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ORIGINAL PAPER

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Growth and magnetosome formation by microaerophilic *Magnetospirillum* strains in an oxygen-controlled fermentor

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Abstract Media and growth conditions were optimized for the microaerobic cultivation of *Magnetospirillum gryphiswaldense* in flasks and in a fermentor, resulting in significantly increased cell and magnetosome yields, compared with earlier studies. A reliable method was established for the automatic control of low dissolved oxygen tensions (pO_2) in the fermentor (oxystat). Growth and magnetosome formation by *M. gryphiswaldense*, *M. magnetotacticum* and *Magnetospirillum* sp. AMB-1 were studied at various oxygen concentrations. Despite differences in their growth responses with respect to oxygen, we found a clear correlation between pO_2 and magnetosome formation in all three *Magnetospirillum* strains. Magnetite biomineralization was induced only below a threshold value of 20 mbar O_2 and optimum conditions for magnetosome formation were found at a pO_2 of 0.25 mbar (1 bar = 10^5 Pa). A maximum yield of 6.3 mg magnetite l^{-1} day^{-1} was obtained with *M. gryphiswaldense* grown under oxystat conditions, which is the highest magnetosome productivity reported so far for a magnetotactic bacterium. In conclusion, the presented results provide the basis for large-scale cultivation of magnetospirilla under defined conditions.

Introduction

Magnetotactic bacteria are capable of synthesizing specific intracellular structures, the magnetosomes (Bazylinski 1995; Schüler 1999). In most magnetotactic bacteria, magnetosomes comprise membrane-bound crystals of the magnetic iron mineral magnetite (Fe_3O_4), which are aligned in chain-like structures within the cell (Balkwill et al. 1980).

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The bacterial magnetosomes are characterized by narrow size distributions and uniform, species-specific crystal habits, which are unknown from magnetite particles produced abiotically (Moskowitz 1995). Because of the unique characteristics of bacterial magnetosomes, their formation is of significant technological interest. Biomineralization may provide a way to produce highly uniform, nano-scaled magnetite crystals (Sarikaya 1999). Their perfect magnetic and crystalline properties, the presence of an enveloping membrane and their large surface-to-volume ratio make magnetosomes potentially useful as a highly ordered biomaterial in a number of applications, e.g., in the immobilization of bioactive compounds, magnetic drug targeting, or as a contrast agent for magnetic resonance imaging (for reviews, see Matsunaga and Takeyama 1998; Schüler and Frankel 1999; Safarik and Safarikova 2002). Although the biotechnological potential of bacterial magnetosomes has been demonstrated, no application has been exploited on a commercial scale, which is mainly due to the problems associated with mass production of magnetosomes. So far, only a few magnetotactic bacteria are available in pure culture; and most of the isolates are difficult to grow and poorly characterized because of their fastidiousness. Consequently, most studies concerned with biomineralization and the isolation of bacterial magnetosomes have focused on a limited number of strains of the genus *Magnetospirillum* that are available from strain collections (Blakemore et al. 1979; Matsunaga et al. 1991; Schleifer et al. 1991; Schüler and Köhler 1992).

M. magnetotacticum was described as an obligate microaerophile, which did not grow in medium with free exchange to air (Blakemore et al. 1979). Magnetosome formation was observed only at low initial atmospheric oxygen tensions, when the cells were cultivated in sealed serum vials (Blakemore et al. 1985). Similar results were obtained with *Magnetospirillum* sp. strain AMB-1. Although originally described as a magnetic bacterium capable of growing aerobically (Matsunaga et al. 1991), magnetosome formation and optimum growth in strain

AMB-1 were later reported to occur only under microaerobic conditions (Yang et al. 2001).

M. gryphiswaldense produces a chain of cubo-octahedral magnetosome particles, which resemble those of other *Magnetospirillum* strains. The strain has been used as a model organism in a number of studies addressing the physiology and molecular genetics of magnetosome biomineralization and for the development of applications of magnetosomes (Schüler and Baeuerlein 1996, 1998; Baeuerlein et al. 1998; Schüler 2000; Grünberg et al. 2001). For *M. gryphiswaldense*, a low dissolved oxygen concentration (pO_2) of 2–7 μM O_2 (equivalent to 1.7–6.0 mbar O_2; 1 bar = 10^5 Pa) was found necessary for the induction of magnetosome formation (Schüler and Baeuerlein 1998). However, the fermentor system used in that study lacked any means for the continuous control of oxygen, which limited both the cell and magnetite yields.

Both for the biochemical and biophysical characterization of magnetosomes and for their application, it is required to obtain high yields of magnetosomes from large quantities of cells cultivated under defined growth conditions. This study was initiated in an effort to establish optimum conditions for the mass cultivation of the microaerophilic *M. gryphiswaldense* in flasks and in a fermentor. To determine and control optimum conditions for magnetosome production, we developed a reliable oxystat system for the automatic control of low oxygen tensions, which allowed the precise regulation of low pO_2 values independent from the oxygen consumption of the growing culture in the fermentor. In addition, we assessed the potential for mass cultivation and magnetosome production of the three strains of *Magnetospirillum* that have been used in cultivation experiments before.

Materials and methods

Bacterial strains

M. gryphiswaldense strain MSR-1 (DSM 6361; Schleifer et al. 1991; Schüler and Köhler 1992), *Magnetospirillum* sp. strain AMB-1 (ATCC 700264; Matsunaga et al. 1991), and *M. magnetotacticum* strain MS-1 (ATCC 31632; Blakemore et al. 1979) were used in this study (referred to as MSR-1, AMB-1, and MS-1, respectively).

Flask cultivation

All *Magnetospirillum* strains were cultured in the following flask standard medium (FSM), which was optimized during this study. It contained (per liter deionized water) 0.1 g KH_2PO_4, 0.15 g $MgSO_4 \cdot 7H_2O$, 2.38 g Hepes (Biomol, Hamburg, Germany), 0.34 g $NaNO_3$, 0.1 g yeast extract (Serva, Heidelberg, Germany), 3 g soy bean peptone (Merck, Darmstadt, Germany), and 1 ml EDTA-chelated trace element mixture (Widdel and Bak 1992). Unless indicated otherwise, the medium contained 27 mM potassium L-lactate as carbon source. Iron was added before autoclaving as ferric citrate (100 μM) and the pH of the medium was adjusted to 7.0 with NaOH. If not specified otherwise, all chemicals (analytical grade) were purchased from Fluka (Neu Ulm, Germany). Gases were from Messer-Griesheim (Krefeld, Germany).

Cultivation of all *Magnetospirillum* strains was carried out under aerobic and microaerobic conditions in 1-l flasks containing 500 ml FSM. For aerobic cultivation, cells were incubated in free gas exchange with air. To generate microaerobic conditions, flasks were sealed before autoclaving with butyl-rubber stoppers under a microaerobic gas mixture containing 1% O_2 in 99% N_2. The microaerobic cultures were inoculated by injection through the stopper. Non-magnetic cells, which had been pre-grown under microaerobic conditions in FSM devoid of ferric citrate and trace element mixture, were used as an inoculum. Initial cell densities after inoculation were approximately 6.0×10^6 cells ml^{-1}. All cultures were cultivated at 28 °C and agitated at 100 rpm for 24–48 h in an incubator shaker.

Oxystat cultivation

For cultivation in the fermentor, Hepes, yeast extract, and trace element mixture were omitted from FSM, resulting in a medium termed large-scale medium (LSM). Iron was added to the LSM as ferric citrate in the range 100–150 μM. Foaming was suppressed by the addition of 0.02 ml polypropylene glycol (P2000; Fluka, Neu Ulm, Germany) per liter of medium. Growth experiments of *Magnetospirillum* strains at various oxygen tensions were performed in a modified dual-vessel laboratory fermentor system (Biostat A Twin; B. Braun Biotech International, Melsungen, Germany) equipped for the automatic control of pH, temperature, and dissolved oxygen concentration. The pH of the culture was controlled by the addition of either H_2SO_4 (1 M) or NaOH (1 M) via peristaltic pumps. The oxygen concentration in the liquid phase was continuously measured using an oxygen probe (InPro 6000; Mettler Toledo, Gießen, Germany). After autoclaving the filled vessels (121 °C for 30 min), nitrogen was sparged through the medium during cooling overnight to obtain oxygen-free conditions. After the desired pH and temperature values were adjusted, the nitrogen flow was discontinued and the medium was gassed up to saturation with either a microaerobic gas mixture (1% O_2) or air (flow rate 3 l min^{-1}), to calibrate the dissolved oxygen probe and detector. After the desired pO_2 was adjusted, growth experiments were initiated by the inoculation of 400 ml non-magnetic flask-cultures into 4 l LSM. Initial cell densities after inoculation were approximately 9.0×10^6 cells ml^{-1}. The inoculum was transferred to the medium via the harvest pipe, using a peristaltic pump. To prepare uniform non-magnetic inocula, cells of the *Magnetospirillum* strains were pre-incubated under microaerobic conditions in FSM devoid of ferric citrate and trace element mixture. Agitation (150 rpm), temperature (28 °C), and pH (7.0) were kept constant.

The fermentor system used in this study was specifically adapted for the microaerobic cultivation of microaerophilic bacteria under oxystat conditions, by several modifications described in the following. Previous experiments showed that the standard types of integrated amplifier for the measurement of dissolved oxygen tensions were not sensitive enough for our application. The installation of more susceptible oxygen amplifiers (input responsivity was increased from 200 nA to 20 nA) in combination with the integration of accessory equipment for the gas supply allowed the establishment of a highly sensitive pO_2-controlling regime in the microaerobic range. Defined low pO_2 tensions were regulated automatically by a cascade control via separate and independent gassing with nitrogen and air. For control of the nitrogen supply, a flowmeter (flow rate 25–250 l h^{-1}; DK 46 N; Krohne, Duisburg, Germany) in line with a pulsed solenoid gas control valve (Bürker, Ingelfingen, Germany) were integrated via a bypass line in the gas inlet pipe. The air supply was regulated via a thermal massflow controller (flow rate 0.1–3 l min^{-1}; BRA-001F; Bronkhorst, Ruurlo, Netherlands) in combination with a further pulsed solenoid control valve. All control valves were connected to and operated by the oxygen control system. The switch between nitrogen and oxygen gassing occurred in response to the actual pO_2 in the medium. When the actual pO_2 was 0.5% higher than the set value, nitrogen was sparged through the medium at a constant flow rate of 150 l h^{-1} by triggering the pulsed solenoid control valve. When the actual

pO_2 fell below 99.5% of the set value, the oxygen concentration in the medium was regulated by varying the aeration rate via the massflow controller, which allowed an automatic, continuous control of the air supply, according to the actual oxygen consumption in the process. To achieve precise regulation of low pO_2 tensions during the early growth phase, it was necessary to introduce a further pulsed solenoid gas valve in line with the massflow controller. When oxygen was required, the solenoid valve was activated to open the gas line and then the pO_2 in the medium was fine-tuned via the massflow controller as described.

Analytical methods

Cell growth and magnetism were measured turbidimetrically, using the optical density at 565 nm (OD_{565}). The average magnetic orientation of cell suspensions (magnetism) was assayed by an optical method, as described by Schüler et al. (1995). Briefly, cells were aligned at different angles relative to the light beam by means of an external magnetic field. The ratio of the resulting maximum and minimum scattering intensities (C_{mag}) was previously demonstrated to be well correlated with the average number of magnetic particles per cell and can be used for semi-quantitative assessment of magnetite formation (for practical purposes, C_{mag}=0 was assumed for non-magnetic cells).

Iron measurements were made using an atomic absorption spectrometer (model 3110; Perkin-Elmer, Überlingen, Germany). All measurements were made by using an acetylene/air flame as described by Grünberg et al. (2001). For iron analysis, 1-ml culture samples were harvested by centrifugation (11,300*g* for 5 min) in polypropylene screw-cap tubes (Eppendorf, Hamburg, Germany). The culture supernatants were decanted, acidified to pH 2–3 with 10 µl of nitric acid (65% wt/vol) and analyzed directly. After washing with 20 mM Hepes/4 mM EDTA, pH 7.4, cell pellets were resuspended in 100 µl nitric acid (65% wt/vol) and incubated at 98 °C for 2 h in sealed screw-cap tubes. The resulting clear cell digest was brought up to 900 µl with distilled water before analysis. A reagent blank was prepared in the same manner.

The dry mass of *M. gryphiswaldense* cells was determined after washing with ammonium acetate (20 mM) and drying the cell pellet to constant weight at 60 °C. A culture density of OD_{565}=1 corresponded to 0.28 g dry weight l^{-1}. The dry weights of the other *Magnetospirillum* strains were calculated based on the same ratio.

For electron microscopy, *M. gryphiswaldense* cells from concentrated suspensions were adsorbed onto a 300-mesh carbon-coated copper grid (Plano, Wetzlar, Germany) and rinsed twice with distilled water. Samples were viewed and recorded without staining, using an EM 301 transmission electron microscope (Philips, Eindhoven, Netherlands) at an accelerating voltage of 80 kV.

Results

Growth of different *Magnetospirillum* strains in flasks

The composition of FSM used for flask cultivation was optimized for maximum cell and magnetite yield in a number of growth experiments with *M. gryphiswaldense* (data not shown). Modifications of the previously reported medium (Schüler and Baeuerlein 1996, 1998; Grünberg et al. 2001) include the use of lactate and nitrate as sources of carbon and nitrogen, respectively. In addition, growth at 28 °C and pH 7.0 was found to be optimal for magnetite production, whereas cultivation at higher temperatures (28–34 °C) or at higher initial pH values (7.0–7.5) led to a slightly improved growth, but a significant reduction in magnetite formation. To optimize the oxygen supply, we studied the effect of different initial atmospheric oxygen concentrations on growth and magnetite formation in the three selected *Magnetospirillum* strains. Results of the growth experiments are summarized in Table 1.

Under microaerobic conditions, all three strains reached comparable final cell densities after 24 h cultivation. All cultures were magnetic and accumulated significant amounts of iron, corresponding to a consumption of about 20% of the dissolved iron from the growth medium. The highest intracellular iron accumulation (2.2% of dry weight) was obtained under these conditions for strain MSR-1. Under aerobic conditions, growth was detected only for strain MSR-1. The aerobic MSR-1 culture grew with a comparable rate and reached a similar OD_{565} value after 24 h, compared with the microaerobic culture. In contrast, aerobically grown cells were non-magnetic at that time (data not shown). However, after 48 h cultivation, weak magnetism could be detected, probably indicating that microaerobic conditions arose in the medium at higher cell densities by oxygen consumption of cells.

Establishing of an oxystat for the controlled microaerobic cultivation of *M. gryphiswaldense*

Growth of strain MSR-1 in flasks was significantly enhanced by increased oxygen supply. However, increased aeration led to a decrease in magnetite formation

Table 1 Final cell yield and magnetism in cells of *Magnetospirillum* strains (MSR-1, AMB-1, MS-1) grown under aerobic and microaerobic conditions. Cells were cultivated in 1-l flasks containing 500 ml flask standard medium, either with 1% O_2 in the headspace (microaerobic; final cell yields were determined after 24 h), or in free gas exchange with air (aerobic; final cell yields were determined after 48 h). – No growth, C_{mag} magnetic scattering intensity, OD_{565} optical density at 565 nm

	Microaerobic (1% O_2)			Aerobic		
	MSR-1	AMB-1	MS-1	MSR-1	AMB-1	MS-1
Growth (ΔOD_{565})	0.20	0.23	0.21	0.58	–	–
Magnetism (C_{mag})	1.2	1.1	0.9	0.7	–	–
Iron concentration of the medium after growth (µM)	81.0	80.0	83.0	69.0	100.0	100.0
Iron content of the biomass (mg g^{-1} dry weight)	21.9	14.7	16.7	10.8	–	–

Table 2 Effect of various medium compounds on final cell yield and magnetism of *M. gryphiswaldense* grown in the oxystat at a pO_2 value of 2 mbar. Unless otherwise indicated, the medium contained 27 mM lactate, 4 mM nitrate and 100 μM ferric citrate. Growth and magnetism were determined after 24–26 h cultivation. Cultures were initiated with an inoculum that had been grown by three sequential transfers on the respective carbon or nitrogen source

Nutrient	Concentration	Growth (ΔOD_{565})	Magnetism (C_{mag})
Carbon source			
Lactate	17 mM	0.84	1.2
Lactate	27 mM	1.36	1.2
Lactate	40 mM	1.33	1.2
Pyruvate	27 mM	1.34	1.2
Acetate	27 mM	0.83	0.7
Succinate	27 mM	0.36	0.8
Nitrogen source			
NH_4Cl	4 mM	1.48	0.5
$NaNO_3$	2 mM	1.19	1.2
$NaNO_3$	4 mM	1.33	1.1
$NaNO_3$	10 mM	1.39	0.8
$NaNO_3$	20 mM	1.36	0.6
Iron source			
Fe(III)citrate	0 μM	1.34	0.3
Fe(III)citrate	100 μM	1.35	1.1
Fe(III)citrate	150 μM	1.40	1.1

in this organism. In contrast, low initial oxygen concentrations clearly limited the growth of higher cell densities in static microaerobic cultures. Thus, to achieve optimum conditions for both growth and magnetite formation, it was necessary to establish cultivation conditions providing the continuous supply of low pO_2 concentrations, which is extremely difficult to realize with flask cultures. Therefore, we established an oxystat system for the cultivation of microaerophilic magnetospirilla under defined microaerobic pO_2 tensions.

By the development of this sophisticated gas control regime, as described in Materials and methods, pO_2 tensions ranging over 0.25–212 mbar (i.e., air saturation) could be maintained exactly over a wide range of cell densities during exponential growth, with fluctuations below 5% of the set value. For the lowest pO_2 (0.25 mbar), a slightly higher deviation of 8% was obtained. During the growth experiments (incubation up to 56 h), the oxygen probe showed no significant drift, even at low pO_2 tensions. These values were in the same range as obtained by Becker et al. (1996) for the oxystat cultivation of *Escherichia coli*, which, however, grew much faster and required shorter incubation times. Compared with the oxystat setup described by Becker et al. (1996) and Arras et al. (1998) using static regulation by alternating valves, the dynamic control of the air supply by use of thermal massflow controllers allowed compensation for increasing oxygen consumption over a wide range and made manual regulation dispensable. Thus, this method proved appropriate to cultivate microaerophilic bacteria at controlled low pO_2 values.

Optimization of growth medium for large-scale cultivation of *M. gryphiswaldense*

Preliminary experiments showed that growth characteristics in the fermentor significantly differed from those observed in flask experiments. Therefore, the medium composition was specifically optimized for fermentor cultivation of strain MSR-1 by testing the growth response to several carbon, nitrogen, and iron sources, and other supplements. These experiments were performed at a constant low pO_2 tension of 2 mbar. The results are summarized in Table 2.

The addition of yeast extract and trace elements was found to be dispensable under oxystat conditions and therefore was omitted from the medium in further experiments. However, both growth rate and final cell yield were significantly improved by supplementing the medium with soy bean peptone, although peptone apparently could not be used as a single source of carbon, nitrogen, vitamins, or amino acids (data not shown). The enhancement of growth by complex medium components, such as peptone and casein hydrolysates, has been also described for other microaerophilic bacteria (Krieg and Hoffman 1986; Yang et al. 2001). Although the reason for the growth-stimulating effect of peptone is not clear, it might be speculated that its effect is in modulating the redox potential, scavenging toxic forms of oxygen, or chelating iron in the medium.

Optimum growth was obtained with either lactate or pyruvate as the sole source of carbon. Concentrations higher then 27 mM did not further increase the cell yield. Both ammonium and nitrate could serve as sole nitrogen source for growth of strain MSR-1. However, the presence of nitrate significantly increased magnetite formation, with an optimum concentration of 4 mM. Higher concentrations (10–20 mM) resulted in a reduced magnetite formation but did not affect cell yield (Table 2). For comparison, initial nitrate concentrations above 4 mM inhibited growth of strain AMB-1 (Matsunaga et al. 1996).

In the absence of an added iron source, the LSM contained iron only in amounts of approximately 5 μM. While growth rates and final OD_{565} values were similar to iron-enriched LSM (Table 2), cells were only weakly magnetic under these conditions, indicating that mag-

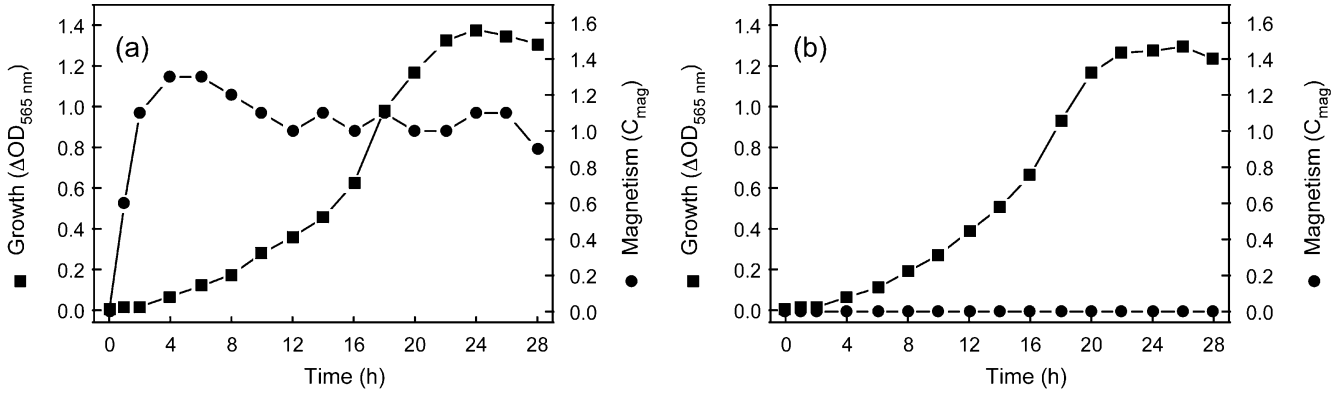

Fig. 1a, b Growth and magnetite formation in oxystat cultures of *Magnetospirillum gryphiswaldense* incubated at controlled dissolved oxygen tensions of 2.0 mbar (**a**) and 20 mbar (**b**). The experiments were started by inoculation of non-magnetic cells. C_{mag} Magnetic scattering intensity, *mbar* millibars (1 bar = 10^5 Pa), $OD_{565\ nm}$ optical density at 565 nm

netite biomineralization was limited by the extracellular iron concentration, as described by Schüler and Baeuerlein (1996). An iron supply of 100 μM ferric citrate was found to be saturating for both growth and magnetite formation, when cells were cultivated at a pO_2 tension of 2 mbar. The addition of higher iron concentrations (Table 2) and the use of alternative iron sources (data not shown) did not further improve growth and magnetite formation. However, as 90% of the medium iron was depleted after growth, the initial iron concentration in the LSM was increased to 150 μM to avoid iron limitation in further mass cultivation experiments.

Despite our efforts to further improve growth yields in the fermentor, neither supplementation with higher amounts of various nutrients nor the increased supply of oxygen ever resulted in higher cell densities. This indicates that the reason for growth limitation apparently was neither nutrient deficiency nor accumulation of toxic waste products, as spent culture fluids of oxystat cultures supported further growth of strain MSR-1. In summary, under oxystat conditions using the LSM, maximum cell yields of about 0.40 g dry weight l^{-1} were obtained with strain MSR-1, compared with 0.33 g dry weight l^{-1} as reported earlier for this organism (Schüler and Baeuerlein 1997).

Effect of oxygen tension on growth and magnetite formation in *M. gryphiswaldense*

After growth conditions for the large-scale cultivation of strain MSR-1 were established, the effect of pO_2 tension on growth and magnetite formation was studied in more detail. In an initial experiment, cells of strain MSR-1 were cultivated in the oxystat at constant pO_2 tensions of 2 mbar and 20 mbar, respectively (Fig. 1). At both pO_2 values, growth proceeded after a lag of 4 h, with a similar generation time of about 5.2 h, and comparable final cell densities were reached. However, magnetite was formed only in cells grown at pO_2 values of 2 mbar (Fig. 1a). In this culture, non-magnetic cells of the inoculum started to produce magnetite immediately upon transfer into the fermentor. A transient maximum in cellular magnetism was detectable at the end of the lag phase, which gradually decreased during the exponential growth phase. In contrast, the culture grown at pO_2 values of 20 mbar did not produce magnetite at any stage of growth (Fig. 1b).

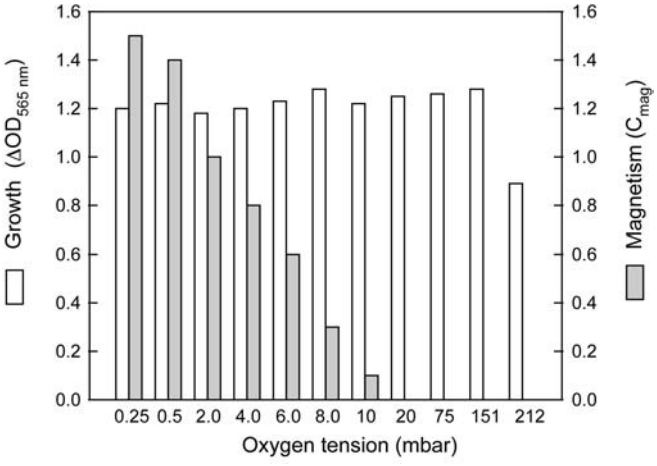

Fig. 2 Effect of various constant dissolved oxygen tensions on growth and magnetite formation in oxystat cultures of *M. gryphiswaldense*. Growth and magnetism were determined after 22 h cultivation in large-scale medium (LSM)

To determine the optimum pO_2 tension for both growth and magnetite formation, cells of strain MSR-1 were cultivated in the oxystat at various pO_2 tensions, ranging over 0.25–212 mbar. Cell densities and cellular magnetism determined at the end of the exponential growth phase (after 22 h) are shown in Fig. 2. Despite their microaerophilic character, cells grew under all conditions tested; and no differences in growth rates or final cell yields could be detected over a wide range of pO_2 tensions. High pO_2 tensions (>151 mbar) resulted in a prolonged lag phase. Under these conditions, cells were

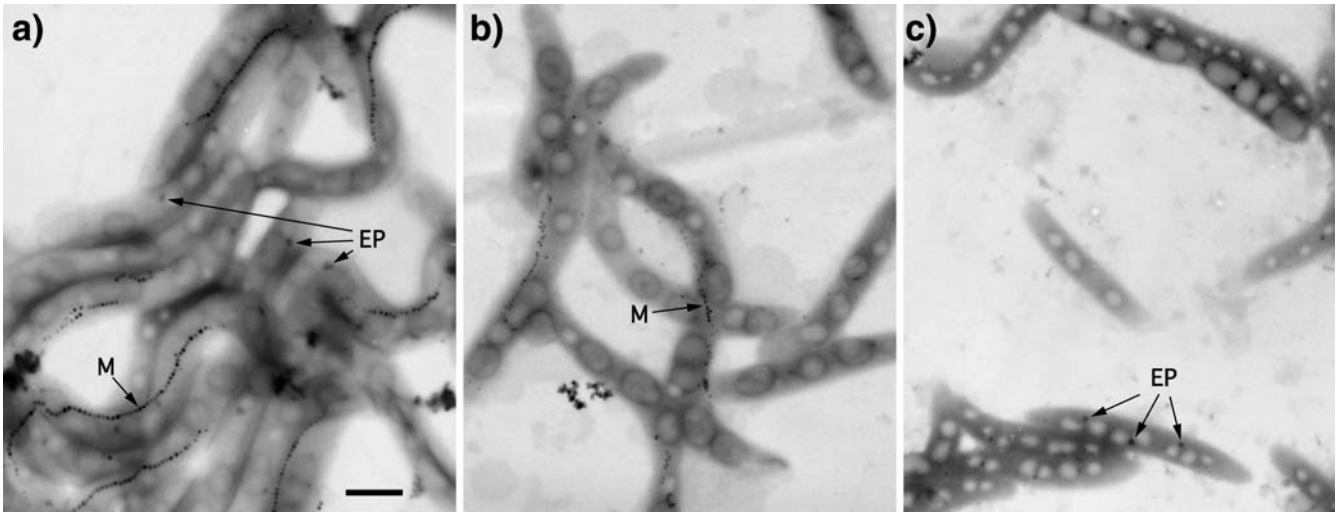

Fig. 3a–c Electron micrographs of *M. gryphiswaldense* cultures grown in the oxystat at defined pO_2 tensions of 0.25 mbar (**a**), 10 mbar (**b**), and 20 mbar (**c**). *Arrows* indicate the magnetosome chains (*M*) and the electron-dense particles (*EP*) found in magnetic and non-magnetic cells. The bright inclusions represent polyhydroxyalkanoate globules. *Bar* 0.5 μm

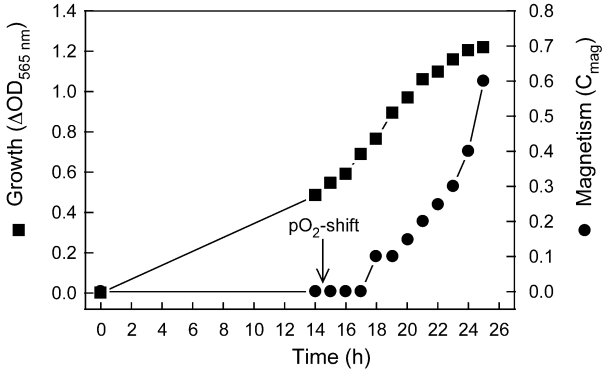

Fig. 4 Growth and magnetism of *M. gryphiswaldense* during oxystat cultivation at different pO_2 tensions in LSM. After 14 h, the pO_2 tension was shifted from 20 mbar to 2 mbar (*arrow*)

abnormally long and less motile. Magnetite formation was detectable only at pO_2 values below 20 mbar. Cultivation at lower pO_2 tensions resulted in a gradually increased cellular magnetism. Maximum magnetite formation was found at a pO_2 tension of 0.25 mbar. Electron microscopy revealed that these cultures on average contained 35 mature magnetosomes (42 nm in diameter) per cell, with a maximum of 54 crystals (Fig. 3a). In contrast, cells from weakly magnetic cultures grown at a pO_2 tension of 10 mbar contained only 14 magnetosome particles on average (Fig. 3b). Under these conditions, the magnetic crystals also were smaller (approximately 20 nm) and distributed irregularly in the cell. In electron micrographs of non-magnetic cultures grown at pO_2 values of 20 mbar, no magnetosome-containing cells could be detected (Fig. 3c).

Both magnetic and non-magnetic cells grown in the fermentor were occasionally found to contain a variable number (0–10) of electron-dense particles, which are likely to be identical with polyphosphate granules, because of their appearance (Fig. 3).

To closer investigate the induction of magnetite formation by low pO_2 concentrations, cells of strain MSR-1 were shifted from a pO_2 tension of 20 mbar to 2 mbar during exponential growth in iron-rich LSM (Fig. 4). As observed before, cells of strain MSR-1 did not produce magnetite in the presence of a pO_2 tension of 20 mbar. After shifting to a pO_2 value of 2 mbar, growth proceeded at an apparently unchanged rate and cells remained non-magnetic for several hours. Weak cellular magnetism became detectable only after 4 h; and this increased until stationary growth. The observed lag in magnetite formation suggests that the machinery involved in magnetite biomineralization is repressed by a high oxygen partial pressure and its induction is likely to require protein synthesis. In contrast, the presence of high extracellular iron concentrations apparently is not required for this induction, since non-magnetic cells that were pre-cultivated under microaerobic but iron-limited conditions immediately started to produce magnetite without a detectable lag upon inoculation into iron-rich, microaerobic (2 mbar pO_2) LSM, as shown in Fig. 1.

Growth of different *Magnetospirillum* strains under oxystat conditions

In a further set of oxystat experiments, we investigated the potential for mass cultivation and magnetite production of *Magnetospirillum* strains AMB-1 and MS-1 in comparison to strain MSR-1 under conditions of oxygen-controlled fermentation. All strains were grown at defined microaerobic pO_2 tensions in the range 0.25–20 mbar in the LSM previously modified for mass cultivation of strain MSR-1. Growth parameters were determined at the end of growth (after 30 h or 48 h of cultivation). The

Table 3 Results of oxystat experiments with *Magnetospirillum* strains. All strains were cultured at various pO_2 values in 4 l large-scale medium. Non-magnetic cells pre-cultivated in flasks under microaerobic conditions without an added iron source were used as inoculum. *dw* Dry weight

Strain	pO_2 (mbar)	Growth (ΔOD_{565})	Magnetism (C_{mag})	Average growth rate (h^{-1})	Cultivation time (h)	Biomass productivity (g dw l^{-1} day^{-1})	Iron content after growth	
							Medium (μM)	Cells (mg g^{-1} dw)
MSR-1	0.25	1.42	1.4	0.129	30	0.32	40	14.2
	2.0	1.43	1.0	0.131	30	0.32	64	10.5
	10	1.42	0.1	0.132	30	0.32	92	6.2
	20	1.45	0.0	0.131	30	0.33	109	5.3
MS-1	0.25	0.40	1.3	0.027	48	0.06	91	28.2
	2.0	1.08	1.1	0.092	48	0.15	97	9.5
	10	1.16	0.2	0.091	48	0.16	124	4.9
	20	1.05	0.0	0.086	48	0.15	121	4.8
AMB-1	0.25	1.70	1.2	0.111	48	0.24	64	9.8
	2.0	1.47	1.0	0.078	48	0.21	90	7.9
	10	1.28	0.2	0.072	48	0.18	115	5.7
	20	1.35	0.0	0.076	48	0.19	116	4.9

results of the comparative growth experiments are summarized in Table 3.

Growth of all *Magnetospirillum* strains occurred at all pO_2 tensions tested. However, growth characteristics differed significantly. Growth rates of strain MS-1 were relatively low, reaching high final cell densities of about OD_{565}=1.1 only after 48 h of cultivation. Growth of strain MS-1 was limited by low pO_2 concentrations of 0.25 mbar (Table 3). In contrast, the fastest growth of strain ABM-1 was found at a pO_2 of 0.25 mbar. After 52–56 h, final cell densities of about OD_{565}=2.5 were detected for this strain at the different pO_2 tensions (data not shown). Of all three strains, strain MSR-1 grew with the highest rate at all pO_2 tensions tested and gave approximately two-fold higher average final cell yields (0.4 g dry weight l^{-1}), compared with strains AMB-1 and MS-1 after 30 h cultivation (Table 3).

Like strain MSR-1, magnetite was produced by strains MS-1 and AMB-1 only at pO_2 tensions below 20 mbar and cellular magnetism gradually increased with further decreased oxygen supply (Table 3). In all three strains, the increase in cell magnetism coincided with a tremendous increase in iron accumulation. A maximum iron content of 2.8% on a dry weight basis was detected in the growth-limited culture of strain MS-1 at a pO_2 value of 0.25 mbar (Table 3). Magnetic cells of all three *Magnetospirillum* strains cultivated at a pO_2 of 0.25 mbar contained about two to five times more iron on a dry weight basis than non-magnetic cells grown at a pO_2 of 20 mbar. By the end of growth, more than 70% of the iron was consumed from the growth medium.

Discussion

Problems in the mass cultivation of magnetospirilla have arisen from their microaerophilic and fastidious growth characteristics. Initial studies indicated that optimum growth and magnetite formation occurred within a narrow range of oxygen concentrations in strains of *Magnetospirillum*, which required the use of elaborate microaerobic techniques. Reports of cultivation of microaerophiles at larger scales are rather scanty in the literature (e.g., Deshpande et al. 1995; Schüler and Baeuerlein 1998; Sabra et al. 1999; Yang et al. 2001). In most of these studies, the establishment of low oxygen levels were adjusted empirically and did not involve the continuous measurement and control of dissolved oxygen concentrations in the medium. In the current study, we established a method for the control of variable, low oxygen concentrations in the culture by the use of a fermentor system modified for oxystat operation. While growth of *M. gryphiswaldense* was unaffected by the oxygen concentration over a wide range, we found a clear correlation between the pO_2 tension and magnetite formation. Most favorable conditions for magnetosome formation were found at the lowest pO_2 of 0.25 mbar. Consistent with earlier studies (Blakemore et al. 1985; Schüler and Baeuerlein 1998; Yang et al. 2001), these results indicate

that magnetite biomineralization strictly depends on a low oxygen partial pressure. In a previous study using a fermentor system lacking oxygen control, the critical pO_2 was estimated as <10 mbar during a transition from aerobic to microaerobic conditions at high cell densities (Schüler and Baeuerlein 1998). In this study, we found a slightly higher pO_2 value of 10–20 mbar during cultivation at a constant low pO_2 tension and using more precise oxygen measurements. Although the molecular mechanism for the low-oxygen dependence of magnetite formation is not clear, the results of pO_2-shift experiments in *M. gryphiswaldense* indicate that oxygen is likely to serve as regulatory signal for metabolic induction of biomineralization. Interestingly, the threshold value for the induction of magnetite formation was found to be nearly identical in all three *Magnetospirillum* strains tested, despite differences in their growth characteristics in response to various oxygen concentrations.

Although all three magnetospirilla displayed a characteristic microaerophilic behavior (e.g., formation of microaerophilic bands in semi-solid agar, etc.), the individual levels of oxygen tolerance apparently are different. In flask experiments, strain MSR-1 was the most oxygen-tolerant organism and grew exposed to air, from large inocula. The inability of strain MS-1 to grow exposed to air is in agreement with earlier reports for this organism, which has been described as an extreme microaerophile (Blakemore et al. 1979, 1985). However, under medium and cultivation conditions used in this study, we were unable to confirm earlier reports describing strain AMB-1 as an organism capable of growing aerobically (Matsunaga et al. 1991). Surprisingly, strain MSR-1 eventually grew at concentrations equivalent to air saturation in the oxystat after a prolonged lag phase with otherwise nearly unaffected growth rates and cell yields, although this strain failed to grow from small inocula in aerobic flasks and did not form colonies on plates exposed to air using the same medium. This indicates that obligate microaerophiles like *Magnetospirillum* are capable of adaptation to higher pO_2 concentrations, possibly by alteration of the growth conditions in the medium by the inoculum.

Almost three-fold higher cell yields were obtained from strain MSR-1 in the fermentor compared with maximum yields of only weakly magnetic cells obtained from aerobic flasks. Although the cellular iron content of strain MSR-1 was slightly higher in microaerobic flask cultures, significantly higher magnetosome yields were obtained from oxystat-grown cultures because of the seven-fold higher cell yields reached under these conditions. The highest cell yield (0.7 g dry weight l^{-1}) was obtained from strain AMB-1, while the highest content of accumulated iron was found in strain MS-1 (2.8% of dry weight at a pO_2 of 0.25 mbar). If it is assumed that magnetosome-bound iron constitutes more then 99% of the total cellular iron in highly magnetic cells (Grünberg, personal communication), this iron content is equivalent to an amount of 39 mg magnetite (Fe_3O_4) per gram dry weight. Accordingly, the highest magnetite yields were obtained from strain MSR-1 (7.9 mg l^{-1} at a pO_2 of 0.25 mbar). The differences between the three strains in their capacity to produce magnetosomes become more obvious, when productivity (amount of magnetite produced per liter per day) is considered. Based on magnetite yields calculated from intracellular iron content, 6.3 mg magnetite l^{-1} day^{-1} were produced by strain MSR-1 in the oxystat at a pO_2 of 0.25 mbar, compared with 3.3 mg and 2.0 mg for strain AMB-1 and MS-1, respectively. This is a significant increase in productivity, compared with the highest value of 1.85 mg magnetosomes l^{-1} day^{-1} reported so far (Yang et al. 2001) during a fermentation experiment with strain AMB-1. In summary, the results of the growth experiments indicate that MSR-1 is the most appropriate magnetotactic strain for the production of magnetosomes under fermentation conditions.

In conclusion, the results presented in this study have solved major problems associated with the growth of microaerophilic magnetospirilla by the determination of optimum growth conditions and the establishment of a reliable method for mass cultivation in flasks and the fermentor. The growth rates, cell crops, and magnetosome yields are the highest reported so far for a magnetotactic bacterium. The method has the potential to be adapted to larger scales and could be applied for other microaerophilic organisms. By overcoming the limitations caused by the poor availability of magnetosomes, this may further support the development of their technological application in the future.

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