

ORIGINAL ARTICLE

Novel magnetite-producing magnetotactic bacteria belonging to the *Gammaproteobacteria*

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Two novel magnetotactic bacteria (MTB) were isolated from sediment and water collected from the Badwater Basin, Death Valley National Park and southeastern shore of the Salton Sea, respectively, and were designated as strains BW-2 and SS-5, respectively. Both organisms are rod-shaped, biomineralize magnetite, and are motile by means of flagella. The strains grow chemolithoautotrophically oxidizing thiosulfate and sulfide microaerobically as electron donors, with thiosulfate oxidized stoichiometrically to sulfate. They appear to utilize the Calvin–Benson–Bassham cycle for autotrophy based on ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) activity and the presence of partial sequences of RubisCO genes. Strains BW-2 and SS-5 biomineralize chains of octahedral magnetite crystals, although the crystals of SS-5 are elongated. Based on 16S rRNA gene sequences, both strains are phylogenetically affiliated with the *Gammaproteobacteria* class. Strain SS-5 belongs to the order Chromatiales; the cultured bacterium with the highest 16S rRNA gene sequence identity to SS-5 is *Thiohalocapsa marina* (93.0%). Strain BW-2 clearly belongs to the Thiotrichales; interestingly, the organism with the highest 16S rRNA gene sequence identity to this strain is *Thiohalospira alkaliphila* (90.2%), which belongs to the Chromatiales. Each strain represents a new genus. This is the first report of magnetite-producing MTB phylogenetically associated with the *Gammaproteobacteria*. This finding is important in that it significantly expands the phylogenetic diversity of the MTB. Physiology of these strains is similar to other MTB and continues to demonstrate their potential in nitrogen, iron, carbon and sulfur cycling in natural environments.

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Introduction

Magnetotactic bacteria (MTB) are a metabolically, morphologically and phylogenetically heterogeneous group of mainly aquatic prokaryotes that passively align and actively swim along magnetic-field lines (Bazylinski and Frankel, 2004). This behavior, called magnetotaxis, is due to the presence of intracellular single-magnetic-domain crystals of magnetite (Fe₃O₄) and/or greigite (Fe₃S₄) that are each surrounded by a lipid bilayer and called magnetosomes. Magnetosomes are generally organized

in chain(s) within the cell and impart a permanent magnetic dipole to the cell (Bazylinski and Frankel, 2004). MTB are considered ubiquitous in aquatic habitats that are near neutral pH and have oxygen concentration and redox gradients in the water column or sediments (Bazylinski and Frankel, 2004).

Known cultured and uncultured magnetite-producing MTB are phylogenetically affiliated with the *Alphaproteobacteria* and *Deltaproteobacteria* classes, of the *Proteobacteria* and the *Nitrospirae* phylum (Bazylinski and Frankel, 2004). Greigite-producing MTB have not yet been cultured and include a group of morphologically similar multicellular magnetotactic prokaryotes affiliated with the *Deltaproteobacteria* (Abreu *et al.*, 2007; Simmons and Edwards, 2007) and large rod-shaped bacteria (Pósfai *et al.*, 1998), whose phylogeny, in general, has not been studied in detail.

The *Gammaproteobacteria* class of the phylum *Proteobacteria* is one of the most genera-rich taxa among the prokaryotes, containing about 250 genera (Garrity *et al.*, 2005), while the phylogeny of this

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group is reportedly difficult to resolve (Williams *et al.*, 2010). There is only a single report of a magnetotactic bacterium, a putative greigite-producing rod, in this group (Simmons *et al.*, 2004). However, the mineral present in its magnetosomes was never unequivocally identified and a more thorough examination of the phylogenetic relationship of this organism raised doubts to its affiliation with the *Gammaproteobacteria* (Amann *et al.*, 2007). Amann *et al.* (2007) point out that the probe used by Simmons *et al.* (2004) for fluorescent *in situ* hybridization of the putative greigite-producing rod, the group-specific 23S RNA-targeted probe GAM42a (Manz *et al.*, 1992), does not allow for the unambiguous linkage of any gammaproteobacterial-specific sequences to this bacterium.

Saline lakes are globally distributed on the Earth and constitute 45% of total inland water bodies (Hahn, 2006). Saline and high-saline environments are relatively common in the southwestern United States and have not been thoroughly examined for the presence of MTB or for the presence of magnetofossils. Microbial research in saline environments is important for multiple reasons. Because of the presence of salt deposits and saline environments on Mars (Catling, 1999; Mancinelli *et al.*, 2004; Squyres *et al.*, 2004; Langevin *et al.*, 2005), studies of microbial diversity in terrestrial saline environments may shed light on the forms of extinct and/or extant life on the Mars, if life ever existed on this planet. In addition, primordial life on the Earth might have started in hypersaline environments (Dundas, 1998; Knauth, 1998); thus, research on microbes in saline environments bears relevance to our understanding of the early evolution of the biosphere on Earth.

During an ecological study of inland saline aquatic environments in the deserts of the southwestern United States, we isolated two novel strains of MTB that are unequivocally, phylogenetically affiliated with the *Gammaproteobacteria* class of the phylum *Proteobacteria*. In this report, we describe these organisms, their significance to the study and evolution of magnetotaxis.

Materials and methods

Study sites and sample collection

Water and sediment samples were taken from various parts of Badwater Basin in Death Valley National Park and the Salton Sea, both sites are located in the California, USA. Badwater Basin (36.05°N, 116.63°W) is an endorheic basin (a terminal or closed watershed with no outflow of water), located in the southern part of Death Valley and noted as the lowest point in North America (86 m below sea level). It consists of a small spring-fed pool; groundwater discharge comes from the porous carbonates of the Panamint Range to the west or from fractured metamorphic rocks of the Black

Mountains to the east (Hunt, 1975). The accumulated salts of the surrounding basin make the water extremely saline in certain areas. The Salton Sea is a saline endorheic rift lake located directly on the San Andreas Fault. Like Badwater Basin, it is located below sea level (~69 m). The lake is fed by the New, Whitewater and Alamo Rivers. Principally because of agricultural runoff, the lake has become increasingly saline with a current average salinity of 47–48 ppt (Reese *et al.*, 2008).

Samples were collected from the shore. One liter plastic bottles were filled to about 0.2–0.3 of their volume with sediment, then filled to their capacity with water that overlaid the sediment. Air bubbles were excluded. Once in the laboratory, samples were stored under dim light at room temperature (~25 °C).

Isolation and growth of magnetotactic Gammaproteobacteria

Cells of MTB were magnetically concentrated by placing the south pole of a magnetic stirring bar next to sample bottles at the sediment–water interface for ~30 min, and then further purified using the capillary magnetic racetrack technique (Wolfe *et al.*, 1987). Cells were used as inocula in a modified semisolid, oxygen-gradient, enrichment medium similar to that described by Bazylnski *et al.* (2004). However, the salinity was adjusted to that of the samples by using a basal medium that contained a salt mixture similar to that of artificial seawaters. For the Badwater Basin samples, the formula used was (per liter): 19.45 g NaCl, 5.9 g MgCl₂ • 6H₂O, 3.24 g Na₂SO₄, 1.8 g CaCl₂ • 2H₂O and 0.55 g KCl. For the Salton Sea samples (per liter): 37.8 g NaCl, 5.4 g MgCl₂ • 6H₂O, 5.4 g Na₂SO₄, 0.5 g CaCl₂ • 2H₂O and 0.9 g KCl. To a liter of these mixtures, 5 ml modified Wolfe's mineral elixir (Wolin *et al.*, 1963; Bazylnski *et al.*, 2000), 0.2 ml 1% aqueous resazurin and 0.3 g NH₄Cl were added and the pH was adjusted to 7.0. Two grams of Bacto-Agar (Difco Laboratories, Detroit, MI, USA) was then added after which the medium was autoclaved. After autoclaving, the following was added as sterile stock solutions (per liter): 0.5 ml of vitamin solution (Frankel *et al.*, 1997); 2.8 ml of 0.5 M KHPO₄ buffer (pH 7.0); 3 ml of 40% sodium thiosulfate (Na₂S₂O₃ • 5H₂O) or 5 ml of neutralized 100 mM sodium sulfide (Na₂S • 9H₂O); 3 ml of 10 mM FeSO₄ • 7H₂O (in 0.02 N HCl); 10 ml of a filter-sterilized, freshly made 5.0% sodium bicarbonate (NaHCO₃) solution; and 10 ml of a filter-sterilized, freshly made, neutralized cysteine • HCl • 2H₂O solution. Screw-capped glass culture tubes were filled to ~80% of their volume with medium. The medium was allowed to sit at room temperature for several hours to solidify and to allow the O₂ concentration gradient to form as evidenced by the presence of a pink (oxidized) zone near the surface and a colorless (reduced) zone in the lower part of the tubes.

Axenic cultures were obtained by dilution to extinction three times in succession in the semisolid growth medium containing thiosulfate as the electron donor. Purity of the cultures was determined using light microscopy.

Heterotrophic growth of the strains was determined using the growth medium described above for each strain except that thiosulfate was replaced by an organic carbon source at a concentration of 5 mM. Carbon sources tested included acetate, succinate, pyruvate, lactate and glucose.

Thiosulfate oxidation to sulfate

Thiosulfate and sulfate concentrations in growth media were determined using liquid chromatography on a high-performance liquid chromatography system consisting of a Shimadzu (Kyoto, Japan) model CBM-20A system controller, model LC-20AD dual pumps, a CTO-20AC column oven and a CDD-10A VP detector. The system was linked to a computer containing the Shimadzu LCsolution system program, which was used for analysis. A SeQuant Anion Suppressor column (Shodex IC SI-90G (PEEK) 4,6x10, Phenomenex, Torrance, CA, USA) coupled with a Continuous Anion Regeneration System (CARS) was used to separate the thiosulfate and sulfate. The mobile phase was composed of NaHCO₃ (1 mM) and Na₂CO₃ (3.2 mM) (25:75, v/v) and was pumped at a flow rate of 1.5 ml min⁻¹. Standard concentration curves were made for sulfate and thiosulfate for every experiment with *r*² values > 0.99.

For both strains BW-2 and SS-5, 600 µl of a microaerophilic band of cells formed 6 days after inoculation in a semisolid culture was removed and centrifuged for 3 min at 13 000 *g* to remove agar and cells. The supernate was filtered (0.2 µm), diluted with distilled deionized H₂O (~1:10) and injected into the injection loop (20 µl) of the high-performance liquid chromatography. All analyses were performed on triplicate cultures. Uninoculated growth medium was used as a negative control.

Ribulose-1,5-bisphosphate carboxylase/oxygenase activity

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) activity of cell-free extracts was performed as described by Beudeker *et al.* (1980), except that the dithiothreitol concentration was changed to 6.8 mM and the pH was adjusted to 7.2 (Bazylnski *et al.*, 2004). Radioactive ¹⁴C counts were determined using a Beckman model LS 6500 liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA).

Nitrogenase activity

Nitrogenase activity of the strains grown in semisolid O₂ concentration gradient medium was determined as described by Bazylnski *et al.* (2000) with some minor modifications. Ethylene concentrations were measured using Shimadzu model GC-2014

gas chromatograph equipped with dual-flame ionization detectors, and the stationary phase was a 3-mm × 1.8-m stainless steel column of Porapak N (80/100 mesh).

Generally, to test for nitrogenase activity, cells are grown in a medium lacking a fixed nitrogen source. In this case, we required cysteine as a reducing agent, so a fixed nitrogen source was present. However, the normal nitrogen sources used, NH₄Cl and NaNO₃, were omitted from the growth medium for the tubes used for nitrogenase testing. These compounds were included at 4 mM as a negative control, as they should repress nitrogenase synthesis if they can be used as nitrogen sources for growth. Nitrogenase assays were performed with triplicate cultures for each condition.

Protein concentration determinations

For RubisCO assays, protein in cell-free extracts was measured using technique of Bradford (1976) with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). For nitrogenase assays, total cell protein was determined as described by Bazylnski *et al.* (2000) except that the cells and agar were not washed before digestion with trichloroacetic acid. Bovine gamma globulin was used as the standard for all protein assays.

Light and electron microscopy

The presence and behavior of magnetotactic microorganisms was determined using a Zeiss AxioImager M1 light microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) equipped with phase-contrast, fluorescence and differential interference contrast capabilities. The hanging-drop technique (Schüler, 2002) was used routinely in the examination of samples and for quantifying MTB. The local magnetic field used to determine magnetotaxis was reversed by rotating a large stirring bar magnet by 180° on the microscope stage. Swimming speeds and patterns of cells of both strains were calculated from images of swimming cells taken using dark-field light microscopy and a long exposure time of 200 ms.

The presence of magnetosomes, and the composition of magnetosome crystals and other intracellular inclusions were determined using combinations of transmission electron microscopy (TEM), energy-dispersive X-ray analysis (EDX) and selected-area electron diffraction (SAED) with a Tecnai (FEI Company, Hillsboro, OR, USA) Model G2 F30 Super-Twin TEM. The morphologies and structural details of the magnetosome crystals were studied using electron micrographs obtained with a Philips CM20 TEM (with an attached Noran Voyager EDS detector) and high-resolution images obtained using a JEOL 3010 TEM (JEOL USA, Inc., Peabody, MA, USA).

Determination of 16S rRNA, RubisCO and mam gene sequences, and phylogenetic analysis

16S rRNA genes were amplified using bacteria-specific primers 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-TACGGHTACCTTGTTACGACTT-3' (Lane, 1991). Partial sequences of the RubisCO form I 'green' and form I 'red' (both *cbbL*), and form II (*cbbM*) genes were amplified using the degenerate primer pairs RubIgF-RubIgR, RubIrF-RubIrR and RuIIIF1-RuIIIF3, respectively (Spiridonova *et al.*, 2004). Primers for *mam* genes were designed from aligned *mam* gene sequences of known *Magnetospirillum* species and using the software AmplifX version 1.44 by Nicolas Jullien (<http://ifjr.nord.univ-mrs.fr/AmplifX-Home-page>) (Supplementary Table 1). PCR products were cloned into pGEM-T Easy Vector (Promega Corporation, Madison WI, USA) and sequenced (Functional Biosciences, Inc., Madison WI, USA).

Alignment of 16S rRNA or RubisCO genes was performed using CLUSTAL W multiple alignment accessory application in the BioEdit sequence alignment editor (Hall, 1999). Phylogenetic trees were constructed using MEGA version 4 (Tamura *et al.*, 2007) applying the neighbor-joining method (Saitou and Nei, 1987). Bootstrap values were calculated with 1000 replicates.

Nucleotide sequence accession numbers

16S rRNA gene sequences of the strains BW-2 and SS-5 are assigned to GenBank Accession numbers HQ595728 and HQ595729, respectively. The partial *cbbM* gene sequences of strains BW-2 and SS-5 carry GenBank Accession numbers HQ638677 and HQ638676, respectively and the partial *cbbL* gene sequence of strain SS-5, HQ638675.

Results

Description of sampling sites and samples

Sediment from Badwater Basin in Death Valley was black in color and reducing; the odor of hydrogen sulfide was readily apparent when sediments were disturbed. The salinity of Badwater Basin was 25 ppt at the underground opening of the spring and 67 ppt at the extremity of the pool formed by the spring. Where the salinity was at its maximum, 67 ppt, the sediment was mainly composed of salt. MTB were always present in samples collected from the area where the sediment was black and the salinities were between 25 and 31 ppt. Two different morphotypes of MTB were observed after magnetic enrichment: large rods, some containing numerous intracellular refractile globules, and a small helical bacterium. All the large rod-shaped MTB we examined contained greigite as the magnetic mineral phase as determined by a combination of EDX and SAED in the TEM (data not shown). In the lab, these MTB remained viable and motile in samples kept at 25 °C for several weeks.

Sediments in most areas sampled at the Salton Sea were similar to those of Badwater Basin, although in some beach areas the sediment consisted mainly of coarse sand. Salinities at the specific sampling sites at the Salton Sea varied widely from 2 to 52 ppt. The site from where the sample containing magnetotactic *Gammaproteobacteria* was taken was situated at the southeastern shore of the sea near the boat ramp at Red Hill Marina County Park (33.20°N, 115.61°W). The salinity here was 52 ppt and samples were collected from a reddish pool containing large numbers of halophilic prokaryotes as determined by microscopic observation. Immediately after collection and magnetic enrichment, the samples contained two morphotypes of MTB: magnetotactic multicellular prokaryotes and magnetotactic cocci. After several months of storage at 25 °C, the samples became devoid of multicellular prokaryotes and magnetotactic cocci, and only short, rod-shaped MTB, with north-seeking polarity (swam toward the south pole of the bar magnet), were observed using the hanging-drop technique.

Isolation of the magnetotactic *Gammaproteobacteria*

Magnetically purified cells from the Badwater Basin and the Salton Sea were used as inocula into semisolid, O₂ concentration gradient growth medium. After several weeks of incubation at 25 °C, one tube inoculated with MTB from Badwater Basin and four tubes inoculated with MTB from the Salton Sea showed evidence of growth. Microaerophilic bands of cells were present in these tubes at the pink-colorless, oxic-anoxic interface (OAI). Microscopic examination of the cells in these cultures showed that the cells were magnetotactic and rod-shaped. The MTB from the Badwater Basin tube and from one of the Salton Sea tubes were diluted to extinction three successive times to obtain axenic cultures. The MTB strains from the Badwater Basin and the Salton Sea are designated as strains BW-2 and SS-5, respectively.

Physical description of strains BW-2 and SS-5

Cells of strain BW-2 are relatively large rods with an average length of $4.4 \pm 0.6 \mu\text{m}$ and a width of $2.2 \pm 0.2 \mu\text{m}$ ($n=62$) that contain a number of inclusions observed using light microscopy (Figures 1a and b). Two types of inclusions, based on size, were identified in cells of BW-2 using TEM. The largest inclusions were sulfur-rich as shown by EDX analysis and severely damaged by the electron beam of the TEM (data not shown). EDX analysis and TEM revealed that the smaller inclusions were phosphorus-rich but devoid of sulfur and likely represent polyphosphate bodies (data not shown).

Cells of strain SS-5 are also rod-shaped but smaller than those of BW-2, having an average length of $2.5 \pm 0.5 \mu\text{m}$ and a width of $1.2 \pm 0.1 \mu\text{m}$ ($n=64$) (Figure 1). During stationary phase, very

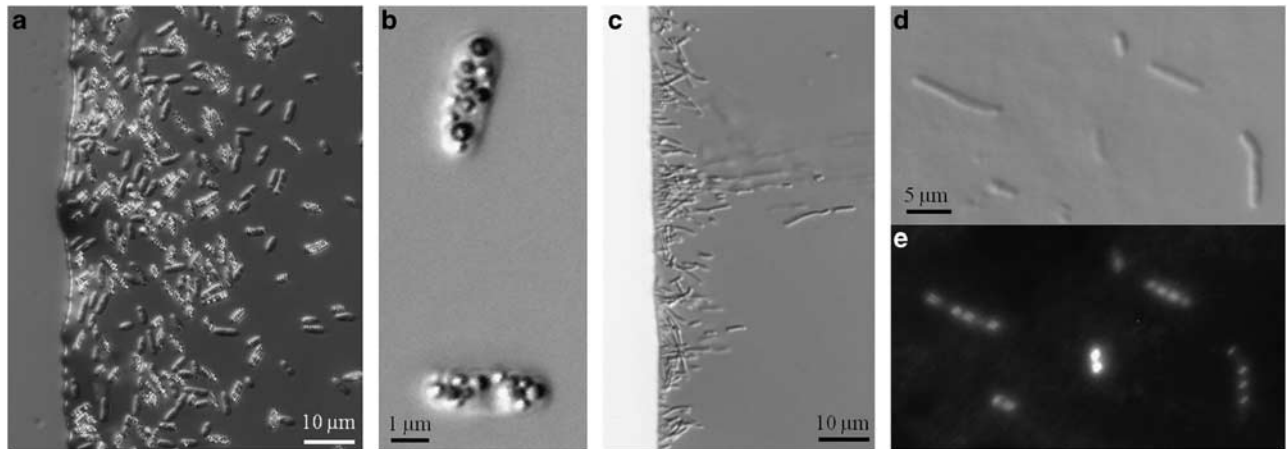


Figure 1 Light microscope images of the novel MTB of the *Gammaproteobacteria*. (a, c) Differential interface contrast (DIC) light micrographs of north-seeking cells of strains BW-2 and SS-5, respectively, at the edge of a 'hanging drop' in a magnetic field. (b) High-magnification DIC micrograph of cells of BW-2 showing the numerous, refractile, intracellular, sulfur-rich globules. (d) High-magnification DIC micrograph of very-long cells of strain SS-5 common in older cultures. (e) Fluorescence microscope image of the same SS-5 cells shown in (d) but stained with 4',6-diamidino-2-phenylindole (DAPI) to show the numerous nucleoids present in very-long cells.

large cells ($\sim 20\text{--}25\ \mu\text{m}$) became apparent suggesting that cells have trouble dividing at this growth stage. Long cells of strain SS-5 stained with 4',6-diamidino-2-phenylindole show numerous nucleoids, which further suggests this to be the case (Figures 1d and e). The long cells were magnetotactic, although they appeared to have trouble reversing their direction of motility when the direction of the local magnetic field was reversed. Examination of strain SS-5 using TEM and EDX revealed that cells contain phosphate-rich inclusions. There was no evidence for the presence of sulfur-containing inclusions.

Chemolithoautotrophic and chemoorganoheterotrophic growth

Cells of the axenic cultures were grown routinely in semisolid O_2 concentration gradient media containing thiosulfate or sulfide as electron donor and in liquid media with thiosulfate. Cells grew in this medium as a microaerophilic band of cells at the OAI evidenced by the presence of the band at the pink–colorless interface when the redox indicator resazurin was present in the growth medium. Sodium bicarbonate was the sole carbon source although a relatively small amount of organic carbon was present as vitamins and as the reducing agent cysteine. Because of the inclusion of this organic carbon, growth in this medium alone is not good evidence for chemolithoautotrophic growth. Thus, we tested the strains for the activity and presence of the gene for the CO_2 -fixing enzyme RubisCO of the Calvin–Benson–Bassham cycle of autotrophy as this pathway is the most widespread autotrophic pathway among the aerobic members of the *Alpha*-, *Beta*- and *Gammaproteobacteria* (Tourova *et al.*, 2010).

Cell-free extracts of both strains BW-2 and SS-5, grown microaerobically under presumably autotrophic conditions with $\text{S}_2\text{O}_3^{2-}$ as the electron donor

and $\text{HCO}_3^-/\text{CO}_2$ as the major carbon source, showed RubisCO activity (Table 1). Carbon dioxide fixation in the assays was always ribulose biphosphate dependent and did not occur when ribulose biphosphate was absent from the reaction mix (Table 1).

Whole-cell PCR of the strains, using primers specific for the various forms of RubisCO genes, resulted in the amplification of partial sequences (812 bp) of putative form I RubisCO, 'green-like' *cbbL* genes from both BW-2 and SS-5. When these *cbbL* sequences were used in a BLAST search using the Genbank database, the most similar sequences to those we obtained were from chemolithoautotrophic species of the *Betaproteobacteria* class including the Fe(II) oxidizer *Sideroxydans lithotrophicus* (77.6% identity with BW-2) and *Cupriavidus metallidurans* (86.4% identity with SS-5).

In addition, a partial sequence (797 bp) of a form II RubisCO *cbbM* gene was obtained from strain SS-5, but not from strain BW-2. The most similar sequence to this gene fragment was from the *cbbM* gene of *Thiobacillus denitrificans* (87.3% identity).

When resazurin was replaced with phenol red in thiosulfate-containing growth medium for both MTB strains, the medium turned from pink to yellow particularly around the band of cells, suggesting that the strains oxidize thiosulfate to sulfuric acid. In experiments where 2 mM thiosulfate was supplied to the cells, the thiosulfate was virtually depleted at the band of cells ($0.025 \pm 0.013\ \text{mM}$ and $0.011 \pm 0.005\ \text{mM}$ thiosulfate remaining after growth by BW-2 and SS-5, respectively). If cells completely oxidize thiosulfate to sulfate then the concentration of sulfate recovered should be about double, $\sim 4\ \text{mM}$, of what was supplied. This is what was observed ($4.23 \pm 0.03\ \text{mM}$ and $4.15 \pm 0.06\ \text{mM}$ at the bands of cells of BW-2 and SS-5, respectively).

Attempts to grow BW-2 heterotrophically on a variety of organic compounds were unsuccessful.

Table 1 RubisCO activities in cell-free extracts of the magnetotactic gammaproteobacterial strains BW-2 and SS-5 grown autotrophically in semisolid (O₂)-gradient medium

Bacterial strain	Growth mode	Electron donor/ carbon source	Electron acceptor	RubisCO activity (nmol CO ₂ fixed min ⁻¹ mg ⁻¹ protein)
BW-2	Chemoautotrophic	S ₂ O ₃ ²⁻ /HCO ₃ ⁻ CO ₂	O ₂	4.6 ± 0.8
Extract heated to 100 °C for 10 min	Chemoautotrophic	S ₂ O ₃ ²⁻ /HCO ₃ ⁻ CO ₂	O ₂	None detected
SS-5	Chemoautotrophic	S ₂ O ₃ ²⁻ /HCO ₃ ⁻ · CO ₂	O ₂	15.1 ± 4.2
Extract heated to 100 °C for 10 min	Chemoautotrophic	S ₂ O ₃ ²⁻ /HCO ₃ ⁻ CO ₂	O ₂	None detected
MV-1	Chemoautotrophic	S ₂ O ₃ ²⁻ /HCO ₃ ⁻ CO ₂	O ₂	3.3 ± 1.4
Extract heated to 100 °C for 10 min	Chemoautotrophic	S ₂ O ₃ ²⁻ /HCO ₃ ⁻ CO ₂	O ₂	None detected
<i>Escherichia coli</i>	Heterotrophic	Tryptone-yeast extract	O ₂	None detected

Abbreviation: RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

Cell-free extract of *Candidatus Magnetovibrio blakemorei* strain MV-1 was used as a positive control (Bazylnski *et al.*, 2004) and cell-free extract of *Escherichia coli* grown heterotrophically was used as a negative control. RubisCO activity was ribulose-1,5-bisphosphate (RUDP)-dependent and no activity was observed in the absence of RUDP. Values represent mean ± s.d. of triplicate analyses.

Table 2 Nitrogenase activity based on C₂H₂ reduction to C₂H₄ by the magnetotactic gammaproteobacterial strains BW-2 and SS-5 grown autotrophically in semisolid (O₂)-gradient medium with thiosulfate as the electron donor

Organism	Fixed N source	Total cell protein per culture (μg)	Rate of C ₂ H ₂ reduction ^a	Duration of assay (hour)
MV-1	None	85.5 ± 8.0	2.0 ± 0.7	5.5
BW-2	None	21.2 ± 1.2	0.7 ± 0.1	17.5
	NH ₄ Cl (4 mm)	59.1 ± 9.8	None detected	17.5
	NaNO ₃ (4 mm)	18.4 ± 4.4	None detected	17.5
SS-5	None	Not determined	None detected	22

Abbreviations: C₂H₂, acetylene; C₂H₄, ethylene.

Cultures of *Candidatus Magnetovibrio blakemorei* strain MV-1 were used as a positive control (Bazylnski and Williams, 2007). Experiments were performed in triplicate for each growth condition, and results are reported as the mean ± s.d.

^anmol C₂H₄ produced min⁻¹ mg⁻¹ cell protein. Rates were determined from linear regression calculations. For *Candidatus Magnetovibrio blakemorei* strain MV-1, $r \geq 0.9826$ ($n = 5$); for strain BW-2, $r \geq 0.9830$ ($n = 5$).

Cells of strain SS-5 appeared to show low levels of growth on succinate.

Nitrogenase activity

When grown in semisolid O₂-gradient medium lacking NH₄⁺ and NO₃⁻, cells of BW-2 exhibited nitrogenase activity by the reduction of C₂H₂ to C₂H₄ (Table 2). The rate was relatively low compared with cultures of *Candidatus Magnetovibrio blakemorei* strain MV-1 used as a positive control (Bazylnski and Williams, 2007). The reason for this low rate is unclear, but activity was completely abolished when the fixed nitrogen sources, NH₄⁺ and NO₃⁻, were present in the growth medium. The total cell protein of cultures grown with NH₄⁺ were about three times higher than those grown with NO₃⁻ or without either compound, consistent with the strain's ability to use NH₄⁺ as a nitrogen source (Table 2). The fact that NO₃⁻ inhibited nitrogenase activity suggests that it can also be utilized as a nitrogen source by this strain although it did not appear to stimulate growth (Table 2).

Cells of SS-5 did not exhibit nitrogenase activity as evidenced by the production of C₂H₄ from C₂H₂ in the standard medium with cysteine as the reducing agent although cells grew in this medium. When

cysteine was replaced by dithiothreitol (1.3 mM) as the reducing agent, growth was observed only when a fixed nitrogen source (for example, NH₄⁺) was provided, suggesting that cells of this strain utilize cysteine as a nitrogen source and cannot fix N₂.

Motility and magnetotaxis

Cells of both strains are motile and magnetotactic (Supplementary Videos S1 and S2); BW-2 possesses a polar unsheathed bundle of seven flagella, whereas SS-5 possesses one unsheathed polar flagellum (Figure 2). The average swimming speed for cells of strain BW-2 in fresh cultures was 92 ± 22 μm s⁻¹ ($n = 56$), whereas strain SS-5 had an average swimming speed of 49 ± 8 μm s⁻¹ ($n = 58$).

Magnetosomes of strains BW-2 and SS-5

Cells of both strains BW-2 and SS-5 biomineralize a chain of octahedral crystals of magnetite in their magnetosomes (Figures 3b and d) although they differ in that those of SS-5 crystals are elongated (Figure 3d) and that some cells of strain SS-5 synthesize two parallel chains (Figure 3e). Cells grown in semisolid O₂-gradient medium with thiosulfate as the electron donor contained 30 ± 9

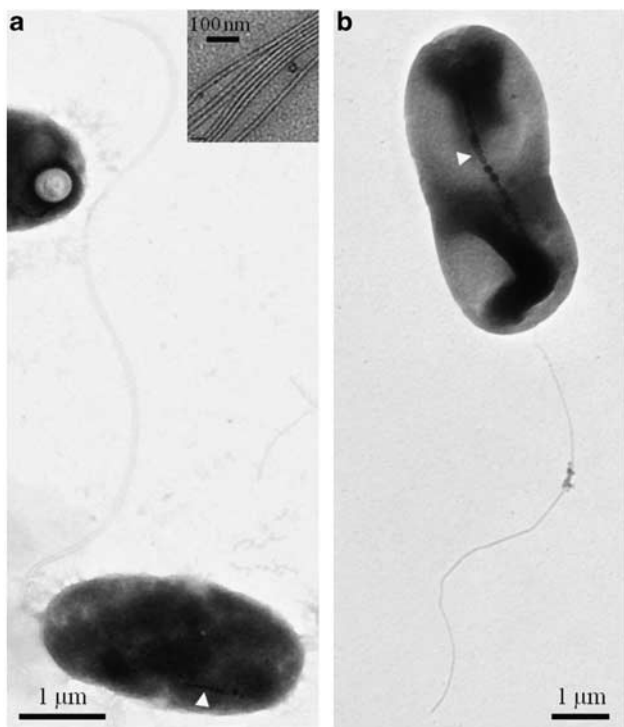


Figure 2 TEM images of negatively stained (1% uranyl acetate) cells of the magnetotactic *Gammaproteobacteria*. (a) TEM image of cells of strain BW-2 showing chain of magnetite magnetosomes (at white arrow) and a polar bundle of flagella. Inset shows that the flagellar bundles consist of seven individual flagellum. (b) TEM of cells of strain SS-5 showing chain of magnetosomes (at white arrow) and a single polar flagellum.

($n = 46$) and 20 ± 7 ($n = 45$) magnetosomes per cell for strains BW-2 and SS-5, respectively. Magnetite crystals of BW-2 averaged $67 \pm 16 \times 63 \pm 15$ nm in size with a shape factor of 0.94 ± 0.04 ($n = 189$). For SS-5, the average length of the crystals was 86 ± 27 nm and the width 63 ± 19 nm with a shape factor of 0.74 ± 0.07 ($n = 171$). Crystals of both strains fit in the theoretical single-magnetic-domain size range (Butler and Banerjee, 1975) as for mature magnetosome magnetite crystals from MTB (Bazylinski and Frankel, 2004) (Supplementary Figure 1).

Phylogeny of the strains BW-2 and SS-5

16S rRNA gene sequences of strains BW-2 and SS-5 clearly indicate that they phylogenetically belong to the *Gammaproteobacteria* class (Figure 4). A phylogenetic tree based on the 16S rRNA gene sequences of the strains shows that SS-5 lies within the order Chromatiales while BW-2 belongs to the order Thiotrichales (Figure 4).

The highest 16S rRNA gene sequence identities to those of BW-2 and SS-5 were from sequences obtained directly from the environment belonging to as yet uncultured bacteria. For strain SS-5, the highest identity sequence (93.4%) was retrieved from a filamentous ectosymbiont on the tail of the

marine oligochaete worm *Tubificoides benedii* collected from sulfidic mudflats at the Wadden Sea (Ruehland and Dubilier, 2010). For BW-2, the highest identity sequence (91.7%) was retrieved from a soil sample that was heavily contaminated with oil collected from the Jidong Oilfield near Bohai Bay, China (Liu *et al.*, 2009). The three organisms in culture with the highest 16S rRNA gene sequence identities to SS-5 belong to the Chromatiales order and include *Thiohalocapsa marina* (93.0%), *Thiohalocapsa halophila* (92.5%) and *Thiorhodococcus pfennigii* (92.0%). The three organisms in culture with the highest 16S rRNA sequence identities to BW-2 are *Thiohalospira alkaliphila* (90.2%; Chromatiales), *Thioalkalispira microaerophila* (89.9%; Thiotrichales) and *Thioalkalivibrio versutus* (89.9%; Chromatiales).

Discussion

Before this work, all magnetite-producing MTB have been found to be phylogenetically associated with the *Alpha-* and *Deltaproteobacteria* classes of the *Proteobacteria* phylum and the *Nitrospirae* phylum (Amann *et al.*, 2007). Here we report the isolation of two novel magnetite-producing MTB that are unequivocally phylogenetically affiliated with the *Gammaproteobacteria*. Strain SS-5 belongs to the order Chromatiales, the bacterium with the highest 16S rRNA gene sequence identity in culture being *T. marina* (93.0%). Although the 16S rRNA gene sequences with the highest identity to that of strain BW-2 belong to species of the Chromatiales (highest sequence identity *T. alkaliphila* (90.2%)), strain BW-2 appears to phylogenetically lie well within the order Thiotrichales. Based on the divergence of the 16 rRNA genes of BW-2 and SS-5, these new MTB unequivocally represent new genera in the *Gammaproteobacteria* class that have a 16S rRNA gene sequence identity between them of 87.9%.

The discovery of magnetotactic *Gammaproteobacteria* is significant for a number of reasons. The *mam*, *mms*, and *mtx* genes are known to encode for a number of magnetosome membrane proteins and other proteins that appear to be involved in mineralizing magnetite magnetosomes and constructing the magnetosome chain (Schüler, 2008). These genes are organized mainly as clusters within a potentially mobile magnetosome gene island (MAI) in many MTB including those of the *Alpha-* and *Deltaproteobacteria* (Jogler *et al.*, 2009) and the *Nitrospirae* (Jogler *et al.*, 2011). For example, in *Magnetospirillum gryphiswaldense*, the magnetosome genes fall within a 35-kb stretch of DNA that is flanked by a larger 130-kb segment containing mobile elements and tRNA genes (Ullrich *et al.*, 2005), characteristics typical of a gene island (Reiter and Palm, 1990). Other MTB lack some of these latter features (for example, *Candidatus Magnetococcus marinus* strain MC-1; Schübbe *et al.* (2009)). Some spontaneous

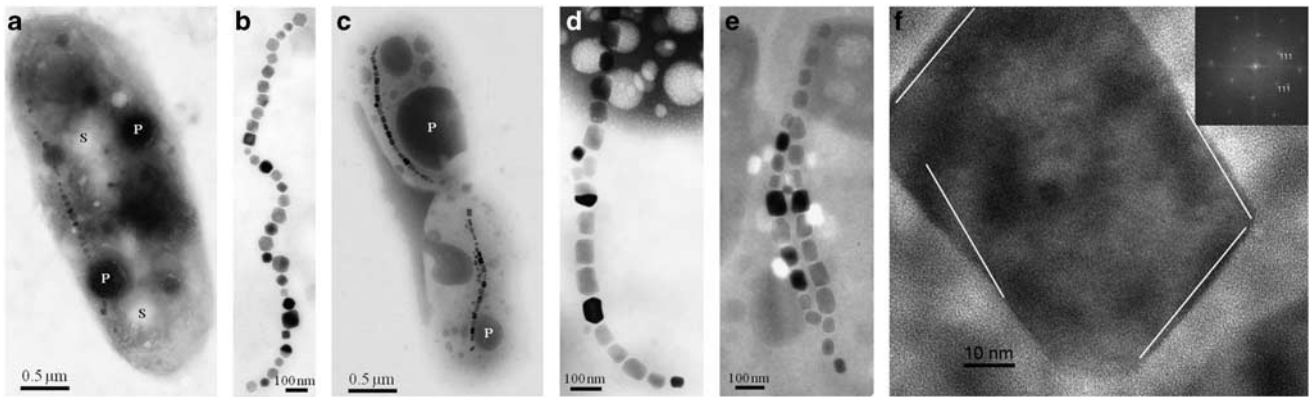


Figure 3 TEM images of inclusions within cells of strains BW-2 and SS-5. (a) TEM image of a whole unstained cell of strain BW-2 showing numerous sulfur- (S) and phosphorus-rich (P) inclusions as determined by EDX (data not shown) and a chain of magnetite magnetosomes. (b) High-magnification TEM image of a chain of magnetosomes from a cell of strain BW-2. The magnetosome crystals were identified as magnetite (data not shown) with octahedral crystal habits. (c) TEM image of a whole unstained cell of strain SS-5 showing phosphorus-rich (P) inclusions as determined by EDX (data not shown) and a chain of magnetite magnetosomes. (d, e) High-magnification TEM image of a chain and double chain, respectively, of magnetite magnetosomes from a cell of strain SS-5. (f) High-resolution TEM image of a magnetosome crystal observed in strain SS-5 identified as magnetite on the basis of its lattice spacings, with the electron beam parallel to a (110)-type crystallographic direction (consistent with the Fourier transform of the image in the upper right inset, indexed according to the magnetite structure). The white lines are parallel to (111)-type, octahedral faces, indicating that the crystal is a slightly distorted octahedron, elongated parallel to (111).

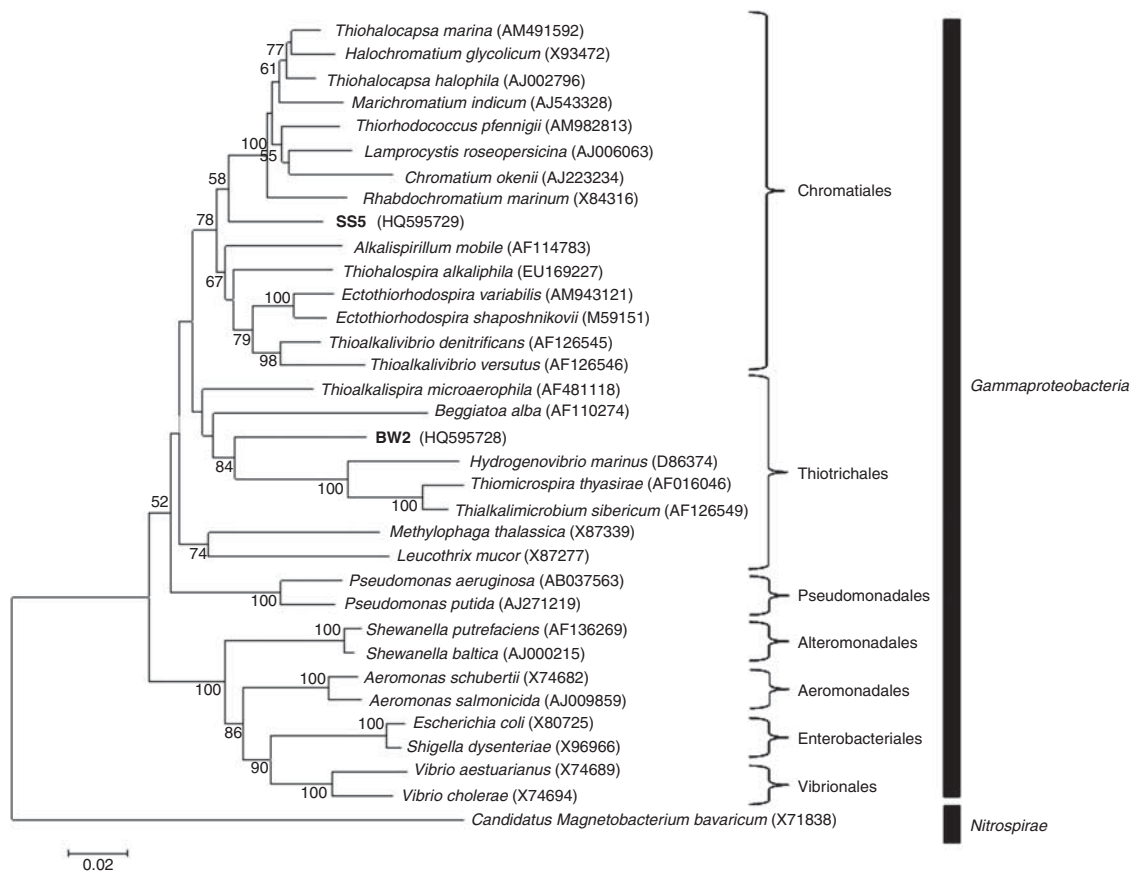


Figure 4 Phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strains BW-2 and SS-5 in the *Gammaproteobacteria* class. Bootstrap values (higher than 50) at nodes are percentages of 1000 replicates. The 16S rRNA gene sequence of the magnetotactic bacterium *Candidatus Magnetobacterium bavaricum*, a member of the *Nitrospirae* phylum, was used to root the tree. GenBank accession numbers are given in parentheses. Bar represents 2% sequence divergence.

non-magnetotactic mutants of *M. gryphiswaldense* that do not biomineralize magnetite have been shown to have the MAI deleted (Schübbe *et al.*, 2003). These findings together with the observed distribution of magnetite-driven magnetotaxis within the *Alpha*- and *Deltaproteobacteria* classes and the *Nitrospirae* strongly suggests that individual MTB in these groups acquired the genes for magnetite magnetosome biomineralization through horizontal gene transfer of the MAI (Jogler *et al.*, 2009, 2011). If *mam*, *mms* and *mtx* genes are present in these new and other magnetotactic *Gammaproteobacteria*, they might have similarly acquired magnetite magnetosome genes. However, degenerate primers designed for nine *mam* genes including *mamA*, *mamB*, *mamJ*, *mamK*, *mamM*, *mamO*, *mamP*, *mamQ* and *mamT*, from conserved sequences of known *Magnetospirillum* species (*Alphaproteobacteria*) were ineffective at amplifying *mam* gene sequences from either strain BW-2 or SS-5, although the primers were effective in amplifying *mam* gene sequences from newly isolated *Magnetospirillum* strains (data not shown). This is most likely due to the fact that the sequence of these genes were not conserved enough at the nucleotide level to amplify the target sequences although it does not exclude the possibility that these genes are completely absent from the genomes of the new gammaproteobacterial MTB. We believe the latter scenario is unlikely based on the fact that the primers were also ineffective with non-*Magnetospirillum* alphaproteobacterial MTB such as *Candidatus Magnetovibrio blakemorei* strain MV-1 and others.

Interestingly, the magnetite crystals found in the gammaproteobacterial strains described here have octahedral crystal habits, one strain producing elongated octahedral habits. Crystals with similar habits have previously been found only in the *Alphaproteobacteria* (Jogler and Schüler, 2009) but not in MTB of the *Deltaproteobacteria* and the *Nitrospirae* that solely produce bullet-shaped crystals (Amann *et al.*, 2007; Lefèvre *et al.*, 2010). These latter groups appear to possess less *mam* genes than MTB of the *Alphaproteobacteria* (Abreu *et al.*, 2011; Jogler *et al.*, 2011) that synthesize cuboctahedral and elongated prismatic crystals of magnetite and possess a greater diversity of *mam* genes (Abreu *et al.*, 2011). Thus, based on the presence of these same magnetite crystal morphologies in the new magnetotactic *Gammaproteobacteria*, we predict that a similar diversity of *mam* genes to those found in the magnetotactic *Alphaproteobacteria* is also present in the MTB of this group.

The proteobacterial classes are thought to have emerged in the following order during evolution (Klenk *et al.*, 1999; Gupta, 2000; Kersters *et al.*, 2006; Emerson *et al.*, 2007): *Epsilon*- and *Deltaproteobacteria* then the *Alpha*-, *Beta*- and *Gammaproteobacteria*. Therefore, it is MTB of the latter diverging classes that biomineralize the apparently more

morphologically consistent, possibly more controlled crystals of magnetite. Abreu *et al.* (2011) suggested that these morphologies are partially a result of the extra magnetosome genes present in these organisms that may have evolved through gene mutation and/or gene duplication, and then were distributed between species of these groups by horizontal gene transfer. If *mam*, *mms*, and *mtx* genes are present in these new and other magnetotactic *Gammaproteobacteria*, determination of the gene sequences and gene arrangement might provide additional evidence for horizontal gene transfer of the MAI and to determine whether there are significant genetic similarities between the *Gammaproteobacteria* and *Alphaproteobacteria* classes as far as magnetite biomineralization is concerned, accounting for the synthesis of similar magnetite crystal habits. In addition, it may also be helpful in determining the origin and evolution of the MAI.

Our pure-culture studies revealed physiological similarities to other cultured MTB of the *Alphaproteobacteria* (Bazyliński *et al.*, 2004; Bazyliński and Williams, 2007; Geelhoed *et al.*, 2010). Both strains grow microaerobically as bands of cells at the OAI in O₂-gradient cultures and chemolithoautotrophically utilizing thiosulfate, oxidizing it stoichiometrically to sulfate, or sulfide as an electron donor, and the Calvin–Benson–Bassham cycle for autotrophy as evidenced by the presence of RubisCO activity of cell-free extracts and fragments of different forms of RubisCO genes. Of the two strains, only SS-5 displays any potential for heterotrophic growth as cells appeared to show meager growth on succinate in the absence of thiosulfate and sulfide. Thus, strain BW-2 may be an obligate chemolithoautotroph as are many other *Gammaproteobacteria* (for example, *T. alkaliphila*) (Sorokin *et al.*, 2008). These physiological findings are consistent with the models of magnetotaxis presented by Frankel *et al.* (1997) and Spring and Bazyliński (2000) in which magnetotaxis functions to make chemotaxis more efficient in aiding chemolithoautotrophic MTB in locating an optimal position in chemical/redox gradients (OAI) where electron donors (for example, reduced sulfur compounds) and acceptors (O₂) overlap or are close to overlapping and are thus relatively easily accessible to the bacteria.

Like all cultured MTB tested (Bazyliński and Williams, 2007), strain BW-2 appears to be capable of dinitrogen fixation based on cell's ability to reduce acetylene to ethylene under microaerobic conditions where a fixed nitrogen source was absent from the growth medium with the exception of cysteine, the reducing agent, and nitrilotriacetic acid was used as a chelating agent in the mineral solution. Cells of strain SS-5 do not appear to possess this ability as nitrogenase activity was lacking under the same culture conditions, as well as when dithiothreitol, which does not contain nitrogen, replaced cysteine as the reducing agent. Although more intensive physiological studies are

required before the strains can be formally described and named, our results shows that, like MTB in other phylogenetic groups, these *Gammaproteobacteria* show potential for carbon, sulfur, iron and nitrogen (strain BW-2) cycling in highly saline, non-marine natural habitats.

The discovery of MTB in inland, saline, aquatic environments is also an important finding because saline lakes are widespread on Earth (Hann, 2006). As MTB have never been reported in these environments, these locales have apparently not been extensively searched for magnetofossils, specific types of magnetite crystals representing putative remains of MTB in terrestrial and extraterrestrial materials (Jimenez-Lopez *et al.*, 2010). Thus, it would be interesting to determine whether magnetite crystals get incorporated and preserved in saline evaporites such as the halite abundant in Badwater Basin (Schubert *et al.*, 2009) as they appear to be in sedimentary carbonates in marine environments (McNeill *et al.*, 1988; Sakai and Jige, 2006).

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