

Growth of magnetotactic sulfate-reducing bacteria in oxygen concentration gradient medium

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Summary

Although dissimilatory sulfate-reducing bacteria (SRB) are generally described as strictly anaerobic organisms with regard to growth, several reports have shown that some SRB, particularly *Desulfovibrio* species, are quite resistant to O₂. For example, SRB remain viable in many aerobic environments while some even reduce O₂ to H₂O. However, reproducible aerobic growth of SRB has not been unequivocally documented. *Desulfovibrio magneticus* is a SRB that is also a magnetotactic bacterium (MTB). MTB biomineralize magnetosomes which are intracellular, membrane-bounded, magnetic iron mineral crystals. The ability of *D. magneticus* to grow aerobically in several different media under air where an O₂ concentration gradient formed, or under O₂-free N₂ gas was tested. Under air, cells grew as a microaerophilic band of cells at the oxic–anoxic interface in media lacking sulfate. These results show that

D. magneticus is capable of aerobic growth with O₂ as a terminal electron acceptor. This is the first report of consistent, reproducible aerobic growth of SRB. This finding is critical in determining important ecological roles SRB play in the environment. Interestingly, the crystal structure of the magnetite crystals of *D. magneticus* grown under microaerobic conditions showed significant differences compared with those produced anaerobically providing more evidence that environmental parameters influence magnetosome formation.

Introduction

Dissimilatory sulfate-reducing bacteria (SRB) are generally considered to be strict anaerobes regarding growth (Rabus *et al.*, 2013). Widespread in marine and freshwater sediments, they constitute a morphologically and metabolically diverse group of prokaryotes, phylogenetically belonging to both the *Bacteria* and *Archaea* domains, and able to use a wide variety of organic compounds. SRB obtain energy for cell synthesis and growth by coupling the oxidation of these organic compounds or molecular hydrogen (H₂) to the reduction of sulfate (SO₄²⁻) to sulfide (H₂S, HS⁻) (Rabus *et al.*, 2013).

Most known SRB belong to the *Deltaproteobacteria* class of the *Proteobacteria* phylum in the domain *Bacteria*. Those in the genus *Desulfovibrio* were described as strictly anaerobic bacteria since their discovery more than 100 years ago (Beijerinck, 1895). *D. vulgaris* and *D. desulfuricans* were used for many years as model organisms to study sulfate reduction (Voordouw and Wall, 1993). Dissimilatory SRB are often present in biotopes where they are exposed to oxic conditions and some have even been shown to be metabolically active in microaerobic environments (Hardy and Hamilton, 1981; Battersby *et al.*, 1985; Sass *et al.*, 1996; 1997; Krekeler *et al.*, 1997; Sass *et al.*, 1998; Lobo *et al.*, 2007) showing that these bacteria are quite resistant to O₂ (Cypionka *et al.*, 1985; Sass *et al.*, 1996; 1997; 1998; Krekeler *et al.*, 1997). Some SRB not only survive exposure to O₂ for at least days, but some even reduce O₂ to H₂O (Dannenberg *et al.*, 1992), coupling aerobic respiration to ATP formation (Dilling and Cypionka, 1990;

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Cypionka, 2000) driving O₂-dependent enhancement of growth (Sigalevich *et al.*, 2000). Johnson *et al.* reported aerobic growth of *D. vulgaris* Hildenborough although growth was meagre at best if even present (Johnson *et al.*, 1997).

To counteract the deleterious effects of highly reactive derivatives of O₂, many anaerobic organisms developed defense systems similar to those found in aerobes (Storz *et al.*, 1990; Dolla *et al.*, 2006). Despite the capacity of some SRB to couple O₂ reduction with energy conservation, the observed chemotaxis of some species toward microaerobic zones, and the detoxification mechanisms developed by some strains, consistent, reproducible aerobic growth by SRB for an infinite number of generations has never been observed (Cypionka, 2000; Dolla *et al.*, 2006; Rabus *et al.*, 2013). Other alternative terminal electron acceptors to sulfate are used by some SRB; for instance, inorganic sulfur species such as sulfite (SO₃²⁻), thiosulfate (S₂O₃²⁻), trithionate (S₃O₆²⁻), tetrathionate (S₄O₆²⁻) and dithionite (S₂O₄²⁻) are respired by some *Desulfovibrio* species while sulfonates, dimethyl sulfoxide (DMSO), nitrate and nitrite are also used by some SRB. Fumarate and malate are also fermented by some *Desulfovibrio* species (Rabus *et al.*, 2013). Thus there is no reason to assume that the capacities for dissimilatory sulfate reduction and aerobic growth in the same bacterium are mutually exclusive.

Magnetotactic bacteria (MTB) biomineralize magnetosomes, intracellular membrane-enclosed magnetic crystals, causing them to swim along the Earth's geomagnetic field lines. MTB are ubiquitous in aquatic environments but are generally localized at the oxic-anoxic interface (OAI) (Bazylinski and Frankel, 2004). Magnetosomes appear to enhance aerotaxis by reducing a 3-dimensional search for the OAI to 1-dimension where cells swim up and down along the Earth's inclined geomagnetic field lines (Bazylinski and Frankel, 2004). MTB belong to three major groups of Gram-negative prokaryotes including the *Proteobacteria*, *Nitrospirae* and *Omnitrophica* phyla (Bazylinski *et al.*, 2013) and possibly the candidate phylum *Latescibacteria* (Lin and Pan, 2015) of the *Fibrobacteres-Chlorobi-Bacteroidetes* (FCB) superphylum (Rinke *et al.*, 2013). Deltaproteobacterial MTB reduce and grow with sulfate as a terminal electron acceptor (Bazylinski *et al.*, 2013). The polyphyletic origin idea of magnetosome biomineralization arose from the discovery of the first magnetotactic *Deltaproteobacteria*, a multicellular magnetotactic prokaryote (DeLong *et al.*, 1993), later found to likely be a SRB (Abreu *et al.*, 2011). Other deltaproteobacterial sulfate-reducing MTB have been isolated and described (Lefèvre and Bazylinski, 2013).

Magnetosome crystals have high chemical purity, narrow size ranges, species-specific crystal morphologies

and exhibit specific arrangements within the cell. These features indicate that the formation of magnetosomes by MTB is under precise biological control. Phylogenetic analysis shows that there is an important correlation between the composition and morphology of the magnetosome mineral crystals produced by MTB and their phylogenetic affiliation (Abreu *et al.*, 2011; Lefèvre *et al.*, 2011a,b, 2012, 2013). Magnetotactic *Alpha-* and *Gammaproteobacteria*, the later-diverging classes of the *Proteobacteria*, biomineralize morphologically consistent, well-defined crystals of magnetite that include cuboctahedral and elongated prisms (Devouard *et al.*, 1998; Lefèvre *et al.*, 2012). In contrast, in the magnetotactic *Deltaproteobacteria*, the most deeply diverging group of the *Proteobacteria*, that biomineralize magnetite, greigite, or both, the magnetite crystals are always bullet-shaped. The magnetotactic *Nitrospirae* and *Omnitrophica*, the more deeply branching phylogenetic groups that contain MTB (Jogler *et al.*, 2011; Kolinko *et al.*, 2012), are known to biomineralize only magnetite crystals whose morphologies are very similar to those found in the *Deltaproteobacteria* (Lefèvre *et al.*, 2011a, b). Thus, based on the phylogeny of MTB and the type of magnetosomes that they biomineralize, it has been suggested that bullet-shaped magnetite crystals represent the first magnetosome mineral phase (Lefèvre *et al.*, 2013). In addition to the genetic control over the type of crystal mineralized in the magnetosome membrane, environmental parameters also play a role in the regulation of magnetosome formation. For instance, it was shown that rates of iron uptake change the morphology of magnetite crystals (Faivre *et al.*, 2008). Culturing experiments also showed that depending on environmental conditions, strain BW-1, preferentially mineralizes magnetite (high redox potential) or greigite (reduced conditions) as a function of the redox conditions (Lefèvre *et al.*, 2011a, b).

Here we show that some magnetotactic SRB of the genus *Desulfovibrio* are capable of reproducible, consistent aerobic growth in semi-solid oxygen concentration gradient ([O₂]-gradient) medium lacking sulfate. In such conditions, the magnetosomes present consistent morphological variations that could be used as biomarkers to understand past environmental conditions present in geological records where such magnetosome morphology was deposited.

Results

Isolation of magnetotactic SRB

MTB were magnetically concentrated and purified from samples from different freshwater environments (Bazylinski *et al.*, 2013) and used as inocula into semi-solid, [O₂]-gradient growth medium containing succinate as

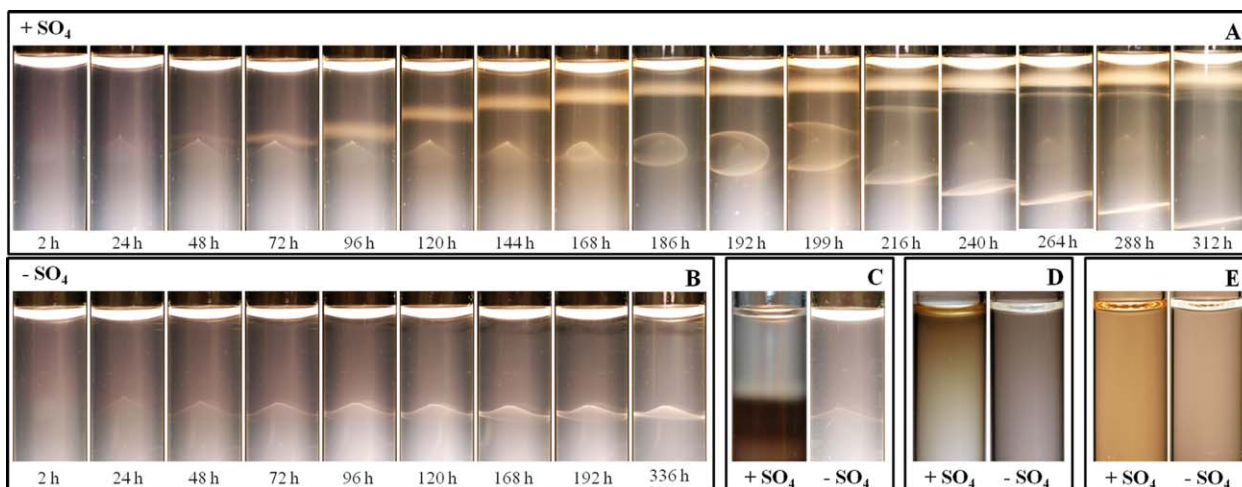


Fig. 1. Growth of *Desulfovibrio magneticus* strain RS-1 in $[O_2]$ -gradient medium in the presence or absence of sulfate. **(A)** Semi-solid $[O_2]$ -gradient medium containing 5.3 mM sulfate. Here growth occurs initially as a microaerophilic band at the oxic-anoxic interface (OAI). After approximately 186 h, the band splits into two, one migrating to the meniscus of the medium apparently seeking O_2 in the microaerobic zone, the other migrating to the anaerobic zone of the tube apparently seeking sulfate. The thick white band, visible after 48 h, is composed of elemental sulfur (S^0) formed by the interaction of H_2S and O_2 (Supporting Information Fig. S2). **(B)** Semi-solid $[O_2]$ -gradient medium without sulfate (except for sulfate contamination in the salts used, determined to be ~ 20 nM). Cells inoculated at the OAI spread and form a microaerophilic band after 24 h. The size and thickness of the band increases while cells grow and stay at the OAI indicating that they are respiring and growing with O_2 as the terminal electron acceptor. **(C)** Same growth conditions as **(A)** (left) and **(B)** (right) with addition of 100 μ M iron to trap H_2S as a black precipitate of FeS indicating that sulfate reduction was occurring. The tube on the right stays clear indicating that no H_2S is formed during growth without sulfate. **(D)** Same growth conditions as **(A)** (left) and **(B)** (right) except that the headspace of the tube was replaced with O_2 -free Ar. Without O_2 , the tube without sulfate (right) shows no growth; when sulfate is present (left), growth occurs as shown by the production of FeS (dark colour). **(E)** Anaerobic liquid culture under Ar. Growth occurs only when sulfate is present in the medium (left) as shown by the production of FeS (dark colour).

the electron donor. After 10–14 days of incubation at 25°C, those tubes containing purified MTB from a fish hatchery pond in Montana and a pond near Zuma Beach, Los Angeles, California, displayed a defined band of cells growing at the OAI. Light microscopic examination of cells in these cultures showed that they were magnetotactic and helical to vibrioid in morphology. MTB from the cultures were diluted to extinction three successive times to obtain axenic cultures. The strains from the fish hatchery in Montana and the pond near Zuma Beach are designated as strains FH-1 and ZBP-1 respectively.

Phylogenetic analysis, based on 16S rRNA gene sequences of FH-1 and ZBP-1, showed that both strains belong to the genus *Desulfovibrio*. The sequence of strain FH-1 showed 99.5% identity with *Desulfovibrio magneticus* strain RS-1^T (Sakaguchi *et al.*, 2002) and strain ZBP-1 showed 99.5% identity with *D. putealis* strain B7-43^T (Basso *et al.*, 2005) (Supporting Information Fig. S1). Based on these high 16S rRNA gene sequence identities, FH-1 and ZBP-1 can be considered as strains of *D. magneticus* and *D. putealis* respectively. Cells of strains FH-1 and ZBP-1 were repeatedly transferred in $[O_2]$ -gradient growth medium without sulfate and growth always occurred as a microaerophilic band of cells formed initially at the OAI. Growth of *D. magneticus* strain RS-1 was indistinguishable from that of

strains FH-1 and ZBP-1 in this medium. Because of the numerous studies on *D. magneticus* strain RS-1 as well as its complete genome sequence available (Nakazawa *et al.*, 2009), we focused our analyses on this strain. We have grown strain RS-1 consecutively since 2009 under microaerobic conditions in $[O_2]$ -gradient media lacking sulfate (>100 transfers). This also applies for strains FH-1 and ZBP-1.

When sulfate (5.3 mM) was present in the growth medium, under an air headspace, initial growth of the strains was as a microaerophilic band of cells at the OAI (Fig. 1A). However, as the band thickened, cells appeared to reduce the sulfate to H_2S which accumulated and diffused toward the surface of the culture. H_2S then reacted with O_2 resulting in a band of a sulfur-rich precipitate (S^0) a few millimeters above the microaerophilic band of cells (Supporting Information Fig. S2). As growth continued, the band split into two with an intermediate step where the cells form a ball-like appearance (Fig. 1A, 186 h). Cells in the upper band migrated toward the surface while those in the lower band migrated to the deeper part of the culture suggesting that those in the upper band were seeking O_2 to respire while those in the lower band were seeking sulfate which was presumably depleted in the upper layers of the medium. When cells from the deeper band in $[O_2]$ -gradient medium containing sulfate were transferred to

the OAI of a tube of the same fresh medium, growth of the strains followed the same scheme as described above and in Fig. 1A.

In the same medium lacking sulfate (except for sulfate contamination in the salts used in the medium estimated at 20 nM by liquid chromatography), cells still formed an initial band at the OAI but did not split in two. H₂S was not detected nor was a layer of S⁰ indicating that sulfate reduction did not occur under these conditions (Fig. 1B). To confirm that sulfate reduction did not occur in medium lacking sulfate, the concentration of iron was raised to 100 µM in order to detect and trap formed H₂S as FeS during sulfate reduction (Fig. 1C). In the presence of sulfate, cells produced H₂S and a black precipitate of FeS was observed, while in the absence of sulfate, no black precipitate was observed. To further demonstrate that O₂ was required for growth in the absence of sulfate, we grew cells in the same medium with O₂-free N₂ in the headspace (Fig. 1D). In the absence of O₂ and the presence of sulfate, cells grew while in the absence of sulfate no growth was observed. We also tested the growth in anaerobic liquid culture medium, with or without sulfate; growth only occurred when sulfate was present in the growth medium (Fig. 1E).

Molybdate has been shown to inhibit sulfate reduction in some SRB (Newport and Nedwell, 1988; Ranade *et al.*, 1999). Indeed, molybdate is a structural analogue of sulfate which is thought to interact with key enzymes involved in sulfate reduction such as the ATP sulfurylase (Peck, 1959). The growth in the anaerobic liquid medium where cells of *Desulfovibrio magneticus* strain RS-1 reduce sulfate was inhibited by a concentration of sodium molybdate of 3 mM. When the same dose of molybdate was added to a semi-solid [O₂]-gradient medium depleted with sulfate, the cells of strain RS-1 continue to grow and to form a band at the OAI. In the same medium with sulfate, a band of cells also formed at the OAI while neither the band of cells in the anoxic zone nor the sulfur precipitates, usually observed in absence of molybdate (Fig. 1A), are formed in such conditions. This indicates that the cells in the band formed at the OAI, in presence or absence of sulfate, are using O₂ as terminal electron acceptor.

Additional evidence of aerobic respiration linked to growth in magnetotactic SRB

A three microelectrode voltammetric probe was used to simultaneously determine O₂ and H₂S concentration profiles (Brendel and Luther, 1995; Luther *et al.*, 2008) (Fig. 2). The microaerophilic band observed in [O₂]-gradient medium without sulfate formed at O₂ concentration of about 28–31 µM. The highest concentration (~550 µM) of H₂S, only detected when sulfate was present in

the medium, appeared at the depth of the second band of cells in the anaerobic zone. H₂S detected close to the upper microaerophilic band in the medium with sulfate likely results from upward diffusion of H₂S produced by the lower band. This diffusion would be expected even if the upper band was not present although the possibility that some sulfate reduction by cells in the upper band that become O₂ starved cannot be excluded. When no sulfate was present in the medium, no H₂S was detected in the culture and only one band formed at the microaerophilic zone.

O₂ respiration rates of cells of strain RS-1

Cells of strain RS-1 grown anaerobically with fumarate completely consumed O₂ (initial concentration 220 µM) in the chamber in the mass spectrometer within 15 min (Supporting Information Fig. S3). The O₂ consumption rate measured on four independent suspensions was 128 ± 27 nmol of O₂ min⁻¹ mg of protein⁻¹.

Growth under microaerobic conditions impacts cell and magnetosome morphologies

Cells of *D. magneticus* strain RS-1 grown in [O₂]-gradient medium lacking sulfate were notably longer than cells grown on sulfate. The average size of the cells grown in the presence of sulfate was 2.3 ± 0.7 µm (*n* = 147) while the cells grown without sulfate had an average size of 4.8 ± 2.0 µm (*n* = 147). Under all conditions tested, species of magnetotactic *Desulfovibrio* had a vibrioid to helical shape with one polar flagellum. Aggregate formation, which is one of the known mechanisms enabling some SRB to survive under periodic exposure to oxic conditions (Sigalevich *et al.*, 2000), was occasionally observed in microaerophilic bands.

The number of magnetosomes per cell in RS-1 was higher in cells grown microaerobically with an average number of 15 ± 6 (*n* = 25) while cells grown anaerobically with sulfate as electron acceptor had an average number of magnetosomes of 8 ± 5 (*n* = 37). Interestingly, the size of the magnetite magnetosome crystals also varied depending upon culture conditions. While the width of the crystals appeared to be relatively constant [30 ± 6 nm (*n* = 113) cells grown anaerobically with sulfate; 29 ± 4 nm (*n* = 258) cells grown microaerobically with O₂], the length of the magnetosome crystals of cells grown under microaerobic conditions appeared to be longer [57 ± 16 nm (*n* = 113) cells grown anaerobically with sulfate; 82 ± 33 nm (*n* = 258) in cells grown microaerobically with O₂] (Supporting Information Fig. S4), with some being longer than 200 nm and present more defects in their morphologies (e.g., kinks) (Fig. 3). Fast Fourier transform (FFT) analysis performed on images obtained from high resolution transmission electron

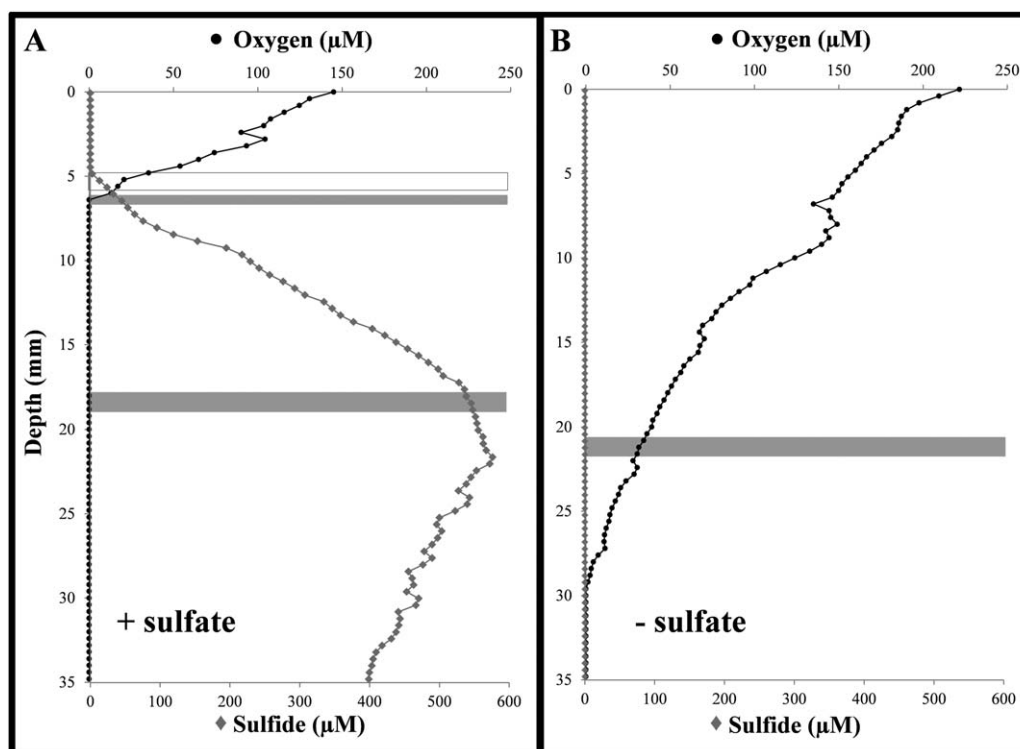


Fig. 2. Vertical concentration profiles of dissolved O_2 (●) and $\Sigma H_2S + HS^-$ (◆) through semi-solid $[O_2]$ -gradient cultures of *Desulfovibrio magneticus* strain RS-1. Profiles in the presence (A) or absence (B) of sulfate in the culture medium. When sulfate is present in the culture (left profile), H_2S is produced through the reduction of sulfate by cells present in the anaerobic zone (band of cells represented by a grey band at ~18 mm). H_2S diffuses upward toward the meniscus in the tube and leading to the formation of a layer of precipitate of sulfur (S^0) in the oxic, upper zone of the culture (S^0 represented by white band at ~5 mm). Although sulfate is present in the culture, a second band of cells formed at the oxic-anoxic interface (OAI) (band of cells represented by grey band at ~6 mm). When sulfate was omitted from the medium (right profile), no H_2S formed and cells grow as a single band (band of cells represented by a grey band at ~21 mm). In this latter tube, the optimal dissolved $[O_2]$ for growth was approximately 28–31 μM . Profiles were obtained from cultures incubated 9 days after inoculation.

microscopy (HRTEM) show that the elongated magnetite crystals produced by RS-1 grown microaerobically are single crystals (Fig. 3A). Although bright-field contrast variations on a highly curved magnetosome indicate two slightly misoriented sub-grains with a misorientation estimated at approximately 2° (Fig. 3B), HRTEM and corresponding FFTs (Fig. 3 B2; B4) indicate that the two sub-grains are monocrystalline. Similar results were found for *D. magneticus* strain FH-1 and *D. putealis* strain ZBP-1 (Supporting Information Fig. S5).

In silico analysis of the mechanisms related to O_2 reduction in *desulfovibrio magneticus*

Several O_2 -reducing mechanisms have been characterized and studied in SRB, mostly from sequence data from the genome of *D. vulgaris* (Lamrabet *et al.*, 2011). Genomic analysis of *D. magneticus* strain RS-1 (Nakazawa *et al.*, 2009) shows that similar mechanisms are present in this species. Three gene clusters coding for oxidases, the enzyme known to be involved in reducing O_2 to H_2O , are present in the genome sequence of strain

RS-1. Two of these clusters are cytochrome *bd* ubiquinol oxidases (*cydA*: DMR_06960 and *cydB*: DMR_06970; *cydA'*: DMR_28300 and *cydB'*: DMR_28310) that share a maximum identity of sequence with orthologous genes from *Desulfovibrio* species (e.g., *CydA* has 78% identity of sequence with a cytochrome oxidase of *D. fructosivorans* and *CydA'* has 85% identity with a cytochrome *d* ubiquinol oxidase subunit I of *D. alcoholivorans*). The third oxidase is a cytochrome *o* ubiquinol oxidase composed by four subunits (DMR_14870 = *cyoA'*, DMR_14880 = *cyoB'*, DMR_14890 = *cyoC* and DMR_14900 = *cyoD*) that shares a maximum sequence identity with orthologous genes from *Desulfovibrio* species (e.g., the subunit II has 66% identity with the sequence of *D. putealis*).

Reactive oxygen species (superoxide, hydrogen peroxide and hydroxyl radical) are intermediates/products formed during O_2 reduction (respiration). Their detoxification involves specific enzymes including superoxide dismutase, superoxide reductase and catalase, generally found in aerobic microorganisms in order to tolerate atmospheric concentrations of O_2 during aerobic respiration. Genes for superoxide dismutase, encoded by the

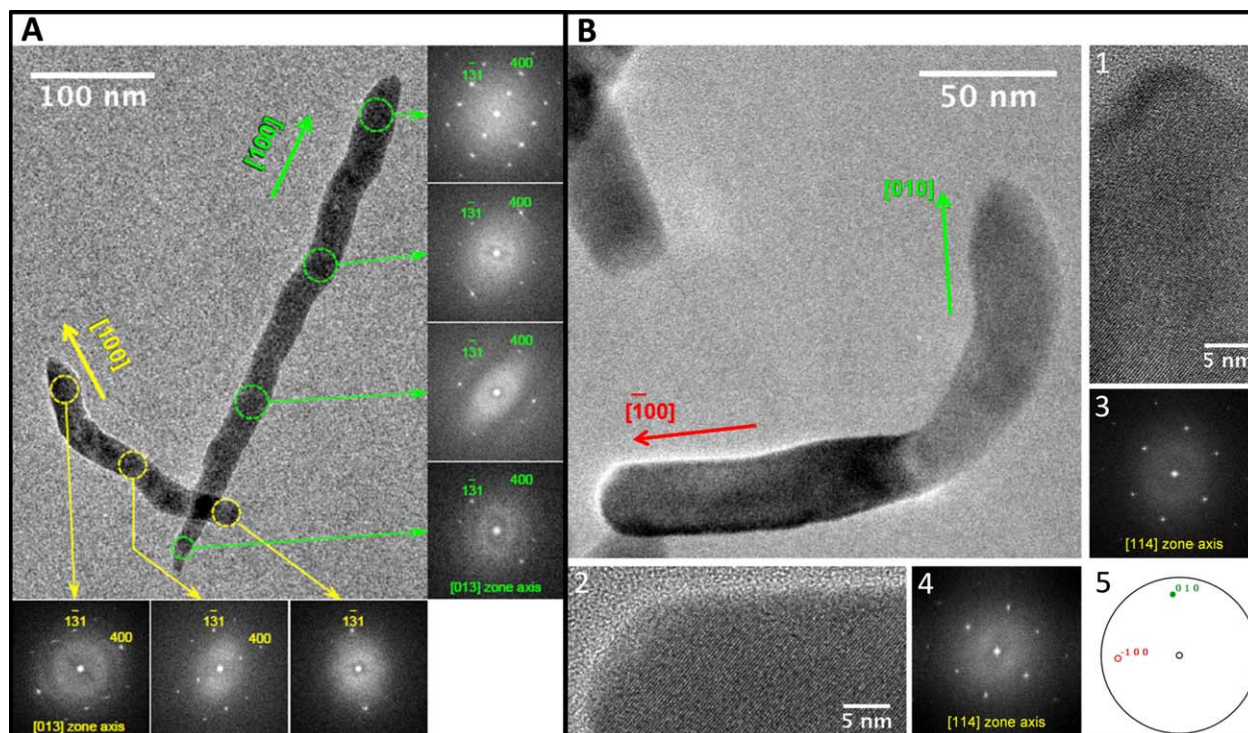


Fig. 3. Crystallographic properties of magnetite magnetosome crystals from cells of *Desulfovibrio magneticus* strain RS-1 grown microaerobically with O_2 as the terminal electron acceptor. (A) Transmission electron microscope (TEM) image of elongated magnetosomes. High resolution TEM (HRTEM) images were obtained on whole crystals and fast Fourier transform (FFT) analysis performed on all HRTEM images. Each FFT is related to the selected area defined by the green or yellow dotted circles. FFTs from the same crystal have the same crystallographic orientation, that is, [013], showing that each magnetite particle is a single crystal. (B) TEM image (larger panel) of a highly curved magnetosome analyzed using bright-field electron contrast variations showing that the magnetosome is formed by two sub-grains slightly misoriented by approximately 2° . (B1 and B3) HRTEM and corresponding FFTs (B2 and B4) indicate that the two sub-grains are monocrystalline. The two elongation directions are respectively [010] and [100] as deduced from the stereographic projection analyses (B5).

sodB gene (DMR_42280), superoxide reductase, a rubredoxin oxidoreductase encoded by the *rbo* gene (DMR_17610) and catalase, encoded by the *katA* gene (DMR_34220), are all present in the genome of *D. magneticus* strain RS-1.

Discussion

Our finding that some SRB respire with O_2 and link this reduction to growth explains many of the puzzling observations regarding these presumably strictly anaerobic organisms over many years. For example, *Desulfovibrio* species routinely exposed to relatively high concentration of O_2 in natural environments such as microbial mats (Canfield and Des Marais, 1991; Krekeler *et al.*, 1997; Teske *et al.*, 1998) and biofilms (Ramsing *et al.*, 1993) survive and may even be metabolically active under these conditions. Results presented here support this contention. The potential of *Desulfovibrio* species and other SRB to reduce and grow with O_2 as a terminal electron acceptor demonstrates another, mostly unrecognized, important ecological role of these organisms.

Why reproducible growth of SRB with O_2 as a terminal electron acceptor had not been demonstrated earlier despite a number of excellent studies focused on this question? Many anaerobes are known to not only require the absence of O_2 for growth but specific redox conditions are also often required. Chemical reducing agents are often added to growth medium to facilitate the growth of many anaerobes not only by scavenging O_2 but also to provide a suitable redox potential for growth. An excellent example of this is the methanogens. These organisms generally will not grow in medium simply where O_2 has been eliminated and require the medium to have a very low redox potential obtained by the addition of a reducing agent such as Na_2S (Huber *et al.*, 1982). The same requirement also applies to SRB where a low redox potential is also needed for optimal growth (Postgate, 1985). To obtain aerobic growth of SRB, a similar situation may apply, that is, for aerobic growth these organisms obviously require microaerobic conditions but perhaps a specific redox potential is also necessary. The $[O_2]$ -gradient medium used in this study provides both microaerobic conditions and an appropriate redox potential for growth. In our medium,

cysteine and the relatively high concentration of Fe^{2+} in the medium (for magnetosome production) act as reducing agents. O_2 in the headspace of the cultures diffuses into the culture resulting in the $[\text{O}_2]$ -gradient which is clearly observable when resazurin, a redox indicator (midpoint reduction potential $E_0' = 380 \text{ mV}$), is added to the medium (oxic zone is pink while anoxic zone is colourless in the presence of resazurin). Moreover, in our semi-solid $[\text{O}_2]$ -gradient medium, cells can swim to their preferred $[\text{O}_2]$ and redox potential. We believe that these specific conditions required for aerobic growth of SRB were not met in the growth media used in the numerous studies that failed to unequivocally demonstrate aerobic growth of SRB in the absence of sulfate (Ramel *et al.*, 2015). It is also possible that the ability to respire O_2 by some *Desulfovibrio* species was lost due to long term transfer in strictly anaerobic conditions and specific approaches such as experimental evolution would be required to obtain oxic growth.

Based on the apparent requirements for a specific $[\text{O}_2]$ and redox potential for growth of the SRB used in our study, it is not surprising that cells grow as a microaerotactic band of cells in the $[\text{O}_2]$ -gradient medium when sulfate is omitted. However, when sulfate was present in the same medium, two bands of cells eventually formed, one migrating upward to the meniscus and the other downward to the anoxic zone of the gradient. Since the band that migrates downward forms secondarily to the original band (even if the medium is inoculated with cells grown anaerobically with sulfate), it is likely that the second band forms only when O_2 becomes limiting by growing cells at the OAI. At this point, many cells in the original band at the OAI may start to switch their metabolism to the reduction of sulfate in the medium resulting in the formation of the second band. These cells eventually use up the sulfate at the band and then swim downward to obtain more sulfate. This result, in particular, suggests an important metabolic feature for the SRB used in this study, that is, the possibility that these SRB prefer to use O_2 as a terminal electron acceptor to sulfate when conditions are appropriate. This explains the formation of the initial band at the OAI when sulfate is present in the growth medium. However, we cannot prove or disprove the possibility that both terminal electron acceptors are used by cells simultaneously in the original band although our results show no evidence of this in the form of H_2S production. Cells might have to reach a certain density in our cultures to initiate sulfate reduction under these conditions.

The O_2 reduction rates for strain RS-1 ($128 \pm 27 \text{ nmol of O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$) and other *Desulfovibrio* species [e.g., *D. vulgaris* Marburg: $222 \text{ nmol of O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$ (Baumgarten *et al.*, 2001) and the highest O_2 reduction rate reported for a SRB thus far, in *D. termittidis*:

$1570 \text{ nmol of O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$ (Kuhnigk *et al.*, 1996)], are comparable to those of aerobic bacteria. The exact mechanism of O_2 reduction by the O_2 -respiring *Desulfovibrio* species used in this study is not yet known although it certainly must involve some of the oxidases whose genes are present in the genome of *D. magneticus*. These include genes for cytochrome *bd* ubiquinol oxidases, cytochrome *d* ubiquinol oxidase and cytochrome *o* ubiquinol oxidase all of which are present in the genomes of other SRB (Dolla *et al.*, 2006). Although the proton motive force generated by the activity of these enzymes appears to contribute at least partially to energy production in *Desulfovibrio* species (Lamrabet *et al.*, 2011), additional studies are required to determine the functions of these oxidases in SRB as well as to elucidate the biochemical mechanism(s) involved in O_2 reduction in these organisms.

A secondary important result of our study is the difference in the number of magnetosomes and the morphology of the magnetosome magnetite crystals produced by the magnetotactic SRB, *D. magneticus* strains RS-1 (Fig. 3) and FH-1 and *D. putealis* strain ZBP-1 (Supporting Information Fig. S5), under different growth conditions. These strains biomineralize more magnetosomes when grown aerobically than when grown anaerobically with sulfate. Sulfate-reducing cells of RS-1 were even once not considered to be an MTB (Pósfai *et al.*, 2006). This is not the case with cells grown microaerobically which produce more magnetosomes per cell and larger magnetosome magnetite crystals than those grown with sulfate anaerobically. Moreover, the ability of magnetotactic SRB to respire with O_2 and grow as a band at the OAI demonstrates that these organisms generally behave as other microaerophilic MTB using magnetotaxis to locate their optimal position at the OAI in $[\text{O}_2]$ -gradients more efficiently (Bennet *et al.*, 2014). The majority of cultured MTB are microaerophiles with some having alternative anaerobic metabolisms [e.g., respiration of NO_3^- by *Magnetospirillum* species (Bazylinski and Blakemore, 1983) or N_2O by *Magnetovibrio blakemorei* (Bazylinski *et al.*, 1988)]. Only cultured MTB of the *Deletaproteobacteria* have been described as strict anaerobes, raising questions regarding the role of magnetosomes and the function of magnetotaxis in strictly anaerobic environments. It thus seems possible that all MTB have the potential to respire with O_2 although the conditions to do so for some MTB only known to grow anaerobically remains to be elucidated.

Environmental conditions affect the composition, morphology and number of magnetosome crystals per cell, both in uncultured MTB in the environment and in cultured species (Bazylinski *et al.*, 1995; Faivre *et al.*, 2008). This also appears to be the case for *D. magneticus*. Magnetosome magnetite crystals appear to be

involved in the elimination of reactive oxygen species in some MTB (Guo *et al.*, 2012) and thus, especially considering the stringent conditions under which the magnetotactic *Desulfovibrio* strains grow microaerobically, might play a similar role in these organisms. Cells of *D. magneticus* strain RS-1, in microcapillary tubes containing an [O₂]-gradient (Lefèvre *et al.*, 2014), act like other microaerophilic MTB, that is, they swim back and forth along magnetic field lines through the band. However, they exhibit a unique behaviour, called unipolar magnetotaxis, when the magnetic field is reversed: all cells swim toward the anoxic zone. It was speculated that this behaviour was favourable to this species because the anoxic side is likely less toxic than the oxic side due to deleterious effects of exposure to high [O₂] (Lefèvre *et al.*, 2014).

Bullet-shaped magnetosomes with a length greater than 200 nm were also reported in MTB from environmental samples such as the Moorsee in Bavaria (Vali and Kirschvink, 1991). MTB have great impacts on geology and paleomagnetism (Bazylinski and Moskowitz, 1997). When a magnetotactic bacterium dies and lyses, its magnetosome crystals are released into the surrounding environment where they can persist or undergo dissolution and/or transformation into other minerals. In habitats where the crystals persist over time, magnetotactic bacterial magnetite is an important, sometimes the primary, carrier of magnetic remanence in oceanic and lake sediments (Oldfield and Wu, 2000). Using isotopic dating and other technologies, investigators can determine when sediments were deposited which in turn provides important information about the origin and the evolution of MTB (Lefèvre *et al.*, 2013). Magnetosomes are considered as indirect bacterial fossils, referred to as magnetofossils (Chang and Kirschvink, 1989). Thus, bullet-shaped particles with a length greater than 200 nm could represent suitable biomarkers of microoxic environments and thus could be used to infer the past environmental conditions in geological records containing such magnetofossils.

Our results pave the way to understanding the molecular mechanisms and metabolic pathways responsible for O₂ respiration in SRB and the ecological roles these organisms play in biotopes where O₂ is either temporarily or permanently present. Dissimilatory SRB have been dated back to 3.5 billion years ago, and along with the phototrophic iron oxidizing bacteria, are considered to be among the oldest microorganisms on Earth (Battistuzzi *et al.*, 2004). Although speculative, our findings suggest that these organisms may have been the first prokaryotes to develop the ability to respire with O₂ when microaerobic conditions developed and became common. Additional evidence for this could come from systematic examination of all SRB in both the *Bacteria*

and the *Archaea* for the ability to respire and grow with O₂ as the terminal electron acceptor, something that can now be done given the growth conditions that support aerobic growth of SRB described here.

Experimental procedures

Growth conditions, medium composition and isolation of magnetotactic strains

Cells of MTB from samples were concentrated using the magnetic "capillary racetrack" technique (Wolfe *et al.*, 1987) and used as inocula in a modified semi-solid [O₂]-gradient enrichment medium similar to that described previously (Bazylinski *et al.*, 2004). Modifications were that the basal medium contained (per liter): 5 ml modified Wolfe's mineral elixir (Wolin *et al.*, 1963; Bazylinski *et al.*, 2000); 0.2 ml 1% (wt/vol) aqueous resazurin; 0.25 g NH₄Cl; 0.1 g MgSO₄ · 7H₂O and the pH adjusted to 7.0. 2.0 g of Bacto-Agar (Difco Laboratories, Detroit, MI) 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); of 0.5 M KHPO₄ buffer pH 6.9; 2.67 ml freshly made 0.8 M NaHCO₃ (the NaHCO₃ is autoclaved dry); 2.5 ml of 10 mM ferric quinate (Blakemore *et al.*, 1979) and 0.4 g of freshly made neutralized filtered sterilized cysteine · HCl · 2H₂O. Screw-capped glass culture tubes were filled to approximately 60% of their volume with medium. The medium was allowed to sit at room temperature for several hours to solidify and to allow the [O₂]-gradient to form as evidenced by the presence of a pink (oxidized) zone near the surface and a colourless (reduced) zone in the deeper portion of the tubes. Cultures were incubated at 25°C. An axenic culture of strain FH-1 was obtained from colonies by a shake tube technique while the axenic culture of strain ZBP-1 was obtained by dilution to extinction three times in succession in the semi-solid growth medium.

Cells of *Desulfovibrio magneticus* strain RS-1 (ATCC 700980; DSM 13731) and strains FH-1 and ZBP-1 were grown routinely in a semi-solid [O₂]-gradient medium containing (per liter): 5 ml modified sulfate-free Wolfe's mineral elixir (all sulfate salts were replaced with chlorides), 0.2 ml 1% aqueous resazurin; 0.17 g NaNO₃ (used as nitrogen source only), 0.75 g sodium succinate · 6H₂O and 0.082 g MgCl₂ · 7H₂O and the pH adjusted to 7.0. 1.0 g of Agarose (Difco Laboratories, Detroit, MI) 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; 2.8 ml of 0.5 M KHPO₄ buffer pH 7.0; 1.5 ml of 0.8 M NaHCO₃ solution; 3.5 ml of 10 mM FeCl₂ · 4H₂O (in 0.02 N HCl); and 0.2 g of freshly made

neutralized filtered sterilized cysteine · HCl · 2H₂O. Screw-capped glass culture tubes were filled to approximately 60% 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 colourless (reduced) zone in the low part of the tubes.

The same medium with 0.75 g of sodium sulfate was used as a control allowing bacteria to grow anaerobically using sulfate as electron acceptor. Liquid anaerobic cultures were done in test tubes sparged with O₂-free N₂. The inhibition of sulfate reduction of strain RS-1 by molybdate was tested by the addition of increasing doses of sodium molybdate in the liquid anaerobic medium. At a concentration of 1 mM, sulfate reduction still occurred. Inhibition of sulfate reduction was observed at 2 and 3 mM and the medium was clear. At 4 mM molybdate, a brownish precipitate occurred in the medium and thus a concentration of 3 mM was chosen as the concentration to inhibit sulfate reduction by strain RS-1.

Chemical measurements

A three microelectrode voltammetric cell was used to determine O₂ and sulfide concentrations in semi-solid [O₂]-gradient cultures (Brendel and Luther, 1995; Luther *et al.*, 2008). Cells of strains RS-1 and FH-1 were grown in the same medium described above, in large test tubes to allow the electrodes to fit in the width of the tube. About 135 ml of medium was added in these large test tubes. An Ag/AgCl reference electrode and a Pt counter electrode were used in conjunction with an Au/Hg working electrode. Preparation of the solid state Au/Hg working microelectrode was performed as previously described (Brendel and Luther, 1995). Voltammetric measurements were made with an Analytical Instrument Systems DLK-100 analyzer (Analytical Instrument Systems, Inc., Flemington, NJ) and recorded to computer. The microelectrode was controlled by a micromanipulator.

Sulfate concentrations in growth medium was determined using liquid chromatography on a high-performance liquid chromatography (HPLC) 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) 4.6 × 10, Phenomenex, Torrance, CA] coupled with a CARS™ Continuous Anion Regeneration System was used to separate the 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 with *r*² values greater than 0.99.

Mass spectrometry measurements of O₂ respiration

Cells were grown anaerobically with pyruvate and fumarate in liquid medium (Byrne *et al.*, 2010) to an optical density (OD) = 1.1 after which aliquots of the cells were placed in the measuring chamber (1.5 ml) of a mass spectrometer (model PrimaδB; Thermo Electron). The bottom of the chamber (Hansatech electrode type) was sealed by a Teflon membrane, allowing dissolved gases to be directly introduced through a vacuum line into the mass spectrometer ion source. The chamber was maintained at 30°C, and the cell suspension stirred continuously magnetically. Air was then introduced into the suspension, reaching an [O₂] of 220 μM, before closing the chamber. O₂ consumption was followed at *m/e* = 32.

Light and electron microscopy

Images of the culture tubes were taken every 24 h, less when necessary, in triplicate for each condition with a camera Nikon model D70 with the macro lens AF MICRO NIKKOR 60 mm. The photographic chamber was black with a Fiber-L-Lite high-intensity illuminator series 180 (Dolan-Jenner Industries, Boxborough, MA) as only light source for optimal contrast and visualization of growth.

Light microscopy imaging was performed with a Zeiss Axiolmager M1 light microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) equipped with phase-contrast and differential interference contrast capabilities. The hanging-drop technique (Schüler, 2002) was used routinely in the examination of MTB and to determine whether cells were magnetotactic.

Imaging and composition of the sulfur precipitate was performed and determined by combinations of transmission electron microscopy (TEM), scanning-transmission electron microscopy (SEM) and energy-dispersive X-ray analysis (EDX) with a Tecnai (FEI Company, Hillsboro, OR) Model G2 F30 Super-Twin transmission electron microscope.

TEM of whole cells was done with a Tecnai (FEI Company, Hillsboro OR) model G2 F30 Super-Twin and G2 Biotwin and a JEOL (JEOL, Ltd., Tokyo, Japan) model 3010 transmission electron microscope. High-resolution TEM (HRTEM) and scanning transmission electron microscopy (STEM) were performed on whole cells and magnetosomes with a JEOL model 2100F (Field Emission Gun) operated at 200 kV and equipped with a high resolution pole piece and a Gatan (Gatan, Inc., Pleasanton, CA) model US4000 (4k × 4k) Charge-Couple Device (CCD) camera. Energy-dispersive X-ray (XEDS) elemental maps were acquired using energy-filtered

TEM (EFTEM) with the JEOL 2100F in the STEM dark-field mode with a focused electron beam (1 nm). Selected-area electron diffraction (SAED) in the TEM was performed with the Tecnai and the JEOL model 2100F electron microscopes.

Determination of 16S rRNA gene sequence and phylogenetic analysis

16S rRNA genes were amplified with Bacteria-specific primers 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-TACGGHTACCTTGTTACGACTT-3' (Lane, 1991). PCR products were cloned into pGEM-T Easy Vector (Promega Corporation, Madison WI) and sequenced (Functional Biosciences, Inc., Madison WI).

Alignment of 16S rRNA genes was performed with CLUSTAL W multiple alignment accessory application in the BioEdit sequence alignment editor (Hall, 1999). Phylogenetic tree was constructed with MEGA version 5 (Tamura *et al.*, 2011) applying the neighbour-joining method (Saitou and Nei, 1987). Bootstrap values were calculated with 1000 replicates.

Nucleotide sequence accession numbers

16S rRNA gene sequences of strains FH-1 and ZBP-1 carry GenBank accession numbers JF330268 and JN015508 respectively.

Acknowledgements

We thank the Héliobiotec platform (funded by the European Union, the region Provence Alpes Côte d'Azur, the French Ministry of Research and the Commissariat à l'Energie Atomique et aux Energies Alternatives) for the mass spectrometer, and D. Pignol for helpful discussions and suggestions. This work was supported by U.S. National Science Foundation (NSF) grants EAR-0920718 and EAR-1423939. P.A.H. and M.L.S. were recipients of an award from the NSF Research Experience for Undergraduates (REU) program A Broad View of Environmental Microbiology at the University of Nevada at Las Vegas (Award NSF-0649267). C.T.L. was supported by the French National Research Agency (GROMA: ANR-14-CE35-0018-01) and G.W.L. acknowledges support from U.S. National Aeronautics and Space Administration Exobiology Grant NNX12AG20G.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the phylogenetic positions of *Desulfovibrio magneticus* strain RS-1, strain FH-1 and strain ZBP-1, in the *Deltaproteobacteria* class of the *Proteobacteria*. Bootstrap values at nodes are percentages of 1000 replicates. *Escherichia coli* was used to root the tree. GenBank accession numbers are given in parentheses. Bar represents 2% sequence divergence.

Fig. S2. Light and electron microscope images and analysis of globular precipitates from a culture of *Desulfovibrio magneticus* strain RS-1 growing in a tube of [O₂]-gradient medium containing 5.3 mM sulfate. **(A)** and **(B)** light microscope images obtained using dark-field and differential interface contrast respectively. **(C)** scanning-transmission electron microscope (STEM) image showing a single globule on a copper grid. **(D)** elemental spectrum of a single globule (left) compared to that of background (right; area of grid without a

globule present) using energy dispersive X-ray spectroscopy analysis showing that the globular precipitate is sulfur-rich and likely represents elemental sulfur (S^0). Other elements, for example, Na, K, are from the growth medium.

Fig. S3. O_2 consumption by cell suspensions of *Desulfovibrio magneticus* strain RS-1 in growth medium. The cell suspensions had an optical density of 1.1 and were sparged with air until the dissolved O_2 concentration was 220 μ M, before closing the spectrometer chamber. The monitoring of N_2 is a control showing that the correction of gas consumption from the mass spectrometer has properly been done, that is, there is no consumption of N_2 by the cells.

Fig. S4. Comparison of magnetite crystal size in magnetosomes in cells of *Desulfovibrio magneticus* strain RS-1 grown in medium with (left panels) or without (right panels) sulfate. From top to bottom: plot of crystal width versus length; crystal length; crystal width; crystal shape factor distributions; and transmission electron microscope images of cells of strain RS-1.

Fig. S5. Magnetosome shapes of strains FH-1 and ZBP-1 respiring oxygen. TEM images of cells of strain FH-1 (**A**) and ZBP-1 (**B**) grown in semi-solid $[O_2]$ -gradient medium lacking sulfate showing defects in magnetosome magnetite morphologies.