ON THE COVER: TEM image from a bacterial sample showing an intracellular magnetosomal chain arranged like the "mark of Zorro". The scale bar represents 100 nm. Inset: TEM image from a sample of magnetotactic bacteria, *M. gryphiswaldense*, showing two cells with intracellular magnetosomal chains. The two cells, presumably one on top of the other, appear in form of a mermaid. The scale bar represents 500 nm. For more technical details, see Mohit Naresh, Vivek Hasija, Megha Sharma, and Aditya Mittal, pp. 4135–4144 of this issue.
Synthesis of Cellular Organelles Containing Nano-Magnets Stunts Growth of Magnetotactic Bacteria

Mohit Naresh¹, Vivek Hasija¹, Megha Sharma¹, and Aditya Mittal²,*,†

¹Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India
²School of Biological Sciences, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India

Magnetotactic bacteria are unique prokaryotes possessing the feature of cellular organelles called magnetosomes (membrane bound 40–50 nm vesicles entrapping a magnetic nano-crystal of magnetite or greigite). The obvious energetic impact of sophisticated eukaryotic-like membrane-bound organelle assembly on a presumably simpler prokaryotic system is not addressed in literature. In this work, while presenting evidence of direct coupling of carbon source consumption to synthesis of magnetosomes, we provide the first experimentally derived estimate of energy for organelle synthesis by Magnetospirillum gryphiswaldense as ∼5 nJoules per magnetosome. Considering our estimate of ∼0.2 μJoules per bacterial cell as the energy required for growth, we show that the energetic load of organelle synthesis results in stunting of cell growth. We also show that removal of soluble iron or sequestration by exogenous compounds in the bacterial cell cultures reverses the impact of the excess metabolic load exerted during magnetosomal synthesis. Thus, by taking advantage of the magnetotactic bacterial system we present the first experimental evidence for the presumed energy consumption during assembly of naturally occurring sub-100 nm intra-cellular organelles.

Keywords: Magnetosome, Metabolism, Cellular Energy, Biomineralization, Biosynthesis, Bioenergetics.

1. INTRODUCTION

Since their discovery over three decades ago¹⁻³ magnetotactic bacteria have invoked significant interest. The key feature of these bacteria is presence of aligned intracellular chains of 30–40 nm nanomagnets encapsulated by biological membranes.⁴⁻⁵ These intracellular organelles are called magnetosomes. It is remarkable that on one hand these bacteria are considered ancient forms of prokaryotic life with their intracellular features serving as bio-signatures for life on Mars,⁶⁻⁹ on the other hand they have specialized organelle assembly comparable to eukaryotic systems. While magnetosomes are unlike sophisticated eukaryotic organelles like mitochondria or chloroplasts, they do possess qualities of other organelles such as peroxisomes and endosomes, being membrane bound functional compartments rich in redox reactions and specific intracellular locations. Several interesting genetic and biochemical studies have been carried out to elucidate the mechanisms behind iron uptake leading to synthesis of intracellular nanomagnets by different magnetotactic bacteria.⁴, ¹⁰⁻¹² However organelle biology inside this presumably ancient prokaryotic life form is yet to be elucidated. In this work, we recognize for the first time that the magnetotactic-bacterial experimental system provides an exceptionally unique link between simpler prokaryotes and highly evolved eukaryotes for studying organized organelle assembly. Primary goal of this work was to investigate distribution of intracellular energetics to support specialized organelle assembly. We divided the total energy requirements of cells into two: (i) for growth (ii) for magnetosomal synthesis. We were able to serendipitously culture phenotypes of magnetotactic bacterial cells showing absence and presence of magnetosomes by regulating both soluble iron and carbon sources in the culture medium.¹³⁻¹⁶ Energy requirements for growth were measured in terms of kinetics of cell growth and the total cell mass obtained after exhausting the carbon sources. Thus, faster growth kinetics and/or higher cellular yields
reflected more input of energy into growth. Energetic input higher than that required for growth was expected to result
in magnetosomal synthesis. With these functional mea-
sures of energy distributions, we report for the first time
that magnetosomal synthesis exerts sufficient metabolic
load on the bacterial cells resulting in stunted growth. We
also show the rescue of stunted growth by either simply
removing soluble iron from the culture medium or by uti-
lizing exogenous means that direct the cellular energy for
the desired purpose.

2. MATERIALS AND METHODS

2.1. Materials, Bacterial Growth and Analytics

*M. gryphiswaldense* (DSM6361) was procured from
DSMZ (Germany). We confirmed purity of cell cul-
ture (lack of contamination) by three approaches:17,18
(i) spirillum cell morphology and motility (using video
microscopy,18–20) (ii) gram-staining and (iii) transmission
electron microscopy (TEM). Activated Charcoal Agar
(ACA) medium culturing of *M. gryphiswaldense* on plates
was done as described previously.15 All materials were
procured from Sigma-Aldrich (Germany), Merck (Darm-
stadt, Germany), Himedia (Mumbai, India), Loba Chemie
(Mumbai, India). Three well established media were uti-

defying magnetosomal synthesis, we grew the bacterial
strain R81 broth were utilized to sequester the soluble
iron.21 For 100 ml working volume in 250 ml flask,
5 ml R81 broth supernatant was added (9.35 μM final
siderophore concentration). For purifying siderophores,
10 g Amberlite XAD4 was drenched in 30 ml supernatant
broth (pH 6) of R81 and the mixture was stirred overnight.
Filtered solids were washed with water and saturated with
50% methanol (60 ml) by stirring for 60 min. Methanol
was removed using vacuum evaporator from filtrate. The
solution obtained contained 0.135 mM of siderophores.
7 ml purified siderophore solution was added to the work-
ing volume (9.45 μM final siderophore concentration).
The final iron siderophore ratio in both cases was 10:1.

2.2. Addition of Exogenous Siderophores

Siderophores in form of supernatant from *Pseudomonas*
strain R81 broth were utilized to sequester the soluble
iron.21 For 100 ml working volume in 250 ml flask,
5 ml R81 broth supernatant was added (9.35 μM final
siderophore concentration). For purifying siderophores,
10 g Amberlite XAD4 was drenched in 30 ml supernatant
broth (pH 6) of R81 and the mixture was stirred overnight.
Filtered solids were washed with water and saturated with
50% methanol (60 ml) by stirring for 60 min. Methanol
was removed using vacuum evaporator from filtrate. The
solution obtained contained 0.135 mM of siderophores.
7 ml purified siderophore solution was added to the work-
in volume (9.45 μM final siderophore concentration).
The final iron siderophore ratio in both cases was 10:1.

3. RESULTS AND DISCUSSION

3.1. *M. gryphiswaldense* Cell Cultures Show Two
Phenotypes, with Magnetosomal Synthesis
Leading to Heavier Cell Pellets

Figures 1(a and b) show ACA plates of
*M. gryphiswaldense* cultures with two distinct phenotypes
of cell colonies. Some colonies were white/cream and the
other were brown. Gram-staining did not show any mor-
phological differences for cells in both types of colonies
(Fig. 1(c)). TEM showed absence of nanomagnets in cells
from white colonies. However, cells from brown colonies
showed chains of nanomagnets (Figs. 1(d, e)). For inves-
tigating magnetosomal synthesis, we grew the bacterial
cells in three different liquid media. These media were

\[
A_{565} = \frac{X_0 e^{kt}}{1 - X_0/X_M (1 - e^{kt})}
\]

where \( k \) represents the growth rate, \( X_0 \) and \( X_M \) represent
the initial and maximum achievable values of \( A_{565} \). For wet
cell weight, culture broth was collected and centrifuged at
10000 rpm for 10 min. The supernatant was discarded and
pellet was gently washed three times in 10 ml of 0.1 M
phosphate buffer (pH 7) before weighing. Dry cell weight
was found by keeping the wet cell pellet overnight in oven
at 60 °C and 15 in Hg (6.59 psi) vacuum. For cell counting,
1 ml of culture broth was fixed in 4% formaldehyde and
the fixed cells were counted using a haemocytometer.

Iron estimation was done by converting ferric form
of iron into ferrous ions (using sulphuric acid, hydroxyl
ammonium chloride and sodium acetate) which bind to
2,2’ Dipyridyl resulting in development of spectrophoto-
metric signal at 520 nm, using a standard solution of
FeSO$_4$·(NH$_4$)$_2$SO$_4$·6H$_2$O. For TEM, cell pellets obtained
by centrifuging at 10000 rpm for 15 min was washed five
times in phosphate buffer 0.1 M, pH 7. The culture was
stained with 2% ammonium molybdate (pH 7 for 5 min)
and examined using CM-10 TEM, (Philips, Eindhoven,
Netherlands) operating at 100 KV at the All India Institute
of Medical Sciences, New Delhi.
Fig. 1. Culturing of *M. gryphiswaldense* on solid medium. (a), (b) show presence of white colonies (solid arrows) and brown colonies (dashed arrows) in ACA petri-plates. (c) shows representative microscopy slides of gram negative cells from both white and brown colonies with spirillum morphology. Transmission electron microscopy confirms similar morphology but shows the absence of magnetosomes inside bacterial cells from white colonies (d), whereas bacterial cells from brown colonies contain magnetosomal chains (e). (f) shows growth profiles (normalized: see text for details) for cells grown in DSMZ380 (gray □), DSMZ512 (orange △) and HS medium (brown ○). The smooth curves are fits of Eq. (1) to the data for estimating the growth rates. The inset shows wet cell weights obtained from 100 ml cultures, harvested after the onset of stationary phase. (g), (h), (i) show the wet pellets harvested after the onset of stationary phase for DSM380, DSM512, and HS medium respectively. (j), (k) show transmission electron microscopy data from white/cream and brown cells respectively. The scale bars in (d), (e), (j), (k) represent 500 nm. All data is shown as mean ± standard deviation of three independent triplicates.
chosen since (i) they are established to support growth of otherwise fastidious *M. gryphiswaldense* and, (ii) energy available to the bacterial cells from carbon sources in the media could be followed both in terms of varying levels of the same carbon source (citrate) as well as in terms of varying the carbon source itself (citrate vs. lactate, Table I). Figure 1(f) shows the bacterial growth kinetics for the three media, with the inset showing bacterial wet cell weight obtained in the media. For meaningful kinetic comparison, the growth curves were normalized by the asymptotic value of absorbance data for each medium. Smooth curves are fits to experimental data obtained by normalizing Eq. (1) with $X_M$. The wet cell pellets (WCPs) obtained subsequent to reaching the stationary phase are also shown (Fig. 1(g) for DSM380, 1H for DSM512, H for HS). Clearly the WCP from DSM380 is predominantly creamish. WCP from DSM512 cultures is also creamish, with a localized brown cell mass next to the wall of the centrifuge tube. However, the WCP from the HS medium is reddish-brown, with a dense dark brown pellet at the bottom. In all the experiments, the different WCPs were found to contain only magnetotactic bacterial cells (and not any other microbial contaminants) by microscopy (Figs. 1(c, d, e)). Therefore, the reason behind the two different colony phenotypes in agar plates, i.e., presence or absence of magnetosomes inside the magnetotactic bacterial cells, was also responsible for the different color of WCPs (Figs. 1(j, k)).

Clearly, presence of more cells (containing magnetosomes) leads to heavier pellets as observed for the HS medium (Fig. 1(k), inset of Fig. 1(f)). Further, HS medium also supports the fastest growth of the bacterial cells ($k = 0.22 \pm 0.010 \text{ hr}^{-1}$). Interestingly, the predominantly creamish WCP from DSM380 has the lowest wet cell weight (inset of Fig. 1(f)) and minimum cells containing nanomagnets (Fig. 1(j)), but has faster growth kinetics ($k = 0.19 \pm 0.009 \text{ hr}^{-1}$) compared to DSM512 ($k = 0.15 \pm 0.014 \text{ hr}^{-1}$). Since ferric citrate is known to be a preferred soluble iron source in several bacterial systems presumably because of role of citrate in iron-transport, the heavier wet cell weights in DSM512 and HS media, compared to DSM380, could be attributed to its presence in the two media (inset Fig. 1(f)). More ferric citrate in HS medium compared to DSM512 also supports (i) more soluble iron uptake resulting in more magnetosome-containing heavier cell pellet and (ii) faster growth of cells in HS medium. Further, metabolic intermediates other than citrate, i.e., tartrate and succinate, allow cells to grow faster in DSM380 compared to DSM512, but do not assist in significant iron biominalization. However, lactate in the HS medium (along with citrate) allowed more iron uptake in *M. gryphiswaldense*. Interestingly, lactate has been shown to directly facilitate iron uptake and metabolism in some eukaryotic systems including plants and mammalian cells. Thus we found that magnetosomal synthesis while not essential for cellular growth, is coupled not only to type of carbon sources, but also the amount of carbon sources in the culture medium. These observations provided us with an experimental system where organelle (magnetosomal) synthesis could be decoupled from the growth of *M. gryphiswaldense*. Therefore, we hypothesized that total energy supplied from the carbon sources in the culture media could be utilized for (a) growth of cells and (b) magnetosomal synthesis. To test this hypothesis, we needed to find out how much energy is made available from the different carbon sources in the culture media.

### 3.2. Energetics of Cell Growth and Organelle Synthesis

The total available energy from the various carbon sources present in the media is shown in Table I. It is well-established that energy (from carbon) in form of one available electron results in $\sim 3.14$ grams dry cell weight of microbial cells, assuming utilization towards only cell growth. Therefore, we were able to calculate the expected dry cell weight from consumption of the available moles of each carbon sources in our different media (based on electrons available per carbon source, as shown in Table I). Assuming that microbial cells are $\sim 80\%$ of water by weight, we were then able to predict the wet cell weight ($P_{\text{WCW}}$) from consumption of carbon sources (for cell growth) in different media. Since it is known that microbial cultures attain stationary phase only after exhausting the carbon sources, we measured the wet cell weights subsequent to stationary phase onset to compare the experimental data with $P_{\text{WCW}}$. While ($P_{\text{WCW}}$) was obviously expected.

### Table I. Carbon sources used in the different culture media.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Chemical formula</th>
<th>Mol. Wt.</th>
<th>$e^-$/mol</th>
<th>$\Delta H_{\text{C}}$ (KJ/mol)</th>
<th>Total millimoles in 100 mL</th>
<th>Energy available (KJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe–Quininate</td>
<td>$\text{C}<em>{24}\text{H}</em>{12}\text{O}_{6}$</td>
<td>247</td>
<td>28</td>
<td>$-3484$</td>
<td>0.002</td>
<td>0.007</td>
</tr>
<tr>
<td>Fe–Citrate</td>
<td>$\text{C}<em>{24}\text{H}</em>{12}\text{O}_{6}$</td>
<td>247</td>
<td>18</td>
<td>$-1962$</td>
<td>0.044</td>
<td>0.043</td>
</tr>
<tr>
<td>Na–Thioglycollate</td>
<td>$\text{C}<em>{11}\text{H}</em>{12}\text{O}_{8}$</td>
<td>114</td>
<td>12</td>
<td>$-1446$</td>
<td>0.247</td>
<td>0.284</td>
</tr>
<tr>
<td>Tartrate</td>
<td>$\text{C}<em>{10}\text{H}</em>{12}\text{O}_{7}$</td>
<td>124</td>
<td>10</td>
<td>$-1150.11$</td>
<td>0.341</td>
<td>0.349</td>
</tr>
<tr>
<td>Succinate</td>
<td>$\text{C}<em>{6}\text{H}</em>{12}\text{O}_{7}$</td>
<td>118</td>
<td>14</td>
<td>$-1492$</td>
<td>0.406</td>
<td>0.469</td>
</tr>
<tr>
<td>Na–Acetate</td>
<td>$\text{C}<em>{2}\text{H}</em>{4}\text{O}_{2}$</td>
<td>82</td>
<td>8</td>
<td>$-875.12$</td>
<td>0.581</td>
<td>1.067</td>
</tr>
<tr>
<td>K–Lactate</td>
<td>$\text{C}<em>{3}\text{H}</em>{6}\text{O}_{5}$</td>
<td>88</td>
<td>12</td>
<td>$-1368.3$</td>
<td>—</td>
<td>2.70</td>
</tr>
</tbody>
</table>

to be linearly correlated the total energy supplied in form of carbon sources, we were interested to obtain the possible relationship of the experimental data with the total energy supplied in the media. Figure 2(a) shows that measured wet cell weight ($M_{WCW}$), that includes magnetosomal synthesis, is also well correlated to the total energy supplied in the culture media. Theoretically, $M_{WCW}$ and $P_{WCW}$ are expected to be equal, if all the energy from carbon consumption was directed only towards cell growth. Inset in Figure 2(a) shows the ratio of measured and predicted wet cell weights for the three media. Remarkably, the ratio decreases in the same order in which we see appearance of a heavier wet cell pellet. The ratio is close to 1 for cells grown in DSM380, in which we observed only cell growth without magnetosomal synthesis (cream pellet).

Based on the total energy in form of carbon sources in the culture media, $P_{WCW}$ as well as the $M_{WCW}$ were expected to be highest for HS medium followed by DSM512 followed by DSM380. However, the $M_{WCW}$ was not as high as predicted for HS and DSM512 media. Thus, for cell cultures in media without magnetosomal synthesis, carbon source metabolism was indeed contributing only towards cell growth ($M_{WCW} \sim P_{WCW}$). However, for cell cultures in media supporting magnetosomal synthesis, utilization of energy from carbon source metabolism was being directed towards the organelle synthesis thereby stunting the growth compared to predicted values ($M_{WCW} < P_{WCW}$).

### 3.3. Energy Calculations for Cell Growth and Organelle Synthesis

Clearly, energy balance for carbon metabolism in our experiments indicated the possibility for a straightforward estimation of energy required for synthesis of magnetosomes. $A_{565}$ is a well established indicator of magnetotactic-bacterial cell growth in solutions, regardless of the culture medium as well as the presence or absence of magnetosomes. Nevertheless, we measured the cell numbers corresponding to respective $A_{565}$ values for cells grown in DSM512 ($r = 0.6, 13, 30$ and $36$ hrs) and HS media ($r = 0, 4, 8, 12, 24, 32$ hrs), in five independent experiments. Note that DSM512 was chosen since it contained cell and pellet phenotypic features observed for both DSM380 and HS. This yielded a cell growth medium independent relationship of $A_{565} = (3.2 \times 10^{-9} \pm 1.6 \times 10^{-9}) N + (0.04 \pm 0.006)$, where $N$ is the cell number (Fig. 2(b) shows a sample correlation between $A_{565}$ and cell numbers for cells grown in DSM512 given by $A_{565} = 4.3 \times 10^{-9} N + 0.036$; similar data, not shown to maintain visual clarity, was obtained for cells grown in HS medium with $A_{565} = 2.0 \times 10^{-9} N + 0.044$, $r^2 = 0.99$). Based on the above, we found that DSM380, that contains only growing cells without magnetosomal synthesis, had a total number of cells $N_{DSM380} = 4.69 \times 10^9$ (in the total culture volume of $100$ ml) immediately subsequent to onset of stationary phase. Assuming all carbon sources have been exhausted, the total energy that had been available to achieve this cell number was $E_{Growth} = 0.875$ KJ. Therefore, energy required for only growth of $M. gryphiswaldense$ is given by $E_{Cell-Growth} = E_{Growth}/N_{DSM380} = 1.87 \times 10^{-7}$ J/cell $\sim 0.2 \mu J$ per cell. For the HS medium, that contained cells with magnetosomes, the total energy available from the carbon sources is $E_{Total} = 3.718$ KJ. The total number of cells immediately subsequent to stationary phase is $N_{HS} = 1.28 \times 10^{10}$. Assuming 20 magnetosomes per cell, $E_{Magnetosome} = (E_{Total} - (E_{Cell-Growth} \times N_{HS}))/20N_{HS} = 5.2 \times 10^{-9}$ J/magnetosome $\sim 5$ nJ per magnetosome. To our knowledge, this is the first ever estimate for energy required for any type of organelle synthesis inside a living cell.

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**Fig. 2.** (a) Experimentally measured wet cell weight ($M_{WCW}$) depends on the energy supplied by carbon consumption in different media (see text and Table I for details). Inset shows the ratio $M_{WCW}/P_{WCW}$ for cells grown in different media. The theoretical energy available on complete combustion of carbon-sources in DSM380, DSM512 and HS media is calculated to be 0.875, 1.705 and 3.718 KJ respectively. (b) A sample calibration curve for obtaining cell numbers from $A_{565}$ for growth in DSM512 medium. The values of $A_{565}$ are well correlated to cell count ($r^2 = 0.98$).
Here it is interesting to note that Figure 1(f) inset shows that the wet cell weight obtained in HS medium is about 1.2 times higher than the wet cell weight obtained in DSM380, in the units of grams per liter. However, the cell numbers used above show \( N_{\text{HS}} / N_{\text{DSM380}} = 2.72 \). Thus an obvious question is how can 2.72 times the number of heavier bacterial cells with magnetosomes (HS medium) yield only 1.2 times wet cell weight in grams per liter. This difference implies that magnetosome-containing cells obtained from HS medium are \( \sim 40\% \) lighter than the cells without magnetosomes obtained from DSM380 medium, assuming a straight-forward relation between the measured wet cell weight and weight of single cells/dry cell weight. Interestingly enough, the inset in Figure 2(a) shows that the measured wet cell weight for HS medium is \( \sim 35\% \) of the predicted wet cell weight on assuming 80\% water content for microbial cells. Thus, while the experimental results obtained by us are self-consistent, they do point towards possible errors in interpretations arising out of correlating measured wet cell weight and weight of single cells/dry cell weight. Considering only the dry wet cell weights, one would obviously expect cells containing nano-magnet crystals to be heavier than cells without the crystals. However, the same may not apply for wet cell weight. The counter-intuitive inference above that wet cell weight of magnetosome-containing cells in HS medium is \( \sim 40\% \) lighter than the wet cell weight of cells without magnetosomes in DSM380 medium, assumes that the wet cell weight (that includes extracellularly bound/associated water) is a direct indicator of single cell weight. At this point we do not have a straight-forward explanation for this. The simplest, but less plausible reason can be a consistent error in cell counting leading to an incorrect correlation between cell numbers and absorbance. However an elegant study\(^{31}\) may provide a better alternative explanation. Using isotope studies, it was experimentally established that the iron–oxide crystal formation inside magnetotactic bacteria results from consumption of oxygen from water molecules and not molecular dissolved oxygen in the culture medium.\(^{31}\) This implies that the intracellular and/or extracellular water content of cells synthesizing iron–oxide crystals would be expected to be lesser than the cells that do not synthesize the crystals. Intracellular water is expected to be un-affected due to no observable morphological differences in cells with and without magnetosomes. Thus, lowering of extracellularly bound/associated water content due to magnetosomal synthesis could lead to lighter wet cell weight than expected. This explanation is also supported by the difficulty experienced by us in obtaining suspensions of brown colonies of cells grown on solid media. Detailed cell compositional studies including measurements on extracellular hydration, which are beyond the scope of this work, would be required to directly address this issue. Nevertheless, it is important to consider that this does not affect our estimates on energy required for magnetosomal assembly inside cells, since the calculations are independent of wet cell weight measurements and are based on energy available on consumption of carbon sources.

Most importantly, regardless of possible variations in cell numbers and/or minor differences in overall cellular compositions (except for presence or absence of iron–oxide crystals), the order of magnitude of energy required for growth (\( \mu\)Joules per cell) and organelle synthesis (nJoules per organelle) obtained by us is not expected to be much variable.

### 3.4. Iron Uptake Leading to Magnetosomal Synthesis

**Stunts Bacterial Growth**

To further investigate the impact of increasing iron metabolic load on cell growth, we cultured *M. gryphiswaldense* with and without soluble iron, but keeping the major carbon source the same. Since HS medium had provided the best nanomagnet-synthesizing cellular yield, we cultured the cells in HS medium only. The idea behind this experimental design was that absence of free iron in the medium should lead to absence of magnetosome formation. Thus, more energy was expected to be diverted to growth (and hence faster growth kinetics), but of lighter cells (without magnetosomes) in absence of iron. Further, since it has been established that magnetotactic bacteria do produce “empty” magnetosomes, i.e., vesicles without iron–oxide crystals, in absence of iron,\(^{32}\) it was expected that the stationary phase cell numbers would not be substantially different in absence of iron.

Figure 3(a) shows that growth rate of cells in HS medium without soluble iron (bar denoted by 1) is statistically
higher \((p = 0.043 < 0.05)\) than the growth rate in presence of soluble iron (bar 2). The soluble iron concentration in the medium used for these experiments was 100 \(\mu M\) based on a recent study.\(^{33}\) No significant difference was observed in cell numbers (indicated by \(A_{565}\)) at the onset of stationary phase with and without iron (not shown). However, Figure 3(b) shows that in spite of slower growth rate in presence of iron, and similar number of cells with and without iron at the respective stationary phase, the dry cell weight (DCW) was higher in presence of iron \((p = 0.021 < 0.05)\), indicating heavier cells due to presence of magnetosomes. These results clearly confirmed our hypothesis that energy consumption for magnetosomal synthesis results in stunted cellular growth (in form of slower growth kinetics).

### 3.5. Exogenous Siderophores Assist in Reducing the Metabolic Load for Synthesizing Magnetosomes

Having confirmed that magnetosomal synthesis stunts bacterial cell growth, it was important to test whether we could rescue the stunted growth without compromising organelle synthesis. While two other strains of magnetotactic bacteria, namely MS-1 and AMB-1, have been shown to utilize self-secreted hydroxamate and catechol siderophores for iron uptake, no siderophores have been detected in the cultures of \(M.\ gryphiswaldense\).\(^{14}\) Thus, introduction of exogenous siderophores in our cultures was expected us to allow investigations for possible reduction of metabolic load using soluble iron-chelation without affecting the metabolic pathways leading to magnetosomal formation. Recently, we showed that cell-free culture broth from \(Pseudomonas\) (strain R81) is rich in siderophores with high affinity for soluble iron.\(^{31}\) Thus, we introduced siderophores in our HS medium cultures, to sequester soluble iron, in two forms: R81 cell free culture broth, and, purified hydroxamate siderophores from the culture broth. Figure 3(a) shows that both forms of siderophores significantly enhanced growth rate of \(M.\ gryphiswaldense\) (bar 3 vs. bar 2, \(p = 0.002 < 0.01\); bar 4 vs. bar 2, \(p = 0.001 < 0.01\)). Surprisingly, while the dry cell weight of cells grown with R81 cell free broth was significantly higher than that obtained in control experiments (Fig. 3(b), bar 3 vs. bar 2, \(p = 0.0002 < < 0.01\)), the dry cell weight of cells grown with purified siderophores was similar to that obtained in control experiments (Fig. 3(b), bar 4 vs. bar 2, \(p = 0.92\)). These results serendipitously provided an

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**Fig. 4.** Iron uptake by \(M.\ gryphiswaldense\) in HS medium. (a) shows growth rates \((k)\) for cells grown with different initial iron concentrations. Stars show a statistically significant difference in the growth rate with no iron in the medium compared to 100 \(\mu M\) \((p = 0.017)\), 200 \(\mu M\) \((p = 0.009)\) and 300 \(\mu M\) \((p = 0.005)\) respectively, using single tailed type 2 \(t\)-tests. (b) shows the dry cell weight (DCW) obtained from 100 ml of cultures with different initial iron concentrations and harvested immediately after the onset of stationary phase. Stars show a statistically significant difference in the dry cell weight with no iron in the medium compared to iron concentration of 100 \((p = 0.011)\) and 200 \(\mu M\) \((p = 0.015)\), using single tailed type 2 \(t\)-tests. There was no difference in the dry cell weight for cells grown in absence of iron and with 300 \(\mu M\) iron \((p = 0.20)\). (c) shows kinetics of iron uptake by cells grown at initial iron concentrations of 0 (gray ■), 100 (brown △), 200 (▲), 300 (red □) and 500 (blue ◊) \(\mu M\). (D) shows net iron uptake for different initial iron concentrations. All data is shown as mean ± standard deviation of three independent triplicates.
interesting insight into the molecular mechanisms leading to magnetosomal synthesis in *M. gryphiswaldense*. Clearly, pure hydroxamate siderophores reduce the metabolic load on cell growth by sequestering soluble iron and making it unavailable for biomineralization, thereby mimicking cell growth conditions similar to absence of iron. In contrast, non-hydroxamate siderophores in the R81 cell free culture broth reduce the metabolic load by assisting soluble iron uptake leading to magnetosomal synthesis.

3.6. Carbon is the “Limiting Reactant” During Iron Uptake Leading to Organelle Synthesis

Having explored the distribution of energetics in our bacterial cells between growth and magnetosomal synthesis, we wanted to gain some insights into iron uptake by the cells. Thus, we cultured *M. gryphiswaldense* with different initial iron concentrations, but keeping the major carbon source the same. The idea behind this experimental design was the same as that for the experiments shown in Figure 3, but with the point of view of "titrating" iron uptake. Since HS medium had provided the best nanomagnet synthesizing cellular yield, we cultured the cells in HS medium only (as for experiments shown in Fig. 3). By doing so, our experimental design essentially ruled out any other complex effects that may arise because of different components of different media. Thus, as for experiments shown in Figure 3, while cell growth was expected to reach the stationary phase on exhaustion of the carbon source, the rate of reaching the stationary phase was expected to be faster in absence of iron. Further, it was expected that the stationary phase cell numbers would not be substantially different in absence of iron. Therefore, by keeping the same quantity of the same major carbon source in the medium, while varying the iron concentration, we expected similar cell numbers at stationary phase with or without iron but with slower growth rate in presence of iron. At the same time, we expected heavier cells in presence of iron.

Figure 4(a) shows that the growth rate of cells in HS medium without soluble iron is statistically higher than that in presence of any iron concentration. No significant difference was observed in cell numbers (indicated by *A*455) at the onset of stationary phase (*t* = 25 hrs) for 0 (*A*455 = 0.38 ± 0.010), 100 (*A*455 = 0.41 ± 0.010), 200 (*A*455 = 0.40 ± 0.006) or 300 (*A*455 = 0.39 ± 0.009) μM iron. Further, Figure 4(b) shows that in spite of the highest growth rate of cells in the HS medium in absence of iron, the dry cell weight (DCW) is higher in presence of iron (compared to absence of iron), below 300 μM. While the WCP appearance in absence of iron was creamish (indicating lack of nanomagnets), there was no observable difference in the WCP in presence of any iron concentration less than 500 μM (i.e., reddish-brown WCP, with a dense dark brown pellet at the bottom, same as shown in Fig. 1(i)). Therefore, these experiments further consolidated our previous findings of magnetosomal synthesis resulting in stunted (in terms of rate) bacterial growth, but of heavier cells. Here it is important to mention that we did not observe any morphological or phenotypic differences (including cell motility) for cells cultured in presence or absence of iron, as also observed in Figure 1.

We also compared the iron uptake kinetics by *M. gryphiswaldense* in the HS medium for the different initial concentrations of soluble iron and found that iron uptake was fastest at 200 μM (Figs. 4(c and d)). At 500 μM, since there is complete inhibition of cell growth, no iron is consumed from the medium. Further, at 300 μM in spite of sufficient iron consumption the DCW is lower than that obtained with 100 μM and 200 μM initial iron concentration. While these observations are in agreement with previously reported inhibitory effects of initial iron concentrations above 200 μM, there is no clear reason for this inhibition at this point. The most interesting observation was that regardless of the iron concentration (below 500 μM), iron was never consumed completely. Only 30–60% (30% for 300 μM and 60% for 100 μM) of the initial soluble iron in the medium was consumed by the cells, presumably for magnetosomal synthesis. This indicates that carbon is the “limiting reactant” in terms of iron uptake leading to organelle synthesis.
4. CONCLUSIONS

Carbon sources in the medium are utilized by cells both as a direct source of energy as well as for synthesis of various cellular components of the cells during growth. This utilization of carbon (along with nitrogen sources) for growth is expressed stoichiometrically as:

\[
aC_P\text{H}_4\text{O}_1 + b\text{O}_2 + c\text{NH}_3 \\
\rightarrow dC_n\text{H}_2\text{O}_2\text{N}_6 + e\text{H}_2\text{O} + f\text{CO}_2
\]

where \(C_n\text{H}_2\text{O}_2\text{N}_6\) represents the empirical cell formula. This stoichiometric representation includes the utilization of nutrient resources including carbon for both cellular growth/synthesis of organelles and other intracellular components, as well as directly as energy sources via metabolic breakdown.\(^{30}\) Thus, considering complete combustion of the carbon source, expressed in terms energy released from the combustion, has been of immense use in applied microbiology. On a single cell level, it is obvious that organelle synthesis requires energy. However, there is no quantitative experimental evidence showing energy utilization to create specialized compartments in biological systems till date. Magnetotactic bacteria are unique prokaryotes that synthesize specialized organelles (like highly evolved eukaryotes) called magnetosomes (membrane bound 40–50 nm vesicles entrapping a magnetic nanocrystal of magnetite or greigite) arranged in chains (as shown in Fig. 5(a)). Thus, they can be viewed as excellent model systems for organelle synthesis in biology. In this work, we provide the very first experimentally derived estimates of energy required for organelle synthesis in the bacterium \(M.\) gryphiswaldense. We experimentally show that investing this energy results in (recoverable) stunted bacterial growth. Considering energy of formation of \(\text{Fe}_3\text{O}_4\) as \(1118\, \text{KJ/mol,}^{34}\) the simplest estimate for energy of formation of a 30–40 nm crystal of \(\text{Fe}_3\text{O}_4\) (as those shown in Fig. 5(b)) yields the requirement of \(\sim 10^{-12}\) Joules. This is at least 3 orders of magnitude lower than our estimated energy for magnetosomal synthesis. Thus, while magnetic nano-crystal formation does utilize cellular energy, the major requirement actually comes from the organelle assembly entrapping the crystal. This is in agreement with previously observed questions regarding the ability of bacterial cells to synthesize 30–50 nm diameter intracellular organelles from biological membranes while compensating for/providing energetic input towards stabilizing membranes that are “ready to explode elastically”.\(^{9}\) Interestingly, our results (Figs. 3, 4) show that stunting of growth in presence of iron is not as substantial as would be predicted, given the conditions when empty organelles are still synthesized (no iron in the medium, data shown in gray). Thus, we believe that a major portion of the energy estimated by us for synthesis of magnetosomes is actually invested in synthesis of a stable cellular organelle rather than just the magnetic nanocrystal entrapped in it. Finally, we are hopeful that the direct approach used by us to arrive at energy of synthesis for a specialized organelle in a living cell is simple enough for gaining such important insights into all cellular systems.

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References and Notes

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