Isolation, cultivation and genomic analysis of magnetosome biomineralization genes of a new genus of South-seeking magnetotactic cocci within the Alphaproteobacteria

Viviana Morillo1, Fernanda Abreu1, Ana C. Araujo1, Luiz G. P. de Almeida2, Alex Enrich-Prast3, Marcos Farina4, Ana T. R. de Vasconcelos5, Dennis A. Bazylinski5 and Ulysses Lins1*

1 Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
2 Laboratório Nacional de Computação Científica, Departamento de Matemática Aplicada e Computacional, Petrópolis, Brazil
3 Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
4 Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
5 School of Life Sciences, University of Nevada at Las Vegas, Las Vegas, NV, USA

Although magnetotactic bacteria (MTB) are ubiquitous in aquatic habitats, they are still considered fastidious microorganisms with regard to growth and cultivation with only a relatively low number of axenic cultures available to date. Here, we report the first axenic culture of an MTB isolated in the Southern Hemisphere (Itaipu Lagoon in Rio de Janeiro, Brazil). Cells of this new isolate are coccoid to ovoid in morphology and grow microaerophically in semi-solid medium containing an oxygen concentration ([O2]) gradient either under chemoorganoheterotrophic or chemolithoautotrophic conditions. Each cell contains a single chain of approximately 10 elongated cuboctahedral magnetite (Fe3O4) magnetosomes. Phylogenetic analysis based on the 16S rRNA gene sequence shows that the coccoid MTB isolated in this study represents a new genus in the Alphaproteobacteria; the name Magnetofaba australis strain IT-1 is proposed. Preliminary genomic data obtained by pyrosequencing shows that M. australis strain IT-1 contains a genomic region with genes involved in biomineralization similar to those found in the most closely related magnetotactic cocci Magnetococcus marinus strain MC-1. However, organization of the magnetosome genes differs from M. marinus.

Keywords: Magnetofaba australis strain IT-1, magnetite, magnetosome, South-seeking magnetotactic bacteria, biomineralization genes

INTRODUCTION
Magnetotactic bacteria (MTB) are a morphologically, metabolically, and phylogenetically diverse group of prokaryotes that share the ability to synthesize intracellular, nano-sized magnetic particles called magnetosomes. Each magnetosome consists of a magnetite (Fe3O4) or greigite (Fe3S4) crystal enveloped by a lipid-bilayer membrane derived from the cytoplasmic membrane (Bazylinski and Frankel, 2004). Magnetosomes are generally organized in linear chains and orient the cell body along geomagnetic field lines while flagella actively propel the cells, resulting in so-called magnetotaxis (Bazylinski and Frankel, 2004; Schüler, 2008). MTB from the Southern Hemisphere swim antiparallel to the vertical component of the geomagnetic field toward the South and are termed South-seeking MTB (SS-MTB). In contrast, MTB from the Northern Hemisphere swim parallel to the vertical component of the geomagnetic field lines and are predominantly North-seeking (NS-MTB) (Blakemore et al., 1980). The inclination of the geomagnetic field lines is believed to direct cells downwards away from toxic concentrations of oxygen in surface waters, thereby helping them locate and maintain an optimal position in vertical gradients which is usually at or near the oxic-anoxic interface (OAI) (Blakemore, 1982; Frankel and Bazylinski, 1994; Bazylinski and Frankel, 2004). However, there are reports of SS-MTB and NS-MTB in both hemispheres (Simmons et al., 2006).

MTB are considered fastidious microorganisms (Schüler, 2008), although there has recently been a considerable increase in available cultures, including the first cultivation of a greigite producer (Lefèvre et al., 2011). The recent availability of MTB cultures has contributed to a better characterization of the physiology and biochemistry of these microorganisms. It has also contributed to an improved understanding of the evolution of MTB and of the biomineralization processes involved since differences in the sequences of magnetosome biomineralization genes in different MTB, particularly the mam genes, revealed a strong correlation between these magnetotaxis-related genes and phylogeny based on the 16S rRNA gene (Lefèvre et al., 2013a). Studies of magnetosome biomineralization genes in uncultivated MTB require unique approaches (Abreu et al., 2011; Jogler et al., 2011) that do not usually reveal the complete organization of biomineralization genes or genes involved in magnetotactic behavior unless the entire genome is sequenced. Moreover,
because not all the magnetosome-related genes may be recog-
nized, a direct correlation with phylogeny based on 16S rRNA
gene sequences cannot be made with total accuracy.

The most characterized cultivated MTB strains are phylo-
genetically affiliated with the Alphaproteobacteria and include
Magnetococcus marinus strain MC-1 (Bazylinski et al., 2013a),
Magnetovibrio blakemorei strain MV-1 (Bazylinski et al., 2013b),
the magneto-ovid bacterium strain MO-1 (Lefèvre et al., 2009),
Magnetospirillum magneticum strain AMB-1, Magnetospirillum
gryphiswaldense strain MSR-1, Magnetospirillum magnetotacticum
strain MS-1, Magnetospira thiophilla strain MMS-1 (Williams et al., 2012) and Magnetospira sp. QH-2 strain 1 (ji et al., 2014). Cultivated strains belonging to Deltaproteobacteria
include the sulfate-reducer Desulfovibrio magneticus strain
RS-1, (Sakaguchi et al., 2002), Candidatus Desulfamplus
magnetomortis strain BW-1 (Lefèvre et al., 2011) and enrich-
cultures of the magnetotactic multicellular prokaryotes
Candidatus Magnetoglobus multicellularis (Abreu et al., 2013).
Two cultivated strains, BW-2 and SS-5, both belonging to
Gammaproteobacteria, have also been reported (Lefèvre et al.,
2012).

The biomineralization of magnetosomes is controlled by a set
of highly conserved genes in magnetite-producing MTB (Richter
et al., 2007; Jogler and Schüler, 2009; Jogler et al., 2009) and, as
demonstrated more recently, in greigite-producing MTB as well
(Abreu et al., 2011, 2013; Lefèvre et al., 2011, 2013b). In some
species, the magnetosome biomineralization genes are clustered
on a genomic magnetosome island (MAI), which partially sup-
ports the hypothesis of horizontal gene transfer (HGT) between
various MTB presumably leading to the wide distribution of these
genes among members of different phylogenetic groups (Jogler
and Schüler, 2009; Jogler et al., 2009; Abreu et al., 2011). However,
certain components of typical genomic islands (transposons, t-RNA sequences, integrases), such as those observed in M. mag-
neticum strain AMB-1, M. gryphiswaldense strain MSR-1 and
D. magneticus RS-1, are not universally shared within the MAI
of all MTB (e.g., M. marinus; Schübbe et al., 2009). Moreover,
phylogenetic analysis based on the amino acid sequences of
magnetosome proteins from MTB are congruent with the phylo-
genetic tree based on the 16S rRNA gene sequences of the same
microorganisms (Lefèvre et al., 2013a). Therefore, the evolu-
tion and divergence of magnetosome proteins and the 16S rRNA
gene occurred similarly, suggesting that magnetotaxis originated
monophyletically in the Proteobacteria phylum (Lefèvre et al.,
2013a). Additional genome sequences and culture of MTB species
are necessary to understand the evolution of biomineralization in
Bacteria. Moreover, the availability of new cultures of MTB allows
a better characterization of the physiology and biochemistry of
these microorganisms, enabling the correlation of these features
to magnetosome formation.

Despite being the most prevalent and diverse morphotype of
MTB in the environment (Spring et al., 1998; Schübbe et al.,
2009), there are currently only two cultivated strains of magneto-
tactic cocci: M. marinus strain MC-1 (Bazylinski et al., 2013a)
and the magneto-ovid bacterium strain MO-1 (Lefèvre et al., 2009).
The complete genome sequence of the NS-MTB M. marinus has
been reported (Schübbe et al., 2009), but further study is required
to better understand the full diversity of the magnetotactic cocci
as well as the ecological function and evolution of magnetosome
biomineralization in the Alphaproteobacteria. Here, we describe
both the isolation in axenic culture and the characterization of
a new magnetotactic coccus, provisionally named Magnetofaba
australis strain IT-1 that represents a new genus. We also con-
ducted whole genome sequencing and functional annotation of
genes related to magnetosome formation to gain insight into the
phylogeny, physiology and biochemistry of this SS-MTB. This
strain is the first cultivated SS-MTB, and the genomic data pre-
vented here are the first report of biomineralization genes in mag-
netotactic cocci capable of synthesizing elongated cuboctahedral
magnetosomes.

**MATERIALS AND METHODS**

**ISOLATION AND CULTIVATION OF Magnetofaba australis STRAIN IT-1.**

Samples of water and sediment were collected from the Itaipu
Lagoon (22°57′51.90″ S 43°2′45.41″ W), a brackish to marine
coastal lagoon near Rio de Janeiro, Brazil, and stored under
dim light at room temperature. MTB were magnetically concen-
trated using a magnetic isolation apparatus described by Lins
et al. (2003). After 20 min, cells were collected in a polypropylene
tube. Concentrated South-seeking MTB were magnetically puri-
ified repeatedly using the racetrack technique (Wolfe et al., 1987)
and inoculated at the OAI of culture tubes. Approximately 4/5
of the tubes were filled with an autotrophic semisolod oxygen
concentration gradient ([O2]-gradient) medium. The medium was
used to isolate M. marinus (Frankel et al., 1997) and contained
bicarbonate as the major carbon source. The medium contained
5 mL of modified Wolfe’s minerals elixir, 3.75 mM NH4Cl, 0.2 mL
of 0.2% resazurin and 2 g of Bacto-Agar diluted in 1 L of artifi-
cial seawater (ASW). The medium was autoclaved, followed by
the addition of 1.5 mL of 0.5 M KH2PO4, pH 7.1, neutral fresh
L-cysteine (final concentration of 0.2 g/L) and 2.68 mL of 0.8 M
NaHCO3, 0.5 mL of vitamin solution and 2 mL of 0.01 M ferric
nitrate (final concentration of 20 μM). The pH was adjusted to
7.2. Cultures were incubated at 28°C until a microaerophilic band
of cells was observed at the OAI and, subsequently, the bands
were inoculated into a solid heterotrophic [O2]-gradient medium
applying the dilution-to-extinction technique and shake-tubes
(Seeley et al., 1991). Briefly, a band of cells were inoculated into
the solid medium before it solidified (approximately at 45°C),
followed by 7 serial 10-fold dilution steps. After inoculation and
agitation by inversion, each tube was put on ice to solidify the
medium quickly without killing a significant number of cells.
Colonies grown on shake tubes were individually transferred to
semi-solid heterotrophic medium. Each colony in culture was
re-inoculated in fresh medium over 10 times to ensure that a
pure culture was obtained. Purity of the culture was evaluated
by light and electron microscopy and sequencing of the 16S
rRNA gene.

The medium chosen for growth and maintenance of M. aus-
tralis strain IT-1 was designed for heterotrophic growth, because
cells grew faster and the number of magnetosome per cell
was higher than in the autotrophic medium. The heterotrophic
medium contained 5 mL of modified Wolfe’s minerals
(Frankel et al., 1997), 3.75 mM NH4Cl, 0.2 mL of 0.2% resazurin, 12 mM
HEPES, 12 mM sodium acetate, 3.7 mM sodium succinate and 2 g of Bacto-Agar in 1 L of ASW. The medium was autoclaved, followed by the addition of 1.5 mL of 0.5 M KHPO₄, pH 7.1, neutral fresh L-cysteine (final concentration of 0.2 g/L), 2.68 mL of 0.8 mM NaHCO₃ and 4.8 mM Na₂O₃S₂·5H₂O. The pH was adjusted to 7.2, and 0.5 mL of a vitamin solution (Frankel et al., 1997) and 2.5 mL of 0.01 M ferric quinate were added. Cells were inoculated at the OAI, and the cultures were incubated at 28°C for at least 15 days.

Oxygen concentrations were measured using a Unisense OX 100 oxygen microsensor, with a detection limit of 0.3 μM for at least 15 days. The O₂ microsensor was stabilized for 2–3 h before any measurement. Calibration was done by submerging the sensor in a 0.1 M of 0.8 mM NaHCO₃ and 4.8 mM Na₂O₃S₂·5H₂O. The pH was adjusted to 7.2, and 0.5 mL of a vitamin solution (Frankel et al., 1997) and 2.5 mL of 0.01 M ferric quinate were added. Cells were inoculated at the OAI, and the cultures were incubated at 28°C for at least 15 days.

Oxygen concentrations were measured using a Unisense OX 100 oxygen microsensor, with a detection limit of 0.3 μM, coupled to a micromanipulator MM33 (Unisense, Aarhus, Denmark). Measurements were carried in duplicate tubes at 24 h intervals for 8 days in semi-solid heterotrophic medium. Calibration was done by submerging the sensor in a 0.1 M of ascorbate and 0.1 M of NaOH solution (0% O₂ saturation) and oxygenated water (100% O₂ saturation). The oxygen concentration profile was determined to a depth of 11 mm from the culture medium surface in 200 μm steps taking 5 s for each measurement. The O₂ microsensor was stabilized for 2–3 h before any measurement. Data were recorded in the software SensorTrace Pro v3.0.2 (Unisense)

LIGHT AND ELECTRON MICROSCOPY

For light microscopy imaging, drops of ASW containing magnetically-enriched MTB were placed onto coverslips and imaged with Zeiss Axioplan 2 or Zeiss Axioimager microscopes (Carl Zeiss, Göttingen, Germany), both equipped with differential interference optics. A bar magnet was used to direct MTB to the edge of the drop where they accumulated. Transmission electron microscope (TEM) imaging of cells and elemental analysis of both magnetosomes and cell inclusions were performed in unfixed and unstained samples with a Jeol 1200 EX transmission (Jeol, Peabody, MA, USA) electron microscope equipped with a Noran accessory for energy-dispersive X-ray analysis (EDS) (Thermo Scientific, Palm Beach, FL, USA). Cells were placed onto formvar-coated electron microscope 300 mesh copper grids, rinsed with distilled water and air-dried. Observations were performed at 100 kV, and spectra were acquired using a spot size of approximately 80 nm in diameter. For magnetosome measurements, the grids were observed with a Morgagni TEM (JEOL Company, Hillsboro, OR, USA) operating at 80 kV, and spectra were acquired using ImageJ software (rsb.info.nih.gov/ij/). Crystal size and shape factor were calculated as (length + width)/2 and width/length, respectively. Analyses of variance were performed using Graphpad InStat version 3.0.

For energy-filtering transmission electron microscopy (EFTEM), unstained ultra-thin sections were imaged with a Zeiss EM902 (Carl Zeiss, Göttingen, Germany) TEM equipped with a mirror-prism. Iron and oxygen maps were calculated with 1000 replicates. Other sequences used in this work include Ca. M. multicularis (HQ336745 and HQ336746) (Abreu et al., 2011), D. magnetiscus strain RS-1 (AP007255) (Matsunaga et al., 2005), M. gryphiswaldensis strain MRS-1 (AM085146) (Lohlke et al., 2011), M. magnetotacticum strain MS-1 (NZ_AAP01003731) (Bertani et al., 2001), M. marinus strain MC-1 (NC_008576) (Schübbe et al., 2009), M. blakemorei strain MV-1 (EP102531) (Jogler et al., 2009), Gammaprotoebacteria strain SS-5 (AFX88993–AFX88992) and M. australis strain IT-1 were used for identity, positives and E-value analysis through Blastp. Other sequences used in this work include Ca. M. multicularis (HQ336745 and HQ336746) (Abreu et al., 2011), D. magnetiscus strain RS-1 (AP010904) (Nakazawa et al., 2009), Ca. D. magnetomortis strain BW-1 (HF547348) and strains ML-1 (JX869936–JX869937) and FH-1 (KC196864—KC196902) (LeFèvre et al., 2013b). A phylogenetic tree of concatenated MamABEIKMQP amino acid sequences was constructed using the maximum likelihood statistical method based on WAG (Whelan and Goldman, 2001) with frequencies and gamma distribution (WAG+G+F) for analyses. Bootstrap value was calculated with 1000 replicates. The sequence of the MAI region has been submitted to GenBank/NCBI under the accession number KF933436.

RESULTS

ISOLATION, GROWTH AND PHYLOGENETIC ANALYSES OF STRAIN IT-1

Magnetotactic cocci were the dominant MTB morphotype in the environmental samples. Occasionally, we detected magnetotactic multicellular prokaryotes, as previously described (Lins et al., 2007). After separation using the magnetic “racetrack” (Wolfe et al., 1987), magnetically-enriched cocci were inoculated...
at the OAI of the semisolid autotrophic medium. Four weeks later, microaerophilic bands of coccoid MTB were observed and were then inoculated in semisolid heterotrophic [O2] gradient medium in which the culture was maintained. Cells formed individual colonies in shake tubes of heterotrophic medium (see experimental procedures for details). Single colonies were re-inoculated in fresh semisolid medium and resulted in pure cultures of a magnetotactic coccus with an average size of 1.4 ± 0.3 × 1.1 ± 0.3 μm (n = 130) as observed by light microscopy (Figure 1A) and confirmed by TEM (Figure 1B). The morphology of cells observed by TEM resembles a “faba” bean, showing well-defined convex and concave surfaces (Figure 1B). Cells contain intracellular granules (Figure 1B) filled with phosphorus as detected by EDS (Figure 1E).

The 16S rRNA gene of the culture was amplified, cloned, and sequenced for phylogenetic analyses. Approximately 50 clones were sequenced. These sequences were 99% similar, confirming the culture was pure. A consensus sequence was generated (accession number: JX534168) and phylogenetic analysis showed that strain IT-1 is phylogenetically affiliated with the Alphaproteobacteria (Figure 2). The 16S rRNA gene sequence of strain IT-1 is 93% similar to the sequence of an uncultured magnetotactic coccus collected from intertidal sediments of the Yellow Sea in China (Zhang et al., 2012; accession number JF421219) and 92% similar to sequences of the cultured species M. marinus strain MC-1 and MO-1 (accession numbers CP000471 and EF6435202, respectively). Thus, strain IT-1 represents a new genus of the magnetotactic cocci (and MTB in general). The name *Magnetofaba australis* gen. nov., sp. nov., is proposed for strain IT-1 (Ma. gne. to. faba Gr. n. magnêtos -êtos, a magnet; N.L. pref. magneto-, pertaining to a magnet; N.L. fem. N. faba, a faba bean; aus.tra’lis. L. masc. australis of Southern or of the south, which refers to the polar south-seeking magnetotaxis behavior and because the bacterium was isolated from South hemisphere).

**FIGURE 1 | Characterization of *Magnetofaba australis* strain IT-1. (A) Differential interference contrast microscopy of a pure culture showing coccoid to ovoid cells. (B) Whole-mount transmission electron microscopy image of strain IT-1 showing a chain of elongated octahedral magnetosomes (m) and three conspicuous granules containing phosphorus (G). Oxygen concentration over time (C) and band formation (D) during strain IT-1 growth in semisolid heterotrophic medium. The points in the lines represent the position of the band in the culture medium at a given time. Control is represented by a non inoculated tube. Note the band with magnetotactic cells (arrow) after 6 h of inoculation. (E) Energy dispersive X-ray microanalysis spectrum of the phosphorus-rich granules. Ca, Zn, and K are cations associated with the granules. Cu peaks come from the supporting grid. The silicon peak is an artifact of the Si (Li) solid state detector used to collect X-rays.**
Magnetofaba australis strain IT-1 grows as a microaerophilic band of cells in semisolid medium (Figure 1D). It grows slowly chemolithoautotrophically, using thiosulfate as electron donor and sodium bicarbonate as the major carbon source, forming a fine band of cells at the OAI at least 4 weeks after incubation. Under these conditions, cells bio mineralize 6 ± 4 magnetosomes/cell (n = 100). Heterotrophic growth was also observed using sodium acetate and sodium succinate as the carbon source; cells grown under these conditions produced 9 ± 4 and 7 ± 3 magnetosomes per cell, respectively (n = 100 for both). When cells were grown in heterotrophic medium containing both sodium acetate and succinate, a band of magnetotactic cells, which contained 10 ± 3 magnetosomes/cell (n = 100), was observed at the OAI after 24 h. This band gradually moved toward the surface of the culture medium after 8 days of incubation (Figure 1D).

The oxygen concentration in the band was measured over 8 days (Figure 1C) in the heterotrophic medium; cells were initially inoculated at the OAI, in which [O2] was less than 3 μM. During the first 6 h after inoculation, cells moved up approximately 3 mm, forming a “bell-shaped” band (Figure 1D) in the medium ([O2] = 50 ± 5 μM). After 24 h, the band was positioned between 24.6 ± 0.7 and 43 ± 1 μM O2, with a less bent bell-shape. 48 h later, the band was located in 29.2 ± 2.7 μM of [O2]. After 72 h, the bell-shaped band became a flat band positioned at [O2] between 9.4 ± 1.5 μM. Until this time, the band did not reach the meniscus of the culture medium. As the cells grew (up to 168 h), O2 was consumed, and the dense population of cells reached the meniscus, presumably to use oxygen present in the headspace of the tube (Figure 1D). At 72 h of incubation the cells of M. australis have consumed near 90% of oxygen ([O2] < 9.4 ± 1.5 μM), and the band appears thicker than in 24 and 48 h. With 72 h, it is likely that the magnetite production also increased, given the higher number of cells and that the population remained responding to the magnetic field at the end of the experiment. Therefore, M. australis strain IT-1 can grow and synthesize magnetite with [O2] below 10 μM, similar to the Magnetospirillum species, which requires microaerobic conditions (2–7 μM O2) to grow and synthesize magnetite (Schüler and Baeuerlein, 1998).

In hanging drop assays under oxic conditions, M. australis strain IT-1 exhibited South-seeking polar magnetotaxis swimming under the magnetic field of a bar magnet with a fast back and forth swimming pattern near the edge of the drop. M. australis swims at average speeds of 186 μm·s−1 ± 63 (n = 50) and can reach 300 μm·s−1. Cells are propelled by two bundles of lophotrichous flagella, each at one extremity of the cell. A helical trajectory was observed when movement was recorded with a CCD camera using dark-field microscopy.

MAGNETOSOMES
Cells of M. australis strain IT-1 each produce a single chain of magnetosomes (see Figure 1B). Each chain consists of 10 ± 3 magnetosomes (n = 100) in cells grown heterotrophically in semi-solid [O2] gradient medium. Energy-dispersive X-ray analysis (Figure 3A) and elemental mapping by EFTEM (Figure 3B) confirmed that the magnetosomes contain iron (Figure 3C) and oxygen (Figure 3D). Electron diffraction (Figure 3E) of isolated magnetosome crystals (Figure 3F) were indexed based on standard cubic system for magnetite. Distances and angles between spots were consistent with magnetite (Fe3O4). Approximately 4% defective twins and multiple twin magnetosomes are observed in M. australis strain IT-1. The crystals are octahedral particles
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FIGURE 3 | Magnetosome biomineralization in *Magnetofaba australis* strain IT-1. (A) Energy dispersive X-ray spectrum showing Fe and O as the main elements in the magnetosomes. Cu originates from the grid bar. (B) Elemental mapping by EFTEM of a magnetosome showing the distribution of iron (C) and oxygen (D). (E) Electron diffraction pattern of isolated magnetosomes shown in (F). (G) Size distribution, (H) shape factor distribution, and (I) scatter plot of length and width of magnetosomes in *Magnetofaba australis* strain IT-1 grown in heterotrophic medium.

elongated along the <111> axis. Figure 3G shows the size distribution of magnetosomes (*n* = 100), estimated by calculating the best fit of an ellipse (major axis = length; minor axis = width). The average size ([length + width]/2) was 78 ± 24 nm (average length = 83 ± 26 nm; average width 74 ± 23 nm). Figure 3H shows the shape factor distribution (average of width/length = 0.89 ± 0.05), and Figure 3I shows the scatter plot of length and width (adjustment *r*² = 0.962). Magnetosomes in *M. australis* strain IT-1 are each enveloped by a membrane, as shown in TEM images of ultra-thin sections (Figure 4A).

MAGNETOSOME GENES

Comparative genome analysis of *M. australis* strain IT-1 with other magnetotactic *Alphaproteobacteria* based on genes related to magnetotaxis and magnetosome synthesis revealed a genomic region of 40.399 Kb that contained both genes associated with magnetosome biomineralization as well as those that encode some hypothetical proteins present in the putative MAI of *M. marinus* and four that are not found in any known MTB. A feoAB gene cluster was also identified in this region, similar to that found in *Magnetospira* sp QH-2, *Ca. Mg. multicellularis* and *Ca. D. magnetomortis*. Figure 4B shows the organization of the genes in *M. australis* strain IT-1. The mamAB-like gene cluster has the same gene organization found in *M. marinus* (*mamK*, *mamF*, *mamL*, *mamM*, *mamO*, *mamP*, *mamA*, HP, *mamQ*, *mamB* and *mamS*) (Schübbe et al., 2009), except that *mamT* is absent. The *mamC* gene is located in the *mamCXZ* gene cluster, similar to *M. blakemorei* (Jogler et al., 2009), and not in the *mamHIEC* gene cluster as in *M. marinus*. The *mms*6 gene cluster and the 10 genes encoding MamF-like protein, chemotaxis protein, signal transduction proteins and three hypothetical proteins are located between the *mamAB* and *mamCXZ* gene clusters. At the end of the *mamCXZ* gene cluster, genes encoding MamD-like, FeoB, FeoA, MamA-like and MamD proteins are present. All predicted proteins related to biomineralization and magnetotaxis genes described here for *M. australis* strain IT-1 share the highest
similarity to those of *M. marinus*, including MamA-like, MamD-like, MamF-like, FeoB and FeoA proteins. A notable exception is MamC, which is more related to that from *M. magneticum* strain AMB-1 (coverage 86%, identity 63%, E-value 5e-038).

The coverage, identity and E-value of the Blastp analysis of predicted Mam proteins from magnetotactic *Alphaproteobacteria* and the *Gammaproteobacteria* strain SS-5 were analyzed (Table 1). *M. australis* MamA-like, MamD-like and MamF-like proteins
### Table 1 | Comparative analysis of biomineralization genes found in *Magnetofava australis* strain IT-1 and other cultivated magnetotactic bacteria with their genomes sequenced.

| Protein (locus number) | IT-1 | MC-1 | MV-1 | AMB-1 | MS-1 | MSR-1 | SS-5 |
|------------------------|------|------|------|-------|------|-------|------|
| **E-value** | **Coverage** | **Identity** | **E-value** | **Coverage** | **Identity** | **E-value** | **Coverage** | **Identity** | **E-value** | **Coverage** | **Identity** | **E-value** | **Coverage** | **Identity** | **E-value** | **Coverage** | **Identity** |
| MamS (2777) | 1E-061 | 90 | 58 | 1E-035 | 65 | 44 | 1E-023 | 64 | 40 | 5E-024 | 64 | 40 | 2E-023 | 62 | 38 | – | – | – |
| MamB (5564) | 6E-169 | 98 | 73 | 2E-112 | 98 | 52 | 5E-100 | 97 | 46 | 2E-063 | 92 | 38 | 3E-101 | 100 | 45 | 2E-128 | 98 | 59 | – | – | – |
| MamQ (2780) | 6E-128 | 96 | 62 | 2E-053 | 91 | 34 | 7E-048 | 84 | 34 | 2E-043 | 67 | 37 | 2E-050 | 91 | 33 | 6E-053 | 67 | 44 | – | – | – |
| MamA (2782) | 6E-089 | 98 | 58 | 2E-048 | 98 | 35 | 2E-054 | 95 | 39 | 6E-060 | 97 | 38 | 1E-054 | 96 | 39 | 1E-031 | 96 | 29 | – | – | – |
| MamP (5510) | 3E-123 | 98 | 64 | 4E-062 | 67 | 52 | 6E-047 | 67 | 43 | 1E-047 | 67 | 43 | 9E-048 | 67 | 42 | 2E-060 | 76 | 46 | – | – | – |
| MamO (5499) | 0.0 | 99 | 57 | 1E-033 | 42 | 27 | 6E-121 | 97 | 35 | – | – | – | – | – | – | – | – | – | – | – | – |
| MamM (5508) | 8E-170 | 95 | 69 | 2E-110 | 96 | 47 | 2E-101 | 89 | 48 | 1E-090 | 83 | 47 | 2E-102 | 84 | 50 | 4E-127 | 86 | 59 | – | – | – |
| HP Similar to MamL (2787) | 5E-031 | 93 | 74 | 7E-016 | 96 | 40 | 6E-007 | 95 | 28 | 1E-006 | 95 | 28 | 7E-006 | 98 | 32 | – | – | – | – | – | – |
| MamF (2788) | 6E-045 | 80 | 70 | – | – | – | 3E-029 | 96 | 46 | 9E-002 | 74 | 52 | 5E-002 | 93 | 48 | – | – | – | – | – | – |
| MamK (2789) | 0.0 | 100 | 82 | 5E-113 | 97 | 51 | 7E-132 | 99 | 51 | 1E-122 | 92 | 51 | 6E-129 | 99 | 50 | 5E-121 (K1) | 96 | 52 | – | – | – | – |
| MamE (2791) | 0.0 | 100 | 48 | 4E-092 | 98 | 32 | 1E-095 | 98 | 35 | 6E-096 | 98 | 35 | 1E-066 | 90 | 41 | 6E-064 | 97 | 50 | – | – | – | – |
| MamJ (2792) | 9E-029 | 66 | 81 | 2E-025 | 78 | 62 | 4E-014 | 82 | 51 | 4E-014 | 82 | 51 | 2E-014 | 71 | 58 | 2E-013 | 63 | 65 | – | – | – | – |
| MamH (5506) | 0.0 | 100 | 70 | 4E-160 | 97 | 53 | 3E-157 | 95 | 55 | 1E-129 | 81 | 54 | 1E-155 | 96 | 54 | – | – | – | – | – | – |
| HP similar to Mms6 (2798) | 9E-011 | 83 | 76 | 5E-039 | 81 | 52 | 1E-036 | 79 | 52 | 8E-035 | 79 | 53 | 2E-035 | 85 | 52 | – | – | – | – | – | – |
| MmsF (2799) | 7E-056 | 83 | 76 | 5E-039 | 81 | 52 | 1E-036 | 79 | 52 | 8E-035 | 79 | 53 | 2E-035 | 85 | 52 | – | – | – | – | – | – |
| MamF-like (2806) | 6E-032 | 77 | 47 | 3E-026 | 96 | 39 | 2E-025 | 91 | 45 | 7E-026 | 91 | 44 | 1E-022 | 83 | 40 | – | – | – | – | – | – |
| MmsF-like | 0.0 | 95 | 61 | 1E-171 | 94 | 51 | 0.0 | 93 | 51 | 0.0 | 93 | 51 | 0.0 | 93 | 51 | – | – | – | – | – | – |
| MamZ (2814) | 6E-104 | 90 | 48 | 3E-025 | 54 | 45 | 2E-056 | 99 | 35 | 2E-010 | 31 | 29 | 1E-057 | 99 | 34 | – | – | – | – | – | – |
| MamC (2817) | 4E-028 | 93 | 62 | 1E-015 | 82 | 62 | 5E-032 | 86 | 63 | 6E-032 | 82 | 63 | 4E-034 | 87 | 62 | – | – | – | – | – | – |
| MamD-like (2822) | 1E-018 | 97 | 42 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| FeoB (2827) | 81-144 | 99 | 59 | 6E-112 | 97 | 50 | 5E-087 | 98 | 45 | 3E-090 | 97 | 45 | 1E-087 | 98 | 45 | 2E-092 | 98 | 44 | – | – | – | – |
| FeoA (2828) | 3E-030 | 55 | 61 | 7E-015 | 61 | 40 | 4E-018 | 58 | 40 | 1E-012 | 52 | 39 | 2E-012 | 60 | 40 | – | – | – | – | – | – |
| MamA-like (2830) | 9E-041 | 65 | 47 | 1E-013 | 57 | 30 | 1E-020 | 66 | 31 | 1E-020 | 66 | 31 | 1E-020 | 66 | 31 | 6E-013 | 56 | 30 | – | – | – | – |
| MamD (2833) | 4E-038 | 92 | 38 | 3E-014 | 96 | 39 | 6E-029 | 63 | 35 | 3E-029 | 63 | 35 | 1E-028 | 68 | 35 | – | – | – | – | – | – |

Bold values are related to most similar proteins between *M. australis* strain IT-1 and other MTB according to blastp.
were compared to MamA-like, MamD-like and MamF-like of \textit{M. marinus}, and MamA, MamD and MamF proteins of other MTB. The \textit{M. australis} MamA-like predicted protein is closely related to the \textit{M. marinus} protein sequence (coverage 65\%, identity 47\%, \textit{E}-value 9e-041), while MamD-like is only related to the MamD-like from \textit{M. marinus} (coverage 97\%, identity 42\%, \textit{E}-value 1e-018). For some MTB, \textit{M. australis} MamF-like sequences were more similar to MmsF or MmsF-like proteins (e.g., from \textit{M. gyphiswaldense} and \textit{M. blakemorei}). MamF and MmsF of \textit{M. gyphiswaldense} MSR-1 have already been reported to share 65\% identity (Murat et al., 2012). \textit{M. australis} strain IT-1 have already been reported to share 65\% identity with \textit{M. marinus} strain MC-1. The \textit{Gammaphotobacteria} strain SS-5, which synthesizes cuboctahedral magnetite magnetosomes, groups with the \textit{Alphaproteobacteria}. Interestingly, after \textit{M. marinus}, MamB, MamQ and MamM of \textit{M. australis} strain IT-1 have the most similarity with MamB of strain SS-5 (coverage 98\%, \textit{E}-value 2E-128, identity 59\%), MamQ (coverage 67\%, \textit{E}-value 6E-053, identity 44\%) and MamM (coverage 86\%, \textit{E}-value 4E-127, identity 59\%). The phylogenetic tree (Figure 4C) with concatenated conserved \textit{mam} genes does not show a clear evolutionary event that divides magnetotactic strains producing cuboctahedral and prismatic hexagonal crystals because bacteria such as \textit{M. blakemorei} and \textit{M. marinus} do not form a separate branch. The evolutionary relationship between \textit{M. australis} and \textit{M. marinus} suggests a recent divergence between the cellular magnetosome biomineralization machinery in these species.

\section*{DISCUSSION}

The number of MTB isolated in culture has recently increased (from 1978 to 2009, 11 MTB were available in axenic cultures; in 2012 this number was 25; Lefèvre and Long-Fei, 2013). However, all cultured MTB were isolated in the Northern Hemisphere and originally showed NS magnetotaxis. This work presents the first isolation of a SS-MTB from the Southern Hemisphere. The new isolate is phylogenetically affiliated with the \textit{Alphaproteobacteria} class of the \textit{Proteobacteria} phylum, a division that contains almost all known \textit{Fe$_3$O$_4$}-producing MTB (DeLong et al., 1993; Spring et al., 1998), and clearly represents a new genus based on 16S rRNA gene sequence similarities. This new coccus represents a third phylogenetic group of MTB occurring in the Itaipu Lagoon (Spring et al., 1998). \textit{M. australis} strain IT-1 is distinct from all the other cultivated magnetotactic cocci examined to date because of its South-seeking polar magnetotactic behavior, it has “faba bean” cell morphology and elongated cuboctahedral magnetite magnetosomes. Based on its 16S rRNA gene sequence, \textit{M. australis} is more related to an uncultured magnetotactic coccus found in the intertidal sediments of the Yellow Sea in China (93\% similarity; Zhang et al., 2012). This uncultured bacterium also shows a bean-like morphology and produces magnetite magnetosomes (Zhang et al., 2012). However, magnetosome crystal morphology, size, shape factor, magnetosome number and swimming speed in \textit{M. australis} are different from the coccus described by Zhang et al. (2012). The close phylogenetic relationships may not be significantly associated to the biomineralization genes, which may result in variations in the Regulation of crystal morphology between these MTB. Hopefully, physiological studies and genomic analysis of these MTB will result in information that advances the understanding of biomineralization in bean-like magnetotactic cocci.

\textit{Magnetofaba australis} strain IT-1 has a swimming speed similar to that observed in strain MO-1 (Lefèvre et al., 2009), higher than speeds found in other magnetotactic cocci (Zhang et al., 2012). Possibly, a highly coordinated flagella rotation is necessary to allow this high swimming speed. The high swimming speed would be advantageous for the survival of \textit{M. australis} strain IT-1 because it would enable the cell to escape quickly from unfavorable environment conditions. Most cells of \textit{M. australis} strain IT-1 (over 80\%) has a South-seeking behavior when observed in hanging drop assays under oxic conditions, but we have also found North-seeking cells in the culture flasks. Further studies are necessary to compare the swimming behavior and orientation of magnetotactic cocci, along with their flagellar apparatus at the genetic and structural levels. We believe that such studies can now be performed because of the available SS-MTB cultures.

The role of biomineralization and magnetotaxis genes in MTB is not only key in the determination of how magnetosomes are formed in MTB but also important in understanding the evolution of magnetotaxis (Lefèvre and Bazylnski, 2013). Although several recent reports have addressed this issue (Lefèvre et al., 2013a,b), only a relatively small number of MTB species have been considered thus far. However, advances in the culturing of new strains promises to improve the low number of species available for evolutionary studies. \textit{M. australis} strain IT-1 is the first MTB isolated in axenic culture that produces cuboctahedral magnetite magnetosomes whose magnetosome biomineralization genes have been sequenced. New data on the magnetosome biomineralization genes of cocoid or ovoid MTB increases our understanding of the biomineralization processes in MTB in general. For example, \textit{M. marinus} and \textit{M. australis} share several hypothetical proteins, not found in other MTB that may have key functions in biomineralization or magnetotaxis like the hypothetical protein between MamE and MamK (locus 02790), the hypothetical protein between MmsF and the Amino acid carrier protein (locus 02801), the Amino acid carrier protein (locus 02803), a hemerythrin-like (locus 02811), and a ferritin-like (locus 02816).

The analysis of the putative functions of \textit{mam} genes is also important in the interpretation of the evolution of magnetotaxis. Variations in both the order and sequence of \textit{mam} genes between \textit{M. australis} and the closely related \textit{M. marinus} could explain differences between magnetosome crystal morphology in the two species. The MamC predicted protein sequence of \textit{M. australis} is more similar to that of \textit{M. magneticum} strain AMB-1, which is particularly interesting because cultivated \textit{Magnetospirillum} species described thus far produce cuboctahedral magnetite.
crys

its that are not elongated (Amann et al., 2007). Scheffel et al. (2008) showed that the protein MamC and other proteins in the same operon (mamGFDC) are not essential for magnetosome formation but are involved in controlling crystal size and morphology in M. gryphiswaldense. In M. australis, mamC is organized in a mamCXX operon, similar to M. blakemoirei. The other proteins involved in the size and shape of magnetosomes (MamD, MamF, Mms6, and MmsF) are more closely related to those found in M. marinus. Therefore, the fact that M. australis MamC is related to cuboctahedral magnetite-producing bacteria suggests that this protein might be responsible for crystal morphology in this case. Additionally, based on the similarity of mamXYC gene organization between M. australis and M. blakemoirei, we speculate that gene organization and/or preferential expression of mamCXX could be involved in crystal elongation. MmsF has been shown to be involved in the geometry of magnetosome maturation, as the deletion of mmsF resulted in elongated magnetosomes in M. magneticum strain AMB-1 (Murat et al., 2012). However, we did not identify a close similarity between MmsF from M. australis strain IT-1 and other MTB that synthesize elongated octahedral crystals. The expression level of MmsF may influence crystal morphology, which could explain how closely related mam genes from different species (i.e., M. australis and M. marinus) produce magnetosomes with different characteristics. Variation in the expression level of the mamFGDC operon in M. gryphiswaldense resulted in crystals exceeding the size of those of the wild-type (Scheffel et al., 2008). The absence of mamT in M. australis strain IT-1 reveals a new group of 19 genes common to cultivated magnetotactic Alphaproteobacteria: mamA, B, C, D, E, F, H, I, K, L, M, N, O, P, Q, R, S, X and Z, in addition to the mms6 and mmsF genes. Although mamT is present in the Alpha- and Deltaproteobacteria, it is not essential for biomineralization. Proteins with similar function (MamP or MamE) are likely sufficient to control the balance between Fe(II) and Fe(III) in the magnetosome. In M. magneticum (Murat et al., 2010) and M. gryphiswaldense (Lohße et al., 2011) mamT is not essential for magnetosome synthesis.

Considering that both M. australis strain IT-1 and M. marinus strain MC-1 have a common magnetotactic ancestor and that biomineralization proteins apparently evolved together in both strains, it is reasonable to assume that a common ancestor exists among all freshwater and marine MTB from the Magnetococcales order. No non-MTB belonging to the Magnetococcales order has ever been reported, but this fact does not preclude HGT among Alphaproteobacteria because strains phylogenetically closer to Magnetospirillum do not have the magnetotactic phenotype (Jogler and Schüler, 2009). Thus, magnetosome biomineralization genes common to all MTB (mamABEIKMPQ) might have been acquired from an ancestor common to all MTB (Abreu et al., 2011; Lefèvre et al., 2013a). However, genes such as mamCDF, mamL, mamXX, mms6, and mmsF could have been acquired by descent of magnetotactic Alphaproteobacteria and magnetotactic cocci, which appear to emerge as the most basal lineage of the Alpha- and Gammaproteobacteria (Singer et al., 2011; Lefèvre and Bazylinksi, 2013). mamG, mamR, mamV, mamU, and mamY genes were likely acquired recently by Magnetospirillum species, given that the magnetotactic cocci studied so far, M. marinus strain MC-1 and M. australis strain IT-1, do not contain these genes. Differences observed in the biomineralization genes between M. australis strain IT-1, M. marinus strain MC-1 and the other Alphaproteobacteria are possibly a result of gene rearrangements, deletions or insertions of new genes through the evolution or a post-acquisition of the biomineralization genotype among MTB. Culture and sequencing of new species of magnetotactic cocci from freshwater or marine water are needed to improve the understanding the evolutionary events that occurred in the Alphaproteobacteria and magnetotactic cocci and will more precisely define the Magnetococcales family in the Magnetococcales order as either the earliest diverging order in the Alphaproteobacteria class or as a new class of Proteobacteria, as proposed by Singer et al. (2011). M. australis strain IT-1 is now the third cultivated magnetotactic coccos that represents a second new genus in the Magnetococcales family and is the first cultivated SS-MTB.

AUTHOR CONTRIBUTIONS
All authors contributed to the analysis of data and composition of the paper; Viviana Morillo, Fernanda Abreu and Ana C. Araujo: experimental data acquisition and cultivation; Luiz G. P. de Almeida and Ana T. R. de Vasconcelos: pyrosequencing and bioinformatics; Alex Enrich-Prast: microelectrode measurements and interpretation; MP: high-resolution transmission electron microscopy, Viviana Morillo, Fernanda Abreu, Dennis A. Bazylinski and Ulysses Lins: analyzed data and wrote the paper.

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