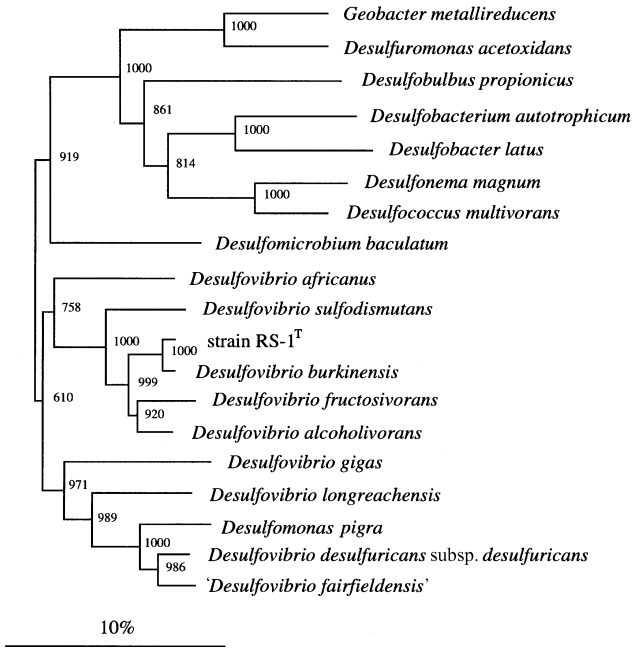

Fig. 2. 16S rDNA-based phylogenetic relationships of strain RS-1^T to members of the sulfate-reducing bacteria. Numbers indicate statistical significance of the respective interior nodes in a bootstrap analysis based on neighbour-joining tests. Bar, 10 nt changes per 100 nt. All sequences were extracted from the DDBJ database.

1988), a feature differentiating it from extracellular magnetite produced by dissimilatory Fe(III) reduction (Lovley *et al.*, 1993).

Nitrate, sulfite, elemental sulfur, iron (III) citrate and oxygen could not be reduced by strain RS-1^T. Strain RS-1^T is apparently a sulfate-reducing rather than sulfur-reducing bacterium. Strain RS-1^T is the only dissimilatory sulfate-reducing micro-organism known to synthesize intracellular magnetite. RS-1^T could metabolize pyruvate to acetate, H_2 and CO_2 in the absence of sulfate. The ability to utilize H_2 as an electron donor in the presence of CO_2 and sulfate was not sufficient to support growth. Optimum growth of RS-1^T occurred with less than 0·5% (w/v) NaCl. Optimum pH was 7·0 and optimum temperature was 30 °C. Doubling time with lactate or pyruvate and sulfate was 12 and 9·3 h, respectively.

Pigments

Cell extracts exhibited a characteristic absorption band at 630 nm indicating desulfoviridin (Skyring & Jones, 1976). Desulfoviridin has been detected in *Desulfovibrio*, *Desulfonema* and *Desulfococcus* spp., where it acts as a dissimilatory bisulfite (or sulfite) reductase (Suh & Akagi, 1969; Lee *et al.*, 1973). Dithionite-reduced minus air-oxidized redox difference spectra of cell extracts exhibited absorption maxima at 553, 523 and 420 nm, which is typical of *c*-type cytochromes (Ouattara & Vincent, 1992). HPLC analysis showed that RS-1^T contained menaquinone MK-7(H_2) as a major component and menaquinone MK-6(H_2) and MK-7(H_4) as minor components. Menaquinone MK-7(H_2) is also the major menaquinone in the completely oxidizing species, *Desulfobacter* and *Desulfobacterium* (Devereux *et al.*, 1989). Strain RS-1^T is the only incompletely oxidizing sulfate-reducing bacterium that contains menaquinone MK-7(H_2). *c*-Type cytochromes and menaquinones predominate in Gram-negative sulfate-reducing and sulfur-reducing bacteria (Collins & Jones, 1981).

Phylogenetic position

A total of 1540 bases of the 16S rDNA sequence of strain RS-1^T corresponding to *E. coli* positions 8–1542 (Winker & Woese, 1991) was determined. On the basis of this sequence data, RS-1^T affiliated with the *δ-Proteobacteria* (Fig. 2); it was most closely related to *Desulfovibrio burkinensis* (similarity of 98·7%). To clarify the phylogenetic relatedness between strain RS-

1^T and *Desulfovibrio burkinensis*, DNA–DNA hybridization was performed. The result showed 51·0% relatedness and indicated these are different. RS-1^T also showed high similarity to *Desulfovibrio alcoholivorans* and *Desulfovibrio fructosivorans* based on 16S rDNA sequencing (levels of similarity were 95·8 and 94·7%, respectively). Based on our sequence analysis, RS-1^T belonged to a cluster with the *Desulfovibrio* species (Fig. 2). The iron reducers, *Desulfuromonas acetoxidans* and *Geobacter metallireducens* cluster with complete oxidizers, which include *Desulfobacter* and *Desulfobacterium*, and show no similarity with strain RS-1^T in 16S rDNA sequences or metabolism. There is no significant sequence similarity to the magnetotactic bacterium *Magnetospirillum* spp., marine magnetotactic vibrio MV-1 or a magnetotactic coccus (DeLong *et al*., 1993; Spring *et al*., 1994; Thornhill *et al*., 1995). Furthermore, strain RS-1^T differed significantly from *Magnetospirillum* species metabolically and morphologically. These results strongly suggest that magnetotactic bacteria have different evolutionary origins.

Cellular fatty acid analysis

Fatty acid profiles indicate that branched-chain saturated fatty acids account for approximately 40% of the total fatty acids, with the predominance of anteiso-$C_{15:0}$ (25% of the total fatty acids). RS-1^T contained high percentages of $C_{16:0}$, iso-$C_{17:1}$ (13 and 10%, respectively) and an extremely low percentage of iso-$C_{15:0}$ (5%). Anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{16:0}$ and iso-$C_{17:1}$ are major components of *Desulfovibrio* species (Vainshtein *et al*., 1992; Dzierzewicz *et al*., 1996; Tardy-Jacquenod *et al*., 1996). Fatty acid profiles of strain RS-1^T were in good agreement with those from *Desulfovibrio*. Fatty acid composition differed from those of iron reducers, *Desulfuromonas acetoxidans* and *Geobacter metallireducens* (Vainshtein *et al*., 1992; Lovley *et al*., 1993). Data confirm that strain RS-1^T can be classified as a member of the genus *Desulfovibrio*.

In spite of 16S rDNA similarity, strain RS-1^T was physiologically distinct from *Desulfovibrio alcoholivorans* (Qatibi *et al*., 1991). Morphological and physiological characteristics of RS-1^T were extremely similar to those of *Desulfovibrio burkinensis* and *Desulfovibrio fructosivorans* (Ollivier *et al*., 1988; Ouattara *et al*., 1999). Unlike *Desulfovibrio burkinensis* and *Desulfovibrio fructosivorans*, strain RS-1^T could not utilize H_2 as an electron donor in the presence of CO_2, acetate and sulfate; it could not use elemental sulfur or sulfite as electron acceptors. Furthermore, strain RS-1^T contained menaquinone MK-7(H_2), instead of MK-6 or MK-6(H_2) observed in other members of *Desulfovibrio*. Based on phylogenetic analysis by 16S rDNA sequencing, DNA–DNA hybridization and G+C content, the phylogenetic distance between strain RS-1^T and other members of the genus *Desulfovibrio* is enough to consider strain RS-1^T as a new member in this genus. Therefore, it is proposed that strain RS-1^T be classified as a novel species, *Desulfovibrio magneticus* sp. nov.

Description of *Desulfovibrio magneticus* sp. nov.

Desulfovibrio magneticus (mag.ne′ti.cus. N.L. masc. adj. *magneticus* magnetic).

Vibrio-shaped, Gram-negative cells, 3–5 × 1 μm, motile by a single polar flagellum, without spores. Cells synthesize variable numbers of irregular bullet-shaped magnetite particles within the cytoplasm. Cells synthesize a mean of six magnetite particles when pyruvate and fumarate are used as carbon source and electron acceptor, respectively. Strictly anaerobic chemo-organotroph that utilizes lactate, pyruvate, malate, oxaloacetate and glycerol as electron donors and carbon sources, and sulfate, thiosulfate and fumarate as electron acceptors. Pyruvate is fermented in the absence of an electron acceptor. Other carboxylic acids, sugars, amino acids and aromatic hydrocarbons are not oxidized. Optimum growth occurs in 30 °C freshwater medium at pH 7·0. NaCl is not required for growth. Cells contain *c*-type cytochromes and desulfoviridin. Major menaquinone is MK-7(H_2). DNA G+C content is 66 mol%. Type strain of *Desulfovibrio magneticus* sp. nov. is RS-1^T (= ATCC 700980^T = DSM 13731^T). Isolated from sediments from the Kameno River, Wakayama Prefecture, Western Japan.

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