Evidence for a copper-dependent iron transport system in the marine, magnetotactic bacterium strain MV-1

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Cells of the magnetotactic marine vibrio, strain MV-1, produce magnetite-containing magnetosomes when grown anaerobically or microaerobically. Stable, spontaneous, non-magnetotactic mutants were regularly observed when cells of MV-1 were cultured on solid media incubated under anaerobic or microaerobic conditions. Randomly amplified polymorphic DNA analysis showed that these mutants are not all genetically identical. Cellular iron content of one non-magnetotactic mutant strain, designated MV-1nm1, grown anaerobically, was ~20- to 80-fold less than the iron content of wild-type (wt) MV-1 for the same iron concentrations, indicating that MV-1nm1 is deficient in some form of iron uptake. Comparative protein profiles of the two strains showed that MV-1nm1 did not produce several proteins produced by wt MV-1. To understand the potential roles of these proteins in iron transport better, one of these proteins was purified and characterized. This protein, a homodimer with an apparent subunit mass of about 19 kDa, was an iron-regulated, periplasmic protein (p19). Two potential ‘copper-handling’ motifs (MXM/MX2M) are present in the amino acid sequence of p19, and the native protein binds copper in a 1:1 ratio. The structural gene for p19, chpA (copper handling protein) and two other putative genes upstream of chpA were cloned and sequenced. These putative genes encode a protein similar to the iron permease, Ftr1, from the yeast Saccharomyces cerevisiae, and a ferredoxin-like protein of unknown function. A periplasmic, copper-containing, iron(II) oxidase was also purified from wt MV-1 and MV-1nm1. This enzyme, like p19, was regulated by media iron concentration and contained four copper atoms per molecule of enzyme. It is hypothesized that ChpA, the iron permease and the iron(II) oxidase might have analogous functions for the three components of the S. cerevisiae copper-dependent high-affinity iron uptake system (Ctri, Ftr1 and Fet3, respectively), and that strain MV-1 may have a similar iron uptake system. However, iron(II) oxidase purified from both wt MV-1 and MV-1nm1 displayed comparable iron oxidase activities using O2 as the electron acceptor, indicating that ChpA does not supply the multi-copper iron(II) oxidase with copper.

INTRODUCTION

Motile bacteria that align and navigate along the Earth’s geomagnetic field lines are referred to as magnetotactic bacteria (Blakemore, 1975; Bazylinski & Frankel, 2004). This behaviour, termed magnetotaxis, is due to the presence of magnetosomes, which are intracellular membrane-bound crystals of magnetite (Fe3O4) and/or greigite (Fe3S4) (Frankel et al., 1997). Magnetosome synthesis appears to be a complex process that is highly regulated and controlled, and is considered to be a biologically controlled mineralization rather than a biologically induced one (Bazylinski & Frankel, 2003; Frankel & Bazylinski, 2003).

Cellular acquisition of iron is the obvious first step in the biomineralization of the bacterial magnetosome. However,
relatively little is known about the biochemistry and genetics of iron acquisition in magnetotactic bacteria and what is known is solely based on Magnetospirillum species. Schüler & Baeuerlein (1996) described two iron uptake systems in Magnetospirillum gyrophilswaldense. The major portion of the iron in Fe$_3$O$_4$ in this organism is taken up as iron(III) in an energy-dependent, low-affinity, high-velocity system. Iron(II) is also taken up by these cells but apparently only by a slow, non-specific, diffusion-like process. Although no evidence for siderophore production by this organism was found, spent culture fluid appeared to enhance iron uptake. Paolotti & Blakemore (1986) found evidence for the production of a hydroxamate-type siderophore by cells of Magnetospirillum magnetotacticum grown under high iron conditions ($\geq 20 \mu$M) but not at iron concentrations $\leq 5 \mu$M. Nakamura et al. (1993) reported molecular evidence for the involvement of a periplasmic binding protein, sfuC, in iron transport by 'Magnetospirillum magneticum' strain AMB-1, although this species was recently found to produce both hydroxamate and phenolide siderophores (Calugay et al., 2003). Finally, an iron-regulated gene, magA, from 'M. magneticum' strain AMB-1 was shown to encode a protein that transports iron in an energy-dependent manner into membrane vesicles prepared from Escherichia coli cells that expressed this gene (Nakamura et al., 1995a, b).

We are studying magnetite magnetosome biomineralization in a marine magnetotactic vibrio referred to as strain MV-1 (Bazylinski et al., 1988). Like members of the genus Magnetospirillum and almost all other magnetite producing magnetotactic bacteria, strain MV-1 is phylogenetically affiliated with the $\alpha$ subdivision of the Proteobacteria, but it is not closely related to Magnetospirillum spp. (DeLong et al., 1993). Cells of strain MV-1 grow chemoorganoheterotrophically utilizing organic and amino acids as carbon and energy sources or chemolithoautotrophically with carbon dioxide as the sole carbon source and thiosulfate or sulfide serving as the source of energy (Bazylinski & Frankel, 2000). Despite the absence of a defined genetic system at the present time, strain MV-1 was chosen as a model organism for several reasons. First, like other magnetotactic bacteria examined, the magnetosomes in this organism appear to be surrounded by a lipid bilayer membrane that contains unique proteins not found in other cell fractions, some of which are believed to be involved in magnetosome biosynthesis. Second, cells of strain MV-1 grow anaerobically, using nitrous oxide (N$_2$O) as the terminal electron acceptor, to high cell densities ($\sim 1-2 \times 10^9$ cells ml$^{-1}$) (Bazylinski et al., 1988), and produce more Fe$_3$O$_4$ per litre of culture than better characterized strains of magnetotactic bacteria. Under anaerobic conditions, magnetosome formation in strain MV-1 appears to be an obligate process and cells continue to synthesize magnetosomes even under iron-limited growth conditions. Third, stable, spontaneous, non-magnetotactic mutants of strain MV-1 appear to occur at a high frequency when cultured on solid media. Lastly, strain MV-1 biomineralizes Fe$_3$O$_4$ crystals with a very unusual morphology, referred to as elongated truncated hexa-octahedrons (Thomas-Keprta et al., 2001). Magnetospirillum spp. biomineralize cubic/octetahedrons, the equilibrium form of Fe$_3$O$_4$ commonly found in inorganically synthesized Fe$_3$O$_4$ (Palache et al., 1944). The MV-1 magnetosome crystal particle morphology is unusual and has been used to distinguish biogenic magnetosome Fe$_3$O$_4$, so-called ‘magnetofossils’, from detrital Fe$_3$O$_4$ using transmission electron microscopy of magnetic extracts from sediments (Bazylinski & Moskowitz, 1997). In addition, elongated Fe$_3$O$_4$ crystals morphologically identical to those of MV-1 are associated with carbonate globules in the Martian meteorite ALH84001 and used as a line of evidence for the presence of life on ancient Mars (McKay et al., 1996; Thomas-Keprta et al., 2000, 2001, 2002).

Recently, evidence of a role for copper has been demonstrated in Pseudomonas aeruginosa, where a multi-copper iron oxidase catalyses the oxidation of iron(II) to iron(III) for transport into the cell (Huston et al., 2002). Here we present molecular and biochemical evidence for a copper-dependent iron transport system similar to that observed in the yeast Saccharomyces cerevisiae (Dancis et al., 1990, 1994a, b; Eide et al., 1992; Askwith et al., 1994; Stearman et al., 1996; Van Ho et al., 2002) that appears to be associated with magnetosome synthesis in the marine, magnetotactic bacterium strain MV-1.

**METHODS**

**Bacterial strains and culture conditions.** Cells of wild-type (wt) and mutant strains of MV-1 were grown anaerobically in liquid culture as previously described by Dean & Bazylinski (1999) except that the cysteine concentration was raised to 0.5 g l$^{-1}$. Cells were also grown on solid medium prepared by the addition of 20 g Agar Noble 1$^{-1}$ (Difco) to liquid medium. Large Petri plates (100 x 25 mm) were poured aerobically and allowed to solidify before being placed inside an anaerobic chamber (Coy Laboratory Products) with a 5 % H$_2$ and 95 % N$_2$ (v/v) atmosphere, where the medium became reduced. Spread and streak plating on reduced anaerobic agar plates was carried out aerobically. The plates were then quickly placed in Oxoid Anaerobic Jars (Oxoid) made anaerobic by exchanging the air atmosphere five times with N$_2$O and leaving a positive pressure of 69 kPa inside the jar. Microaerobic conditions were similarly established by replacing N$_2$O with a 1 % O$_2$ and 99 % N$_2$ atmosphere.

Cells of E. coli strains B and DH5a were grown at 37 °C on Luria-Bertani agar (1-5 % agar) or broth supplemented with the appropriate antibiotics, ampicillin (100 µg ml$^{-1}$) or kanamycin (50 µg ml$^{-1}$), when required. Competent cells of E. coli DH5α were prepared as described by Inoue et al. (1990). Cells of Methylococcus capsulatus Bath were grown as previously described (Zahn & DiSpirito, 1996).

**Isolation of spontaneous, non-magnetotactic mutants of strain MV-1.** A liquid culture of wt MV-1 was serially diluted in shake tubes of growth medium containing 13 g Agar Noble 1$^{-1}$. After the agar solidified, the headspace of the tubes was aseptically purged with N$_2$O for 15 min and over-pressurized to approximately 138 kPa. Tubes were incubated for about 2 weeks at 28 °C in the dark, by which time black lens-shaped colonies typical of wt MV-1 were observed. A cream-to-pink-coloured colony was found in one of the tubes, removed aseptically and transferred to liquid medium. Morphological, genetic and biochemical characteristics showed this strain to be identical to wt MV-1 except that cells were not
magnetotactic and did not produce magnetosomes, as demonstrated by transmission electron microscopy. This strain was designated MV-1nm1. A more detailed characterization of this strain is in progress.

Cells of wt MV-1 spread-plated on the surface of solid media routinely gave rise to colonies of two distinct colours. Some were brown-to-black in colour and contained magnetotactic cells. Others were pink-to-cream-coloured and contained non-magnetotactic cells. Magnetism was determined microscopically using the method of Blakemore et al. (1979). Twenty of these pink-to-cream-coloured colonies were patched onto reduced anaerobic plates with sterile toothpicks. Small vials of liquid media were inoculated with single colonies of either wt MV-1 or non-magnetotactic mutants and then cultivated in liquid cultures of larger volume.

**Estimation of growth.** Growth was determined by direct cell counts using a Petroff-Hauser (Arthur H. Thomas) cell-counting chamber on a Zeiss standard research phase-contrast microscope (Carl Zeiss). Growth was also estimated by protein concentration as described below.

**Preparation of cell fractions and protein determination.** Cell-free extracts of wt MV-1 and the non-magnetotactic mutants were prepared by harvesting cells by centrifugation at 10 000 g for 25 min at 4 °C. Cells were washed once with 10 mM Tris/HCl buffer (pH 7-2; buffer A) in dilute artificial sea water (ASW) (Bazylinski et al., 1994) and centrifuged at 10 000 g for 25 min at 4 °C. They were resuspended in buffer A, and lysed by three passes through a French pressure cell at 124 MPa. The cell lysates were centrifuged at 10 000 g for 25 min at 4 °C to remove whole cells, cell debris and magnetosomes.

Periplasm was prepared by glycine extraction (McCoy et al., 1975), osmotic shock (Thompson & MacLeod, 1974) and chloroform extraction techniques (Ames et al., 1984). Proteins released during the chloroform treatment were recovered with buffer A in ASW to minimize cell lysis. The integrity of the periplasmic fraction was determined by the presence of iron(III) reductase activity and MV-1 cytochrome c-551.

Magnetosomes were purified according to the method of Gorby et al. (1988). Purified magnetosomes were suspended in 20 mM HEPES (pH 7-2) containing 1% SDS and incubated at 25 °C for 2 h with occasional stirring to extract the magnetosome membranes. Magnetosomes were then removed by centrifugation at 10 000 g for 2 min.

Soluble and membrane fractions were prepared by centrifugation of cell-free extracts at 150 000 g for 2 h at 4 °C. The pellet was resuspended with a Dounce homogenizer in buffer A containing 1 M KCl and centrifuged at 150 000 g for 2 h at 4 °C. The membrane pellet was again Dounce homogenized in buffer A and designated the washed membrane fraction. Protein concentrations of cellular fractions were determined by the method of Bradford (1976) as modified by Nelson et al. (1982).

**Purification of p19 and an iron(II) oxidase.** p19 and an iron(II) oxidase were purified to homogeneity from the soluble and/or periplasmic fractions of wt MV-1 and MV-1nm1 by ammonium sulfate fractionation and anion exchange and gel filtration chromatography. The purified proteins were stored at −80 °C.

**Preparation of antibodies against p19 and iron oxidase.** Polyclonal antisera against MV-1 p19 and iron oxidase were obtained from New Zealand White rabbits by Animal Pharm Services. Individual antisera were loaded onto a Protein-A Sepharose CL4B column (Amersham Pharmacia Biotech) (2 x 5 cm) equilibrated with five column volumes of 50 mM Tris (pH 7-0) and bound immunoglobulin G was eluted with 100 mM citric acid (pH 3-0). The individual purified antibody fractions were pooled and stored at −80 °C.

**Electrophoresis and immunoblot analysis.** SDS-PAGE was car- ried out on 12% polyacrylamide gels using the method of Laemmli (1970), or the NuPAGE gel system from Novex on 10% Bis-Tris gels using MES and SDS (pH 7-3) as the running buffer. Prior to separation, samples were reduced and denatured in the appropriate SDS sample buffer containing 50 mM dithiothreitol and heated to 90 °C for 5 min, unless the gel was stained for haem. Gels were stained for total protein with Coomassie brilliant blue R250 or for c-type cytochromes with diaminobenzidine (McDonnel & Staehelin, 1981). Proteins were transferred to TransBlot PVDF membranes (Bio-Rad) using the NuPAGE transfer system (Novex). Membranes were subjected to treatment with antisera containing antibodies to purified p19 or the iron oxidase. Detection of antibody reaction was performed with the Opti 4CN system (Bio-Rad) according to the manufacturer’s directions. The isoelectric points for p19 and the iron oxidase were determined using Novex IEF pH 3–10 gels as directed by the manufacturer.

**N-terminal amino acid sequence analysis.** N-terminal amino acid sequence of proteins was determined by Edman degradation using an Applied Biosystems 492 Protec Protein Sequencer coupled with a model 140C Analyser by the Protein Facility at Iowa State University.

**Analytical ultracentrifugation, haem c determination and spectroscopy.** Sedimentation equilibrium experiments on purified p19 were carried out with a Beckman Optima XL-A analytical ultra- centrifuge equipped with a Beckman An-60 Ti rotor. The reference and sample cell assemblies were monitored at a wavelength of 280 nm with rotor speeds of 10 000 and 13 000 r.p.m. at 4 °C. Haem c concentrations were measured by the pyridine ferrohaemochromogen method (Furhop, 1975; DiSpirito, 1990). Optical absorption spectroscopy was performed with an SLM Amino DW-2000 spectrophotometer in the split beam mode.

**Determination of iron concentration in growth medium and cells.** All glassware used in iron measurements was immersed overnight in 0-1 M HNO3 and rinsed five times with Nanopure water (Millipore). Iron concentrations in growth medium were determined by aseptically removing 10 ml media by syringe and immediately adding concentrated HCl to a final concentration of 1% (v/v). For whole cell total iron analysis, cells of MV-1 and MV-1nm1 were harvested and washed as described above except that the procedure was carried out in an anaerobic chamber. Cell pellets were digested by adding 10 ml concentrated HNO3, and the volume reduced to approximately 1 to 2 ml by heating at 95 °C. The digests were allowed to cool and the process was repeated with 5 ml concentrated HCl. After cooling, 5 ml 70% HClO4 was added and the volume reduced to between 0-5 to 1 ml by heating at 95 °C. Each digest was diluted to either 50 or 100 ml with 1% HCl in a volumetric flask. Acids for cell pellet digestion were trace metal grade from Fisher Scientific. Total iron was assayed using the ferrozine reagent (Stokey, 1970). Each reaction contained 1-25% (w/v) hydroxy- lamine hydrochloride (Acros), 0-5 mM ferrozine, 260 mM ammonium acetate buffer (pH 10-0) and, when necessary, buffer A to a volume of 1 ml. Absorbance of the reactions was measured at 562 nm after 10 min.

**Metal analysis of p19 and iron oxidases.** Purified p19 (5 μM) was mixed with an equimolar amount of copper or iron from 5 mM stock solutions of CuCl2 or FeSO4, respectively, in buffer A. The total volume of each sample was 1 ml. The samples were incubated at room temperature for 1 h and individually passed through a Sephadex G-25 column (2 x 6 cm), equilibrated with three column volumes of buffer A, to remove the excess copper and iron salts from protein. The protein fractions and unbound metal fractions were collected from the desalting column and volumes adjusted to 5 ml with buffer A. The samples above, a 5 μM solution of purified
p19 and 1 μM solutions of each metal salt were each measured for copper and iron content using a Perkin Elmer model 1100B atomic absorption spectrometer. Purified iron(II) oxides from MV-1 and MV-1nm1 were each diluted to 1 nmol ml⁻¹ in 10 mM Tris/HCl (pH 8-2; buffer B) and measured for copper content using a Hewlett Packard 4500 Series ICP-MS. Standards for metal analyses were obtained from Fisher Scientific.

**Determination of enzyme activities.** Enzyme activities were measured aerobically at room temperature on an SLM Aminco DW-2000 spectrophotometer in the split beam mode. Iron oxides assays were performed as described by de Silva et al. (1997), except that reaction volumes were 1 ml.

Iron reduction was measured by the method of Dailey & Lascelles (1977). The 1 ml assay mixture contained 0.4 mM NADH, 0.2 mM ferric citrate and 1 μM flavin mononucleotide (FMN) in buffer A. Following 10 min incubation in the dark, ferric was added to 0.5 mM and the reaction was initiated by the addition of protein. The reaction was monitored for an increase in absorbance at 562 nm.

**Detection of siderophore activity.** Liquid medium for siderophore assays was prepared as described above except that nitrotroicarboxylic acid and resazurin were omitted. Uninoculated media for each individual iron condition were prepared for use as references. Spent culture media were passed through 0.22 μm filters after centrifugation of cells as described above. The chrome-azuro sulfonate (CAS) shuttle assay (Schwyn & Neilands, 1987) for siderophore detection was performed as modified by Payne (1994) and expressed as per cent siderophore units. The ferric perchlorate assay for the presence of hydroxamate (Atkins et al., 1970) and the Arnow (1937) assay for the presence of catechols were performed as previously described by Payne (1994).

**PCR.** Degenerate primers were used to amplify a 255 bp region of the p19 gene in strain MV-1. The forward primer, 5’-ATGGARGTNATGGG-3’, was designed from the N terminus of p19 from MV-1 and the reverse primer, 5’-GCNCCRTARTGNGG-3’, was designed from a well-conserved region found downstream in p19 homologues from *Campylobacter jejuni* (Janvier et al., 1998), *Treponema pallidum* (Swancutt et al., 1989) and *Yersinia pestis* (Buchrieser et al., 1997). The PCR reaction contained 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 2 mM MgCl₂, 0.1% (w/v) Triton X-100, 0.2 mM dNTPs, 25 pmol of both forward and reverse primers, 5 units of *Pfu* DNA polymerase (Stratagene) and 10 ng of MV-1 genomic DNA in a total volume of 50 μl. This PCR mixture was subjected to the following parameters: one cycle at 94°C for 1 min followed by 25 cycles of 95°C for 30 s, 51°C for 30 s and 72°C for 30 s, with no final extension step at 72°C. PCR amplification of a 900 bp fragment from genomic DNA of MV-1nm1 and four non-magnetotactic mutants isolated from agar plates with primers (5’-GATGTCGCTGTTGTTGAAAG-3’ and 5’-AATAGACCAACGGACAGA-3’) specific for the p19 structural gene in strain MV-1 was accomplished by subjecting a 100 μl PCR [10 mM Tris/HCl (pH 9.0), 50 mM KCl, 2 mM MgCl₂, 0.1% (v/v) Triton X-100, 0.2 mM dNTPs, 25 pmol of both forward and reverse primers, 5 units of *Taq* DNA polymerase (Promega) and 10 ng of non-magnetotactic mutant genomic DNA] to the following parameters: one cycle at 95°C for 1 min followed by 15 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s and a final extension step at 72°C for 10 min.

**Randomly amplified polymorphic DNA analysis (RAPD).** RAPD analysis was performed generally using the protocol of Dassanayake & Samaranayake (2003). Genomic DNA from MV-1, MV-1nm1 and four non-magnetotactic mutants isolated from agar plates was quantified using the PicoGreen dsDNA quantification kit (Molecular Probes) and a fluorometer (Turner Quantech Digital Filter Fluorometer). The random primer GTAGCCCTAC was used as both a forward and reverse primer. PCRs (25 μl) contained 16 mM (NH₄)₂SO₄, 10 mM β-mercaptoethanol, 2.5 mM MgCl₂, 0.01%, (v/v) Tween-20, 100 μg bovine serum albumin ml⁻¹, 0.3 mM dNTPs, 25 ng genomic DNA and 1 unit of *Taq* polymerase (Bionline) in 67 mM Tris/HCl (pH 8.8). PCRs for RAPD were subjected to the following treatment: one cycle at 94°C for 2 min followed by 45 cycles of 93°C for 30 s and 36°C for 1 min, followed by a final extension step at 72°C for 6 min. The Iowa State University DNA Synthesis and Sequencing Facility synthesized all primers used in this work.

**Recombinant DNA techniques.** A genomic library of MV-1 was prepared in the cosmid vector, SuperCos 1 (Stratagene), according to manufacturer’s directions and modifications by Dean (1999). The 255 bp PCR product was electrophoresed on a 3% NuSieve 3:1 agarose gel (FMC BioProducts), purified using the QIAquick gel extraction kit (Qiagen), and cloned using the Zero Blunt PCR cloning kit (Invitrogen). A cosmid clone (c2E9), containing the p19 gene, was digested with EcoRI (Life Technologies) and subcloned into EcoRI-restricted, dephosphorylated pUC18 (Bayou Biolabs), using T4 DNA ligase (Life Technologies) according to Sambrook et al. (1989), and transformed into *E. coli* DH5α. The plasmid pBD619 was isolated from this subclone library by Southern hybridization (see below). For sequence analysis, plasmid DNA was purified using the Plasmid Midiprep kit (Qiagen) and sequenced on an ABI 377 automated sequencer (Perkin-Elmer/ABI). The 900 bp fragment amplified from the non-magnetotactic mutant strains was cloned using the pGEM-T Easy Vector System I (Promega), after gel purification. DNA sequencing was performed at the Iowa State University DNA Synthesis and Sequencing Facility.

**Southern hybridizations.** Genomic DNA from all bacterial strains was isolated as previously described by Kimble et al. (1995). Genomic DNA (~10 μg) was digested with several restriction endonucleases as directed by the manufacturers. Agarose gel electrophoresis, preparation of colony blots and transfer of denatured DNA onto nylon membranes via capillary action were performed as previously described by Sambrook et al. (1989). DNA probes were derived from plasmid pBD619, which contains a 5.7 kb EcoRI DNA fragment that includes a 3.4 kb region with six ORFs, some of which encode a putative inner permease, a putative ferredoxin and the p19 structural gene. Probes were prepared by digesting pBD619 with the restriction endonucleases indicated by gel purification to give: the entire 3.4 kb region (*Smal*, EcoRI), putative inner permease (*AfII*, BsrGI) and putative ferredoxin (*Ncol*). The DNA probe used for detection of the p19 gene was the cloned 255 bp PCR product. The double-stranded probes were radioactively labelled by the random hexamer priming method (Feinburg & Vogelstein, 1983), using the Random Primers DNA Labelling System. Removal of unincorporated nucleotides and buffer exchange was accomplished with Microspin S-300 HR columns according to the manufacturer’s directions. Prehybridization of genomic and colony blots was done in 6× SSPE (150 mM NaCl, 10 mM NaH₂PO₄, 10 mM NaEDTA), 0.5% SDS, 100 μg denatured, fragmented salmon sperm DNA ml⁻¹ and 5× Denhardt’s reagent (Sambrook et al., 1989). Genomic and colony blots were hybridized in 6× SSPE containing 10 μg denatured, fragmented salmon sperm DNA ml⁻¹ at 42°C. All Southern blots were imaged using standard autoradiography (Sambrook et al., 1989).

**Small-subunit rRNA gene sequencing.** The 16S rDNA of MV-1nm1 was amplified from genomic DNA, using primers *F1* and *R1* according to the method of Weisburg et al. (1991), with the annealing temperature being changed to 56°C. The amplified products were purified on MicroSpin S-300 HR spin columns. PCR
products were cloned using the pGEM-T Easy Vector System I and transformed into chemically competent cells of *E. coli* DH5α (Inoue et al., 1990). Plasmid DNA for insert analysis and sequencing was prepared as described above.

**Transcript analysis.** Total RNA was isolated from MV-1, MV-1nm1 and *Methylbacterium capsulatus* Bath using the polyethylene glycol (PEG) 6000 extraction protocol of Hepinstall (1998), followed by purification on a cushion of CsCl (Sambrook et al., 1989). Formaldehyde gel electrophoresis of total RNA was carried out according to the method of Bryant & Manning (1998) and transferred to nylon membranes by the alkaline capillary transfer procedure of Löw (1998). Northern hybridization analysis and probe labelling was performed as described above for Southern hybridizations except all solutions were made with 1% (w/v) diethyl polycarbonate (DEPC)-treated water and autoclaved prior to use (Löw, 1998). Northern blots were imaged using standard autoradiography (Sambrook et al., 1989). All glassware used for RNA isolation and manipulation was baked overnight at 180 °C.

**RESULTS**

**Isolation and growth of a spontaneous, non-magnetotactic mutant strain of MV-1**

While cells of strain MV-1 typically produced black, lens-shaped colonies in shake tubes, pink-to-cream-coloured, lens-shaped colonies were occasionally observed in shake tubes of solid medium. One of these was aseptically removed and inoculated into sterile growth medium for further characterization. With respect to cell morphology, specific growth characteristics (e.g. anaerobic growth on N2O, growth as microaerophilic bands of cells in oxygen gradient cultures) and motility, cells of this isolate, strain MV-1nm1, looked and behaved identically to MV-1, except they did not exhibit magnetotaxis. Transmission electron microscopy revealed that these cells did not produce magnetosomes (data not shown). Results from specific biochemical tests (e.g. acetylene reduction, carbon source utilization) were also identical to those for MV-1 (data not shown). Sequence analysis of the 16S rDNA of MV-1nm1 showed 99% sequence identity to wt MV-1 (data not shown). The strain has not shown reversion to the magnetotactic phenotype over a period of several years of continuous transfer. This strain thus appears to be a stable, spontaneous, non-magnetotactic mutant of MV-1.

Growth as colonies of MV-1 and MV-1nm1 on solid media was accomplished by streaking or spread-plating cells onto the surface of solidified growth medium and incubating under microaerobic or anaerobic conditions. However, conventional Petri plates (15 mm deep) did not support growth of either strain on solid media. Colony formation on plates required the use of Petri plates (25 mm) that could hold more growth medium, where the thickness of the agar (16 to 20 mm) was 2–3 times that of a conventional agar plate. Cells of wt MV-1 formed black colonies on these plates and those of MV-1nm1 pink-to-cream-coloured colonies on these plates. Pink-to-cream-coloured colonies are typical of non-magnetotactic mutants that do not biomineralize magnetosomes. Formation of colonies was visible on plates after 15–20 days of incubation at 28 °C, regardless of which terminal electron acceptor was supplied.

**Effect of media iron concentration on biomass and iron content of cells**

When cultured under low iron conditions (i.e. ~3 μM iron from mineral solution; the major source of iron omitted from the growth medium), the growth characteristics of MV-1 and MV-1nm1 differed. When subjected to 10 consecutive transfers in low-iron media, final cell yields of MV-1nm1 at stationary phase (as cell numbers) were comparable to those of cultures grown in high-iron media (~1×10^9 cells ml⁻¹). However, when MV-1 was treated similarly, cells remained magnetotactic and produced magnetosomes but the final cell yield based on cell counts was only 23 ± 10% of that for MV-1nm1 grown under low iron conditions. Cells of MV-1nm1 grown anaerobically in high-iron media (i.e. ≥25 μM; major iron source added as FeSO₄) did not display magnetotaxis nor did they synthesize magnetosomes.

Cell yields were also measured as total cell protein. Table 1 shows the total protein and iron content of cell-free extracts of MV-1 and MV-1nm1 grown under anaerobic conditions. Protein yields of MV-1 grown under low iron conditions were approximately 30% that of MV-1nm1 and cells were magnetotactic. This result is consistent with the direct cell counts of the strains grown under the same conditions. Although we did not count the number of magnetosomes per cell in this particular experiment, electron microscopy of MV-1 cells grown under low iron conditions in previous experiments showed they contained about half the mean number of magnetosomes per cell (~5) compared to cells grown under high iron conditions (~10) (D. A. Bazylinski, unpublished results). At higher iron conditions (~28 μM Fe), protein yields of MV-1 and MV-1nm1 were comparable. The haem c content per mg protein of the soluble fractions of wt MV-1 and MV-1nm1 grown under low iron conditions was measured and compared. The soluble

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<th>Strain</th>
<th>Initial Fe (μM)</th>
<th>Protein (mg)</th>
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fraction of wt MV-1 grown under low iron conditions contained roughly 50% of the haem c per mg protein of that found in MV-1nm1 (data not shown). Cellular iron content of MV-1 (Table 1) increased as the concentration of iron in the growth medium rose from 3 to 106 \( \mu \text{M} \) and values were \( \sim 20-80 \)-fold greater than the iron content of MV-1nm1 for those same media iron concentrations.

**Siderophore activity and response to media iron concentration**

To determine if siderophore activity could account for the differences in iron content of the strains, liquid spent culture fluids of MV-1 and MV-1nm1 were compared. Spent medium from both strains showed CAS reactivity (Fig. 1). The amount of siderophore activity of either strain was affected by media iron concentration, with an apparent repression of this activity beginning at different iron concentrations for the two strains. Cultures of MV-1 exhibited a consistent level (mean of 17.6% siderophore units) of siderophore activity when media iron concentrations were \( \leq 25 \mu \text{M} \) and an apparent repression of this activity at iron concentrations of \( \geq 50 \mu \text{M} \). The siderophore activity in cultures of MV-1nm1 was highest at 5 \( \mu \text{M} \) iron, which was approximately 50% of the highest activity found in MV-1 cultures and decreased as media iron concentration increased.

Attempts to determine the chemical nature of this siderophore activity were made by employing the ferric perchlorate assay for hydroxamates (Atkins et al., 1970) and the Arnow (1937) assay for catechol/phenolate types of siderophores. When spent medium was tested in the ferric perchlorate assay, the reaction mixture quickly turned blue indicating the presence of a hydroxamate type of siderophore, although the colour faded within minutes suggesting the siderophore was unstable at the pH of the assay. There was no visible reaction with spent medium in the Arnow assay.

**Protein profiles of MV-1 and MV-1nm1**

Protein profiles of soluble and membrane fractions of wt MV-1 and MV-1nm1 showed several differences, the most prominent being the absence of a major protein with an apparent molecular mass of 19 kDa in the soluble fraction of MV-1nm1 (Fig. 2). Because the soluble fraction contains both soluble cytoplasmic and periplasmic proteins, the cellular location of p19 in strain MV-1 was determined. Three methods for the extraction of periplasmic proteins were examined and qualitatively compared by SDS-PAGE. Periplasmic protein profiles obtained through osmotic shock (Thompson & MacLeod, 1974) and glycine extraction (McCoy et al., 1975) were very similar, each containing 10–15 protein bands. Chloroform extraction (Ames et al., 1984) was less effective and resulted in considerable cell lysis. While glycine extraction was a simple procedure and worked well, subsequent lysis of the spheroplasts of MV-1 by disruption in a French pressure cell was not possible.

![Fig. 1.](image1.jpg)  
*Fig. 1.* Siderophore activity in response to media iron concentration in spent culture fluid of wt MV-1 and that from the non-magnetotactic mutant MV-1nm1, as measured by the CAS assay. Wt MV-1, filled bars; MV-1nm1, open bars. The x-axis represents the concentration (\( \mu \text{M} \)) of the major source of iron, added as a FeSO\(_4\) solution to the growth medium. The background iron concentration was \( \sim 3 \mu \text{M} \), mainly from Fe in the mineral solution. Data shown are mean values of triplicate measurements; error bars represent 1 SD.

![Fig. 2.](image2.jpg)  
*Fig. 2.* Protein profiles by SDS-PAGE of different cell fractions of wt MV-1 and MV-1nm1. Lanes: 1, wt MV-1 soluble fraction; 2, MV-1nm1 soluble fraction; 3, wt MV-1 membrane fraction; 4, MV-1nm1 membrane fraction; 5, wt MV-1 magnetosome membrane fraction; M, molecular mass standards. Arrow in lane 1 denotes a soluble 19 kDa protein (p19) not present in MV-1nm1 and those in lane 5 denote several proteins unique to the magnetosome membrane in wt MV-1. All lanes were loaded with \( \sim 10 \mu \text{g} \) protein.
Therefore, the preferred method for periplasm extraction from MV-1 was with osmotic shock (Fig. 3). Periplasm from MV-1 derived from this method accounted for 6.9% of the total cell protein, a value consistent with other Gram-negative species (Ames et al., 1984).

Iron(III) reductase activity, mediated by a known cytoplasmic enzyme in M. magnetotacticum (Noguchi et al., 1999) and cytochrome c-551 (which initially co-purified with p19 from the periplasmic extract) were used as cytoplasmic and periplasmic markers, respectively, in strain MV-1 (Table 2). As soluble, low-molecular-mass c-type cytochromes are not known to exist in the cytoplasm of bacteria or mitochondria (Hooper & DiSpirito, 1985) and the iron reductase activity requires NADH (which is not present in the periplasm or outer membrane), p19 is assumed to be primarily located in the periplasm in strain MV-1. Moreover, once the amino acid sequence of p19 was determined, it was located in the periplasm in strain MV-1. Moreover, once the amino acid sequence of p19 was determined, it was analysed by the program PSORT which predicted a periplasmic location for this protein (Gardy et al., 2003).

p19 and the iron(II) oxidase in periplasm ran slightly differently in PAGE than when purified (Fig. 3). This is likely due to the presence of additional proteins in the periplasmic fraction and/or to the much higher amount of p19 in the purified sample than in the periplasm. The N-terminal sequence of the band in question from the periplasm matched the sequence from purified p19 exactly (data not shown).

**Purification and properties of p19**

Soluble p19 was purified to homogeneity from the periplasm of MV-1 (Fig. 3). Ammonium sulfate was added to periplasmic or soluble fractions of MV-1 to 50% saturation. This solution was stirred for 1 h at room temperature and then centrifuged at 10 000 g for 30 min. The supernatant was removed and ammonium sulfate added to 90% saturation. This solution was stirred at room temperature for 1 h and recentrifuged at 10 000 g for 30 min. The resulting pellet (50–90% ammonium sulfate fraction) was resuspended in a minimal volume of buffer A containing 150 mM KCl (buffer C), dialysed against three changes of buffer C and concentrated in a stirred cell containing a YM-10 membrane (Amicon). The concentrated sample was loaded onto a Superdex G-75 FF (Amersham Pharmacia Biotech) column (2.6 × 60 cm) equilibrated with two column volumes of buffer C. Fractions were monitored at 280 nm and peaks assayed by SDS-PAGE for the presence of p19, after dialysis and concentration in Centricon YM-10 (Amicon) concentrators with buffer A. Fractions containing p19 were again concentrated by ultrafiltration in a stirred cell (YM-10 membrane) and applied to the Superdex G-75 FF column. Purified p19 eluted from this column at a mass corresponding to about 30 to 40 kDa suggesting that p19 exists as a dimer in its native state. Fractions containing p19 were pooled and dialysed by ultrafiltration in buffer A and concentrated in a stirred cell (YM-10 membrane). The sample was loaded on a DEAE-Sephrose FF (Amersham Pharmacia Biotech) column (2.6 × 30 cm) equilibrated with two column volumes of buffer A. A linear gradient (500 ml) of 400–700 mM KCl was passed through the column. p19 eluted from the column at a KCl concentration of approximately 460 mM. Fractions containing p19 were pooled and dialysed against three changes of buffer A. Following dialysis, the sample was concentrated in a stirred cell (YM-10 membrane) and applied to the DEAE-Sephrose FF column, equilibrated with buffer A. A linear gradient (100 ml) of 300–400 mM KCl was passed through the column and p19 eluted at approximately 370 mM KCl. Pooled fractions containing p19 were dialysed against three changes of buffer A and concentrated in a Centriprep YM-10 (Amicon).

Analytical centrifugation experiments confirmed purified

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Iron(III) reductase [µmol iron(II) produced min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
</table>
| MV-1
| Cell-free extract | 6.9 ± 0.3 |
| Periplasm fraction | 1.8 ± 0.1 |
| Cytoplasmic fraction | 20.2 ± 1.1 |
| Membrane fraction | NM |
| MV-1nm1
| Cell-free extract | 7.3 ± 0.4 |

**Table 2.** Localization of iron(III) reductase activity in cell fractions of strain MV-1

Values represent means ± SD of triplicate measurements. NM, No measurable activity.

![Fig. 3. SDS-PAGE of periplasmic fraction, purified p19 and purified iron oxidase from wild-type MV-1. Lanes: 1, osmotic shock periplasmic fraction (~2 µg); 2, purified p19 (~1 µg); 3, purified iron oxidase (~1 µg); M, molecular mass standards.](http://mic.sgmjournals.org)
p19 was homodimeric with a molecular mass of 34.6 kDa. With the exception of the absorption maximum at 280 nm, p19 had no UV-visible absorption spectral characteristics. Purified p19 contained 0.5 copper atoms per dimer. However, when analysed after incubation with an equimolar amount of copper and desalted by gel filtration, p19 was found to contain 1 copper atom per dimer (Table 3). p19 was also found to contain 0.15 atoms of Fe per dimer. The N-terminal sequence of p19 showed homology to a previously described Fur-regulated, 19 kDa periplasmic protein of C. jejuni (Janvier et al., 1998; van Vliet et al., 1998). Isoelectric focusing showed p19 to be an acidic protein with a pI of 4.8, consistent with the C. jejuni homologue (Janvier et al., 1998).

Cloning of the p19 structural gene, chpA (copper handling protein)

To isolate the structural gene for p19, Southern hybridization of an EcoRI subclone library of cosmid c2F9 in pUC18 with the 255 bp p19 gene probe was performed. This led to the isolation of plasmid pBD619 that contains a 5.7 kb EcoRI DNA fragment. Sequencing of this fragment revealed the presence of an approximately 3.5 kb region containing six ORFs. The first four ORFs encoded for peptides that showed homology to a family of iron permease proteins known as the Ftr1p permease family. Proteins of the Ftr1p permease family share the EXXE motif that has been shown to be involved in iron transport (Stearman et al., 1996). The fifth ORF encoded for a protein of unknown function; however it contained three ferredoxin motifs (CXXXCP and CXXCXXC) (Bruschi & Guerlesquin, 1988). The sixth ORF was chpA, the gene encoding p19. Comparison and alignment of the complete amino acid sequence of p19 and homologous sequences showed the following identities and similarities, M. magnetotacticum (47, 59%), Actinobacillus actinomycetemcomitans (57, 67%), Bordetella pertussis (53, 67%), C. jejuni (48, 62%) and Y. pestis (57, 67%) (Fig. 4a). The 3.5 kb region also contained a 19 bp sequence, 90 bp upstream of the start codon for the first ORF, with an 89-5% sequence identity to the E. coli Fur box consensus sequence (Escolar et al., 1999). Another Fur box showing 78.9% identity was found within the N-terminal region of chpA.

A comparison of the genetic organization of putative operons containing chpA from MV-1, C. jejuni, Y. pestis and T. pallidum is shown in Fig. 4(b). chpA was shown to be immediately downstream of the FTR1 iron permease homologues from C. jejuni, Y. pestis and T. pallidum.

Regulation of expression of p19 by iron concentration

Immunoblot analysis of soluble and salt-washed (1 M KCl) membrane fractions from MV-1 grown under low to high iron concentrations showed the expression of p19 to be regulated by iron concentration (Fig. 5). Levels of p19 were highest under low iron, and responded inversely to media iron concentration to 50 μM. At concentrations ≥50 μM, levels of p19 appeared to plateau. The presence of periplasmic p19 in the salt-washed membrane fraction suggests that some form of p19 might be peripherally associated with the outer or cytoplasmic membrane in MV-1 (Fig. 5).

Purification and properties of an iron oxidase

During the purification of p19, a bluish-green coloured band was observed on a gel filtration column with a molecular mass between 70 and 90 kDa. This band represented a single protein that exhibited iron(II) oxidase activity. The iron(II) oxidase was purified to homogeneity as follows. During the purification of p19, a bluish-green coloured band was observed on a gel filtration column with a molecular mass between 70 and 90 kDa. This band represented a single protein that exhibited iron(II) oxidase activity. The iron(II) oxidase was purified to homogeneity as follows.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value(s) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>p19</td>
<td>Fe(II) oxidase</td>
</tr>
<tr>
<td>Molecular mass (kDa)</td>
<td>34.6 ± 0.5</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>19-0</td>
</tr>
<tr>
<td>Translated amino acid sequence</td>
<td>17-6</td>
</tr>
<tr>
<td>Copper content (atoms (molecule)⁻¹)</td>
<td>1.00 ± 0.02</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>4.8</td>
</tr>
<tr>
<td>Absorption maxima (nm)</td>
<td>280</td>
</tr>
<tr>
<td>Molar absorptivity (mM⁻¹ cm⁻¹)</td>
<td>–</td>
</tr>
</tbody>
</table>

ND, Not determined.
DEAE-Sepharose FF column (5-2 × 6-2 cm) equilibrated with two column volumes of buffer B. The column was washed with 200 ml buffer B, followed by a step gradient with increments of 200 mM KCl for one column volume each, with a final concentration of 1 M KCl. Fractions were dialysed, concentrated and assayed by SDS-PAGE and UV- visible absorption spectroscopy. The fractions containing iron oxidase were dialysed and concentrated in a stirred cell with a YM-30 membrane and applied to a Superdex G-75 FF column (2-6 × 60 cm) equilibrated with two column volumes of buffer B plus 150 mM KCl (buffer D). The iron oxidase eluted in the 70–90 kDa fraction. The iron oxidase fraction was diluted with buffer B and applied to a DEAE-Sepharose FF column (5-2 × 6-2 cm) equilibrated with two column volumes of buffer B. The void volume was blue in colour and was concentrated with a stirred cell containing a YM-30 membrane. The iron oxidase was applied to a Superdex G-200 FF column (2-6 × 28 cm) equilibrated with two column volumes of buffer D. A blue-coloured fraction, migrating between 70 and 90 kDa, was dialysed with buffer B and concentrated with a Centriprep YM-10.

In NuPAGE gels, the iron(II) oxidase migrated as a single band with an apparent molecular mass of 81 kDa (Fig. 3), consistent with the molecular mass of 77–85 kDa determined by gel filtration. Table 3 shows some characteristics of the iron(II) oxidase. The enzyme from strain MV-1 showed a specific iron oxidase activity of 609 ± 7 nmol Fe(III) produced min⁻¹ (mg protein)⁻¹ (Table 3). The MV-1nm1 enzyme was also active with a specific iron(II) oxidase activity of 358 ± 58-8 nmol Fe(III) produced min⁻¹ (mg protein)⁻¹. The molecular and spectral characteristics and enzymic activities of the iron(II) oxidases from both strains of MV-1
are similar to those of the Fet3p iron oxidase from *S. cerevisiae* (de Silva et al., 1997; Hassett et al., 1998). The N-terminal sequence of the MV-1 iron oxidase was determined by Edman degradation to be ADSLDDVMKRRGLTEKV... A BLAST search of the *M. magnetotacticum* genome revealed a putative ORF encoding an amino acid sequence with significant similarity to the MV-1 iron(II) oxidase. This information can be found at the Joint Genome Institute website (http://www.jgi.doe.gov/JGI_microbial/html/index.html) where the incomplete genome sequence of *M. magnetotacticum* is available.

**Characterization of non-magnetotactic mutants of MV-1**

Twenty new spontaneous, non-magnetotactic mutants from strain MV-1 were isolated from agar plates. All of these new isolates displayed the normal vibrioid cell morphology typical of wt MV-1 and were motile, but showed no magnetotactic response.

Cell-free extracts of wt MV-1, MV-1nm1 and the 20 new non-magnetotactic mutants were screened for the presence of p19 and the iron(II) oxidase. p19 was not produced by any of the non-magnetotactic mutants (Fig. 7). However, as in MV-1nm1, immunoblot analysis showed the presence of the iron(II) oxidase in all of the mutants. Genomic DNA of wt MV-1 and MV-1nm1 was hybridized with a 3-4 kb probe specific to the six ORFs (comprising the chpA region) and probes specific to the putative iron permease gene, the putative ferredoxin-like gene and the iron oxidase and p19 were used to screen proteins from cell-free extracts (10 μg protein lane−1). Lanes: 1, wt MV-1; 2, MV-1nm1; 3–10, eight non-magnetotactic mutants of MV-1 isolated from agar plates.

Interestingly, the chpA probe for p19 hybridized to a differently sized transcript in both MV-1 and MV-1nm1. The size of the 3-4 kb transcript was large enough to encode for all three of the proteins, but these results indicate that the genes were not present on a single polycistronic mRNA. The 1-6 kb transcript was large enough to encode for p19. The level of hybridization to each individual probe indicates that the 3-4 and 1-6 kb transcripts were present in the same amounts in both strains. Hybridization of the chpA probe to the 1-6 kb transcript in MV-1nm1 was surprising because the synthesis of p19 appears to be completely absent in this mutant.

A 900 bp PCR product of the chpA region was amplified from genomic DNA of MV-1nm1 and four of the non-magnetotactic mutants of MV-1 isolated from solid media showed the presence of chpA. The primers for this PCR product were designed to include a region starting 226 bp upstream of the p19 start codon and ending 119 bp downstream of the stop codon. Sequencing of the PCR products confirmed the identity of the target region. However, the sequences revealed two bp changes within the structural gene of p19 (Fig. 9) in all five non-magnetotactic mutants analysed. The two transversions occurred at base pairs +110 (AT to TA) and +145 (GC to TA) upstream of the chpA start codon in wobble positions and did not result in amino acid substitutions in the encoded peptide. The MV-1 and MV-1nm1 chpA sequences surrounding the two transversions contained the sequence 5′-ACGGAAGGAGGT-3′ and 12 bp downstream was the

![Fig. 6. UV-visible spectrum of the periplasmic iron oxidase purified from wt MV-1 in 10 mM Tris/HCl, pH 8.2. The spectrum was recorded at room temperature and the iron oxidase concentration was ~ 33 μM.](image)

![Fig. 7. Western blot analysis of p19 and iron oxidase in wt MV-1 and non-magnetotactic mutants of MV-1. Antibodies to the iron oxidase and p19 were used to screen proteins from cell-free extracts (10 μg protein lane−1). Lanes: 1, wt MV-1; 2, MV-1nm1; 3–10, eight non-magnetotactic mutants of MV-1 isolated from agar plates.](image)
DISCUSSION

In this paper we describe the first non-magnetotactic mutants from a marine, magnetotactic bacterium strain MV-1 that do not produce magnetosomes, and have identified physiological, biochemical and genetic differences between wt MV-1 and these mutants. Although the spontaneous mutation(s) responsible for the non-magnetotactic, non-magnetosome-forming phenotype are potentially pleiotropic, this phenotype is significant because such mutants will likely be invaluable in determining the mechanism of magnetosome biosynthesis in strain MV-1. This is proving to be the case for the magnetotactic spirillum M. gryphiswaldense (Schübbe et al., 2003; Grünberg et al., 2004).

In addition to the non-magnetotactic mutant phenotype, cells of MV-1nm1 lack an iron uptake system that appears to be related to magnetosome biomineralization. Both wt MV-1 and MV-1nm1 appear to synthesize a hydroxamate type of siderophore but differ in their patterns of putative siderophore production. Wt MV-1 produces the highest levels of siderophore, based on the CAS assay, between initial media iron concentrations of about 8–28 μM. Siderophore production appeared to be repressed above and below this concentration range. Although this siderophore production pattern is unusual for most non-magnetotactic bacteria, for which siderophore production is induced at very low concentrations of iron, usually 0–1 μM (Neilands, 1995), it is apparently not unusual for magnetotactic bacteria. For example, Paoletti & Blakemore (1986) demonstrated hydroxamate siderophore production by cells of M. magnetotacticum at growth medium iron concentrations of ≥20 μM but not at concentrations ≤5 μM. Recently, Calugay et al. (2003) observed hydroxamate and catechol siderophore production by ‘M. magneticum’ strain AMB-1 under conditions where the concentration of iron would be considered sufficient, if not high, for most microbes. A concentration of at least 6 μM was shown to be required to initiate siderophore synthesis by cells of this species. Calugay et al. (2003) suggested that cells of ‘M. magnetis’ strain AMB-1 rapidly assimilate the iron in iron-sufficient medium, causing the amount of external iron to drop to iron-deficient levels, to explain the siderophore production pattern. It does not, however, explain why cells do not synthesize siderophores at low initial concentrations of iron (≤6 μM). The siderophore production pattern of the complimentary sequence 5’-CCTCCAGCCCGT-3’ (Fig. 9) which might cause a stem–loop secondary structure in the p19 transcript.
Cells of MV-1nm1 do not synthesize an iron-regulated periplasmic protein, p19, produced by wt MV-1. The periplasmic protein p19 contains two amino acid motifs (MXM, MX2M) characteristic of copper transport proteins found in *S. cerevisiae*, as well as an MX3M motif. The best characterized of these copper transport proteins is Ctr1p, an integral plasma membrane component of a copper-dependent, high-affinity iron transport system (Dancis *et al.*, 1994a, b; Koch *et al.*, 1997). This permease is responsible for the transport of copper across the plasma membrane for eventual incorporation into the multi-copper iron oxidase Fet3p, which is involved and required in the secretory pathway for high-affinity iron transport in this organism (Askwith *et al.*, 1994). Cells of wt MV-1 and MV-1nm1 both produce a periplasmic, copper-containing iron oxidase that has similar molecular, spectral and enzymic activities to Fet3p. This secretory pathway for iron transport also includes Ftr1p, a high-affinity plasma membrane iron permease that forms a complex with Fet3p in *S. cerevisiae* (Stearman *et al.*, 1996). Directly upstream of *chpA*, the gene encoding p19, are five ORFs, the first four encode amino acid sequences that show strong homology to members of the FTR1 family of iron permeases (Stearman *et al.*, 1996). In addition, there is a Fur (ferric uptake regulator) box (89-5 % identity to consensus) starting 90 bp upstream of the start codon of this ORF, suggesting that the expression of the two upstream ORFs is iron regulated, as is *chpA*. The presence of a periplasmic iron(II) oxidase, an FTR1 homologue and Fur box linked p19 to a possible role in iron uptake in strain MV-1.

Although we did not discover a specific function for p19 in strain MV-1, the protein binds one atom of copper per dimer. Given its cellular location, p19 might have a similar copper transport function to Ctr1 in *S. cerevisiae*, providing copper to an Fe(II) oxidase for the formation of an active oxidase–permease complex for the transport of iron(III) across the cell membrane. Thus far, p19 has only been studied in *C. jejuni* (van Vliet *et al.*, 1998). The gene encoding p19 in *C. jejuni* is under transcriptional control of the Fur protein. However, the function of p19 has not been determined in this organism and a knock-out mutant of the p19 gene shows no obvious phenotypic change from the wild-type (C. W. Penn, University of Birmingham, UK, personal communication). Because an obvious phenotypic change appears in MV-1nm1 when *chpA* is not expressed (i.e. non-magnetotaxis and the absence of magnetosomes), strain MV-1 may be an excellent model organism for the determination of the physiological function of p19. In contrast with the expression of the p19 gene homologue from *C. jejuni*, which is repressed at 40 μM iron (van Vliet *et al.*, 1998), expression of *chpA* is not repressed even at 100 μM iron in MV-1. If *chpA* is involved in a copper-dependent iron uptake system in magnetotactic bacteria and its expression is not repressed at high iron concentrations, the result might be the uptake of extremely high amounts of iron, which could be toxic. Forming magnetite may be a way of detoxifying the intracellular accumulation of free iron ions since the compound is relatively inert. How specific magnetite crystal morphologies and sizes are achieved is unknown but thought to be related to the magnetosome membrane (Bazylinski & Frankel, 2004). Protein and microbial genome sequence databases contain several p19 homologues, most from Gram-negative pathogens, and one from another magnetotactic species, *M. magnetotacticum*, thus indicating that many other different bacteria including non-magnetotactic species possess an iron uptake system like that found in *S. cerevisiae*.

Based on the high-affinity iron uptake system of the yeast *S. cerevisiae*, we hypothesized that the periplasmic copper handling protein, ChpA, supplies a periplasmic, multicopper iron(II) oxidase with copper. This iron(II) oxidase, in turn, could provide an iron permease with iron(III) that facilitates the transport of iron(III) across the cytoplasmic membrane of MV-1, where it is available for incorporation into the growing magnetosome magnetite crystal. Because magnetite is a mixed valence iron oxide, iron(II) might be provided by a cytoplasmic iron(III) reductase, an enzymic activity present in the cytoplasm of both MV-1 and MV-1nm1. It might be expected, based on the above hypothesis, that the iron(II) oxidase in MV-1nm1 and the other non-magnetotactic mutants might be inactive due to a lack of copper. However, iron(II) oxidase purified from both wt MV-1 and MV-1nm1 displayed comparable iron oxidase activities. Therefore, although the absence of ChpA is correlated with the inability to synthesize magnetosomes, it seems very unlikely that the role of ChpA is to supply the multicopper iron(II) oxidase with copper. The iron(II) oxidase might utilize another copper chaperone or may not require one. In addition, modifying the growth medium with high levels of copper failed to reverse the non-magnetotactic phenotype (data not shown). These results might be an indication that the lack of ChpA in the non-magnetotactic mutants might be an effect rather than a cause of the inability to biomineralize magnetite magnetosomes.

In contrast to *M. magnetotacticum* and *M. gryphiswaldense*, species that grow to normal cell yields and do not produce magnetosomes when the major source of iron is omitted from the growth medium (Blakemore *et al.*, 1979; Schuler & Baueulein, 1996), anaerobically grown cells of MV-1 continue to produce magnetosomes under conditions of low iron. Under low iron conditions (~3 μM), the continued production of magnetosomes appears to cause cells to be starved of iron, thereby limiting their growth. This idea is supported by an observed limitation in the incorporation of
iron into cellular constituents based on soluble haem c content per mg protein. These results suggest that the magnetite in magnetosomes is not a form of iron storage for cells and MV-1 cannot use iron in magnetite for growth.

Because of the difference in growth yields under low iron concentrations and the spontaneous nature of the non-magnetotactic phenotype, we expected that the non-magnetotactic phenotype would dominate the culture under low iron conditions. However, the vast majority of cells of MV-1 in liquid culture appeared to remain magnetotactic even after 10 transfers under low iron conditions. These results suggest that either the spontaneous mutation(s) described in this report do not occur in liquid culture, or the presence of magnetosomes provides an unrecognized competitive advantage to this strain under laboratory conditions.

We have previously determined that MV-1nm1 is a deletion mutant (Dean, 1999) and that this strain and some of the other non-magnetotactic mutants isolated from agar plates are not genetically identical, as demonstrated by RAPD analysis (Williams et al., 1990) (data not shown). The latter result suggests that different deletions and/or chromosomal rearrangements occur in strain MV-1. Schübbe et al. (2003) recently described the frequent spontaneous loss of the magnetic phenotype in mutants apparently formed in stationary-phase cultures of _M. gryphiswaldense_. One of these mutants, designated strain MSR-1B, was isolated and characterized and was shown not to synthesize magnetosomes, as well as internal membrane vesicles. Unlike strain MV-1nm1 which grows well under low and high iron conditions, the growth of strain MSR-1B was impaired under all growth conditions tested. However, like strain MV-1nm1, cellular uptake and accumulation of iron were drastically reduced under iron-replete conditions. MSR-1B has a chromosomal deletion of about 80 kb that contains a number of insertion sequences, many of the genes that encode for magnetosome membrane proteins (the _mam_ genes), and additional genes. The _mam_ genes are localized in a 35 kb region of the deletion that may represent a magnetosome genomic island (Schübbe et al., 2003). We have not yet determined whether the _mam_ genes are absent from MV-1nm1, although it is clear that if they are present, they are not expressed in this strain. However, our results show that the structural gene for p19, _chpA_, is present in MV-1nm1 but is not expressed. Examination of the DNA sequence data and transcript analysis suggests that the MV-1 FTR1 homologue is expressed. The conserved two bp transversions within _chpA_ in MV-1nm1 and the other genetically different, non-magnetotactic mutants examined lead us to conclude that their appearance is not likely to be the result of random mutations and that a physiological mechanism might exist for these mutations. How these mutations directly affect the expression of _chpA_ in the non-magnetotactic mutants is unclear. Our results show that _chpA_ is transcribed in MV-1nm1 and sequence analysis revealed a region of the transcript potentially capable of forming a stem–loop structure that might block initiation and translation of the transcript. The mechanism responsible for this seemingly complex regulation deserves further study. Transcriptional and translational fusions with p19 in a heterologous system (e.g. in _E. coli_) to test what elements are responsible for this phenomenon should help to elucidate how this regulation works.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


Biochemical characterization, regulation by copper, and physiologic


