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Effects of Dissolved Oxygen Concentration and Iron Addition on Immediate-early Gene Expression of *Magnetospirillum gryphiswaldense* MSR-1

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Abstract

We report effects of dissolved oxygen (DO) concentration and iron addition on gene expression of Magnetospirillum gryphiswaldense MSR-1 cells during fermentations, focusing on 0.25-24 h after iron addition. The DO was strictly controlled at 0.5% or 5% O\textsubscript{2}, and compared with aerobic condition. Uptake of iron (and formation of magnetosomes) was only observed in the 0.5% O\textsubscript{2} condition where there was little difference in cell growth and carbon consumption compared to the 5% O\textsubscript{2} condition. Quantitative reverse transcription PCR analysis showed a rapid (within 0.25 h) genetic response of MSR-1 cells after iron addition for all the genes studied, except for MgFnr (oxygen sensor gene) and fur (ferric uptake regulator family gene), and which in some cases was oxygen-dependent. In particular, expression of sodB\textit{1} (superoxide dismutase gene) and feoB\textit{1} (ferrous transport protein B1 gene) were markedly reduced in cultures at 0.5% O\textsubscript{2} compared to those at higher oxygen tensions. Moreover, expression of kat\textit{G} (catalase-peroxidase gene) and feoB\textit{2} (ferrous transport protein B2 gene) was reduced markedly by iron addition, regardless of oxygen conditions. The data provides a greater understanding of molecular response of MSR-1 cells to environmental conditions associated with oxygen and iron metabolisms, especially relevant to immediate-early stage of fermentation.

Keywords: magnetotactic bacteria; cell growth; carbon consumption; magnetosome; oxygen; iron metabolism

Introduction
Magnetotactic bacteria are a group of Gram-negative prokaryotes capable of migrating along magnetic field lines (Blakemore 1975). This function depends on a specific intracellular structure, namely magnetosome, which contains a number of crystals of magnetic iron minerals in form of magnetite or greigite (Schüler 2002). The magnetosomes have attracted much attention for potential biomedical applications such as magnetic resonance imaging, magnetic hyperthermia and drug targeting (Alphandéry 2014), as well as the development of immunoassay and magnetic markers (Arakaki et al. 2008). To understand the formation of magnetosomes, *Magnetospirillum gryphiswaldense* MSR-1 has been used as a model microorganism (Schüler and Baueulein 1996; Heyen and Schüler 2003; Sun et al. 2008; Liu et al. 2010; Zhang et al. 2011; Li et al. 2014; Loehr et al. 2016), most likely as it is not a strict anaerobe, thus simplifying its cultivation. This strain was isolated from a mud sample in Germany (Schleifer et al. 1991), and requires a low dissolved oxygen (DO) concentration for magnetosome formation during cultivation (Heyen and Schüler 2003).

Biosynthesis of magnetosomes is controlled by a complex machinery that includes genes located in a single chromosomal region, the genomic magnetosome island (Ullrich et al. 2005), as well as a number of genes outside of the island. A thorough review of the biosynthesis process was recently published (Uebe & Schüler, 2016). Key genes are involved in metal ion activation and substrate recognition (Deng et al. 2015), iron utilization (Rong et al. 2008; Uebe et al. 2010; Rong et al. 2012; Qi et al. 2012), oxidative stress protection (Rong et al. 2012; Qi et al. 2012), as well as regulation of denitrification (Li et al. 2014). Expression profiles of 13 such genes were previously examined during high-iron (20 μM ferric citrate) and low-iron (without ferric citrate) cultivations of MSR-1 cells (Wang et al. 2013). The finding revealed that these genes were coordinated to a balance level during magnetosome biosynthesis. Recently, the same group reported a transcriptome analysis of MSR-1 cells under aerobic (high oxygen) and micro-aerobic (low oxygen) conditions (Wang et al. 2016). Samples were collected at 24 h during a fed-batch fermentation and consequently the authors proposed a regulatory network to reflect the complexity of the physiological metabolism in MSR-1. On the other hand, Staniland et al. 2007 demonstrated rapid magnetosome formation during cultivation of MSR-1. This was observed 0.25 h after the formation was initiated by the addition of an iron source, shown by real-time x-ray magnetic circular dichroism. These studies ultimately suggested that
DO concentration and iron addition have a rapid impact on molecular and physiological response of MSR-1 cells. Despite this, precise influence of DO concentration and iron supply on cellular response of MSR-1 is not well understood. In particular, immediate-early gene expression associated with oxygen and iron metabolism has not been studied.

The purpose of this study was to investigate the effect of DO concentration and iron addition on rapid genetic response of MSR-1 cells over time. To achieve this, fermentations were conducted at DO concentration of 0.5% or 5% O₂, and compared with aerobic fermentations. The genes *sodB1, katG, MgFnr, feoB1, feoB2* and *fur* were examined at multiple time points during each set of fermentations. It is anticipated that the data presented here will provide a greater understanding of cell response to environmental conditions, potentially paving the way to strain engineering and process optimization in the future.

**Materials and Methods**

**Bacterial strain and growth media**

*M. gryphiwaldense* MSR-1 was purchased from Leibniz Institute DSMZ. Cells were grown and maintained on solid activated charcoal agar media as described (Schultheiss and Schüler 2003).

Liquid cultures of MSR-1 were grown in a media described previously (Heyen and Schüler 2003), containing 0.1 g/L KH₂PO₄, 0.15 g/L MgSO₄·7H₂O, 0.34 g/L NaNO₃, 3 g/L soy peptone, 3.5 g/L potassium lactate and 100 µM ferric citrate. The media was adjusted to pH 7.0 with 2 M NaOH. All chemicals were purchased from Sigma (DK). The media devoid of ferric citrate, namely DFM, was used for preparation of non-magnetic inoculum and for the initial phase of fermentation.

**Preparation of non-magnetic inoculum**

Non-magnetic cells were grown by three sequential transfers at a ratio of 10% (v/v) in shaking flasks containing the DFM media as described above.
Fermentation

Fermentation was carried out in 1 L BIOSAT® B plus bioreactors (Sartorius, DK) with a working volume of 0.8 L. Temperature was maintained at 28°C, agitation at 120 rpm, and gas flow rate was 1 L/min. pH 7.0 was controlled via a pH electrode (Mettler Toledo, USA) by addition of either H₂SO₄ (2 M) or NaOH (2 M) via peristaltic pumps. DO was measured using an oxygen probe (Hamilton, USA) and controlled by gas blending, automatically varying the proportion of nitrogen and air in the inflowing gas by cascade mode. The bioreactor was also equipped with a condenser to avoid evaporation from the media.

The bioreactors were loaded with the DFM media as described above. After sterilization (121°C for 15 min), the oxygen probe was calibrated by sparging the media with sterile nitrogen and air. When the media was saturated with air, all the fermentations were initiated by inoculation of non-magnetic cells to reach a starting OD₅₆₅ of 0.1, and fermentation was allowed to proceed with constant aeration. When the OD₅₆₅ reading reached 0.3, the DO concentration was set to 5% or 0.5% O₂ in randomly selected bioreactors, whereas aeration (i.e. sparging with air continuously) remained in the rest of the bioreactors as control. Once the required DO level was reached, an identical volume of sterile ferric citrate solution was added into each fermenter to give a final concentration of 100 μM. Immediately after the addition, samples were collected and the sampling point was set as ‘0 h’. Thereafter samples were also collected at 0.25, 2, 15 and 24 h. At each time point, cell density was measured in a spectrophotometer at a wavelength of 565 nm. Approximately 30 mL of culture was collected and placed on ice immediately. Cell fractions and spent media were separated by centrifugation at 5000 g for 5 min at 4°C (Thermo Scientific, DK). Subsequently cell pellets were prepared with RNAProtect Bacteria Reagent (Qiagen, DK) according to manufacturer’s instructions and stored at -80°C, whilst the spent media was passed through a membrane filter with a pore size of 0.22 μm (VWR, DK) and stored at -20°C prior to analysis. Each fermentation condition was performed in duplicate.

Determination of iron and lactate concentration in the media
Iron content was determined by following the European Brewing Convention iron determination method (Analytica EBC 9.13.1, 2000). Briefly, ferric iron in the samples was reduced to ferrous form (Fe$^{2+}$) by ascorbic acid (Sigma, DK). Subsequently a complex ([Fe(bipy)$_3$]$^{2+}$) was formed between Fe$^{2+}$ and 2,2'-bipyridine (Sigma, DK), which was used for the colorimetric analysis of iron contents at the wavelength of 520 nm (Thermo Scientific, DK).

Concentration of potassium lactate was determined by a HPLC method: 20 µL of each sample was injected onto an HPLC column (Aminex HPX-87H, 300×7.8 mm, Bio-Rad, DK) preheated to 60ºC. The samples were eluted using 5 mM sulfuric acid at a flow rate of 0.5 mL/min. Peak signals were detected by a refractive index detector and analyzed using Millenium software. Quantification of compounds was carried out by comparison with known external standards.

**RNA isolation and quantitative reverse transcription-PCR**

Cells were disrupted using a Tissue-Lyser LT (Qiagen, DK) and total RNA was isolated using a RNeasy plus kit. The purity of the total RNA was determined using a NanoDrop Lite (Thermo Scientific, DK) and the quantity was measured with a Qubit Fluorometer (Invitrogen, DK). cDNA was generated from 0.9 µg of the total RNA using a QuantiTect Reverse Transcription Kit. The subsequent qRT-PCR was performed in a Real-Time PCR detection system (Bio-Rad, DK) by Quantifast SYBR Green PCR Kit. PCR amplification was carried out in a volume of 20 µL with following conditions: 9ºC for 5 min and 40 cycles of 95ºC for 10 s and 60ºC for 30 s. A melting curve from 65ºC to 95ºC with reads every 0.15 min was generated at the end of the program to evaluate the specificity of the PCR products. Gene *rluB* (encoding a 16Sr related synthase) was used as the internal standard for normalization of expression levels. All primers are shown in Table 1 and all kits were purchased from Qiagen, DK. The relative expression levels were approximated based on $2^{\Delta\Delta Cq}$, with $\Delta\Delta Cq = \Delta Cq_{\text{normalized}} - \Delta Cq_{\text{calibrator}}$, where $\Delta Cq_{\text{normalized}} = \Delta Cq_{\text{target gene}} - \Delta Cq_{\text{MGR_2835}}$. The calibrator Cq values are those from the cells that were collected at ‘0 h’. Consequently results were represented as relative expression (log2-ratio) at 0.25, 2, 15 and 24 h compared to ‘0 h’ when iron was added.
Transmission electron microscopy

A transmission electron microscope (FEI Titan 80-300ST, Netherlands) was used at an acceleration voltage of 120 kV to investigate the morphology and size of magnetosomes. For specimen preparation, aqueous cell samples were dropped onto carbon-coated copper grid (Agar Scientific, UK), and dried in air using an infrared lamp.

Statistical analysis

The mean and standard deviation of a data set was calculated using Excel (Microsoft, USA). Statistical analyses were performed using Origin V8.6 (Massachusetts, USA). At every time point, the expression difference of each gene between the three fermentation conditions was accessed by the Analysis of Variance (ANOVA) coupled with Tukey’s test.

Results and discussion

Fermentation performance

As shown in Fig. 1A, cell density of MSR-1 reached OD_{665} of 0.3 at ‘0 h’ of each fermentation prior to iron addition. At 2 h following the addition, similar cell growth was observed between the conditions studied; however, after 15 h cultivation, higher growth were observed in the cultures conducted at 5% and 0.5% O_{2}, compared to those under aerobic conditions. At 24 h, while the latter resulted in the highest cell growth, cell densities dropped to a similar level under the two oxygen-controlled fermentations. The reduction is likely due to limitation of the carbon source. Indeed, Fig. 2A shows that the entire carbon source was consumed by 15 h in the cultures except for aerobic cultures, which showed slower carbon consumption until 24 h. This observation was consistent with DO profile of the aerobic fermentation (Fig. 1B). Here the DO level decreased gradually as cell growth increased until approximately 22 h, followed by a rapid increase when cell growth ceased. For cultures under defined oxygen conditions, the DO level was well controlled with average values of 5.0 (± 0.20) % or 0.5 (± 0.07) % O_{2} prior to the addition of iron at ’0 h’. Similar values were measured after ’0 h’ (Fig. 1B). The results indicate there was a minor difference in cell growth between
5% and 0.5% O₂ conditions, which in both cases was faster compared to the aerobic condition. The finding is in agreement with Heyen and Schüler 2003, who observed a prolonged lag phase under a dissolved oxygen tension of 212 mbar, corresponding to a fully aerobic condition.

Iron was only consumed under the 0.5% O₂ condition (Fig. 2B) and formation of magnetosomes was confirmed at 24 h by microscopy analysis (Fig. 3). The cell pellets obtained from this condition were observed to be totally black at the end of fermentation while the others remained cheese-white. This is expected since Zhang et al. 2011 reported that DO concentration < 1% is required for magnetosome formation in cultures of MSR-1. Staniland et al. 2007 showed rapid magnetosome formation 0.25 h after the addition of ferric quinate at a growth condition with 0.5% O₂, which was the same as we used; however, an immediate uptake of iron was not detected at 0.25 h in the current study, probably due to limitation of the iron quantification method in detecting minor changes of iron content. In addition, it should be noted that the data presented here only shows the level of external iron, since measurement of internal iron would include magnetosomes, in which the iron is not in a soluble form and thus not directly accessible for metabolism.

Nevertheless, between 2 and 15 h of cultivation, a drastic uptake of iron was observed followed by a plateau until 24 h under the 0.5% O₂ condition, indicating the utilization of iron ceased after approximately 15 h in spite of the availability of iron in the media (Fig. 2B). This could probably be explained by the depletion of carbon source in the culture, since carbon is crucial for magnetosome formation as an energy source (Naresh et al. 2012). Indeed, carbon is particularly important in the transport process of magnetite synthesis (Schüler and Baeuerlein 1996), and is the limiting source for consumption of nitrogen and iron in the later phase of growth (Naresh et al. 2012).

**Gene expression**

*Genes associated with oxygen metabolism*

Oxygen is a major factor in magnetosome formation and changes in oxygen tension can induce adaptive transcriptional changes in MSR-1 cells (Wang et al. 2016). *katG* and *sodB* are two typical oxidative stress response genes, encoding catalase-peroxidase and superoxide dismutase, respectively (Qi et al. 2012).
Interestingly in the current study, the addition of iron resulted in dramatic reduction in katG expression throughout fermentation, regardless of oxygen conditions (Fig. 4). This observation indicated that iron repressed expression of the katG gene in MSR-1 cells is probably used as an indirect protection against oxidative stress and the real mechanism needs to be investigated further. In contrast, the addition of iron led to a rapid influence on sodB expression (Fig. 4A). Just 0.25 h after addition, the gene was ca. 1.5 fold overexpressed in the aerobic fermentation, ca. 0.5 fold reduced in the 0.5% O₂ fermentation and essentially unaffected in the 5% O₂ fermentation. These differences became less as the fermentation progressed (Fig. 4). This suggests that sodB is involved in the rapid response to DO concentration and this effect was trigged by iron addition, likely to be associated with iron uptake in MSR-1 cells. It would be interesting to investigate the exact role of sodB in the future.

MgFnr was reported as an oxygen sensor with the role of controlling magnetite biomineralization by regulation of denitrification in MSR-1 cells (Li et al. 2014). In the current study, however, there is no significant (p < 0.05) difference in expression level of MgFnr between the three oxygen conditions throughout fermentations. This suggests that the expression of MgFnr was neither affected by iron addition nor by different DO levels studied, indicating that the magnetite biomineralization may also be regulated by other unknown oxygen sensors. Our finding is in agreement with Li et al. 2014, where MgFnr was only involved in the expression of denitrification genes but not genes encoding oxygen respiration enzyme. The authors therefore concluded that MgFnr plays an indirect role in maintaining proper redox conditions required for magnetite biomineralization.

Genes associated with iron metabolism

Iron is another major factor during magnetosome formation. The cells accumulate a total iron content that is at least 100 times higher than that found in commonly studied bacteria such as Escherichia coli (Komeili 2012). Previous studies reported two ferrous iron transport genes in MSR-1, namely feoB1 (Rong et al. 2008) and feoB2 (Rong et al. 2012). The feoB1 was reported to have a major role in transporting ferrous iron compared to feoB2 (Rong et al. 2008). The latter only participates in magnetosome formation when the
feoB1 is deleted (Rong et al 2012). In the current study, there were significant difference across different conditions for expression of feoB1 at p < 0.05; that is, the expression was increased dramatically in the aerated culture and was reduced in the 0.5% O₂ culture at each time point except for 24 h (Fig. 4). In contrast, under 5% O₂ condition the addition of iron had little impact on feoB1 expression throughout the fermentation. These finding indicate the expression of feoB1 is greatly affected by oxygen conditions and feoB1 may play a significant role in sensing oxygen immediately following the presence of iron. Although the regulation mechanism is not clear, the result is partly consistent with the findings of Xu et al. 2016, who reported a down-regulation of feoB1 in 24 h cells grown in low oxygen condition (0.5% dO₂) compared to highly aerated cells (30% dO₂). In contrast, expression pattern of feoB2 showed reduced expression after the addition of iron, irrespective of oxygen conditions. At 0.25 and 2 h, the expression level was significantly (p < 0.05) reduced under 5% and 0.5% O₂ conditions compared to aeration cultivation, respectively, whilst the difference was less at 15 and 24 h. This result was in agreement with the observation of Rong et al. 2012, indicating feoB2 is not active in the process of magnetosome synthesis in the presence of feoB1. Despite this, the findings here are unexpected according to previous studies, since expression levels of both feoB1 and feoB2 (in very low oxygen condition) were observed to be reduced following iron addition. Nevertheless, it should be noted that we use a different approach to study gene function than previous workers which might therefore give different results compared to previous studies. The difference in the results may be explained by the way of cultivating cells and controlling oxygen levels. For example, in our study gene expression was analysed after the addition of iron to non-magnetic MSR-1 cells whereas a constant iron source was present in Wang et al 2016; we conducted fermentation at controlled oxygen level of 5% or 0.5% O₂ whilst shaking flasks (i.e. aerobic conditions) were used in an earlier study (Wang et al 2013). In addition, Rong et al 2008 and Rong et al 2012 studied the role of feoB1 and feoB2 by construction of feoB1-deficient and feoB2-deficient mutants, respectively. In view of this, these findings may suggest that another level of genetic regulation may exist in MSR-1 cells to regulate the uptake and transport of ferric iron during biosynthesis of magnetosomes.
Fur was identified as a regulator involved in global iron homeostasis (Uebe et al. 2010) and Qi et al. 2012 reported that this protein can directly regulate the four genes including _feoB1, feoB2, katG_ and _sodB_. The authors also demonstrated that Fur interacts with these genes via binding to their promoters. Previous studies have shown an increased expression of _fur_ in a high-iron fermentation using shaking flask (Wang et al. 2013), and an up-regulation of a _fur_ family gene (MGMSRv2_1721) in a fed-batch fermenter with constant iron supply (Wang et al. 2016). Unlike the experimental set-up in previous studies (Wang et al. 2013 and 2016), we showed the expression pattern of _fur_ gene following the addition of iron in the current study, where interestingly a depressed expression of _fur_ gene was observed at immediate-early stage and throughout different fermentations. In particular, at 0.25 h expression of _fur_ was significantly (p < 0.05) decreased at 5% O₂ compared to aeration condition, whilst at 2 h and 15 h the expression was significantly (p < 0.05) reduced at 0.5% O₂ than aeration condition. Nevertheless, the expression was observed to become reduced in general as the fermentation progressed irrespective of oxygen conditions, indicating that the Fur protein may be more important in other cellular biochemical processes. Moreover, this may suggest that the binding of Fur to the promoters of genes as _katG_ and _sodB_ (Qi et al., 2016) is not directly controlled by the transcription of _Fur_, but that other proteins could be involved in the immediate regulation of the binding of Fur to these promoters.

Moreover, the carbon source was depleted in all cases by 24 h, and by 15 h in the case of the 5% and 0.5% O₂ conditions (Fig. 2A). For some of the genes studied such as _sodB, MgFnr, feoB1_ and _Fur_, expression in general declined during the fermentation (Fig. 4CD), and seemed to coincide with the exhaustion of carbon source, which is to be expected for many genes. In view of this, the large error observed in a couple of samples at 24 h (Fig. 4D) may be explained by cell lysis or declined cell viability. Furthermore, our study is not exhaustive, and future research should continue to refine relationships investigated in the present study.

Firstly, although the present study conducted fermentations at controlled DO level of 5% and 0.5% O₂, a direct correlation of the actual concentration of oxygen with DO in the fermentation medium used would be helpful to understand the absolute oxygen level in the culture and to investigate the discrepancy of different studies. However, very sensitive oxygen electrodes would be needed for such a study. Secondly, there is a
difference in sampling handling time before quenching as we centrifuge the sample for 5 min compared to 1 min (Wang et al. 2013). This could therefore have an impact on RNA transcripts and may partly explain the differences found compared to other work. Nevertheless, the RNA samples were handled minimally and kept cold at all times. Internal reference gene was also used for normalisation. These can partly limit the effect of change during sample handling. It should also be noted other workers also seem to have longer processing times than 1 min (e.g. Schübbe, 2006 and Qi, 2012).

**Conclusion**

To the best of our knowledge, we show for the first time the impact of iron addition on the genetic response of *M. gryphiswaldense* MSR-1 cells during fermentations and the significance was observed from just 0.25 h following the addition of iron. In particular, expression of *sodB* and *feoB1* were dramatically reduced at 0.5% O₂ conditions compared to higher oxygen conditions, whereas no clear effect was observed on the expression of *MgFnr* and *fur*. In contrast, expression of *feoB2* and *katG* was negatively affected by iron addition, irrespective of oxygen conditions. These findings demonstrated immediate-early gene expression in MSR-1 cells associated with environmental changes (*i.e.* iron addition and DO concentration), and the specific role of these genes in bacterial magnetite biomineralization needs to be further investigated.

**Conflict of interest** The authors declare no conflict of interest.

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Fig. 1. Effect of dissolved oxygen (DO) concentration and addition of iron on cell growth of *M. gryphiswaldense* MSR-1. Cell growth (A) was determined using optical density reading at 565 nm, and DO was monitored continuously over time. Blue: aerobic; red: 5% O₂; green: 0.5% O₂. In each instance, agitation (120 rpm), temperature (28 °C), gas flow rate (1.0 L/min) and pH (7.0) were kept constant. Each fermentation condition was performed in duplicate.
Fig. 2. Utilisation of lactate (A) and iron (B) during different fermentations. Blue bar: aerobic; red bar: 5% O₂; green bar: 0.5% O₂. Data represents the mean with range from two independent experiments, each performed in duplicate.
**Fig. 3.** TEM images of whole cells (A) and magnetosomes (B) at 24 h under 0.5% O$_2$ cultures. More than 20 micrographs were captured and two representative images were presented here.
**Fig. 4.** Relative expression level of key genes associated with oxygen metabolism (katG, sodB and MgFnr) and iron metabolism (feoB1, feoB2 and fur) at 0.25 h (A), 2 h (B), 15 h (C) and 24 h (D) under different fermentations. Blue bar: aerobic; red bar: 5% O2; green bar: 0.5% O2. Data represents the mean with range.
from two independent experiments, each performed in duplicate. At every time point, the expression difference of each gene between three fermentation conditions was accessed by the Analysis of Variance (ANOVA) coupled with Tukey’s test. Bars bearing different letters for one gene are significantly different, same letters indicate no differences (p<0.05).

Table 1 Summary of genes, functions, and primer sequences (5’ to 3’) used in this study.

<table>
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<th>Gene</th>
<th>Locus tag</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
<th>Reference</th>
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<td>MgFnr</td>
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<td>GGAAACCGTGTGGGCGA</td>
<td>AACTGGGTAAGGTCCGCGG</td>
<td>Li et al, 2014</td>
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<td>feoB2</td>
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