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Siderophore production by the magnetic bacterium *Magnetospirillum magneticum* AMB-1

Ronie J. Calugay, Hideaki Miyashita, Yoshiko Okamura, Tadashi Matsunaga *

Department of Biotechnology, Tokyo University of Agriculture and Technology, 2-24-16 Naka-Cho, Koganei, Tokyo, Japan

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Abstract

Siderophore production by the magnetic bacterium *Magnetospirillum magneticum* AMB-1 is elicited by sufficient iron rather than by iron starvation. In order to clarify this unusual pattern, siderophore production was monitored in parallel to iron assimilation using the chrome azurol sulfonate assay and the ferrozine method respectively. Iron concentration lowered approximately five times less than its initial concentration only within 4 h post-inoculation, rendering the medium iron deficient. A concentration of at least 6 μM Fe^{3+} is required to initiate siderophore production. The propensity of *M. magneticum* AMB-1 for the assimilation of large amounts of iron accounts for the rapid depletion of iron in the medium, thereby triggering siderophore excretion. *M. magneticum* AMB-1 produces both hydroxamate and catechol siderophores.

Keywords: Magnetic bacterium; Siderophore; *Magnetospirillum magneticum*

1. Introduction

Iron is a requirement for the formation of intracellular, nm-sized magnetic iron minerals magnetite (Fe_3O_4) or greigite (Fe_3SO_4) in magnetic bacteria [1–5]. These membrane-bound crystals aligned in chains parallel to the cell axis are postulated to function mainly as biomagnetic antennae conferring a biological advantage allowing the bacterium to migrate along redox gradients through the Earth's geomagnetic field lines [6,7]. The biomineralization of these highly organized magnetic structures requires the acquisition of large amounts of iron, approximately 100 times more than that in *Escherichia coli* cell [3,8]. Because of the large amount of iron to be transported, a complex system of iron assimilation in magnetic bacteria may exist which is highly efficient and strictly controlled to avoid the generation of toxic radicals from excess intracellular iron [7,9]. Such regulated transport might indicate that iron-uptake systems in magnetic bacteria differ from known transport mechanisms in other microorganisms, which mediate uptake under starvation conditions.

In spite of the abundance of iron in nature, trivalent Fe(III) is very insoluble in aerobic environments at neutral pH [10]. In response, numerous microorganisms evolved an iron scavenging system by synthesizing and excreting low molecular mass, Fe(III)-specific ligands termed siderophores to sequester Fe(III) [10]. Although numerous siderophores have been reported and extensively studied in diverse groups of bacteria, microalgae and fungi [11–17], very few studies have been reported for magnetic bacteria. The microaerobic *Magnetospirillum gryphiswaldense* does not form siderophores [18]. Only the microaerobic *Magnetospirillum magnetotacticum* MS-1 among magnetic bacteria so far was reported to produce siderophores [19]. However, siderophore production by this bacterium is induced by high iron, not iron starvation. This mode of iron uptake and the results of this finding remain to be established.

The *mag*A gene was obtained from a non-magnetic mutant generated by transposon mutagenesis from *Magnetospirillum* sp. AMB-1 and completely sequenced. The *mag*A was found to encode an integral iron translocating protein associated with bacterial magnetic particle membrane and considered to be involved in the synthesis of magnetites

* Corresponding author. Tel.: +81 (423) 88-7020; Fax: +81 (423) 85-7713.
E-mail address: tmatsuna@cc.tuat.ac.jp (T. Matsunaga).

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[20,21]. The non-magnetic mutant lacking *mag*A had reduced ferric uptake compared to wild-type. MagA accumulation in the cytoplasmic membrane is enhanced during iron-limited conditions and its iron-uptake function is ATP dependent. These data taken together called for an investigation of the presence of siderophores in *Magnetospirillum magneticum* AMB-1.

Since magnetic bacteria assimilate large amounts of iron, the siderophore production and iron concentration in the culture supernatants were monitored simultaneously in *M. magneticum* AMB-1 cultured under iron-sufficient and iron-deficient media.

2. Materials and methods

2.1. Cells and culture condition

M. magneticum AMB-1 was maintained microaerobically by sparging argon gas for at least 5 min to 40 ml of chemically defined magnetic spirillum growth medium as previously described [3] in 50 ml Erlenmeyer flasks incubated at 25°C. Ferric quinate was provided as iron source 0.1, 1.0, 5.0, 20, 40 and 80 μM and remaining iron was determined by the spectrophotometric reagent ferrozine, as described below.

2.2. Detection of siderophores

The chrome azurol sulfonate (CAS) assay by Schwyn and Neilands [22] was used as an universal assay for the detection of siderophores. A fast-reacting assay solution was prepared by adding 10 μl 0.2 M 5-sulfosalicylic to the sample–CAS solution mixture (equal volumes of 0.5 ml) prior to measurement of absorbance at 630 nm. The medium was used as a blank, and the medium plus CAS assay solution as a reference. The sample should have a lower reading in the presence of siderophores. Initial detection of siderophores was quantified and defined according to Payne [23] and finally expressed as micromolar equivalents of the iron chelator deferoxamine (DFX).

To monitor siderophore production, cells were inoculated at 1×10^6 cells ml^{-1} in 160 ml of media with different iron concentrations. Aliquots of 2 ml were drawn every 4 h within a 24-h growth period. Cell-free culture supernatants were obtained by centrifugation at $7000\times g$ for 10 min at 4°C and subjected to CAS assay. Determinations were done in triplicate.

2.3. Detection of hydroxamate and catechol types of siderophores

The Arnow test [24] was used for the detection of catechol type of siderophores and ferric perchlorate test [25] was used to detect hydroxamates directly from the culture supernatant aliquots of cells grown with 20 μM Fe(III). Catechol and deferoxamine (Sigma) were used as standards respectively. To confirm the results, rotoevaporated and freeze-dried stationary phase culture supernatant from three volumes of 4-l cultures was resuspended in 2 ml sterile Milli-Q water and applied to gel-filtration chromatography using Sephacryl S-100 (1.6×60 cm) at 1 ml min^{-1} in fast protein, peptide and polynucleotide liquid chromatography (FPLC) system (Amersham Pharmacia, Biotech, Uppsala, Sweden) monitored at $Abs_{280\,nm}$. Collected fractions were tested for specific classes of siderophores.

2.4. Measurement of total iron

Iron content from cell-free culture supernatants was determined by using 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (referred to as ferrozine), a spectrophotometric reagent for iron [26]. A modified method [20] was used in which 50 μl of 60% hydroxylamine hydrochloride was added to 100 μl of the samples as a reductant and argon gas was sealed into the sample tubes. After a 24-h incubation, 100 μl of glacial acetic acid-sodium acetate buffer, pH 6.0 (4 g sodium acetate, dissolved with a small amount of distilled water, plus 3.5 ml concentrated glacial acetic acid, diluted to 100 ml) and 200 μl of 1% ferrozine were added, and the absorbance of sample solutions at 562 nm was measured spectrophotometrically (in triplicate samples).

2.5. Measurement of Fe(II) versus Fe(III) concentrations

Early log phase cells were cultured microaerobically in media with 20 and 80 μM initial Fe(III) concentrations. Initial inoculum concentrations were 1×10^6 cells ml^{-1}.

The concentrations of specific Fe(II) and Fe(III) in the culture supernatants at different times within the initial 4-h growth period were estimated. Fe(II) was measured in the non-reduced supernatant aliquots (the air phase was not replaced with argon so that ferric iron will not be detected) using the ferrozine method. Total iron concentrations in the same samples were measured as described above. The Fe(II) concentrations were subtracted from the total iron concentrations to obtain the approximate Fe(III) concentrations [total Fe−Fe(II) = Fe(III)]. Determinations were done in triplicate.

3. Results

3.1. Detection of siderophores

The initial CAS assay of culture supernatants from early stationary phase of *M. magneticum* AMB-1 revealed the presence of siderophores (13.3 μM deferoxamine equivalents) from cultures grown in medium with 20 μM Fe(III) and not in 0.1 and 1.0 μM Fe(III).

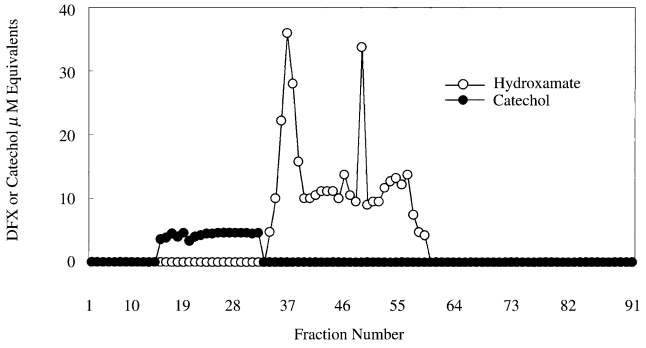

Fig. 1. Siderophores in *M. magneticum* AMB-1 culture supernatants of *M. magneticum* AMB-1 grown in medium with an initial concentration of 20 μM Fe(III) were applied to gel-filtration chromatography using Sephacryl S-100 (1.6×60 cm) at 1 ml min^{-1} in a fast protein, peptide polynucleotide chromatography (FPLC) system monitored at $Abs_{280\,nm}$. Fractions were tested for the presence of catechols (●) and hydroxamate (○) and were expressed as micromolar equivalents of iron chelators catechol and deferoxamine respectively.

3.2. Detection of hydroxamate and catechol types of siderophores

Aliquots directly from the culture supernatants showed that only hydroxamates were excreted into the medium. To confirm this result, concentrated culture supernatants were subjected to gel-filtration chromatography, and both hydroxamate and catechol siderophores were detected from the eluted fractions (Fig. 1). However, the CAS peak activity coincided only with the hydroxamate peaks. Possibly the catechol signals may not be purely siderophores but may be interspersed with high molecular mass molecules since they were eluted out early during the fractionation. The compounds containing catechol showed absorption at 510 nm in the Arnow test. Other bacterial excretions in the supernatant may have been included. The catechol fractions were pooled, washed with phenol–chloroform (1:1), rotoevaporated, freeze-dried, dissolved in water and again injected in size exclusion

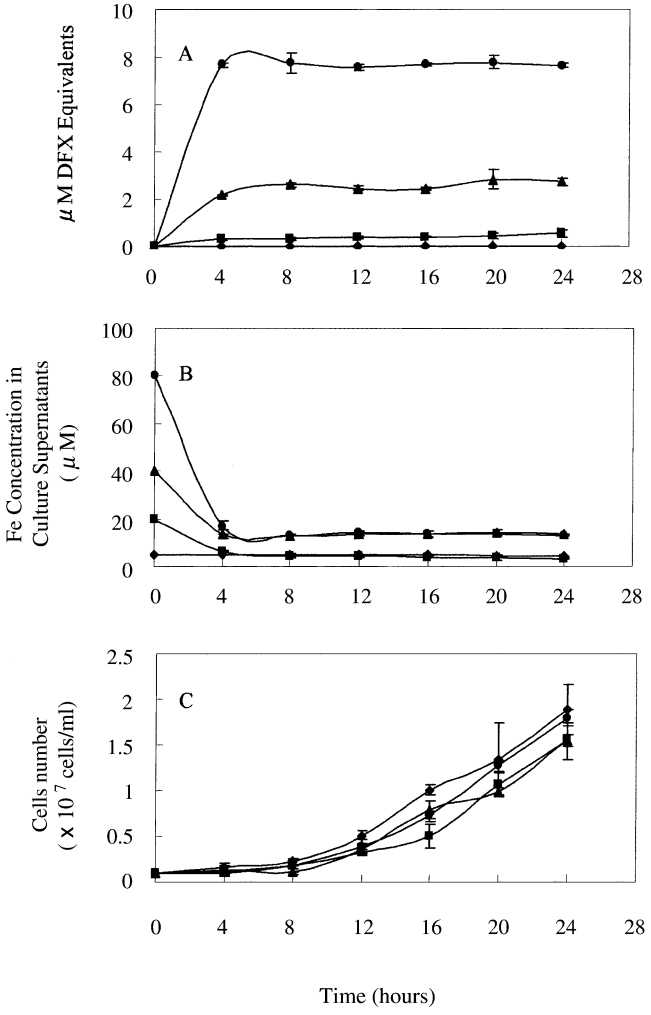

Fig. 2. Siderophore production, iron uptake and growth of *M. magneticum* AMB-1. Cells were grown in media with different initial iron concentrations 5 (♦), 20 (■), 40 (▲) and 80 (●) μM Fe. A: Culture supernatants were aliquoted at 4-h intervals during a 24-h growth period. Siderophores were detected by using CAS assay and expressed as micromolar equivalents of the iron chelator deferoxamine. B: Iron concentrations from the same samples were measured using ferrozine. The data shown are mean values of triplicate measurements. C: Cell numbers were measured by direct cell count using a hemacytometer.

chromatography. As a result, a CAS reactive, low molecular mass catechol peak was finally obtained.

3.3. Measurement of remaining cell-free total iron concentration

The iron concentration in the culture supernatant and the siderophore production of *M. magneticum* AMB-1 were measured at intervals within a 24-h growth period with an initial Fe(III) of 5.0 μM. However, this concentration did not induce siderophore production. When cells were cultured in media with initial iron concentrations of 40 or 80 μM Fe(III), a corresponding increase in siderophore production was observed (Fig. 2A).

The iron concentrations in the culture supernatants were measured and iron concentrations dropped from 80 to 16 μM, from 40 to 13 μM and from 20 to 6 μM (Fig. 2B).

Growth of cells under low iron condition (5 μM initial ferric concentration) was higher than in cells grown under high iron conditions (Fig. 2C).

3.4. Measurement of remaining cell-free Fe(II) and Fe(III) concentration

In cultures with 20 μM initial Fe(III) concentration, there is only a trace amount of Fe(III) remaining. Most of the Fe(III) were taken up or reduced to Fe(II) after 1 h and 4 μM Fe(II) remained up to 4 h (Fig. 3A). When 80 μM initial Fe(III) was employed, Fe(III) in the supernatants was immediately decreased and 4 μM Fe(II) remained after 4 h. The remaining trace Fe(III) concentrations were observed only within the first 2 h (Fig. 3B).

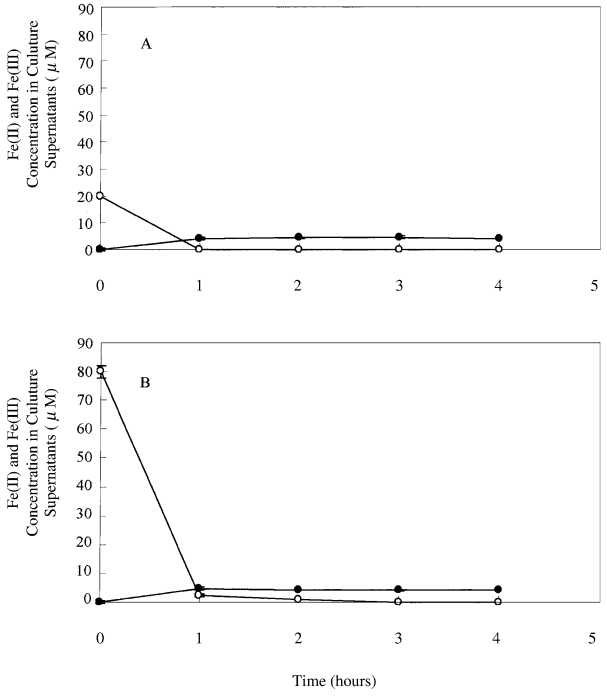

Fig. 3. Fe(III) and Fe(II) concentrations in *M. magneticum* AMB-1 cultures. Early log phase cells were cultured in medium with A: 20 μM and B: 80 μM initial Fe(III) concentration. Fe(II) (●) and Fe(III) (○) were measured using the ferrozine method at different times within the initial 4 h of the cultures.

4. Discussion

M. magneticum AMB-1 produces siderophores, and the observation that production is triggered by sufficient iron is actually only superficial. When cells are inoculated in iron-sufficient medium, iron is rapidly assimilated reaching levels comparable to that in iron-deficient cultures. These results may provide the answer for the unusual pattern of siderophore production observed in *M. magnetotacticum* MS-1 [19]. The conversion of some ferric ions into ferrous under the reducing conditions of the microaerobic cultures renders the iron available to the cell by simple diffusion. This has contributed to the rapid uptake and reduction of iron from the medium.

In other microorganisms, siderophore production is induced from 0 to 1 μM Fe [10]. *M. magneticum* AMB-1 may require more Fe(III) to meet its metabolic requirement for the biosynthesis of magnetite. In *Pseudomonas aeruginosa* PA01, cells grown under succinate as carbon source had iron requirement of about 16 times more than that with other carbon sources [27], thus the growth medium readily became iron deficient. Similarly, succinate is actually a component of the chemically defined media used in this study, serving as a carbon source for *M. magneticum* AMB-1. This may be another reason for the high uptake of iron by *M. magneticum* AMB-1.

M. magneticum AMB-1 cells become non-magnetic and exhibit a higher growth rate when cultured under low iron concentration. Siderophore-mediated ferric transport entails a series of steps for iron translocation into the cytoplasm [28]. Since magnetic cells grown under higher iron concentrations have to synthesize highly organized magnetites and siderophores, their growth can be expected to be slower than non-magnetic cells.

To date, strain AMB-1 is the only isolated magnetic bacterium capable of growing under aerobic condition [8]. Cells grown aerobically become non-magnetic and their growth almost doubled that of their microaerobic counterparts [8]. The cell growth of non-magnetic cells was higher than of the magnetic cells. Under anaerobic conditions, cells produce magnetites, and the production increased linearly with cell growth [8,29].

M. magneticum AMB-1 produces both hydroxamate and catechol types of siderophores. These were confirmed by detecting after size exclusion chromatography. Both hydroxamate and catechol positive peak fractions were obtained.

Knowledge on iron acquisition during iron-deficient conditions in magnetic bacteria is still very limited. So far, only the microaerobic *M. magnetotacticum* MS-1 is reported to produce siderophores [19]. It was proposed

that a sufficient amount of iron in this bacterium induced the production of outer membrane proteins which may serve for iron metabolism or for hydroxamate secretion and/or binding. The results in this study indicate that the propensity of magnetic bacteria for the assimilation of iron accounts for the pattern of siderophore production in *M. magneticum*. Extracellular iron concentration actually drops approximately five times from its initial concentration after 4 h post-inoculation. This characteristic may be the key to the unraveling of the unusual pattern reported in *M. magnetotacticum*.

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