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Disruption of a *fur*-Like Gene Inhibits Magnetosome Formation in *Magnetospirillum gryphiswaldense* MSR-1

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Abstract—In this study, a genomic library of *Magnetospirillum gryphiswaldense* MSR-1 strain was constructed and a *fur*-like gene (encoding Fur protein, ferric uptake regulator) was isolated and sequenced. This gene consisted of 420 bp and encoded 139 amino acid residues. To investigate the function of this gene in MSR-1, a *fur* mutant was generated by double crossover with a kanamycin cassette inserted into its coding region. Iron uptake and magnetosome formation were dramatically inhibited by disruption of *fur*. Iron content analysis of the *fur* mutant indicated that it contained approximately 0.037% by dry weight, which was at least 10-fold less than that observed in the wild type. Electron microscopy revealed the absence of a magnetosome in the *fur* mutant, although it was able to tolerate 1 mM H_2O_2 at 10-fold higher level than wild-type. These data suggest that Fur protein may possess a novel function in magnetic bacteria.

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Magnetic bacteria, most of which are obligate micro-aerobic or anaerobic motile prokaryotes, are able to sense the geo-magnetic field and swim to an appropriate micro-aerobic habitat [1-3]. Magnetosomes, magnetic particles formed in the cytoplasm, are uniform-size (40-120 μm) and membrane-coated magnetite or greigite minerals [4, 5] that are thought to induce magnetotaxis of magnetic bacteria. Magnetosomes have attracted interest because they are excellent immobilization carriers of enzymes, antibodies, and drugs [6-10]. However, they have not yet been used for commercial purposes because the fastidious culture conditions for magnetic bacteria result in inadequate magnetosome yields.

A number of proteins associated with the magnetosome membrane are tightly related to the formation of magnetosomes. Mms6 is involved in Fe_3O_4 crystal morphology [11], MamA is associated with magnetite formation [12], and MamK and MamJ aid in the arrangement of magnetosome chains [13, 14]. At present, genes involved in the bio-mineralization of Fe_3O_4 are thought to be clustered in the magnetosome island region (MAI) [15], but the molecular mechanisms of magnetosome synthesis remain unknown.

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Iron is an essential element for almost all living things because it is employed as a metal cofactor by many enzymes. However, iron forms extremely insoluble ferric oxides at neutral pH. To overcome iron-limitation, many organisms have evolved effective ways to capture the ferric element, including the synthesis of ferric chelators called siderophores that transport iron into the cells. Contrary to expectation, however, *Magnetospirillum magnetotacticum* MS-1 and *M. magneticum* AMB-1 only secrete siderophores in iron-replete conditions [16, 17]. No siderophores are found in the *M. gryphiswaldense* MSR-1 under either high or low iron conditions [18].

Iron is often poorly available and can be toxic to cells at high concentration. Excessive iron can react with superoxide and hydrogen peroxide to form hydroxyl radicals, which can directly damage macromolecules such as DNA and proteins [19]. The ferric uptake regulator (Fur) functions as a global factor to control the iron homeostasis in *Escherichia coli* by mediating expression of iron-transporting genes in response to intracellular free iron content [20]. The Fur–Fe^{2+} complex can bind the "Fur-box" to repress expression of these genes and prevent iron-import in iron-rich states, or induce expression under iron-poor conditions [21]. The first crystal structure of Fur was determined from *Pseudomonas aerugi-*

nosa, which should help in understanding the interaction between Fur–iron complexes and DNA [22].

Genomic sequence data show that *fur* homologs are found in *M. magnetotacticum* MS-1 (http://www.jgi.doe.gov/tempweb/JGImicrobial/html/index.html). However, the function of these homologs and their mechanism of regulating iron uptake in magnetic bacteria remain unknown.

To investigate the mechanisms by which iron uptake is regulated and magnetosomes are formed in magnetic bacteria, a *fur* gene was cloned from the *M. gryphiswaldense* MSR-1 and a *fur* mutant was generated. Results illustrated that disruption of the *fur* gene dramatically inhibited both iron uptake and magnetosome formation.

MATERIALS AND METHODS

Strains, plasmids, and media. The strains and plasmids are listed in Table 1. *Magnetospirillum gryphiswaldense* MSR-1 (DSM6361) was cultured in sodium lactic media (SLM) at 30°C, as previously described [23]. The iron source, ferric citrate, was added after autoclaving. *Escherichia coli* strains were cultured in Luria broth (LB) at 37°C. Ampicillin (Ap) (100 μg/ml), kanamycin (Km) (50 μg/ml), apramycin (Am) (100 μg/ml), and tetracycline (Tc) (12.5 μg/ml) were added to the media. SLM was supplemented with 20 μg/ml kanamycin for the *fur* mutant culture.

Construction of the genomic library. The vector pKC505 [24, 25], harboring the apramycin resistance gene, used to construct the genomic library of MSR-1, was digested with *Hpa*I and *Bam*HI at 37°C to generate two arms. A 50-μg sample of genomic DNA was partially digested with *Sau*3A, and the digestion product was fractionated by sucrose gradient centrifugation to obtain several 23-30 kb inserted fragments. A 5-μl aliquot of the ligation product (arms/inserted fragments = 1 : 1) containing 0.5 μg DNA was packaged in 25 μl of package extract (Promega, USA) for 3 h and added to 100 μl of prepared *E. coli* DH5α cells. A similar method was adapted to construct the sub-clone library of the combined plasmid using pUC18 as a vector.

Cloning the *fur* gene. A 480-bp fragment containing the *fur* gene was amplified using Mmfur-F: 5′-GACTCCATGTTAGCGTTGC-3′ and Mmfur-R: 5′-AGACGAGGGACGTTCAAA-3′ as primers and the genomic DNA of *M. magnetotacticum* MS-1 as a template. The amplified product was labeled with digoxigenin-UTP (Roche, Germany) as a probe, and named DIG-*Mmfur*. Colony blots and southern hybridization were carried out to screen the genomic and sub-clone libraries with DIG-*Mmfur* as previously described [26]. Positive colonies containing the *fur* gene were confirmed by DNA sequencing.

Disruption of the *fur* gene. A plasmid designated pYJH10 that contained the disrupted *fur* gene was constructed using a three-step procedure. First, a 1.4 kb DNA fragment containing the *fur* gene was amplified by PCR using fur-U (5′-GGTCGACCGCTCGCACCGTC-3′) and fur-D (5′-ATCGCGGCGGCAAGGCCATG-3′) as primers and ligated into the pGEM-T Easy vector to produce pGTF21. Second, a 1.3 kb fragment containing the *kan* gene was amplified using kan-U (5′-AAACCGGTCCCAGTCACGACGTTGTAAA-3′) and kan-D (5′-AAACCGGTGGAATTGTGAGCGGATAACA-3′) (the *Age*I site is underlined) as primers and pUC4K as a template, digested with *Age*I, and inserted into the *Age*I site of pGTF21 to produce the plasmid, pTFK22. Third, an *Nco*I-cut 2.7 kb fragment of pTFK22 with *fur::kan* was cloned into the suicide vector, pSUP202, to generate the plasmid, pYJH10. pYJH10 was transformed into *E. coli* S17-1 cells. The bi-parental conjugation was carried out as described previously [9] using nalidixic acid (10 μg/ml) to select against the donor *E. coli* S17-1 cells. Colonies with single resistance to kanamycin and double resistance to kanamycin and tetracycline were used to prescreen potential double crossover mutant strains. The disruptants were confirmed by PCR using fur-F (5′-ATGAGCATGGTAACCG-3′)/fur-R (5′-TCAACCGTCAACCCTG-3′) and fur-U/fur-D as primers and the genomic DNA of the *fur* mutant as a template. The resultant *fur* mutant was validated by sequence analysis of the PCR product that was amplified using the fur-U/fur-D primers.

Iron uptake and content. Cells were grown in SLM medium containing 0, 2, 20, 60, 100, or 150 μM ferric citrate at 30°C for 30 h in an orbital shaker (100 rpm) and were harvested by centrifugation and washed three times with 10 mM HEPES (pH 7.2). The pellets were dried to constant weight at 105°C. The dry mass was weighed and nitrified in 1 ml of nitric acid for 3 h. Iron content was assayed using a 4520TF atomic absorption spectrometer (China) as previously described [27].

Cell growth and magnetism. Cells were grown in bottles containing 50 ml SLM medium with 0, 2, 20, and 80 μM ferric citrate. The bottles were incubated in an orbital shaker (100 rpm) for 30 h at 30°C. The optical density (OD_{600}) of the cells was measured in every 3 h. The number of cells adhering to the bottle wall was used to evaluate cell magnetism after an exterior magnet had been attached to the sample bottles for 24 h, as described previously [28].

Electron microscopy. A bacterial cell suspension was adsorbed onto copper grids and directly observed with a Tecnai F30 transmission electron microscope (Philips, USA) to view the magnetic particles.

Analysis of H_2O_2 tolerance. Five-milliliter aliquots of cells were grown in SLM medium in the absence of ferric citrate to an optical density of 0.5 and inoculated into 50 ml SLM medium containing various concentrations of

Table 1. Strains and plasmids

Strains/vectors	Phenotype	References/sources
Strains		
M. gryphiswaldense MSR-1	wild type	DSM6361
M. magnetotacticum MS-1	wild type	DSM3586
M. gryphiswaldense DFH-1	*fur*-mutant (*fur::kan*)	this study
E. coli S17-1	*rec*A, *thi*, *pro*, *hsd*R, RP4-2, Tc::Mu, Km::Tn7, λpir	[33]
E. coli DH5α	*sup*E44, Δ*lac*U169(Φ80 *lac*ZΔM15), *hsd*R17, *rec*A1, *end*A1	Biolab
Plasmids/vectors		
pGEM T-easy	Ap^r, lacZα, PCR cloning vector	Promega
pUC18	Ap^r, lacZα	GenBank (L08752)
pKC505	Am^r, cosmid vector	[27]
pUC4 K	Km^r, containing kanamycin cassette	GenBank (X06404)
T-simple	Ap^r, lacZα, PCR cloning vector	Takara
pSUP202	Tc^r, Ap^r, Cm^r, Mob	[33]
pTFU1	Ap^r, pGEM T-easy derivative carrying 500-bp *fur* gene of MS-1	this study
pKCC4	Ap^r, pKC505 derivative screened from the genomic library	this study
pGTF21	pGEM T-easy containing 1.4 kb of fragment with *fur*	this study
pTFK22	pGTF21 derivative with disrupted *fur::kan*	this study
pYJH10	pSUP202 containing 2.7 kb of fragment with *fur::kan*	this study

H_2O_2 and ferric citrate. After 24 h incubation, the cell density was measured at 600 nm by using a Beckman DU 640 spectrophotometer (Beckman, USA).

RESULTS

Genomic library of MSR-1. A total of 2880 colonies were obtained, comprising a genomic library with resistance to the apramycin. To test the quality of the library, 10 plasmids were randomly selected and digested with *Pst*I. A fragment of approximately 23-30 kb was present in each recombined DNA, indicating that the library was reliable, and any desired DNA fragment could be theoretically isolated.

Cloning of the *fur*-like gene from the genomic library. To clone the *fur* gene from MSR-1, colony hybridization was used to screen the genomic library with the DIG-*Mmfur* probe. Five positive recombinants were identified, and dot Southern blot confirmed that all of them contained the desired fragments. One of them was designated pKCC4 and a sub-clone library of this fragment was constructed using pUC18 as a vector and transformed into *E. coli* DH5α cells. The sub-clone library was similarly screened. Four positive plasmids were obtained—pUCC4-1, pUCC4-2, pUCC4-3, and pUCC4-4. Four inserted fragments, C4-1, C4-2, C4-3, and C4-4, were sequenced and assembled into a 3663 bp fragment (Fig. 1a; see color insert). Sequence analysis revealed that four ORFs were present in this fragment including a product encoded by ORF1 that shared 57.2% identity with 2,2′-nitropropane dioxygenase (encoded by *npd*) from *M. magneticum* AMB-1 and a product encoded by ORF2 that shared 81.25% identity with the Fur of *M. magnetotacticum* MS-1. No identity was found between the proteins encoded by ORF3 and ORF4 and any other known proteins. The coding region of the *fur* gene was composed of 420 bp and encoded 139 amino acid residues. Its

sequence was deposited in GenBank with the accession number DQ462588.

Analysis of the Fur protein. The amino acid sequence similarity was compared between *Mg*-Fur (Fur of *M. gryphiswaldense*) and other published Fur proteins classified into α-proteobacteria. A Neighbor Joining phylogenetic tree was drawn by the Clustal X program (figure not shown), which clearly showed that the Fur proteins were sorted out into three groups. The tree represents the Fur similarity of α-proteobacteria in accordance with divergence of bacterial relationship. Especially, Fur proteins of magnetic bacteria (MSR-1, MS-1, and AMB-1) are highly similar to that of *R. rubrum*. In fact, *Magnetospirillum* species and *R. rubrum* are classified into Rhodospirillaceae. So, Fur protein seemed to be a potential marker to investigate the phylogenetic relationship.

In addition, *Mg*-Fur contained a modified motif Asp94-His95-His96-His97 that is highly conserved and is believed to bind metal ions [29]. The two metal binding sites are also identified in the crystal structure of *Pa*-Fur, the Fur from *Pseudomonas aeruginosa* [22]. A modified motif, His41-Asp89-His97-His108, located in a corresponding site in *Mg*-Fur, was thought to comprise the Zn-binding site, although Asp residues were located in place of the Glu residue. However, the iron-binding site appeared to be different. Residue divergence at the C-terminus is suitable for functional diversity of the Fur proteins [30]. The tertiary structure of the N-terminus of Fur proteins is predicted to be similar with *Pa*-Fur and *Ec*-

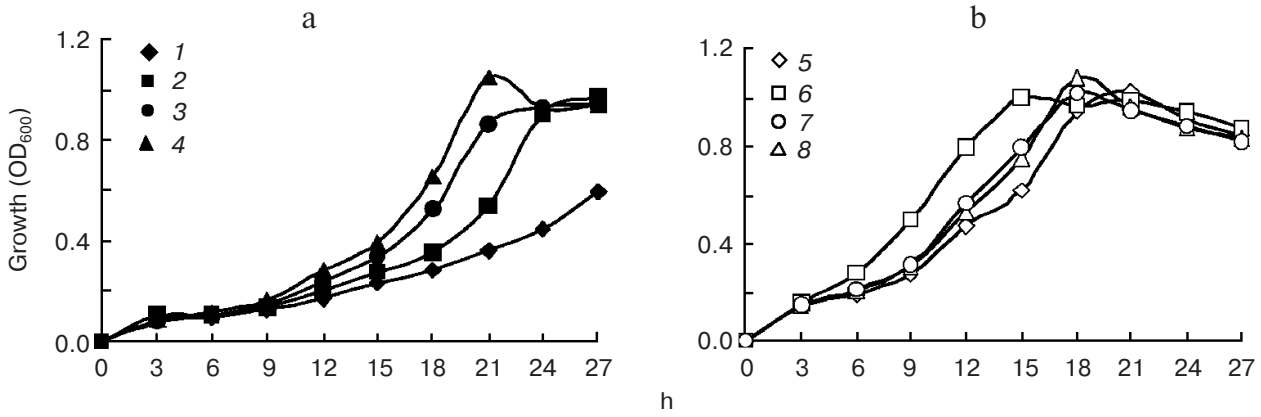

Fig. 2. Effect of iron concentration on the growth of *M. gryphiswaldense* MSR-1 (a) and the *fur* mutant DFH-1 (b). The sodium lactic media was supplemented with 0- (*1* and *5*), 2- (*2* and *6*), 20- (*3* and *7*), or 80-μM ferric citrate (*4* and *8*). Each sample was done in this experiment with two repeats and using the average data to draw the figures.

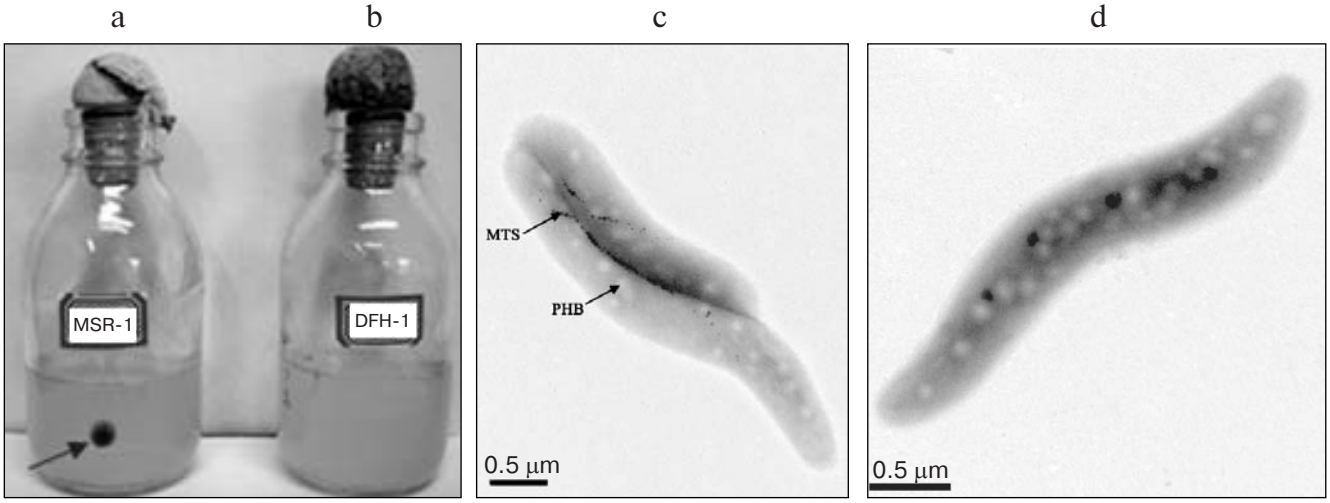

Fig. 3. Magnetism determination and electron microscopy of DFH-1 and MSR-1 cells. a) After magnetic attraction, magnetic MSR-1 cells adhered to the bottle wall (shown with an arrow); b) the magnetism was absent in DFH-1; c) magnetosome chain in MSR-1 cells (PHB, polyhydroxybutyrate; MTS, magnetosome); d) electron micrographs showed the absence of magnetosome in DFH-1.

Fur (the Fur of *E. coli*) by the SWISS-MODEL (http://swissmodel.expasy.org/), though the amino acid sequence of *Mg*-Fur shares only 27.3 or 23.1% identity with *Pa*-Fur or *Ec*-Fur.

Four α-helixes and two reverse β-sheets occurring in the N-terminal of *Mg*-Fur are similar to others, but the C-terminus structure of *Mg*-Fur could not be predicted (Fig. 1b). After analysis with the above information, we presumed that *Mg-fur* is a *fur*-like gene, and the protein encoded by it belongs to the Fur family.

Screening of the *fur* mutant. The *fur* gene was disrupted by insertion of the kanamycin cassette into its coding region (*fur::kan*). The disrupted gene fragment was then subcloned into the suicide vector, pSUP202, to generate the *fur* mutant by homologous exchange with *M. gryphiswaldense* MSR-1. The wild type MSR-1 strain is resistant to nalidixic acid, and this can be used to select against donor *E. coli* S17-1 cells. Thus, 20 μg/ml kanamycin and 10 μg/ml nalidixic acid were used to select the recombinants. After a week, colonies resistant to kanamycin appeared on the plates. To verify the double-crossover mutant, PCR were carried out. Only a 1.8 kb of fragment was produced with the fur-F/fur-R primers using the genomic DNA of the *fur* mutant as a template. The double crossover was also confirmed by sequence analysis of the PCR products using the fur-U/fur-D primers. The *fur* mutant was designated DFH-1.

Phenotypic characteristics and iron content. Transmission electron microscopy revealed no differences between the shape and size of the *fur* mutant DFH-1 and the wild type strain. To investigate the effects of iron on growth, DFH-1 and MSR-1 were grown in SLM containing various concentrations of ferric citrate. The *fur* mutant showed almost the same growth tendency under lower or higher iron conditions (Fig. 2b), even though *fur* inactivation of *E. coli* increases its sensitivity to iron-induced oxidative stress [9]. In contrast, higher iron promoted growth of the wild type strain (Fig. 2a). While MSR-1 cells grown in SLM medium congregated by exterior magnet attraction, no magnetism was observed by DFH-1 cells under the same conditions (Fig. 3, a and b).

Electron microscopy showed that while a magnetosome could be observed in MSR-1 cells, it was not seen in DFH-1 cells (Fig. 3, c and d). These findings suggested that Fur is required for magnetosome formation.

To investigate the effect of the disrupted *fur* gene on iron uptake, the iron content of DFH-1 and wild type were measured after both strains were cultured in SLM medium containing an iron concentration gradient for 24 h. As compared with the wild type strain, the iron content of the *fur* mutant made up only 0.037% (w/w) of the constant level of dry weight under both low and high iron conditions. In contrast, the iron content was enhanced in MSR-1 together with increase in extracellular iron (0-20 μM). A 10-fold higher iron content (0.45%) was reached in MSR-1 compared to DFH-1, and no further increase was observed when 20 μM or more iron was added. Surprisingly, when the wild type strain was cultivated in iron-low SLM, the iron content was 0.033%, which was almost equal to DFH-1 (Fig. 4). It appears that 0.03-0.04% iron is the basal level required to meet the metabolic demands of the cells.

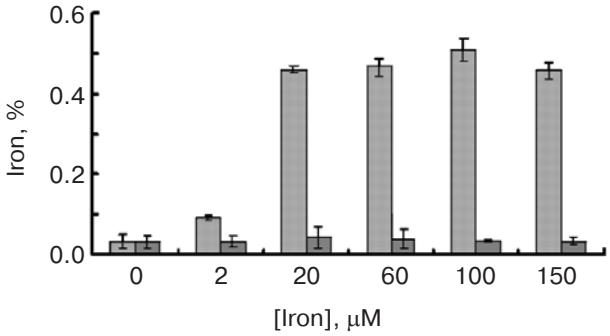

Fig. 4. Iron content percent of dry weight of MSR-1 (light-gray) and the *fur* mutant DFH-1 (dark-gray) using an atomic absorption spectrum.

H_2O_2 tolerance. Iron-dependent toxicity occurs when ferric ion interacts with hydrogen peroxide, generating the Fenton reaction and the production of hydroxyl radicals. The *fur* gene is induced by H_2O_2-mediated redox stress to reduce the cellular free iron [31]. The ability of strains to tolerate H_2O_2 was investigated in SLM medium plus 0, 0.1, 1, and 10 mM of H_2O_2 in the presence or absence of 50 μM ferric citrate (Table 2). The results showed that DFH-1 was able to tolerate 1 mM H_2O_2, while MSR-1 could only tolerate 0.1 mM H_2O_2. In addition, DFH-1 growth was not impaired by ≤1 mM H_2O_2 even in combination with high extracellular iron. In contrast, the growth and magnetism of MSR-1 were obviously harmed by a low concentration of H_2O_2. MSR-1 growth in less than 100 mM H_2O_2 was still more facilitated by iron than DFH-1, even though bacterial sensitivity to redox stress agents is enhanced under iron-rich conditions [32]. The mutant DFH-1 showed a 10-fold higher resistance against hydrogen peroxide when compared to the parent strain MSR-1.

DISCUSSION

In this study, we constructed genomic and a subcloned libraries of MSR-1 and successfully cloned the *fur*-like gene. A *fur* mutant (DFH-1) was obtained. The resulting mutant was then characterized by growth experiments and electron microscopy. The mutant was found to be deficient in magnetosome formation at iron sufficient conditions.

The regulation of iron assimilation is required to maintain iron homeostasis and prevent toxicity. Magnetosome formation is one potentially effective way

Table 2. Effect of H_2O_2 on cell growth and magnetism

H_2O_2, mM	MSR-1		*fur* mutant	
	OD/magnetism			
	0 μM Fe^{3+}	50 μM Fe^{3+}	0 μM Fe^{3+}	50 μM Fe^{3+}
0	0.799 ± 0.017/n.d.	0.918 ± 0.008/++	0.858 ± 0.0060/n.d.	0.859 ± 0.015/–
0.1	0.568 ± 0.040/n.d.	0.629 ± 0.023/+	0.870 ± 0.0076/n.d.	0.866 ± 0.001/–
1	0.050 ± 0.002/n.d.	0.055 ± 0.001/n.d.	0.870 ± 0.011/n.d.	0.873 ± 0.025/n.d.
10	0.045 ± 0.001/n.d.	0.049 ± 0.001/n.d.	0.046 ± 0.006/n.d.	0.050 ± 0.002/n.d.

Note: OD, optical density measured at 600 nm. Magnetism was determined by the size and color of magnetic bacterial coherence adsorbed by a magnet. The number of symbols "+" represented the intensity of the magnetism; "–", non-magnetic; n.d., not determined.

to store iron and reduce intracellular free iron to enhance survival from redox stress. Similar to the *E. coli fur* mutant, the cellular iron concentration is dramatically lower in DFH-1. DFH-1 possesses higher iron content than the *E. coli fur*$^-$ [1], however, indicating that there are other ways to store iron in magnetotactic bacteria, such as bacterioferritin [33].

Surprisingly, *fur* inactivation did not enhance DFH-1 sensitivity to the redox stress factor, H_2O_2, which is also distinct from the *E. coli fur* mutant [34-36]. The redox-active ferrous iron level may be lower in DFH-1, even though the iron uptake system is thought to be constitutively expressed. DFH-1 growth was not affected by increased iron concentration, while nonmagnetic mutant MSR-1B growth was severely hampered by high iron levels [37]. The reason for this difference will require further study.

It remains unknown whether the magnetosome deficiency resulted from the repression of enzymes related to magnetosome synthesis or from the lack of cellular iron. Fur appears to be a global regulatory factor in proteobacteria [19]. However, the role Fur plays in regulating magnetosome biosynthesis requires further investigation.

The Fur in *M. gryphiswaldense* (MSR-1) was distinguished from the Fur proteins of non-magnetic bacteria due to its C-terminal diversity. Disruption of the *fur*-like gene dramatically inhibited iron uptake and prevented magnetosome formation. Thus, Fur likely acts to positively regulate iron uptake in MSR-1. The enhanced resistance to H_2O_2 by the mutant was likely the result of low intracellular iron levels. Lower oxygen and higher iron were able to promote wild type strain MSR-1 growth and magnetosome synthesis. These findings suggest that the Fur protein maintains a tight balance between iron metabolism and oxidative stress.

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