Effect of oxidative stress on the growth of magnetic particles in *Magnetospirillum magneticum*

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Summary. Individual magnetosome-containing magnetic mineral particles (MMP) from magnetotactic bacteria grow rapidly such that only a small fraction (<5%) of all magnetosomes contain dwarf (≤20 nm) MMP. Studies of the developmental stages in the growth of MMP are difficult due to the absence of techniques to separate dwarf from mature particles and because the former are sensitive to extraction procedures. Here, O2 stress was used to inhibit MMP expression in *Magnetospirillum magneticum* strain AMB-1. In addition, defined growth conditions not requiring chemical monitoring or manipulation of the gas composition during growth resulted in the production of cells containing high numbers of dwarf MMP. Cells exposed to different incubation treatments and cells with dwarf MMP were compared to cells with normal MMP with respect to growth, respiration, iron content, and relative magnetite load (RML). The cells were examined by electron microscopy, low temperature magnetometry, X-ray diffraction (XRD), and Mössbauer spectroscopy. In the 0–110 μM O2(aq) range, growth was positively correlated with [O2] and negatively correlated with RML. Most MMP formed during exponential growth of the cells. At 50–100 μM O2(aq) with stirring (150 rpm) and <30% O2 loss during incubation, MMP expression was strongly inhibited whereas MMP nucleation was not. Cells highly enriched (~95%) in dwarf MMP were obtained at the end of the exponential phase in stirred (150 rpm) cultures containing 45 μM O2(aq). Only one dwarf MMP formed in each MMP vesicle and the chain arrangement was largely preserved. O2-stress-induced dwarf MMP consisted of non-euhedral spheroids (~25 nm) that were similar in shape and size to immature MMP from normal cells. They consisted solely of magnetite, with a single domain signature, no superparamagnetic behavior, and magnetic signatures, Fe(II)/Fe(III) ratios, and XRD patterns very similar to those of mature MMP. These results show that O2 stress in liquid cultures amended with an inorganic redox buffer (S2O32−/S0) can be used to produce abundant dwarf MMP that are good proxies for studying MMP development. [Int Microbiol 2009; 12(1):49-57]

Key words: *Magnetospirillum magneticum* AMB-1 · magnetotactic bacteria · magnetosomes · biomineralization · magnetite · dwarf MMP

Introduction

Magnetosomes are membrane-bound organelles containing magnetic mineral particles (MMP). They are present in magnetotactic bacteria (MB) [1,2,6,7,13,19,42] as well as in many eukaryotes [20,46]. When aligned in chains, magnetosomes increase the magnetic momentum of MB and (coupled with chemotaxis) help cells move more efficiently toward interfaces of redox comfort [4,10,43]. MMP are euhedral single-domain crystals with species- and even strain-specific shapes ranging from cuboidal, parallelepipedal, or elongated pseudoprismatic to anisotropic [3,44]. Most MB have MMP that are made of magnetite. The majority of these species belong to the α-subdivision of Proteobacteria (e.g., *Magnetospirillum*, *Magnetococcus*, magnetotactic vibrios),
but *Desulfovibrio magneticus* belongs to the δ-subdivision of Proteobacteria [18,31], and *Magnetobacterium bavaricum* to the family Nitrospiraceae [41,42]. Most of our knowledge on the formation of MMP is derived from cultures of *Magnetospirillum* [16,21,23,29,32,34]. The biomineralization of MMP in MB includes several steps that are probably common to most species, including iron-uptake [23,28,30,33,37], Fe(III)-reduction during or after uptake [11,33,37,39], formation of lipid membrane-based MMP vesicles [3,21,23], accumulation of Fe(II) in the MMP vesicles coupled with an increase in the intra-vesicular pH [23,24,36], oxidation of a portion of the intravesicular Fe(II) [11,37], initiation of the MMP [11,37], growth of immature MMP to full size [8,23,35], and the organization of magnetosomes in chains [21,23,34]. Nonetheless, important questions about the formation of MMP remain unanswered.

We are interested in factors that control the growth of immature MMP to full-size MMP. In general, immature MMP are difficult to study because of their low abundance (<5% of the total MMP population) and the fact that they cannot be distinguished by optical microscopy, are sensitive to extraction procedures (dissolution and oxidation), and participate in magnetic aggregation during centrifugation and thus cannot be quantitatively separated from the overall MMP population. Our approach to the study of MMP is to produce MB cells containing a high abundance of immature (i.e., dwarf) particles.

MMP development is largely controlled by biochemical and molecular mechanisms, but environmental factors can also impair development and thus must be monitored in studies of MMP [16,17,23,36]. However, daily verification of the O₂ levels and re-adjustment of the gas composition are often impractical. In the present study, the effects of various levels of initial O₂ and liquid:gas ratios were monitored in cultures with O₂ concentrations between 17 and 240 μM O₂ in freshwater with air at 760 mmHg and a temperature of 30°C. The amount of O₂ in the incubation tubes was determined after corrections for changes in pressure and verified based on changes in the N₂:O₂ ratio. After injection of 1.67% O₂(g) in the gas phase, the O₂(g) concentration decreased to 1.4–1.5% (~16.7 μM O₂(aq)) ~24 h after the nutrients had been autoclaved, due to chemical oxidation of secondary sulfides (S₂O₃²⁻ + S = H₂S₂ + S²⁻ + H₂O). For rapid initiation of the exponential growth phase (~24 h), 6–10 ml medium were inoculated with ~10⁻¹⁰⁶ cells from liquid cultures of exponentially growing *M. magneticum* strain AMB-1 and incubated at 30°C.

**Monitorization of cell growth.** Cell growth was monitored as the change in A₄₆₅ (using a HP 8452 diode array spectrophotometer); cell density (in cells/ml) was estimated from a calibration of A₄₆₅ against direct cell counts as determined by optical microscopy. Total protein content was determined by the Lowry method [22], and total iron content by the phenantroline method with hydroxylamine reduction [15] after acid extraction of the cell pellets with 5 M HCl. The magnitude of the magnetic field (B) was measured with a triaxial fluxgate magnetometer with a Hall probe (FGM-5DTAA, Walker Scientific) in the 10⁻⁵–10⁻² T range, and with a gaussmeter (Walker Scientific) in the 10⁻¹–10⁻² T range. MMP expression was monitored according to the relative magnetite load (RML), which was derived from changes in light scattering, according to Bₐ (~4 × 10⁻³ T), along the light path during spectrophotometric measurements.

\[
\text{RML} = \frac{(B - A)A}{B}
\]

where: \( A = A_{465} \) without applied B, \( B = A_{465} \) with applied B.

In our opinion, RML is a better quantifier of MMP expression than the direct difference B–A (Eq. 1) [38], because RML includes a correction for cell density. The two methods were compared in the analysis of cumulative data from 4 days of readings of 21 tubes. The results showed that in the 17–90 μM O₂(aq) range there was better correlation between [O₂(aq)] and RML (\( R^2 = 0.7074, n = 54 \)) than between [O₂(aq)] and B−A (\( R^2 = 0.095, n = 54 \)).

**Transmission electron microscopy (TEM).** Cells were fixed in 2.5% glutaraldehyde, post-fixed in 1% OsO₄, and stained with saturated uranyl acetate in 70% EtOH. Samples were subsequently dehydrated in an EtOH series and embedded in LR-White resin (London Resin, England). A Sorval Porter-Blum MT2-B ultramicrotome was used to cut ~75-nm thin sections, which were mounted on carbon-coated copper grids. TEM images were obtained on an Akashi EM-002B microscope at 100 keV.

**Magnetic and XRD measurements.** Exponentially growing cells were harvested by centrifugation under 100% N₂. Magnetic measurements included FC(ZFC) remanent magnetization curves, hysteresis loops, and ZFC/FC induced magnetization curves, and were carried out with a MPMS-XL SQUID magnetometer (Quantum Design). FC(ZFC) remanent magnetization curves and ZFC(FC) remanent magnetization curves were obtained by cooling the samples from 300 to 5 K in a 2.5-T field (FC) and then measuring magnetization, as the temperature was increased stepwise, in a zero field. Then, the samples were again cooled from 300 to 5 K, but in a zero field, subjected to low-temperature isothermal remanence in a 2.5-T field (ZFC), and magnetization was measured during warming of the samples in a zero field. The (FC) and ZFC(FC) remanence curves allowed whole cells, MMP, and synthetic...
magnetite to be distinguished based on the parameter $\delta$, which is a measure of the remanence lost by warming magnetite particles through the Verwey transition ($T_{V}$) at 120 K [26,27].

$$\delta = \frac{M_{im}(80) - M_{im}(150)}{M_{im}(80)}$$

where $M_{im}$ is the initial saturation of Isothermal Remanent Magnetization (SIRM) remaining at 80 and 150K for FC$_{2.5T}$ and ZFC$_{2.5T}$ curves [26].

The $\delta_{FC}/\delta_{ZFC}$ ratio is diagnostic of magnetite magnetosomes. For intact chains of unoxidized magnetite magnetosomes, the $\delta_{FC}/\delta_{ZFC}$ ratio is >2 [26]; for maghemite samples, the $\delta_{FC}/\delta_{ZFC}$ ratio is close to one, but in this case $\delta_{ZFC}$ and $\delta_{FC}$ have values of about 0.05–0.06, while the $\delta_{ZFC}$ values for magnetite magnetosomes are larger (~0.08–0.3). Hysteresis loops, or measurements of magnetization ($M$) as a function of applied field ($H$), were obtained by applying fields up to 5 T at 300 K. To investigate the presence of superparamagnetic (SPM) behavior, ZFC/FC induced magnetization curves were obtained as follows: the samples were cooled in a zero field from a high temperature (in which all particles show SPM behavior) to a low temperature after which magnetization was measured, as the temperature was increased stepwise from 2 to 300 K (ZFC process), in a small applied field ($B_0 = 5 \text{ mT}$). The sample was again cooled in the same small field and FC magnetization curves were obtained by measuring magnetization of the samples in the field during a stepwise increase in temperature. Several distinct features of superparamagnetism can be verified from these ZFC/FC measurements, such as the blocking temperature ($T_{B}$) peak of the ZFC magnetization curve. Mössbauer spectra were acquired at room temperature and a conventional constant-acceleration spectrometer (Wissel) was used in transmission geometry with a $^{57}$Co/Rh source, using $\alpha$-Fe at room temperature to calibrate isomer shifts and velocity scale. Fitting was obtained by considering a distribution of quadrupole splitting values.

**Results**

In many applications involving MB, it is impractical to monitor and correct the $O_2(g)$ concentration daily. It would therefore be very useful to determine specific initial culture and incubation conditions that result in cells with a high abundance of arrested growth magnetosomes after a specific time interval or growth stage, without the need for further manipulation. One approach to this problem is to determine the different initial concentrations of $O_2(g)$ and to stir the cultures to avoid the formation of redox gradients in the liquid column. Accordingly, we verified growth and changes in RML at different initial $O_2(g)$ in stirred (150 rpm) vs. unstirred (27 ml Hungate tubes with 10 ml liquid at ~1 bar) cultures. In the 0–147 $\mu$M initial $O_2(aq)$ range, stirring the cultures at high $O_2$ resulted in faster exponential growth of the cells and larger cell densities in the stationary phase (up to $\sim 5 \times 10^8$ cells/ml, ~0.28 g dry weight/l after 72 h). Growth was inhibited above 13.2 % initial $O_2(g)$ (>147 $\mu$M $O_2(aq)$ in the stirred cultures), while magnetite formation was strongly inhibited in all stirred tubes with >3.9% initial $O_2$ (Fig. 1). This inhibition was attributed to oxidative stress because magnetite growth requires a $\sim 2:1$ Fe(III):Fe(II) ratio, while chemical iron oxidation is fast and has a high equilibrium constant at neutral pH [17,25]. TEM analysis of the non-stirred cultures showed a dominance of large magnetosomes irrespective of the initial $O_2$ concentration, a lower number of MMP per cell at the highest initial $O_2(g)$, and no sizable increase in the abundance of dwarf MMP. In stirred cultures, however, cells incubated in 0.8% initial $O_2(g)$ (~8.9 $\mu$M $O_2(aq)$) formed mostly normal MMP. A high abundance of dwarf MMP and very few mature MMP was obtained at 5.4% initial $O_2(g)$ (~60.3 $\mu$M $O_2(aq)$),

![Fig. 1. The effect of $O_2$ on growth (A) and relative magnetite load (RML) (B) in liquid cultures of *Magnetospirillum magneticum* strain AMB-1 with/without stirring (150 rpm). The initial $O_2$ values are concentrations in the gas phase at 1 bar.](image)
and very few, mostly dwarf, MMP were formed by cells incubated at 10.1% initial O₂(g) [~112 μM O₂(aq)]. These results suggested a simple, practical means to obtain arrested growth in MMP without daily monitoring and manipulation of the gas composition, by adjusting the initial gas composition to the correct [O₂] values and by stirring the cultures.

During incubation, the O₂ concentration decreases due to chemical oxidation and respiration, which makes it difficult to identify O₂ conditions optimal for the expression of dwarf MMP. It was therefore necessary to limit depletion of the O₂ pool and to monitor its evolution. This was accomplished using stirred cultures with a larger gas:liquid ratio (27 ml Hungate tubes with 7 ml of liquid culture) and by increasing the initial gas pressure to 1.2–1.4 bar. Under these conditions the O₂(g):O₂(aq) molar ratio was ~125:1 at 30°C and pH 7. Gas pressure, cell density, RML, O₂(g), and N₂(g) were monitored daily in 21 culture tubes containing AMB-1 at seven initial O₂(g) concentrations in the range 17–225 μM O₂(aq). Controls consisted of 21 tubes treated the same way but without cells. Exponential growth occurred between 24 and 48 h in all tubes containing cells. During this 24-h interval, the O₂ concentration did not decrease by more than 25% and the generation time was shorter at higher O₂ concentrations (Fig. 2). The average O₂(g) values between the 24- and 48-h readings for the seven O₂ treatments were 20, 37, 53, 74, 92, 110, and 190 μM. The negative correlation between growth rate and magnetite production in the 20–92 μM O₂(aq) range (Fig. 2B) indicated that the O₂ conditions optimal for the growth of strain AMB-1 are independent of those optimal for MMP development. The sharpest drop in RML occurred in the 53–74 μM O₂(aq) range.

After 96 h of incubation, O₂ consumption due to respiration was about four times larger than the effect of chemical oxidation (Fig. 3A). Yet, because respiration is sensitive to changes in [O₂], it is generally recommended to avoid interpreting respiration results when [O₂] changes by ≥ 30% between successive readings. For this reason, and because cells in the lag and stationary phases may express different respiration values, O₂ respiration was calculated only during the 24-h interval that corresponded to exponential growth (24–48 h). To calculate the total O₂ respired by cells of strain AMB-1, corrections were made for changes in pressure and for chemical oxidation, predicted from controls at similar [O₂(aq)]. A positive correlation (R² = 0.7609; n = 20) was found between the respiration rate and [O₂(aq)], such that μmol O₂ respired × 10⁻¹⁰ cells/h = 1.00608 + 0.03299 × [O₂(aq)] (in μM). A comparison of the changes in RML during growth at different initial O₂ concentrations showed that cells of strain AMB-1 formed magnetosomes mostly during exponential growth (Fig. 3B) and that a subsequent drop to <100 μM O₂(aq) at 72 and 96 h, after cells entered the stationary phase, did not restore the RML (results not shown). The expression of MMP remained low in all cultures in which the initial [O₂(aq)] was >100 μM. It must be emphasized here that these results are dependent on culture stirring; without stirring, cells form large amounts of MMP even at high [O₂(g)].
Magnetic bacteria also store iron in inorganic deposits other than magnetosomes, such as ferritin granules [5] and vacuoles enriched in amorphous iron phosphate [9]. Although such iron reserves are difficult to quantify, they may play important roles in the ability of the cells to form magnetosomes. In addition, the dynamics of these non-MMP deposits may be controlled by oxidative stress and are thus connected with the growth of MMP. Accordingly, total iron content of *M. magneticum* strain AMB-1 cells was measured in four treatments (18.6 vs. 50 μM O₂(aq), and 0 vs. 150 rpm). To limit changes in [O₂] during incubation, filter-sterilized gas mixtures (O₂ in N₂) containing 1.7 and 4.5% O₂, respectively, were injected every 12 h; the pressure was kept at ~1.3–1.4 bar. Cells were sampled after 48 h (upper exponential phase). The iron content was: 3.8 ± 0.1 mg Fe/mg protein in the 18.6 M O₂(aq)/0 rpm treatment, 2.5 ± 0.2 mg Fe/mg proteins in the 18.6 M O₂(aq)/150 rpm treatment, 5.6 ± 0.5 mg Fe/mg proteins in the 50 mM O₂(aq)/0 rpm treatment, and 1.7 ± 0.2 mg Fe/mg proteins in the 50 μM O₂(aq)/150 rpm treatment. Surprisingly, although RML was larger in the 18.6 μM O₂(aq) treatments, the 50 μM O₂(aq)/0 rpm cultures accumulated the largest amount of iron. Except for the 50 μM O₂(aq)/150 rpm treatment (which resulted mostly in dwarf MMP), all other treatments led to ~95–100% normal MMP. Since dwarf MMP are only 15% of the size of mature MMP (Fig. 4), a significant part of the iron from cells grown at 50 μM O₂(aq) is probably not stored in magnetosomes. However, given the variability of these measurements, the obstacles to exactly measuring the average number of MMP per cell, and the as-yet unclear relationship between RML and the amount of magnetite made, it was difficult to quantify the intracellular non-MMP iron deposits.

The ultrastructure and arrangement of MMP resulting from the different treatments were analyzed by TEM (Fig. 4). The cells were incubated for 4 days at 30°C in 140-ml serum bottles with 50 ml of liquid medium. In the O₂-stress treatments, premixed gas was injected daily to maintain a concentration of 4% in the gas phase (~45 μM). Under 0% O₂ (0 rpm, or 150 rpm) and 4% initial O₂ (at 0 rpm), only normal mature MMP were formed, with no notable differences between treatments. The mature MMP were euhedral, 59 ± 5 nm vs. 42 ± 7 nm in size within the single domain of magnetite. In contrast, MMP formed under O₂ stress (45 μM O₂; 150 rpm), were smaller, non-euhedral spheroids, ~25 ± 4 nm (hence dwarf MMP), with some as small as 10 nm. If dwarf MMP are made solely of magnetite (see below), this size is still within the single domain. A few of these particles (generally <10%) had elongated shapes (1.5:1 length:width ratio) but still were not euhedral. Dwarf MMP were also present in cells with normal MMP, albeit at low abundance (~12–14%). In cells with dwarf MMP, a slightly elevated number (11 ± 2% vs. ~5 ± 2% in cells with normal MMP) of non-aligned MMP were found. The total number of MMP per cell was almost the same between treatments, ranging between 8 and 25 per cell; however, the total number of MMP per cell observed by TEM thin sections has to be taken as an underestimate. Dwarf MMP (≤25 nm) were also found in cells with normal MMP, but they represented ≤5% of the population and were more frequently distributed toward the ends of the chain, suggesting terminal growth of the MMP chain. In cells placed...
under O2 stress (45 μM O2; 150 rpm), the abundance of dwarf MMP was very high (>95%). Without exception, no more than one MMP was ever found per magnetosome vesicle, although a few vesicles (~3–4%) did not contain MMP. In some TEM images, positive identification of some of the vesicles and of the dwarf MMP was difficult. There was no significant difference in the abundance of empty MMP vesicles between the different treatments, indicating that 45 μM O2(aq) stress did not inhibit nucleation but only MMP growth. The morphology of the few dwarf MMP contained in cells with normal MMP was very similar with the morphology of the dwarf MMP from cells subjected to O2 stress. Euhedral MMP <20 nm in size were never found, irrespective of the treatment.

To address whether dwarf MMP are similar in composition to normal MMP, cells with normal and dwarf MMP were compared during two different treatments: ~18.7 μM O2/150 rpm (for normal MMP) and O2-stressed cultures ~45 μM O2/150 rpm (for dwarf MMP). The large amounts of samples needed for the magnetic measurements were obtained by culturing the cells in 1- to 2-l serum bottles with 20–25% liquid medium (as shown in Materials and methods). The medium was autoclaved with ~1.67% O2 in the gas phase and the gas composition adjusted after cooling to the desired [O2], adjusted the pressure at ~1 bar. One ml of inoculum from a culture of AMB-1 in late exponential phase was added per liter and the cultures incubated for 72 h at 30°C. After centrifugation, the pellet obtained from cultures with normal MMP was dark-gray to black, uniform, with a thin white upper layer, while pellets of cells with dwarf MMP were heterogeneous. The upper part of the pellet (~90%) was white, while the bottom part was gray with black spots. We assumed that this heterogeneity was due to cells with different abundances of dwarf MMP, or to a small population of normal size magnetosomes still present in the O2-stressed cultures, or to an agglomeration of MMP as a result of centrifugation and poor chain alignment. TEM analysis of these pellets indicated that dwarf MMP were present, abundant, and similar in both the upper and lower parts of the pellets of O2-stressed cells, and that the sizes of the MMP did not significantly differ between these subsamples.

We then sought to determine whether different parts of the O2-stressed cell pellets contained the same type of magnetic materials. Three types of samples were compared: (A) pellets of cells with normal MMP, (B) the upper, white part of the pellets with dwarf MMP, and (C) the lower, dark part

Fig. 4. Transmission electron micrographs of magnetosomes from cells of *Magnetospirillum magneticum* strain AMB-1. (A) Normal MMP formed under 0% N2 and 150 rpm. (B) Normal MMP observed at ca. 140,000× magnification. (C) Dwarf MMP formed under 4% O2 and 150 rpm. (D) Dwarf magnetosomes observed at ca. 140,000× magnification.
of the pellets with dwarf MMP. The $\text{FC}_{(2.5T)}/\text{ZFC}_{(2.5T)}$ remanent magnetization curves showed very similar patterns between samples B and C, and all samples had Verwey transitions at \~120 K, characteristic for magnetite (Fig. 5). The contribution of paramagnetic (PM) materials was larger in B and C than in normal MMP samples; this was partly expected because cells with dwarf MMP have less Fe and magnetite than cells with normal MMP. All samples showed a $\delta_{\text{FC}}/\delta_{\text{ZFC}}$ behavior between whole cells and extracted magnetosomes; which was also expected since dwarf MMP were not extracted from the cells, in order to limit dissolution, oxidation, and chain breakage. A value of \~1 for $\delta_{\text{FC}}/\delta_{\text{ZFC}}$ indicates isolated magnetite particles while a value of \~2 indicates perfectly aligned magnetite chains [26]. No significant differences in the level of alignment between B ($\delta_{\text{FC}}/\delta_{\text{ZFC}} = 1.59$) and A ($\delta_{\text{FC}}/\delta_{\text{ZFC}} = 1.60$) were found whereas smaller values ($\delta_{\text{FC}}/\delta_{\text{ZFC}} = 1.39$), an indication of poorer alignment, were obtained for C. It was previously observed [26] that the conversion of magnetite MMP to maghemite (such as during oxidation) reduces $\delta_{\text{FC}}$ and $\delta_{\text{ZFC}}$ close to 0.05–0.06 and brings the $\delta_{\text{FC}}/\delta_{\text{ZFC}}$ ratio to \~1. We found $\delta_{\text{FC}}/\delta_{\text{ZFC}}$ ratios <2 and thus inferred no evidence of magnetite alteration.

Samples A, B, and C (Fig. 5) were also compared by hysteresis loop analysis (data not shown). Paramagnetic (PM) and ferrimagnetic contributions in all samples were recorded. In addition, the PM effect was stronger in samples with dwarf MMP, supporting the results presented above (namely, Fe composition and $\text{FC}_{(2.5T)}/\text{ZFC}_{(2.5T)}$ ratio). The hysteresis parameters at 300 K, the saturation magnetization ($M_s$), and the saturation remanent magnetization ($M_r$) were very similar between the A and B samples. Using the known value of $M_s$ for magnetite, we estimated that the dried-pellet samples each contained <1% magnetite. Additional information was obtained from the $M_r/M_s$ ratio, which for whole-cell samples

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**Fig. 5.** $\text{FC}_{(2.5T)}/\text{ZFC}_{(2.5T)}$ remanent magnetization curves showing Verwey transitions and $\delta_{\text{FC}}/\delta_{\text{ZFC}}$ ratios. (A) Pellet of cells with normal MMP. (B) Upper part of the cell pellet containing dwarf MMP. (C) Lower part of the cell pellet containing dwarf MMP. (D) $\delta_{\text{FC}}/\delta_{\text{ZFC}}$ plot of the A, B, and C samples relative to data on other published samples [26].
at room temperature is ~0.5 for a random distribution of uniaxial “single domain chains” [26]. The values obtained for the A and B samples were smaller than expected (~0.4); this may have been due to broken chains, which is a typical artifact of extended centrifugation. It was difficult to determine a precise \( M_s/M \) value for the C samples because of their high PM contribution. Mössbauer spectra showed only doublets, which may have indicated PM or SPM iron phases, thus confirming previous reports [12], but ZFC/FC induced magnetization analysis showed only PM and not SPM behavior. Lastly, XRD analysis showed no significant differences between samples and that the only mineral present was magnetite (results not shown), supporting the previous results.

**Discussion**

It was previously reported that the expression of MMP in _M. magneticum_ strain AMB-1 is optimal at 2.35 \( \mu \text{M} \text{O}_2 \), is partly inhibited at 11.7 \( \mu \text{M} \text{O}_2 \), and totally inhibited at 23.52 \( \mu \text{M} \text{O}_2 \). Without subsequent manipulation of the gas composition, we found that _M. magneticum_ strain AMB-1 cells are more tolerant to \( \text{O}_2 \) stress than previously acknowledged, when an inorganic reductant (such as \( \text{S}_2\text{O}_3 \) mixture) is added to the medium. During these incubations, \( \text{O}_2 \) decreased by 6–45% over 96 h. The initial culture conditions described herein allow a high abundance of dwarf MMP to be obtained without the need for chemical monitoring and periodic adjustment of \( \text{O}_2 \). Cells of _M. magneticum_ strain AMB-1 grew better at 100–225 \( \mu \text{M} \text{O}_2(aq) \) in stirred liquid culture than at lower \( \text{O}_2 \), yet the formation of MMP was repressed at \( \sim 45 \mu \text{M} \text{O}_2(aq) \) and strongly inhibited at \( \geq 100 \mu \text{M} \text{O}_2 \). Under conditions of \( \sim 45 \mu \text{M} \text{O}_2 \) in liquid, 150 rpm, and 30°C, numerous dwarf magnetosomes, representing \( \geq 95\% \) of the total MMP population, formed after 48 h of incubation, and the \( \text{O}_2 \) concentration decreased by \( \sim 20\% \). The higher respiration rate, faster growth, and higher final density at higher \( \text{O}_2 \) supports the conclusion that, in strain AMB-1, the oxidative stress of MMP production does not coincide with the oxidative discomfort of the cells.

The total number of MMP was similar between cells with normal MMP and cells with dwarf MMP grown at 45 \( \mu \text{M} \) initial \( \text{O}_2(aq) \) and 150 rpm. Only a small fraction of all dwarf MMP were not aligned. Magnetite was the only magnetic material or mineral detected in strain AMB-1. The smallest dwarf magnetosomes were \( \sim 10 \) nm in size and were very seldom euhedral, but rather irregular spheroids. MMP vesicles with more than one MMP particle were not observed, implying that MMP are initiated from a sole nanocrystallite and novel magnetosome were added mainly terminally in the MMP chain. Despite the fact that very large populations of dwarf MMP were analyzed (~4 × 10^{11} per sample), there were no signals of SPM behavior; instead, only SD behavior and PM signal. The magnetic signatures of cells with dwarf MMP was nearly the same as that of cells with normal magnetosomes, i.e., no ferryhydrite, aligned SD magnetite, no SPM behavior, and enrichment in PM iron.

In earlier models, it was hypothesized that MMP are initiated via: (i) SPM nanocrystallites of magnetite growing into mature single domain particles; (ii) early granules of crystalline or amorphous ferryhydrite, later replaced by magnetite [11,37]; or (iii) iron-rich organic matrices, later replaced by magnetite [23,45]. The existence of a short-lived SPM magnetite or ferryhydrite phase during MMP growth cannot be excluded, but a method to systematically stop the growth of all magnetosomes in very early SPM stages has yet to be found. The MMP were not in physical contact with the MMP membrane, perhaps indicating that the growth of MMP is controlled via solute chemistry rather than surface contact. The initiation of novel MMP was not observed, probably because the period of growth between early nanocrystallites and dwarf MMP is very short. The culturing conditions proposed herein have a greater effect on the growth of MMP from dwarf to mature MMP than on the formation of dwarf MMP, and can be used to study stages in MMP development.

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