Regulation of magnetosome biogenesis by oxygen and nitrogen

Yuri Alan Gorby
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Regulation of magnetosome biogenesis by oxygen and nitrogen

Gorby, Yuri Alan, Ph.D.
University of New Hampshire, 1989
REGULATION OF MAGNETOSOME BIOGENESIS BY OXYGEN AND NITROGEN

BY

YURI ALAN GORBY

B.S. Biology, Bethany College, 1983

DISSERTATION

Submitted to the University of New Hampshire in Partial Fulfillment of the requirements of the Degree of

Doctor of Philosophy in Microbiology

May, 1989
This dissertation has been examined and approved.

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ABSTRACT

REGULATION OF MAGNETOSOME BIOGENESIS BY OXYGEN AND NITROGEN

by

Yuri Alan Gorby
University of New Hampshire, May, 1989

Magnetosomes were purified from broken cells of *Aquaspirillum magnetotacticum* by a magnetic separation technique. Electron microscopic and chemical analyses revealed they consisted of crystalline magnetite (Fe₃O₄) cores enclosed by a lipid bilayer membrane containing numerous proteins. Two membrane proteins were absent from the non-magnetic (membrane or soluble) cell fractions and were absent from cells of non-magnetic mutant strains. Knowledge of their partial sequence is expected to lead to construction of hybridization probes to identify magnetosome-specific DNA sequences.

Cells of this organism in laboratory culture typically achieved low cell yields and were variably magnetic or non-magnetic. Therefore better understanding of physiological conditions appropriate for growth and Fe₃O₄ production was a prerequisite for studies of molecular biology and membrane biogenesis. The cell and Fe₃O₄ yield in batch-cultured denitrifying cells was previously shown to depend upon culture dissolved oxygen tension (D.O.T.). We developed continuous culture methods for this obligate microaerophile and obtained, with D=0.075 h (T_d=9.2 h), control of pH (6.8), and D.O.T. control (1 - 10 % of saturation), 10-fold higher cell yields with more predictable and reproducible cell growth and Fe₃O₄ production than were previously possible. Our interest has been to use continuous culture to further clarify the effect of D.O.T. in magnetosome biogenesis.

Denitrifying magnetic cells (4 mM NO₃⁻ as the limiting nutrient) and non-denitrifying cells (0.1 mM NH₄⁺ as the limiting nutrient) became non-magnetic as the D.O.T. was
increased from 1 to 5%. Although 5% O$_2$ was toxic for denitrifying cells and cultures washed out, non-denitrifying cultures again became magnetic when the D.O.T. was decreased to 1%. This reversible transition between magnetic and non-magnetic state, regulated by D.O.T, provided a reproducible system for examining magnetosome biogenesis.
This thesis is dedicated to my loving wife, Terry.
ACKNOWLEDGMENTS

Many friends have contributed to this work. I thank Robert Mooney and Alberta Moultan for taking the guess work out of departmental protocol. This would be a pretty shabby place without them. Many thanks to Linda DiBernardo, an exemplary secretary and a lovely person, for the time she gave teaching me about literary etiquette. I am grateful to Drs. Bob Vallari, Andy Laudano, and Bill McGrew for their helpful advice concerning enzymology, protein chemistry, and electrophoresis, as well as the time spent talking about anything but science. Special thanks to each of my committee members who graciously contributed time, expertise, and advice. This work has benefited greatly from their input. My deepest regards and appreciation to Dr. Terry Beveridge for performing freeze-etching while my wife and I Honeymooned in the Great White North. Thanks goes to my friend Larry Paoletti, Master of Design (M.D.) and perfecter the laboratory Sloppy Joe. Not only can he cook, but he showed me many of the techniques used throughout this dissertation.

Richard and Nancy Blakemore, two of the world's finest people. They have provided a unique graduate experience for which I am very grateful. I consider myself very fortunate to be able to call them my friends.

Special thanks to all members of my family. Their interest in my progress proved to be a great motivating force. Much of the power needed to complete this work came from my mother's kind heart.

Finally, I wish to thank Terry Finlay, my dearest love and closest friend. Words will never fully express my feelings for her. She has given me my most fondest memories and the time spent with her has been precious. I wish that each moment would last an eternity. I love you, Terry.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>viii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>7</td>
</tr>
<tr>
<td>I. CHAPTER ONE: Characterization of the Bacterial Magnetosome Membrane</td>
<td>10</td>
</tr>
<tr>
<td>II. CHAPTER TWO: Iron Relieves Nitrate Limitation in Continuous Cultures of <em>Aquaspirillum magnetotacticum</em></td>
<td>31</td>
</tr>
<tr>
<td>III. CHAPTER THREE: Effects of Dissolved Oxygen on Magnetite Production in Continuous Cultures of <em>Aquaspirillum magnetotacticum</em></td>
<td>42</td>
</tr>
<tr>
<td>IV. CHAPTER FOUR: Occurrence of a d-Type Hemoprotein in the <em>Aquaspirillum magnetotacticum</em> Outer Membrane</td>
<td>58</td>
</tr>
<tr>
<td>V. CHAPTER FIVE: Metal Accumulation and Resistance by <em>Aquaspirillum magnetotacticum</em></td>
<td>71</td>
</tr>
<tr>
<td>APPENDIX A: Procedure for Low Temperature Cytochrome Spectra</td>
<td>90</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

FIGURE 1. Electron microscopical evaluation of magnetosome purity .......... 23
FIGURE 2. Freeze-etch preparations of magnetic cells of strain MS-1 .......... 24
FIGURE 3. Freeze-etch preparation of purified magnetosomes .................. 25
FIGURE 4. Freeze-etch preparation of the nonmagnetic mutant strain NM-1. 26
FIGURE 5. Thin section of a magnetic cell ........................................... 27
FIGURE 6. Thin sections of cells cultured under iron limitation .................. 28
FIGURE 7. SDS-PAGE of cell fractions of strain MS-1 ............................. 29
FIGURE 8. Two-dimensional gel electrophoresis of proteins recovered from
the (A) magnetic and (B) nonmagnetic fractions from strain MS-1. 30
FIGURE 9. Effect of nitrate on growth of strain MS-1 cultured with 8μM FeQ. 38
FIGURE 10. Effect of FeQ concentrations on growth of A. magnetotacticum
strain MS-1 in continuous culture ........................................... 39
FIGURE 11. Effect of oxygen deprivation on strain MS-1 cells ................... 40
FIGURE 12. Low temperature (77 K) cytochrome spectra of strain MS-1
cells continuously cultured under NO3⁻-limitation
with 8 or 16 μM FeQ ........................................................... 41
FIGURE 13. Fed batch culture of A. magnetotacticum strain MS-1 grown
with ammonium chloride as the limiting nutrient ............................ 52
FIGURE 14. Continuous culture of ammonium limited A. magnetotacticum
strain MS-1 ............................................................................. 53
FIGURE 15. Effect of D.O.T. on biomass yield and magnetite production of
strain MS-1 in continuous culture ........................................... 54
FIGURE 16. Two-dimensional gel electrophoresis of total cell proteins from
magnetic and nonmagnetic cells cultured with 1 and 10% D.O.T. ....... 55
FIGURE 17. Low temperature (77 K) red-ox cytochrome spectra of cells
continuously cultured with 1, 10, and 25 % D.O.T. ...................... 56
FIGURE 18. Low-temperature (77 K) difference spectrum of whole cells from
continuous culture with sodium nitrate and ammonium chloride
as sole nitrogen source ............................................................. 67
FIGURE 19. Low-temperature (77K) difference spectra of whole cells and
cellular fractions from denitrifying cells ..................................... 68
FIGURE 20. Nitrite reductase specific activities by membrane fractions of A. magnetotacticum strain MS-1 grown with NaNO₃ or NH₄CL as sole N sources ................................................................. 69

FIGURE 21. SDS-PAGE of cellular fraction of cells from continuous culture with sodium nitrate or ammonium chloride as sole nitrogen sources ................................................................. 70

FIGURE 22. Cells of A. magnetotacticum grown with 20 μM added nickel ...... 81

FIGURE 23. Unstained preparation of A. magnetotacticum grown with 20 μM added nickel ................................................................. 82

FIGURE 24. EDAX spectrum of an electron dense inclusion within a cell of A. magnetotacticum grown with 20 μM added nickel ................. 83

FIGURE 25. EDAX spectrum of cytoplasm adjacent to putative polyphosphate inclusions of cells grown with 20 μM nickel ......................... 84

FIGURE 26. Nickel grown cell of A. magnetotacticum exhibiting aberrant magnetosome chain morphology .............................................. 85

FIGURE 27. A. magnetotacticum grown in the presence of 20 μM added lead... 86

FIGURE 28. EDAX spectrum of magnetosomes within a cell cultured with 20 μM added nickel ................................................................. 87

FIGURE 29. EDAX spectrum of cytoplasm which is adjacent to a magnetosome within a cell grown with 20 μM added nickel ......................... 88
INTRODUCTION

Part One

Biogenic minerals are formed in either of two ways. Biologically induced mineralization (BIM) is the process by which metabolic activities of organisms may modify environmental factors such as Eh' and pH thus bringing about reduction and precipitation of inorganic elements to form minerals. It is a non-specific process and a wide range of cations and anions are usually involved. The biomineral thus formed normally exhibits variation in crystal size and morphology and may contain impurities. In contrast, boundary organized biomineralization (BOB) is a more controlled process involving a limited number of cations and anions. The biomineral is formed in close association with an organic surface or membrane which influences and directs the bioprecipitation process. The result is a biomineral with highly structured crystal morphology.

Biogenic magnetite is the fourth most common biogenic mineral (Lowenstam and Weiner, 1982) and is formed by both a BIM and a BOB process. Some bacteria (dissimilatory iron reducers) use iron as an electron sink for the oxidation of reduced organic compounds. Bell et al. (1987), for instance, reported that cocultures of riverined sediment bacteria produced extracellular magnetite in a synthetic iron oxyhydroxide medium by BIM. Addition of glucose in lieu of acetate as the carbon source resulted in a drop in pH during growth and no magnetite was formed. This experiment demonstrated that some bacteria are capable of modifying the Eh' and pH of their environment to favor the formation of extracellular magnetite.

Biogenic magnetite was also produced in a similar manner by axenic cultures of an organism able to use ferric iron as sole terminal electron acceptor anaerobically (Lovley et
Magnetite was not produced in uninoculated media, in cultures incubated at temperatures too high for growth, or in inoculated cultures which were autoclaved prior to incubation; evidence that mineralization was initiated as a result of biological activity. Crystals produced by this BIM process exhibit random, heterogeneous morphology similar to those formed synthetically or abiotically.

Biogenic magnetite is also produced by BOB and was first described by Lowenstam (1962) who studied the radular teeth of chiton. Biomineralization of chiton magnetite was described as a four stage process. Firstly, an organic framework was formed on which the biomineralization eventually occurred. Secondly, a reddish-brown ferrihydrite mineral was transported to the tooth surface as ferritin (Kirschvink and Lowenstam, 1979), where, during the third stage, it was reduced and dehydrated to crystalline magnetite. The fourth stage was marked by progressive thickening of the magnetite tooth.

Magnetotactic bacteria (Blakemore, 1975) contain intracellular linear arrays or clusters of magnetite particles resulting from BOB. In thin section, each magnetite crystal appeared to be surrounded by an organic matrix or covering which was probably involved in biomineralization. The complete structure, including magnetite core and enveloping layers, was termed a magnetosome (Balkwill et al., 1980).

Magnetosome crystal morphology varies in a species-specific manner and has been determined in four organisms. Those within the axenically cultivable species *Aquaspirillum magnetotacticum* are cubo-octahedrons (Mann et al., 1984a). Parallelepiped crystals are produced by two other species (Mann et al., 1984b; Matsuda et al., 1983). Magnetite crystals of these two morphologies can be produced abiotically.

Anisotropic forms have also been identified (Mann et al., 1987). These bullet-shaped inclusions have no abiotic counterpart. Such forms can only be formed by bioprecipitation in close association with a biological matrix which could influence the crystal morphology.

Indirect evidence for the sequence of magnetite deposition in magnetotactic bacteria
was obtained using Mössbauer spectroscopy (Frankel et al., 1983). Low density hydrous ferric oxide and a high density hydrous ferric oxide (ferrihydrite) were detected in magnetotactic cells. The following process of magnetite deposition was proposed. Extracellular chelated iron was reduced and transported into the cell. Ferrous iron may have then been reoxidized and precipitated within magnetosome enveloping layers. This amorphous precipitate was probably dehydrated to a high density ferrihydrite, some (one third) of which was reduced. The resulting ferrihydrite was further dehydrated to magnetite.

Direct evidence for both crystalline and noncrystalline mineral phases within magnetosomes was obtained using high resolution transmission electron microscopy and crystal lattice imaging (Mann et al., 1984a, 1984b, 1987a, 1987b; Matsuda et al., 1983). The well ordered lattice spacing of the crystalline surfaces was consistent with that of magnetite. In addition, noncrystalline phases, presumably amorphous ferric hydroxide or ferrihydrite, occurred at the interface between crystalline magnetite and the organic covering or membrane. These results supported earlier models describing the biomineralization of magnetite within magnetotactic bacteria.

It is likely that the organic matrix or membrane which surrounded each crystal mediated the precipitation process and may have determined the size and shape of the crystalline magnetite. In chapter one of this dissertation, the structure and composition of the magnetosome membrane has been described. Using a magnetic separation technique, magnetosomes were purified and examined for the presence of proteins which may be involved in magnetite deposition.

Part Two

Magnetic bacteria were discovered nearly 15 years ago, yet only three have been isolated and maintained as axenic cultures. *Aquaspirillum magnetotacticum* strains MS-1 and MS-2 are microaerophilic, diazotrophic denitrifiers which use reduced organic acids as
carbon sources. Isolate (MV-1) is Gram-negative with a vibrioid morphology isolated from a marine environment (Bazyliński et al., 1988). The latter bacterium oxidizes reduced organic acids anaerobically with nitrous oxide as sole terminal electron acceptor. Axenic cultures of magnetotactic bacteria have been studied exclusively in batch cultures in which they obtain low final biomass yields.

Cells in batch culture usually exhibit a three phase growth curve (Pirt, 1975). Lag phase describes the interval in which cells increase in mass and volume but do not divide. Exponential growth phase refers to unrestricted growth during which cell numbers increase exponentially. Following exponential growth, the culture becomes limited for an essential nutrient, produces toxic substances, or altered environmental conditions which are unfavorable for growth. The culture is then said to be in stationary phase. Throughout growth, cells in batch culture vary with respect to size, composition and function. Information obtained from these variable populations reflects only the average characteristics of the population at any given time.

Continuous culture is an attractive alternative. Developed in 1950 by two laboratories (Monod, 1950; Novick et al., 1950), continuous culture devices include, at a minimum, delivery of fresh nutrient medium, a reaction vessel containing medium of constant volume in which cells are actively growing and dividing, and an outflow for cells and spent medium. Culture growth rate varies in direct proportion to the concentration of a limiting substrate. As biomass increases to a point at which the concentration of a single essential nutrient is rapidly reduced to zero, the growth rate decreases. Biomass washes from the reaction vessel and, as a result, the concentration of the incoming limiting nutrient increases and the culture growth rate increases accordingly. At the point at which the rate of biomass washout equals the culture growth rate, the system is considered to be in steady state. Cells in a steady state culture are similar with respect to size, composition, and function.

Continuous culture of *A. magnetotacticum* strain MS-1 was described in Chapter...
Two of this dissertation. By determining limiting nutrient concentration and controlling culture parameters such as dissolved oxygen concentration and pH, culture biomass was increased fivefold over those obtained previously with batch cultures. Denitrifying cultures were examined and iron concentration was related to biomass yield, denitrification, physiology, and magnetite production. Results were compared with previous batch culture experiments in which iron concentration influenced hydroxamate production, magnetite yield, and outer membrane protein profiles in *A. magnetotacticum*. (Paoletti and Blakemore, 1986). Modification of cytochrome composition was also investigated since changes in oxygen and iron concentration have been related to differences in the cytochrome composition in other organisms (Rannie and Bragg, 1973; Rice and Hempfling, 1976).

Short and Blakemore (1989) reported that denitrifying cells of *A. magnetotacticum* strain MS-1 accumulated toxic concentrations of nitrite when the initial headspace oxygen tension was about 5% or greater. They attributed this effect to the inhibition of nitrite reductase by oxygen and consequent accumulation of nitrite. Blakemore et al. (1985) also showed that microaerobic conditions were required for magnetite production. Cells in batch culture exhibited optimal magnetite yields with initial headspace oxygen tension of 0.5 to 1.5%. Very little magnetite was produced with either trace or >5% oxygen in the headspace. Chapter Three of this thesis describes the relationship between oxygen, biomass yield, and magnetism in continuous cultures of *A. magnetotacticum*. Toxic effects of nitrite at higher oxygen tensions were avoided by using ammonium chloride as the sole N source. The relationship among oxygen, biomass, and magnetite was evaluated by monitoring and adjusting the input of oxygen to the system. Information concerning the reversible effect of oxygen variation on magnetite production was obtained which would have been impossible to glean from batch culture experiments.

**Part Three**

*A. magnetotacticum* is an obligate microaerophile able to use nitrate as the sole
nitrogen source and terminal electron acceptor. The pathways of denitrification has been determined for this organism (Bazyliński et al., 1984a, 1984b). Indirect evidence for the presence of a nitrite reductase was obtained by detecting the evolution of nitrous oxide from cell suspensions containing sodium nitrite. Inhibition of this enzyme by oxygen has been implicated in accumulation of toxic amounts of nitrite in cells cultured with 5% oxygen. These findings may account for the obligately microaerophilic nature of A. magnetotacticum strain MS-1.

Nitrite reductase catalyzes the reaction of nitrite to nitrous oxide. The enzyme has been purified from a number of organisms and occurs as one of two types (Hochstein and Tomlinson, 1989). One type is a copper-containing metalloflavoprotein. Copper nitrite reductase occurs in species of Achromobacter, Pseudomonas, and Rhodopseudomonas (Iwasaka et al., 1963, 1975; Sawada et al., 1978) and vary from species to species. Nitrite reductases containing the multiheme cd₁ occur in members of Thiobacillus (LeGall et al, 1979; Sawney and Nicholas, 1978), Alcaligenes (Matsubara and Iwasaki, 1971), Paracoccus (Lam and Nicholas, 1969; John and Watley, 1975), and Pseudomonas (Shimada and Orii, 1975). They are composed of two identical subunits each containing a cd₁ multiheme complex.

The cellular location of nitrite reductase is controversial. Early studies suggested the nitrite reductase was a soluble protein (Cox and Payne, 1973; Iwasaki and Matsubara, 1971; Iwasaki et al., 1963). Other studies using immunolabelling techniques suggested it may be associated with the inner leaflet of the inner membrane (Saraste and Kuronen, 1978; Zumpft, 1979). It is possible that the enzyme is membrane associated but easily removed and solubilized during purification.

A. magnetotacticum contains a cd₁-nitrite reductase. Chapter Four of this dissertation addresses the presence and cellular distribution of this heme-containing enzyme in denitrifying cells from microaerobic continuous culture.

6
LITERATURE CITED


CHAPTER ONE
CHAPTER ONE

Characterization of the Bacterial Magnetosome Membrane

ABSTRACT

Intact magnetosomes of *Aquaspirillum magnetotacticum* were purified from broken cells by a magnetic separation technique. Electron microscopical and chemical analyses revealed the magnetite to be enclosed by a lipid bilayer admixed with proteins. Free fatty acids were detected in the membrane, along with glycolipid and phospholipid (in a weight ratio of 1:4:6, respectively). Phospholipids included phosphatidylserine and phosphatidylethanolamine. Of the many proteins detected in the magnetosome membrane, two were not found in other cell membranes or soluble fractions.

INTRODUCTION

The permanent magnetic character of magnetotactic bacteria (4) and magnetotactic algae (1) results from a conspicuous intracellular structure characterizing the group; the "magnetosome" (2). Those magnetosomes which have been studied are enveloped single crystals of the iron oxide, magnetite, commonly arranged in one or more linear arrays within the cytoplasm (9,16,18,28).

Magnetite crystal morphology varies among species. In some bacteria, the crystals are truncated hexagonal prisms as revealed by crystal lattice imaging (18); in others they are bullet-shaped (4). Those within the axenically cultivable species *Aquaspirillum magnetotacticum* (17) are truncated octahedrons (16) which lie in a single helical line along
the cell axis and adjacent to the cytoplasmic membrane. The structure and composition of the magnetosome envelope has not been studied widely, although trilaminate membrane structures have occasionally been observed surrounding magnetosomes of thin sectioned magnetotactic bacteria collected directly from mud (3). Balkwill et al. (2) considered the possibility that magnetite particles of *A. magnetotacticum* were each surrounded by a lipid bilayer. However, due to the high electron density of the magnetite core, it was not possible to discern the electron opaque inner leaflet expected of a closely apposed lipid bilayer in these stained preparations. We have applied magnetic separation methods to disrupted cells as a unique and effective means of purifying magnetosomes for chemical and structural analyses and, from these magnetosomes, obtained definitive proof of an attendant bilayer envelope.

**MATERIALS AND METHODS**

**Media and culture conditions.** *A. magnetotacticum* was grown in 15 L batch cultures as previously described (5). The chemically defined mineral medium contained 4 mM NaNO₃ and lacked organic forms of nitrogen. Iron, at a final concentration of 20 μM, chelated with an equimolar concentration of quinic acid, was added to autoclaved and cooled medium. To study iron limitation, cells were transferred at least three times in medium from which iron compounds were omitted. The total trace iron concentration in media to which no iron was intentionally added was less than 1 μM, as determined with ferrozine (27).

Cells concentrated by filtration were centrifuged at 5000 x g for 10 min at 4°C. They were resuspended and washed three times in buffer A consisting of 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (pH 7.4) containing 10 μg/ml of the protease inhibitor phenylmethyl-sulfonyl fluoride.
**Magnetosome purification.** Approximately $10^{12}$ cells suspended in 30 ml of buffer A were disrupted by three passes through a French pressure cell at 18,000 psi. DNase (50 \( \mu\)g/ml), RNase (100 \( \mu\)g/ml), and MgCl\(_2\) (10 \( \mu\)M) were added to the disrupted cells and incubated for 60 min at 23°C. A centrifuge tube containing disrupted cells were placed in the gap of a large radar magnet (2 Kgauss). The black magnetic fraction accumulated within ten minutes at the sides of the tube nearest the magnet. The nonmagnetic fluid fraction was removed by aspiration and the magnetic phase was resuspended in 100 times its volume of buffer A. This procedure was repeated ten times. The partially purified magnetosome fraction was suspended in 100 times its volume of buffer A containing 1 M NaCl. The salt was added to remove adventitious electrostatically associated proteins. Purified magnetosomes were washed at least ten more times with buffer A.

**Fractionation of nonmagnetic subcellular components.** The nonmagnetic cell fraction was separated into outer membrane, inner membrane, and soluble fractions by methods described by Schnaitman (25). Nonmagnetic cellular debris (approximately 30 ml obtained from $10^{12}$ cells) was centrifuged at 500 x g for 15 min at 4°C to remove unbroken cells. The supernatant fluid was centrifuged at 200,000 x g for 1 h at 4°C to remove membranes. The supernatant fluid from this high speed centrifugation, considered to contain soluble proteins, was stored on ice. The brown pellet, containing outer and inner membranes, was suspended in 30 ml of buffer A containing 2 % (vol/vol) Triton X-100 and 10 mM MgCl\(_2\). The solubilized cytoplasmic membrane proteins were precipitated with cold 95 % ethanol overnight at 0°C and collected by centrifugation at 500 x g for 15 min at 4°C. Fractionation was evaluated by assaying for specific activity of the inner membrane enzyme succinic dehydrogenase (7), and by measuring the quantity of 2-keto-3-deoxy-octonate, a constituent of outer membrane LPS (21).

**Freeze etching.** Cells of *A. magnetotacticum* strain MS-1 and the nonmagnetic mutant, strain NM-1A, were flash frozen in Freon-22 held at near liquid nitrogen temperature. Frozen preparations maintained at -100°C in a Balzers BA 360M freeze etching apparatus.
were fractured by striking the sample with a knife edge. The surface of the sample was etched by passing the knife over the sample for 10 s. The sample was shadowed with platinum and carbon replicas were made. The replicas were floated onto 10% sulfuric acid and incubated overnight to remove organic material. The replicas were then washed ten times in distilled water and mounted on an uncoated copper grid. Magnetically purified magnetosomes were similarly prepared. Replicas were examined at 60 kV using either a Philips EM300 or EM400 electron microscope equipped with a goniometer stage under standard operating conditions.

**Thin sections.** Cells grown with or without 20 \( \mu M \) iron were fixed for 1 h with glutaraldehyde (5 % vol/vol) buffer followed by washing and secondary fixation for 30 min with osmium tetroxide (1 % vol/vol) in 50 mM cacodylate buffer (pH 6.8) containing 10 mM MgCl\(_2\). Samples were dehydrated in ethanol, followed by propylene oxide, and embedded in EPON 812 or EPON 812 - Araldite. Thin sections obtained with an LKB 8800 Ultratome III ultramicrotome were stained with 5 % uranyl acetate and 0.4 % lead citrate (23) and viewed with either a Hitachi H600 or Philips EM400 STEM at 80 kV in the TEM mode.

**Lipid analysis.** Lipids were extracted from purified magnetosomes with chloroform-methanol as described by Bligh and Dyer (6) and purified by the Sephadex bead (Pharmacia Fine Chemicals, Piscataway, N.J.) method of Wurthier (29). The purified total lipids were separated into neutral lipids and fatty acids, glycolipids, and phospholipids with an acid-treated Florisil (Sigma Chemical Co., St. Louis, MO) column (10). Each lipid fraction was dried and weighed. The dry phospholipid fraction was dissolved in 0.3 ml of chloroform-methanol (1:1, vol/vol) and spotted onto a 20 x 20 cm glass TLC plate of silica gel. The plate edge was placed in a mixture of chloroform-methanol-water (65:25:4, vol/vol) until the entirety of the plate was wetted. Subsequently, the plate was air dried, rotated 90\(^\circ\), and placed in a mixture of chloroform-methanol-7 N ammonium hydroxide (60:35:5, vol/vol). The developed plate was air dried. Lipids were
stained with iodine vapors. Each spot was scraped from the TLC plate and transferred to a Pasteur pipet plugged with glass wool. Phospholipids were eluted from the pipets with 2 ml of chloroform-methanol (1:1, vol/vol) followed by 2 ml of absolute methanol. Each sample was collected in a 5 cc glass ampoule and evaporated to dryness in a stream of nitrogen. The residues were each dissolved in 2 ml of 1 N HCl and, after sealing the ampoule, heated to 100°C for 4 h. Cooled ampoules were opened and 2 ml of redistilled hexane added. The mixture was shaken vigorously, allowed to separate, and the aqueous phase removed and lyophilized. The residues were dissolved in 0.1 ml of distilled water and spotted onto Whatman no. 1 chromatography paper. The chromatogram was developed with redistilled phenol-absolute ethanol-glacial acetic acid (50:5:6, vol/vol), air dried, and sprayed with ninhydrin reagent to stain serine and ethanolamine. A duplicate chromatogram was sprayed with Dragendorf reagent (10) to stain choline and dimethyl-ethanolamine. The color and Rf value of each unknown sample were compared to those of lipid standards.

**Gel electrophoresis.** The protein concentration of each subcellular fraction was determined by the method of Lowry et al. (14). Magnetite, liberated from organic material during the Lowry assay, was removed by centrifugation prior to spectrophotometric analysis at A760. Proteins (5 µg) from each subcellular fraction were separated by SDS-polyacrylamide gel electrophoresis through a 4 % stacking gel and a 12 % separating gel as described by Laemmli (11).

Proteins from partially purified magnetosomes and nonmagnetic fractions from cells of strain MS-1 were separated by 2-dimensional gel electrophoresis. Samples (30 µg protein each) were separated in a pH gradient ranging from 3.5 to 10.0 with tube gels as described by O'Farrell (20). At 16 h the constant voltage (400 V) was increased to 800 V for an additional hour to . Each tube gel was fixed with 1 % agarose to the top of an SDS-polyacrylamide gel (10 % to 20 % linear gradient of acrylamide). Proteins were separated
in the second dimension at constant current (15 mA) for 9 h. Gels were stained with silver as described by Oakley et al. (19).

RESULTS

Magnetosome purification. Magnetosomes within *A. magnetotacticum* cells were always arranged in a linear array in the manner described by Balkwill et al. (2). They appeared to be enveloped and were separated from one another by a distance of about 9.0 nm. The interparticle spacing decreased to about 6.8 nm in crude preparations of magnetosomes although particles remained attached end-to-end and were still enveloped (Fig. 1A). After NaCl treatment and extensive washing, magnetosomes appeared free of contaminating cellular components. However, each particle remained enveloped and separated from adjacent particles by a distance of 5.0 nm (Fig. 1B). Purified magnetosomes did not exhibit succinic dehydrogenase activity or contain KDO. SDS detergent treatment, which dissolves lipid bilayers, removed the enveloping material, destroyed the linear arrangement of the electron-dense particles, and allowed them to clump with virtually no interparticle spacing (Fig. 1C).

Freeze etching. In frozen and etched preparations of magnetic cells of strain MS-1 (Fig. 2A), intact magnetosomes appeared as convex shaped protrusions (MM). Cup-shaped depressions with raised rims (MM) were interpreted to be regions through which the fracture plane had passed with removal of magnetite cores. The raised edges comprising the rim were attributed to a magnetosome envelope differing in composition and structure from adjacent cytoplasm. A number of representative fracture surfaces associated with magnetosomes within strain MS-1 cells are shown in Fig. 2B. The results are those expected if a lipid bilayer were present around each magnetite crystal. Some fractures appeared to expose the external surface of intact magnetosomes (MM). In other cases, the magnetite crystal appeared to have been extracted (as revealed by characteristic raised rims) and the internal surface of the magnetosome enveloping layer (MM) was evident.

16
Occasionally, the fracture appeared to have penetrated the magnetosome envelope without removal of the mineral core, thereby exposing a surface which was either the magnetite core or the external face of the internal leaflet of the magnetosome envelope (MMF). The latter would be possible only if the boundary were a lipid bilayer which fractured internally along its hydrophobic region. Frozen and etched magnetosomes, isolated from cells, displayed these same structural features. When the alignment of magnetosomes to the fracture angle was correct, the particles appeared to be arranged in linear arrays (Fig. 3). Replicas of magnetosomes treated with detergent to remove enveloping layers (not shown) did not exhibit fractures characteristic of intact magnetosomes.

Frozen and etched cells of a nonmagnetic mutant strain lacked features associated with magnetite cores or associated enveloping layers (Fig. 4), although fracture surfaces associated with the inner and outer membranes appeared similar to those of other gram-negative bacteria. Fracture surfaces associated with external wall layers, such as capsules or surface arrays, were not observed with either the magnetic or nonmagnetic strains.

Thin sections. Magnetosomes within cells cultured in medium containing 20 μM iron (Fig. 5) appeared as electron-dense crystalline iron cores each enveloped by a 1.7 nm thick electron-transparent layer and a 2.0 nm electron-dense layer. These results were comparable to those of Balkwill et al. (2). Stereo views of our thin sections (not shown) offered additional evidence of the bilayer nature of this envelope and suggested it was not merely an electron phase artifact.

Magnetic cells cultured with no added iron contained some typical magnetosomes (Fig. 6A). In addition, however, numerous 40 nm diameter membranous vesicles (MV) were present. These vesicles lacked electron-dense cores and were adjacent to one another along the long axis of the cell in the position normally occupied by intact magnetosomes within cells cultured with iron. Each "empty" membrane vesicle consisted of two 1.7 nm thick electron-dense layers separated by a 2.2 nm thick electron-transparent layer as
characteristic of a lipid bilayer unit membrane (24). In some sections these vesicles were not filled with crystalline magnetite but (as determined from energy dispersive X-ray analysis and selected area electron diffraction) contained amorphous iron (Fig. 6B) which was presumably a derivative of polyferric hydroxide.

**Lipid analysis.** Magnetically separated and washed magnetosomes from 25 g of wet packed cells yielded 25 mg of purified lipids and 150 mg of purified magnetite. Lipids associated with this magnetosome fraction included free fatty acids (1.5 % of total by weight). The phospholipids included phosphatidylethanolamine and phosphatidylserine, as determined by TLC.

**Gel electrophoresis.** Proteins of the cell outer and inner membranes and of the soluble cell fraction were compared with those associated with the purified magnetosome fraction (Fig. 7). The outer membrane protein profile of this organism was similar to that described by Paoletti et al. (22). Several proteins of identical molecular mass were shared between the magnetosome membrane and either the outer membrane or inner membrane, but the intensity of the SDS-PAGE bands differed reflecting concentration differences. Two proteins with apparent molecular masses of 16,000 and 27,000 daltons appeared to be restricted to the magnetosome membrane fraction.

Characteristic magnetosome protein profiles obtained following two-dimensional gel electrophoresis are shown in Figure 8. Two abundant anodically migrating proteins, with apparent molecular masses of 15,500 and 16,500 daltons, were present only in the magnetosome fraction. In concert, the uni- and two-dimensional gels showed the magnetosome membrane to be distinct from the other cell outer or inner membranes.

**DISCUSSION**

Previous studies (2,3) provided suggestive evidence for a lipid bilayer envelope surrounding the bacterial magnetosome. However, conclusive evidence has been lacking
because of the difficulty in interpreting thin sections and the absence of data on purified magnetosomes. We have extended previous cytological studies and have used a magnetic separation method to recover intact magnetosomes from cellular debris. The data presented here, obtained by freeze-etching and by thin sectioning of both cells and magnetically extracted magnetosomes, indicated the presence of a trilaminate membrane surrounding each magnetite core. This membranous envelope was absent from purified magnetosomes treated with detergent to remove lipids and proteins. Trilaminate membrane vesicles with dimensional and spatial characteristics of magnetosomes, but devoid of magnetite cores, were present in wild type magnetic cells grown without iron. Amorphous iron was occasionally present in small quantity within these vesicles. Magnetosomes, vesicles with amorphous iron, or empty vesicles were not present within cells of the nonmagnetic mutant strain NM-1A. It was apparent, therefore, that these membranes were an integral part of magnetosomes and are now considered to be magnetosome boundary membranes.

Magnetosome membranes do not appear to be contiguous with the cytoplasmic membrane. Connections have never observed between the two membranes in numerous thin sections, including stereo views, of magnetic cells. If the magnetosome membranes were invaginations of the cytoplasmic membrane, freeze-etching would reveal severed connections as pits in the inner surface of this membrane (as observed with freeze-etched preparations of cyanobacteria which possess photosynthetic membranes as vesicular intrusions of the cytoplasmic membrane (12)); it did not. Furthermore, when spheroplasts were made, they did not evert their magnetite crystals as might be expected of particles within surficial invaginations of the cytoplasmic membrane.

The magnetosome membrane does not appear significantly different in overall composition from other cell membranes. We detected proteins, free fatty acids, glycoproteins, and phospholipids as components. The ratio of their abundance is that expected for a biological membrane (24). Although most proteins detected in envelopes of purified magnetosomes were of similar mass (but not quantity) to those of the cytoplasmic
membrane, two were unique to the magnetosome envelope. It is tempting to speculate that these could have a specific role in magnetite production. As enzymes, they could promote the accumulation of supersaturating quantities of iron within vesicles, serve to oxidize iron, or reduce and dehydrate the ferrihydrite precursor (8) of bacterial magnetite. They could also be ferrihydrite-associated proteins such as bacterioferritin (26) apoprotein. As structural proteins, they might contribute to the compartmentalization deemed essential for "organic matrix-mediated" (13) biomineralization. The use of artificial membranous vesicles to study iron biomineralization, as recently initiated by Mann et al. (15), would undoubtedly be advanced by purification and inclusion of these magnetosome-specific proteins.

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LITERATURE CITED


FIG. 1. Electron microscopical evaluation of magnetosome purity. (A) Magnetosomes liberated from cells following three passages through a French pressure cell. Particles, each separated by a distance of 6.8 nm, remain in chains. Note contaminating cellular debris. (B) Purified magnetosomes following treatment with 1 M NaCl and extensive washing. Interparticle spacing has decreased to 5.0 nm, yet magnetosomes remain in chains. Note the covering around each particle and the lack of contaminating cellular debris. (C) Magnetosomes following treatment with 10% SDS. Enveloping material has been removed and particles are randomly oriented. Bar equals 250 nm.
FIG. 2. Freeze-etch preparations of magnetic cells of strain MS-1. (A) An intact magnetosome appears convex with the magnetosome membrane surface (MM) exposed while a magnetosome from which the iron core has been removed by the fracture appears concave revealing the inside of the membrane (MM). Poly-B-hydroxybutyrate, PHB; cytoplasm, cyt. (B) The fracture has penetrated the magnetosome membrane and has exposed what appears as either the face of the magnetite particles or the convex fracture of the inner leaflet of the magnetosome membrane, MMF. Arrow indicates the direction of shadow. Bar equals 250 nm.
FIG. 3. Freeze-etch preparation of purified magnetosomes. Note the chain formation and characteristic fracture surfaces. Outer surface of the magnetosome membrane, MM; inside surface of the magnetosome membrane, MM; either the face of the magnetite particle or the convex fracture of the inner leaflet of the magnetosome membrane, MMF. Bar equals 100 nm.
FIG. 4. Freeze-etch preparation of the nonmagnetic mutant strain NM-1A. Note the lack of magnetic particles. Inner surface of the outer membrane, OM; convex fracture of the inner leaflet of the inner membrane, IMF; poly-B-hydroxybutyrate, PHB; cross fracture of a PHB particle, PHBcf. Bar equals 250 nm.
FIG. 5. Thin section of a magnetic cell. Outer membrane, OM; inner membrane, IM; magnetosome membrane, MM; magnetite, M. Bar equals 100 nm.
FIG. 6. Thin sections of cells cultured under iron limitation. (A) Note the trilaminate structure of the membranous vesicles (MV) which lie along the same axis as complete magnetosomes. Bar equals 250 nm. (B) Note small electron-dense deposits of amorphous iron within the membranous vesicles.
FIG. 7. SDS-PAGE of cell fractions of strain MS-1. Outer membrane proteins, OM; inner membrane proteins, IM; magnetosomes membrane proteins, MM; soluble proteins, SP; molecular weight standards, MW. Arrows indicate positions of the 16,000 and 27,000 dalton magnetosome membrane proteins.
FIG. 8. Two-dimensional gel electrophoresis of proteins recovered from the (A) magnetic and (B) nonmagnetic fractions from strain MS-1. Arrows indicate the position of the 15,500 and 16,500 dalton anionic proteins which are present only in the magnetic fraction.
CHAPTER TWO
CHAPTER TWO

Iron Relieves Nitrate Limitation in Continuous Cultures of

*Aquaspirillum magnetotacticum*

ABSTRACT

Growth of denitrifying *Aquaspirillum magnetotacticum* cells in microaerobic continuous culture was NO$_3^-$-limited over the range 2-8 mM. No differences in the number of magnetosomes per cell, outer membrane protein profiles, or cytochrome composition were detected in cells cultured with iron concentrations ranging from 2 to 32 $\mu$M. As expected, at 4 mM NO$_3^-$, no change in biomass yield was observed when Fe$^{3+}$ was increased from 2 to 4 or from 4 to 8 $\mu$M. However, an increase from 8 to 16 $\mu$M Fe$^{3+}$ at constant (4 mM) NO$_3^-$ produced a doubling of the culture biomass and a five-fold increase in the rate of O$_2$ consumption per cell. No additional increase in cell yield was observed with further increase in Fe$^{3+}$ to 32 $\mu$M. Thus, the relief of NO$_3^-$ limitation triggered by the particular increase in Fe$^{3+}$ concentration from 8 to 16 $\mu$M appeared to be due to a shift in cell respiration from use of principally NO$_3^-$ to O$_2$ as a terminal electron acceptor.

INTRODUCTION

Continuous culture methods provide benefits of precise regulation of oxygen, pH, and nutrients and permit study of cells maintained at constant growth (5). The work presented here was undertaken to establish the parameters necessary to improve the notably poor growth yields of *A. magnetotacticum* strain MS-1 while maintaining cell magnetism.
The relationship of culture available iron concentration to cell growth, siderophore production, magnetite yield, and respiration, particularly with NO₃⁻ or O₂ as oxidant is examined.

MATERIALS AND METHODS

Culture conditions. *A. magnetotacticum* strain MS-1 was grown microaerobically in continuous culture in a two liter, water-jacketed glass vessel (Pegasus, Ontario, Canada) at 30°C. The reservoir and chemostat contained no metal components. The principal carbon and energy sources were tartaric and succinic acids (3 mM each), and 0.36 mM sodium acetate. Ascorbic acid (0.2 mM) was added as a reducing agent. Iron was supplied as ferric quinate (FeQ) and, unless otherwise noted, NO₃⁻ was at a growth-limiting concentration (4 mM as NaNO₃). Culture mass doubling time (Tₐ) was maintained at 9.2 h with D = 0.075 h⁻¹. Dissolved O₂ was monitored with a galvanic electrode (model M1016-0208, New Brunswick Scientific Co., Edison, NJ) and maintained at 1% of saturation by adjusting the nitrogen-to-air ratio of a mixture supplied at a constant rate of 3.3 cc · min⁻¹. The culture pH was continuously maintained at a value of 6.8 by means of a pH controller (model pH40, New Brunswick Scientific Co.) delivering sterile 3 N HCl. The total background iron content of medium to which no FeQ was added was 0.35 μM.

Growth measurements. Samples for dry weight measurements and cell counts were periodically collected from the outflow of the chemostat and fixed with 1% formalin. Cultures were considered to be at steady state when the change in optical density measured at 660 nm was zero for more than 45 h (5 culture doubling times). Steady state samples (500 ml) were collected on ice and immediately cleared of cells by centrifugation at 5,000 x g for 30 min at 5°C. To test for hydroxamate production (4), supernatant fluids were concentrated 50-fold on a rotary evaporator at 30°C, adjusted to pH 7.0, and clarified by filtration. Outer membrane proteins were obtained using Triton X-100 and MgCl₂ (8).
Concentrated cells and fluids were stored at -20°C for analysis. Direct cell counts were make with a Petroff-Hausser bacterial counting chamber. Biomass was determined from dry weight measurements.

Low temperature spectra. Low temperature (77 K) dithionite-reduced minus air-oxidized difference spectra were obtained using intact, steady state cells in 33 % ethylene glycol. Spectra were obtained using a variable temperature cell (0.2 mm pathlength) and a Beckman DU-8 UV-VIS spectrophotometer (see Appendix A).

Oxygen consumption. Oxygen consumption rates of steady state cells from the chemostat were measured polarographically using a Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH).

Electron microscopy. Cells negatively stained with 0.5% uranyl acetate were examined with a Hitachi 600 STEM operating in the TEM mode. Magnetosomes within each of 100 cells collected at each steady state were enumerated.

RESULTS AND DISCUSSION

With the Fe$^{3+}$ concentration constantly maintained at 8μM, *A. magnetotacticum* culture biomass approximately doubled with each doubling of NO$_3^-$ concentration over the range 2 to 8 mM NO$_3^-$ (Fig. 9). This established that NO$_3^-$ (provided as both an electron acceptor and sole N source) was the limiting nutrient at 4 mM, the concentration used in subsequent studies with iron. From these results, however, it was not possible to determine whether NO$_3^-$ was limiting as terminal oxidant or as a source of assimilated nitrogen. Through control of culture pH and dissolved oxygen tension (D.O.T.), the growth yields obtained (1 x 10$^9$ cells · ml$^{-1}$; 78 μg dry weight · ml$^{-1}$) were 10-fold greater than those obtained previously at a similar NO$_3^-$ concentration to that used previously in batch culture. Cells were grown continuously in the magnetic state for more than 22 culture doublings.
Increase in the Fe$^{3+}$ supply from 2 to 4 and from 4 to 8 $\mu$M produced little effect on culture biomass of NO$_3^-$-limited cells (Fig. 10). However, biomass doubled when the culture Fe$^{3+}$ supply was increased from 8 to 16 $\mu$M. Additional biomass increases were not observed when the Fe$^{3+}$ concentration was increased from 16 to 32 $\mu$M (Fig. 10).

The increase in biomass effected by raising the culture Fe$^{3+}$ concentration from 8 to 16 $\mu$M was accompanied within 10 h by a greater culture O$_2$ demand. Unless the culture D.O.T. was maintained at 1% of saturation by providing more O$_2$, growth increase was not observed and the culture became magnetic (Fig. 11). Controlled restoration of microaerobic conditions to such O$_2$-starved cultures (Fig. 11, arrow) allowed for only 35% of the expected steady-state biomass and the cells produced were non-magnetic. This illustrated the obligately microaerophilic nature of this organism and its O$_2$ requirement for growth and magnetite synthesis (1).

Respiration rates for steady state cells cultured with 8 and 16 $\mu$M FeQ were 0.93 and 4.3 ng O$_2$ consumed $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ dry weight biomass, respectively. *A. magnetotacticum* respires with Fe$^{3+}$ (9). However, because Fe$^{3+}$ supplied in micromolar quantities in these experiments was not merely substituting for NO$_3^-$ (present in millimolar amounts) as the terminal electron acceptor, another explanation was sought for its stimulatory role. The increase in biomass and respiration rate triggered by the shift to 16 $\mu$M iron was consistent with a shift in cell respiration from principally NO$_3^-$ to O$_2$ as the terminal electron acceptor. In addition to increasing respiration efficiency, this would also spare NO$_3^-$-N for assimilation. However, despite the fact that iron concentration (2,6) and oxygen supply (7) may influence respiration and cytochrome composition, we detected no differences in the amount or types of cytochromes in intact steady-state cells collected at each iron concentration (Fig. 12). Cytochrome spectra were similar to those of cells from batch culture (3).

The range of Fe$^{3+}$ concentrations effecting the change in respiration rates were similar to those shown previously to effect hydroxamate production and synthesis of a
55,000 dalton iron regulated outer membrane protein (IROMP) in cells grown in batch cultures (4). In batch cultured cells, this IROMP was formed and siderophore synthesis was repressed only at Fe³⁺ concentrations less than 10 μM (4). At each Fe³⁺ concentration used in this study, cells produced the 55,000 dalton IROMP and no detectable hydroxamate. An average of only 7 magnetosomes per cell was present regardless of the iron concentration. Thus, at every iron concentration used in this study, steady state cells appeared, with respect to (i) siderophore production, (ii) the presence of a 55,000 dalton iron-regulated outer membrane protein, and (iii) their magnetite yield, to have experienced iron deficiency.

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FIG. 9. Effect of nitrate on growth of strain MS-1 cultured with 8μM FeQ.
FIG. 10. Effect of FeQ concentrations on growth of *A. magnetotacticum* strain MS-1 in continuous culture.
FIG. 11. Effect of oxygen deprivation on strain MS-1 cells monitored by absorbance ( ) and biomass ( ). Arrow indicates the addition of oxygen to 1% of saturation.
FIG. 12. Low temperature (77 K) cytochrome spectra of strain MS-1 cells continuously cultured under NO$_3^-$-limitation with 8 of 16 $\mu$M FeQ.
CHAPTER THREE
CHAPTER THREE

Effects of Dissolved Oxygen on Magnetite Production in Continuous Cultures of *Aquaspirillum magnetotacticum*

**ABSTRACT**

*Aquaspirillum magnetotacticum* strain MS-1 was maintained in fed-batch and continuous cultures at 1 and 10% dissolved oxygen tensions (D.O.T.) with ammonium chloride as sole nitrogen source. It appeared that only during periods of oxygen limitation, with the D.O.T. at 1% of saturation, cells were magnetic. At a D.O.T. of 10%, cultures consisting of uniformly nonmagnetic cells progressively became magnetic (i.e. produced magnetite in direct inverse proportion to the D.O.T. value) with the D.O.T. decreased to 1%. The specific activity of periplasmic iron reductase of magnetic cells grown at 1% O2 was 61% greater than that of nonmagnetic cells cultured at 10% O2. Two peptides detected in whole cell protein preparations nonmagnetic cells were not detected in those cultured with 1% D.O.T. Magnetic cells produced greater amounts of β- and c-type hemes than did those cultured with excess oxygen.

**INTRODUCTION**

Culture oxygen values influence magnetite yield in *A. magnetotacticum* with optimal production at a dissolved oxygen tension (D.O.T.) of 1% of saturation. However, the repressive effect of higher D.O.T. values has been difficult to study in a controlled manner. Cultures of this organism have usually been grown with NO3⁻ and denitrify. Because of
the inhibition of nitrite reductase by oxygen, cells are poisoned by NO₂⁻ which accumulates at culture D.O.T. values greater than 1% (22). To obviate this impediment to investigating the role of elevated D.O.T. on magnetite formation, we have cultured cells with NH₄Cl as the sole N source. Cells in these continuous cultures have been grown at D.O.T. values as high as 100% of saturation.

MATERIALS AND METHODS

Growth conditions. *Aquaspirillum magnetotacticum* strain MS-1 was grown in continuous culture in a Pegasus 2 liter glass vessel with $D = 0.074 \, h^{-1}$. Mass doubling time ($T_d$) was maintained at 9.2 h. Succinic acid served as principal carbon source in magnetic spirillum growth medium (MSGM) (5). Ascorbic acid (0.2 mM) was included as a reducing agent. Iron was supplied as ferric quinate (FeQ). Ammonium, provided as NH₄Cl, served as sole nitrogen source. Dissolved oxygen was monitored with a galvanic electrode and maintained at desired concentrations by adjusting the nitrogen-to-air ratio of a gas mixture supplied at a constant rate of 3.3 cc min⁻¹. The pH was monitored with and autoclavable electrode and was continuously maintained at a value of 6.8 by means of a New Brunswick model pH 40 pH controller delivering 3 N HCl. Although the culture medium was not deferrated, at no time during growth did the culture or any solutions added to it come in contact with ferrous metals. The total background iron content of the medium to which no FeQ was added was 0.35 μM.

Growth yield. Samples for dry weight measurements and cell counts were periodically collected from the outflow of the chemostat and fixed with 1% formalin. Growth was monitored by absorbance at 660 nm (Spectronic 88). Cultures were considered to be at steady state when the change in optical density measured at 660 nm was zero for more than 45 h (5 culture doubling times). Samples (10 ml) were filtered onto preweighed polycarbonate filters (0.2 μm pore size) and dried to constant weight in a desiccator oven.
Biomass trapped on filters was determined with a Cahn electronic balance. Steady state samples (1 L) were collected on ice, centrifuged (5,000 x g, 30 min, 5°C), washed in 10 mM HEPES buffer (pH 7.4), and frozen at -70°C. Periplasm assayed for iron reductase activity was obtained by a freeze/thaw method (23).

**Magnetosomes per cell.** The average number of magnetosomes per cell was determined using a Hitachi H600 STEM electron microscope. Samples were stained for 10 seconds with 1 % phosphotungstic acid (pH 6.8), applied to copper grids, and blotted dry with filter paper. Magnetosomes within each of 100 cells from each sample were counted.

**Polyacrylamide gel electrophoresis.** Protein (30 µg/µl sample) were separated by two-dimensional gel electrophoresis as described by O'Farrell (18). Periplasmic and membrane proteins, obtained by a freeze/thaw method (20) and the method of Schnaitman (22), respectively, were separated by sodium dodecyl sulfate gel electrophoresis through a 4% stacking gel and a 12% separating gel as described by Laemmli (11). Protein concentration was determined by the method of Lowry et al. (12) using bovine serum albumin as standard. Gels were stained with silver as described by Oakley et al. (16).

**Iron reductase assay.** Iron reductase activity was measured with ferrozine as described by Dailey and Lascelles (7). Reaction mixture consisting of 10 mM Tris-acetate buffer (pH 8.1) containing 10% (vol/vol) glycerol, 0.8 mM β-NADH, 0.5 mM ferrozine, 0.2 mM ferric citrate, and 10 µM flavine mononucleotide (FMN) was added to Thunberg cuvettes. Periplasmic proteins were added to the side-arm. Cuvettes were sparged with O₂-free N₂ for five min. The reaction was initiated by combining protein and assay mixture. Absorbance changes at 562 nm were recorded using a Beckman Instruments DU-8 spectrophotometer.

**Low temperature cytochrome spectra.** Washed whole cells were suspended in 10 mM HEPES buffer (pH 7.4) containing 33% (vol/vol) ethylene glycol to a final protein concentration of 10 mg · ml⁻¹. Dithionite-reduced-minus-air-oxidized 77 K spectra were
obtained using a SPECAC P/N 21.000 variable temperature cell (0.2 mm pathlength) mounted in a Beckman DU-8 UV-visible spectrophotometer (See Appendix A for details).

RESULTS

Ammonium limitation in fed-batch culture. The molar growth yield of strain MS-1 on ammonium was determined using fed-batch and continuous cultures. Figure 13 shows results of a fed-batch experiment in which cells were limited for NH$_4^+$ while oxygen was continuously supplied at non-limiting concentration (10% of saturation). Biomass increased to 21.5 µg • ml$^{-1}$ with 0.1 mM NH$_4$Cl in the medium. Biomass increased another 20 µg • ml$^{-1}$ to 41.5 µg • ml$^{-1}$ following addition of 0.1 mM sterile NH$_4$Cl. Based on these results, the molar growth yield of _A. magnetotacticum_ for ammonium was approximately 207.5 g • mole$^{-1}$. Cells were nonmagnetic throughout.

Ammonium limitation in continuous culture. Continuous cultures of strain MS-1 were maintained with NH$_4$Cl (0.1 mM) as the limiting nutrient. Oxygen was maintained at a D.O.T. of 10 % for the first 190 h. The molar growth yield with ammonium (grams of biomass • mole$^{-1}$ limiting nutrient) was calculated from results presented in Figure 14. Cultures maintained with ammonium at a limiting concentration of 0.1 mM and a D.O.T. of 10% (not limiting for growth, see below) produced 24.75 µg biomass • ml$^{-1}$. The molar growth yield with ammonium was 247.5 g • mol$^{-1}$. An increase in NH$_4$Cl to 0.2 mM relieved nitrogen limitation and allowed for biomass increase. Nitrogen comprised approximately 15% of the dry weight biomass, an amount consistent with that reported for other gram-negative bacteria (12). Cells grown the D.O.T. at 10% or higher were not magnetotactic, did not align in magnetic fields, and lacked magnetosomes. Cells became magnetic, however, within 5 hours after the D.O.T. was decreased to 1%.

Oxygen limitation in continuous culture. Figure 15 illustrates the effect of O$_2$ on growth yield and magnetism of strain MS-1 in continuous culture. Culture biomass (9.5
μg · ml⁻¹) was maintained for 50 h with oxygen limiting for growth (1 % D.O.T.). When O₂ was increased to 10% (a value not limiting for growth with NH₄Cl present at 0.1 mM), biomass increased to 22.5 μg · ml⁻¹ (values expected from previous results). Cells became nonmagnetic (less than 1 mag · cell⁻¹) within 50 h (over 5 culture doublings) of increasing the D.O.T. to 10% or more. Nonmagnetic cells, maintained in continuous culture for over 100 hours (over 10 culture generations), again became magnetic after the D.O.T. was decreased to 1%. By 5 h after decreasing the D.O.T. from 10% to 1%, cells in culture each contained an average of 5 magnetosomes. Cells contained an average of 16 magnetosomes by 30 hours after the decrease in the D.O.T.

Cells in continuous culture were maintained on 0.1 mM NH₄⁺ at D.O.T. values of 25, 50 and 100% of saturation (data not shown). Culture biomass did not vary in response to increased D.O.T. values above 10%, indicating that cells were not limited for O₂.

**Iron reductase.** Periplasm from magnetic cells of strain MS-1 exhibited iron reductase activity. The specific activity of iron reductase in cells continuously cultured with oxygen limitation was 61% greater than that of nonmagnetic cells of the same strain cultured with 10 % O₂ (Table 1).

**Gel electrophoresis.** Results of two-dimensional gel electrophoresis of total cell proteins from magnetic and nonmagnetic cells cultured with 1 and 10% D.O.T., respectively, appear in Figure 16. With the exception of two polypeptides detected in gels of nonmagnetic cells cultured with 10% O₂, protein profiles appeared similar.

**Low temperature cytochrome spectra.** Low temperature (77 K) red-ox cytochrome spectra of cells continuously cultured with 1, 10, and 25 % D.O.T. appear in Figure 17. Maxima indicative of cytochromes b 558 and c 551 were present in spectra collected from cells cultured with 1 and 10% O₂, although peaks were distinctly smaller from cells cultured with 10% O₂. Peaks were not detected in spectra from cells cultured with 25% D.O.T.
DISCUSSION

*A. magnetotacticum* strain MS-1 has been described as an obligate microaerophile, growing optimally in a D.O.T. range of 0.5 to 1.5% of saturation. This is characteristic of denitrifying cells of *A. magnetotacticum*. However, when cells were continuously cultured with NH$_4^+$, the range of D.O.T. values optimal for growth greatly increased. Even though cells of this organism will not grow on the surface of solidified media in air or initiate growth from low inocula in liquid culture incubated at D.O.T. values of about 25% or higher, we have maintained actively respiring cells in dense continuous cultures for more than 200 hours (20 culture doubling times) with D.O.T. values as high as 100% of saturation. Thus, growth of the organism on NH$_4^+$ prevents NO$_2^-$ toxicity and thereby provides a means of investigating growth and production of magnetite at high D.O.T. values.

Excess molecular oxygen reduces the amount of *b-* and *c-* type cytochromes produced by strain MS-1 grown with NH$_4^+$. O'Brien et al. (17) reported a similar effect with denitrifying cells. They showed that cells cultured with 5% O$_2$ produced greater amounts of all cytochromes than did cells cultured with 1% O$_2$. The reason for the inverse relationship between D.O.T. and cytochrome production is unclear, although similar findings have been reported in other organisms. *Escherichia coli* produced a *d-*type heme which functioned as a high affinity oxidase when cultured under oxygen-deficient conditions (21). Cytochrome *d* was replaced by a low affinity oxidase, cytochrome *o*, when oxygen was plentiful.

Magnetosome production varied inversely with available O$_2$. In continuous culture, cells synthesized magnetite only when the D.O.T. was below about 4%. Blakemore et al. (6) demonstrated in sealed batch culture that *A. magnetotacticum* strain MS-1 cells were magnetic when supplied with between 0.5 and 5% O$_2$ in the initial headspace. They also
showed that the number of magnetosomes were drastically reduced in cells cultured with trace amounts of oxygen.

Molecular oxygen does not appear to be a requirement for growth or magnetite production by all magnetotactic bacteria. Bazylinski and Jannasch (1) have recently isolated a marine species which produces magnetite under anaerobic conditions with N$_2$O as the terminal electron acceptor. Additionally, an organism has been identified which reduces iron as the sole oxidant and produces magnetite extracellularly (13).

A consistent characteristic of magnetic bacteria studied to date (including those organisms from sediments not as yet isolated) is that all have been restricted to microaerobic or anaerobic environments. Although the reason remains unclear, magnetosome production appears to be an anaerobic or microaerobic process. Oxygen-sensitive iron reductases, possibly involved in iron transport, may be the key. Not only did nonmagnetic cells cultured with 10% O$_2$ express over 60% less specific activity of this enzyme than magnetic cells cultured with 1% O$_2$, but this enzyme could have been further inhibited by elevated oxygen tensions in vivo. This hypothesis is consistent with the observation that nonmagnetic cells became magnetic 5 hours (slightly more than half the culture doubling time) after oxygen was decreased from 10 to 1% of saturation.

We have developed a system in which magnetite synthesis in *A. magnetotacticum* has been monitored and controlled. We plan to pursue continuous culture experiments using $^{57}$Fe Mössbauer spectroscopy to examine magnetosome biogenesis as cells are converted from nonmagnetic to magnetic. In this way we plan to further understand bacterial iron biomineralization and what role oxygen plays in this process.
LITERATURE CITED


FIG. 13. Fed batch culture of *A. magnetotacticum* strain MS-1 grown with ammonium chloride as the limiting nutrient. Arrows indicate addition of sterile NH$_4$Cl to a final concentration of 0.1 mM. Oxygen was maintained at 10% throughout.
FIG. 14. Continuous culture of ammonium limited *A. magnetotacticum* strain MS-1. The
NH$_4$Cl concentration in the culture vessel and nutrient reservoir was adjusted to 0.2 mM at
150 h of growth as shown. A shift from 10% to 1% D.O.T. at 190 h is also indicated.
FIG. 15. Effect of D.O.T. on biomass yield and magnetite production of strain MS-1 in continuous culture. Note that cultures became nonmagnetic and biomass increased as oxygen became on-limiting. Biomass decreased and cells again became magnetic following return to oxygen-limiting conditions.
FIG. 16. Two-dimensional gel electrophoresis of total cell proteins from (a) magnetic and (b) nonmagnetic cells cultured with 1 and 10% D.O.T., respectively. With the exception of two polypeptides detected in gels of nonmagnetic cells cultured with 10% O₂, protein profiles appeared similar.
FIG. 17. Low temperature (77 K) red-ox cytochrome spectra of cells continuously cultured with 1, 10, and 25 % D.O.T.
Table 1. Characteristics of strain MS-1 continuous culture^a.

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Limiting nutrient</th>
<th>Biomass^b (µg · ml⁻¹)</th>
<th>Magnetosomes (Ave · cell⁻¹)</th>
<th>Fe-reductase (nmol min⁻¹ mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mM NH₄⁺ 1% D.O.T.</td>
<td>O₂</td>
<td>9</td>
<td>16</td>
<td>29</td>
</tr>
<tr>
<td>0.1 mM NH₄⁺ 10% D.O.T.</td>
<td>NH₄⁺</td>
<td>23</td>
<td>&lt;1</td>
<td>18</td>
</tr>
<tr>
<td>4 mM NO₃⁻ 1% D.O.T.</td>
<td>NO₃⁻</td>
<td>75</td>
<td>8</td>
<td>N.D.</td>
</tr>
<tr>
<td>4 mM NO₃⁻ 10% D.O.T.</td>
<td>*N.G.</td>
<td>N.G.</td>
<td>N.G.</td>
<td></td>
</tr>
</tbody>
</table>

^a Medium MSGM modified to contain 8 mM succinate as sole C source and 8 µM FeQ; D=0.075, Td=9.2 h, pH 6.8.

^b µg · ml⁻¹

* No growth
CHAPTER FOUR
CHAPTER FOUR

Occurrence of a d-Type Hemoprotein in the
Aquaspirillum magnetotacticum Outer Membrane

ABSTRACT

Denitrifying cells of Aquaspirillum magnetotacticum strain MS-1 contain a-, b-, c-, d-,
and o-type hemes. Periplasm contained only soluble c-type cytochromes while c- and b-
types were found as components of the inner membrane. Absorption maxima for c- and d-
type hemes were detected in the outer membrane. A 21,000 Dalton protein detected only in
the outer membrane of nitrate grown cells and may be a component of a cd\_1 multiheme
nitrite reductase or a protein involved in its formation. NO\_2 was reduced to N\_2O by outer
membranes but not by periplasm or inner membrane fractions of denitrifying cells. The
data indicate that a cd\_1-type heme is on the outer membrane of denitrifying cells of
Aquaspirillum magnetotacticum.

INTRODUCTION

O'Brien et al. (7) studied the cytochrome composition of Aquaspirillum
magnetotacticum cells grown microaerobically in batch culture. They detected hemes of the
a-, b-, c-, d-, and o-types in denitrifying cells. Because absorption maxima for a- and
d-type cytochromes appear in the same spectral regions (indeed, d-types were formerly
referred to as a\_2-types), O'Brien et al. suggested that maxima which they attributed to a
-type hemes may have, in fact, been those of a d-type.
Reported here are the composition and cellular distribution of cytochromes in *A. magnetotacticum* cells grown in continuous culture (i.e. unchanging physiology) with nitrate or ammonium as sole nitrogen source and limiting nutrient. Our results corroborate and extend those of O'Brien et al. by revealing the presence of nitrite reductase activity and correlating with spectral evidence for the presence of a \( cd_1 \) multiheme complex in the cell outer membrane of denitrifying cell.

**MATERIALS AND METHODS**

**Media and culture conditions.** *Aquaspirillum magnetotacticum* strain MS-1 was grown in continuous culture in a Pegasus 2 liter glass vessel with \( D = 0.074 \) h\(^{-1}\). The culture mass doubling time (\( T_d \)) was maintained at 9.2 h. Cultures were grown in magnetic spirillum growth medium (MSGM) (2) with succinic acid (8 mM) as the principal carbon source. Ascorbic acid (0.2 mM) was included as a reducing agent. Iron was supplied as ferric quinate (FeQ) at 20 \( \mu M \) NH\(_4\)Cl (0.1 mM) or NaNO\(_3\) (4 mM) was the sole nitrogen source and limiting nutrient. Dissolved oxygen was monitored with a galvanic electrode and maintained at desired concentrations by adjusting the nitrogen-to-air ratio of a gas mixture supplied at a constant rate of 3.3 cc min\(^{-1}\). The pH was monitored and continuously maintained at a value of 6.8 with an autoclavable electrode attached to a New Brunswick model pH 40 pH controller delivering 3 N HCl. Although the culture medium was not deferrated, at no time during growth did the culture or any solutions added to it come in contact with ferrous metals. The total background iron content of the medium to which no FeQ was added was 0.35 \( \mu M \).

**Isolation of cellular fractions.** Cells were harvested by tangential flow filtration. The bacterial concentrate was centrifuged at 5,000 x g for 10 min at \( 4^\circ \)C. Cells were washed three times in 50 mM N-2-hydroxyethylpiperizine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) and mechanically disrupted by three passes through a French
pressure cell at 15,000 psi. Magnetosomes and unbroken cells were removed by centrifugation (5,000 x g, 30 min, 4°C). Supernatant fluid containing outer membranes, inner membranes, and soluble material, was carefully decanted. Membrane fractions were removed from suspension by centrifugation (200,000 x g, 1 h, 4°C) in a Beckman L8-70 preparative ultracentrifuge. The reddish supernatant fluid was decanted and incubated for 30 min at 25°C to allow small membrane fragments, not removed by previous centrifugation, to fuse. These aggregates were removed from suspension with a second high speed centrifugation (200,000 x g, 1 h, 4°C). Membranes were washed three times in HEPES buffer (50 mM, pH 7.4) to remove contaminating soluble proteins.

Outer membranes were separated from inner membranes by differential solubilization with 2% (vol/vol) Triton X-100 containing 10 mM MgCl$_2$ (11). Outer membrane fragments, presumably unaffected by the non-ionic detergent, were separated from solubilized inner membrane proteins by centrifugation (21,000 x g, 1 h, 4°C). The outer membrane preparation, treated a second time with 2% (vol/vol) Triton X-100, was washed in at least 1000 pellet volumes in cold HEPES buffer (50 mM, pH 7.4). Inner membrane proteins were precipitated by adding the detergent solution to twice its volume of cold ethanol and washed in at least 1000 times the pellet volume of cold HEPES buffer. Purity of subcellular fractions was evaluated by assaying specific activity of succinic dehydrogenase, an inner membrane enzyme (9), and by measuring the quantity of 2-keto-3-deoxyoctonate, a constituent of outer membrane lipopolysaccharide. The protein concentration in each subcellular fraction was determined by the method of Lowry et al. (5) with bovine serum albumin as standard.

**Low temperature cytochrome spectra.** All spectra were collected at temperatures near 77 K. The protein concentration in each subcellular fraction was adjusted to 10 mg·ml$^{-1}$ with HEPES buffer. Each sample was diluted with ethylene glycol (33 % vol/vol, final concentration). Dithionite-reduced-minus-air-oxidized (red-ox) spectra were obtained at 77 K using a SPECAC P/N 21.000 variable temperature cell (0.2 mm pathlength)
suspended in a vacuum-jacketed dewar filled with liquid N\(_2\) and mounted in a Beckman DU-8 UV-visible spectrophotometer (See Appendix A for details).

**Gel electrophoresis.** Proteins (5 µg) from each subcellular fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) through a 4 % stacking gel and a 12 % separating gel (15 mA, 6 h) as described by Laemmli (4). Gels were stained with silver nitrate as described by Oakley et al. (6).

**NO\(_2^-\)** reduction. Nitrite reductase was assayed by measuring N\(_2\)O evolved from reaction mixtures containing NaNO\(_2\). Headspace gas samples (10 µl) were removed from sealed 10 ml serum vials each containing 6 ml of reaction mixture. Reaction mixture contained (µmoles): potassium phosphate buffer, 462 (pH 6.9); NaNO\(_2\), 1; Na\(_2\)S\(_2\)O\(_4\)·2H\(_2\)O, 3.8; and methyl viologen, 0.6, as electron donor. Acetylene (1 kPa) was added to each reaction vessel to inhibit N\(_2\)O reduction (13). N\(_2\)O was measured by gas chromatography as previously described (1). A Varian Series 2400 gas chromatograph, equipped with a \(^{63}\)Ni electron capture detector, was fitted with two Porapak Q columns (having meshes of 80/100 and 60/80) arranged in series. Carrier gas (O\(_2\)-free N\(_2\)) was delivered at a flow rate of 25 ml/min. Operating temperatures were as follows: detector, 300°C; column oven, 55°C; injector, 70°C. Peaks areas were recorded with a Hewlett-Packard model 3390A computing integrator.

**RESULTS AND DISCUSSION**

**Low temperature cytochrome analysis.** Low temperature (77 K) red-ox spectra of denitrifying whole cells grown with 4 mM NO\(_3^-\) as limiting nutrient in continuous culture (Fig. 18a) were nearly identical to those obtained previously with denitrifying cells from batch cultures (see Fig. of O'Brien et al.). Hemes detected included types \(a\), \(b\), \(c\), and \(d\). O'Brien et al. (7) previously suggested that \(a\)- and \(d\)- type
hemes were inducible within *A. magnetotacticum* and produced only during periods of denitrification. They showed that cells batch cultured with ammonium lacked putative *a-* and/or *d*-type hemes.

We analyzed the cytochromes of O$_2$-respiring cells continuously cultured with ammonium as sole nitrogen source (Fig. 18b). As mentioned, in other denitrifiers some *d*-type hemes may occur together with *c*-types as a *cd$_1$*-multiheme complex which functions as part of a nitrite reductase (3). This enzyme participates in dissimilatory NO$_3^-$ reduction or in NO$_3^-$ detoxification in these bacteria. Maxima for *b*- and *c*-type hemes were detected although those of *d*- or *a*-types were minimal or absent. These results were consistent with the likelihood that maxima previously attributed to cytochrome *a* in this organism were, in fact, those of a *d*-type heme and present only in denitrifying cells.

Nitrite reductases of the *cd$_1$*-type in diverse species generally been found to be soluble proteins, occurring in the cytoplasm or periplasm of denitrifying cells, although much controversy exists concerning their location. Nitrite reductase of *Thiobacillus denitrificans* appears in cytoplasm, periplasm, and on the periplasmic aspect of the inner membrane (3). Nitrite reductase was present on the cytoplasmic aspect of the inner membrane of denitrifying *Pseudomonas halodenitrificans* cells: activity was detected in membrane vesicles but not in spheroplasts. We examined subcellular fractions of denitrifying cells of *A. magnetotacticum* to determine the distribution of *d*-type cytochromes. Spectra collected from intact cells and subcellular fractions of nitrate-grown cells are shown in Fig. 19. The spectrum of intact cells (Spectrum I) described previously is used as a reference. Only soluble *c*-type hemes were detected in periplasm (Spectrum II) collected by a freezing and thawing technique (10). Cytochromes *b* 558 and *c* 551 were detected as components of inner membrane (Spectrum III). Both soluble fractions and inner membranes of other Gram negative bacteria have been reported to contain hemes of these types (3). Unexpectedly, the outer membrane fraction of *A. magnetotacticum*
showed absorption maxima for cytochromes b 558, c 551, and relative to other cytochromes, an abundance of d hemes (608, 464, 455). Thus, outer membrane appeared selectively enriched in c- and d- type cytochromes which is consistent with the presence in this fraction of a cd1-multiheme component of nitrite reductase.

**NO3- reduction.** Results from nitrite reductase assays for the various cell fractions are presented in Figure 20. Nitrite reductase activity was present only in outer membranes of cells cultured with nitrate. No N2O was detected in the headspace of vials containing inner membrane or soluble proteins. Boiled outer membrane samples did not contain nitrite reductase activity. Membranes of cells cultured with NH4+ exhibited no nitrite reductase activity, as previously reported (1). These results were consistent with spectral data which suggested that cd1-nitrite reductase was restricted to the cell outer membrane.

We separated membrane proteins of nitrate and ammonium-grown cells using SDS-PAGE. In examining these cells for proteins which might be components of a cd1-nitrite reductase, we sought evidence for a protein (hemoprotein) present in the outer membranes of nitrate-grown cells, absent from the inner membrane of these same cells, and absent from both membranes of ammonium-grown cells. A 21 KD protein fulfilling these requirements was identified (Fig. 21). It was detected only in the outer membrane of nitrate-grown cells and was absent from the inner membrane of nitrate-grown cells, and from both inner and outer membranes of ammonium-grown cells. The coincidental occurrence of this protein along with nitrite reductase activity in only the outer membrane of denitrifying cells suggested that it may be part of a cd1-nitrite reductase or a protein involved in its formation. It is unlikely that this band represents a nitrate reductase; an enzyme reported to be a soluble, constitutively produced protein in denitrifying and nondenitrifying cells of this organism (1). The presence of a cytochrome in the outer membrane of Gram negative bacteria is unusual. Most bacterial cd1-cytochrome nitrite reductases are soluble. Nitrite reductases loosely bound to the inner membrane of a few
species have been reported. These results suggest that denitrifying cells of *A. magnetotacticum* contain *cd1*-nitrite reductase tightly bound to their outer membranes. It is unlikely that the presence of this hemoprotein in the outer membrane is an artifact of the cell preparation procedure. Each subcellular fraction was shown to be at least 95% free of contaminating material from other cell fractions using known markers and established separation techniques. Moreover, the *d*-type heme was detected *only* in the outer membrane fraction. Had it been merely a contaminant from a different fraction, it should have been detected in more than one fraction.

A nitrite reductase in the outer membrane presents significant implications concerning denitrification and microaerophilicity. It may serve to exclude toxic NO₃⁻ or keep it at subtoxic concentration for sensitive sites on the cytoplasmic membrane. A location on the outer membrane might also be detrimental by limiting growth of denitrifying cultures at elevated D.O.T.
LITERATURE CITED


FIG. 18. (I) Low-temperature (77 K) difference spectrum of whole cells from continuous culture with sodium nitrate as sole nitrogen source. Maxima for cytochrome $c$ occur at 547, 519, and 418 nm ($\alpha$, $\beta$, and $\gamma$ peaks, respectively). Cytochrome $b$ is identified by shoulders at 553, 525, and 420 nm. Cytochrome $d$ is indicated by maxima at 608, 464, and 556 nm. (II) Low-temperature (77 K) difference spectrum of whole cells continuously cultured with ammonium chloride as sole nitrogen source. Note the lack of peaks attributable to $d$-type hemes.
FIG. 19. Low-temperature (77K) difference spectra of whole cells and cellular fractions from denitrifying cells. Spectrum I = whole cells; spectrum II = periplasmic fraction; spectrum III = inner membrane; spectrum IV = outer membrane. Peaks attributable to $d$-type hemes are present only in outer membrane preparations.
Specific activity
(μmol N₂O formed · mg protein⁻¹ · hr⁻¹)

<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>NO₃⁻</th>
<th>NH₄⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer membrane</td>
<td>4.8</td>
<td>0</td>
</tr>
<tr>
<td>Inner membrane</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Periplasm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Boiled outer membrane</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

FIG. 20. Nitrite reductase specific activities by membrane fractions of *A. magnetotacticum* strain MS-1 grown with NaNO₃⁻ or NH₄CL as sole N sources.
FIG. 21. SDS-PAGE of cellular fraction of cells from continuous culture with sodium nitrate or ammonium chloride as sole nitrogen sources. Arrow indicates protein band present only in the outer membrane fraction of denitrifying cells.
CHAPTER 5
CHAPTER 5

Metal Accumulation and Resistance by
Aquaspirillum magnetotacticum

INTRODUCTION

The quest to recover metallic elements from the environment has recently intensified. Dwindling deposits of high grade metals have prompted extensive research for improved extraction from low grade metal ores, principally sulfide complexes. Additionally, soluble metal species produced from mining, industrial processes, and municipal waste water are being released into the environment in high quantities. The solubility and retention of metals in soil and water is determined by the total amount released, the content of mobilizing and immobilizing organic matter, and the concentration of salts and ligands (12). The ultimate fate of metals released is still uncertain, but the toxic nature of metallic ions poses a very real threat to all forms of life on this planet.

Historically, the recovery of precious and toxic metals has involved a variety of purely physico-chemical processes, including precipitation, ion exchange, solvent extraction, and electrowinning. These are inherently expensive and inefficient (16).

Advancements in microbial technology have provided incentives for both the industrially and environmentally inclined investor to examine the feasibility of biological metal recovery from both waste water and sulfide ore deposits.

At present, microorganisms play their premier metal recovery role in the leaching process of low grade metal sulfide ores. Thiobacillus ferrooxidans is the primary prokaryote involved in this process. Hutchins et al. (14) present an excellent review of
recent developments in this field, especially with respect to leaching of uranium, copper, and precious metals, as well as the biogenic desulfurization of coal.

The ability of microorganisms to remove soluble metal ions from industrial and municipal waste waters is also currently under investigation. Two systems exist which account for the accumulation of metal cations from solution. Passive accumulation (or biosorption) refers to the electrostatic association of positively charged metals ions with the negatively charged walls of bacteria and other microorganisms. The second system of metal bioaccumulation does not involve electrostatic association of metal ions with the outer layers of cells. Alternatively, cells selectively transport metal ions across their cell membrane systems and accumulate them intracellularly.

Biosorption by bacteria appears to differ among morphological types. Gram positive bacteria have been shown to accumulate divalent cations by the negatively charged carboxyl groups of the peptidoglycan within the cell walls (5). Investigators have also reported metal complexation by the phosphoryl substituents of the Gram negative lipopolysaccharide (6). In either case, biosorption of metals by microorganisms offers feasible possibilities for recovery of metals from solution.

Biosorptive systems have mainly been studied with regard to heavy metal recovery. Lead, plutonium, uranium, and mercury are of particular interest because of their potentially toxic nature as well as their relatively high content in mine tailings and industrial effluents. In a recent experiment, Nakajima et al.(18) compared the absorptive capacity of immobilized cells from a variety of microorganisms. They report the preferential accumulation of uranium by all tested species of bacteria, yeasts, and fungi when saturated with a mixed metal solution. The bacterial and fungal species absorbed an average of 80 to 90 mg of uranium per gram of dry biomass while the tested yeast strains accumulated only half as much. The amount of biosorbed lead and copper was comparable among the tested microorganisms, averaging about 35-45 mg per gram of dry biomass. The lighter
transition metals, such as cadmium, zinc, nickel, cobalt, and manganese were absorbed only in trace amounts.

Friis et al. (10) have shown that the dry biomass of *Streptomyces longwoodensis* is an extremely effective biosorbent material. Columns containing these organisms complex up to 0.44 g of uranium per gram of dry weight biomass at pH 5.0. Determining the relationship between the phosphorous content and uranium uptake, these investigators propose that the metal ions are bound to the phosphodiester residues within the Gram negative wall.

Algae have also been shown to be effective biosorbents. Hasset et al. ( ) reported that high levels of lead, zinc, copper, mercury, and cadmium in a lead mine discharge stream were reduced to acceptable drinking levels by in situ algal biomass.

Compared to the large biosorptive capacity of microbial cells, intracellular accumulation is quite minimal and probably not feasible as a means of metal recovery. A group of unique bacteria do accumulate large amounts of metals as intracellular inclusions. Magnetic bacteria recover solubilized iron from their environment and deposit it in the form of a crystallized iron oxide. In cells of *Aquaspirillum magnetotacticum*, the crystalline inclusions are composed of the iron oxide magnetite and account for about 2% of the cell's dry weight. This amount is comparable to the passive accumulation systems of the biosorbents discussed above. In this investigation we wished to determine whether cells of *A. magnetotacticum* can accumulate metals other than iron as partial constituents of their metallic inclusions. Incorporation of toxic metals in a magnetic moiety could prove very valuable as a tool for metal reclamation.

**MATERIALS AND METHODS**

**Culture conditions.** Cells of *Aquaspirillum magnetotacticum* were cultured with 0 μM, 20 μM, and 200 μM concentrations of each of the following compounds; Cu(CHOO)₂
2H$_2$O, CrCl$_2$ 6H$_2$O, CoCl$_2$ 6H$_2$O, NiCl$_2$, TiCl$_3$, HgCl$_2$, and Pb$_3$(C$_6$H$_5$O$_7$)$_2$ 3H$_2$O. All cultures were passed three times in the various media prior to sampling.

**Electron microscopy.** Unstained cells and cells negatively stained with 0.5% uranyl acetate were visually examined using a Hitachi H600 scanning-transmission electron microscope operating in the transmission mode at 80 kV accelerating voltage. Cells were also examined using a VG Microscopes HB5 scanning-transmission microscope operating in the STEM mode at an accelerating voltage of 100 kV. Elemental dispersive X-Ray analysis (EDAX) was performed with a Link System LZ5 detector.

**Purification of magnetite from cells.** Cells were harvested from 15 1 growth vessels through tangential flow filtration followed by centrifugation at 5000 X g for 10 min. Cells were washed 10 times in 10mM HEPES buffer (pH 7.4) and mechanically disrupted using a French pressure cell. Magnetic material was separated from non magnetic debris with the application of a strong magnetic field for one hour. The magnetite was liberated from associated organic matter by suspending the intact magnetosomes in 5N NaOH. The mineral phase was washed excessively with deionized distilled water and magnetically separated from the supernatant fluid. This purified magnetite (approximately 10 mg wet weight) was solubilized in 10 ml of 0.1N HCl by incubating the suspension at room temperature for 7 days. The acidic metal solutions were analyzed using an Instrumentation Laboratories atomic absorption spectrophotometer operating with an acetylene-air flame for all metals except uranium which was combusted with an acetylene-nitrous oxide flame.

**RESULTS**

**Cell morphology.** As recorded in Figure 22 and 23, cells cultured with each of the tested metals exhibited extensive production of poly-B-hydroxybutyric acid which appear as electron transparent intracellular deposits which also deeply with Sudan black by light microscopy. Adjacent to the PHB granules were electron dense inclusions. As evidenced
by scanning electron microscopy and elemental dispersive X-ray analysis (Fig. 24), these deposits contain large amounts of sodium and phosphorus, consistent with the composition of polyphosphate inclusions. They also appear as red deposits in a blue cytoplasm when stained with toluidine blue which is also indicative of polyphosphate. Positioning the electron probe next to these inclusions, the X-Ray signal for sodium and phosphorous decreases to background level (Fig. 25). Other metals were not detected within the polyphosphate inclusions.

Unusual magnetosome chain morphology was observed within many cells cultured at 20 μM and 200 μM concentrations of the investigated metals. The number of cells exhibiting aberrant magnetosome arrangement appeared to increase with each successive passage. By the eighth passage roughly 30% of the population contained unusual chains. Figure 26 is a photomicrograph of an unstained cell grown with 20 μM Ni in which the magnetosomes do not line in a single chain. Figure 27. represents a cell which was cultured with 20 μM lead. The divergent chain formation is quite apparent. Mercury grown cells as well exhibit aberrant magnetosome chain morphology. Multiple magnetosome chains assumed no consistent orientation within the cell but were divided equally between the daughter cells of an apparently dividing cell.

Composition of magnetite particles. Results from elemental dispersive X-Ray analyses of the magnetite grains within intact cells grown with 20 μM nickel are presented in Figure 28. As expected, iron is the predominant compositional element within the magnetic inclusions. Nickel was not detected within the magnetite grains, nor was detectable within the surrounding cytoplasm (Fig. 29). As evidenced by atomic absorption, none of the investigated metals were detected within the purified magnetic fraction.
DISCUSSION

Cells of *A. magnetotacticum* are affected by relatively high concentrations of metals other than iron in at least three ways. Intracellular accumulation of PHB is normally associated with nitrogen or energy limitations. The production of this carbon storage polymer observed during this study suggested that metals affected either nitrogen assimilation or some form of cellular catabolism. Likewise, the occurrence of polyphosphate granules was another indication that the tested metals were somehow affecting cellular metabolism, although at this point the mechanisms remain unknown.

Aberrant chain morphologies in magnetic cells were particularly interesting. Normally cultured cells contained about 20 octahedron shaped crystals of magnetite each measuring about 42 nm on a side and appeared as a single chain along the central axis of the cell (3). What determines the relatively consistent size of magnetosomes or why magnetosomes normally appear in chains is still a mystery. It has been speculated that the envelope surrounding each of the crystals may somehow be involved (7). The effects of metals on aberrant chain morphology may prove beneficial for the elucidation of the controlling mechanisms of magnetosome formation.

This study has shown that cells of *A. magnetotacticum* do not accumulate metals other than iron in their magnetic inclusion bodies. However it would be a grave error to assume that there exists no magnetic species which can accumulate metals other than iron as intracellular inclusions. Environments rich in toxic metals have been repeatedly shown to influence the population of indigenous microflora (2, 13). Duxbury et al. (8) have compared relative tolerances between bacteria isolated from metal laden waters to those found in waters which were free from high levels of metal contaminants. They report that tolerance to nickel by adapted bacteria was nearly four times as great as by cells from unpolluted environments. In a study by Aiking et al. (1), cells of *Klebsiella aerogenes* were maintained at a steady state in a continuous culture system free from high levels of
toxic metals. Upon the addition of $6 \times 10^{-4} M$ cadmium, growth ceased but resumed (presumably reflecting adaptation) within 5 hours. The adapted cells were more resistant to cadmium and could be maintained at a steady state in the presence of this metal. These cells also formed extracellular deposits of cadmium sulfide which accounted for about 2.5% of their dry weight. It was proposed that these sulfide deposits accounted for cadmium tolerance. Large cadmium resistant populations also occur naturally in soil to which metal-rich sewage sludge has been applied (4).

Environmentally adapted organisms which concentrate toxic metals as intracellular deposits may exist. Magnetic bacteria both provide a unique handle by which bacterially adsorbed metals may be recovered. Their ubiquitous nature supports the possibility that they may indeed be recovered from the sediments of metal rich environments. The discovery of a magnetic bacterium which can incorporate toxic metals intracellularly, or which can be made to by genetic manipulation of uptake systems for metals other than iron, should be quite valuable for the advancement of biologically mediated metal recovery.
LITERATURE CITED


FIG. 22. Cells of *A. magnetotacticum* grown with 20 μM added nickel. Note the electron transparent regions which have previously been identified as poly-B-hydroxybutyrate granules. Electron dense inclusions are also evident.
FIG. 23. Unstained preparation of *A. magnetotacticum* grown with 20 μM added nickel. As shown in the previous figure, electron dense and electron transparent regions are quite evident.
FIG. 24. EDAX spectrum of an electron dense inclusion within a cell of *A. magnetotacticum* grown with 20 μM added nickel. Note the high sodium and phosphorus peaks which are consistent with polyphosphate inclusions. Also note the absence of a peak for nickel.
FIG. 25. EDAX spectrum of cytoplasm adjacent to putative polyphosphate inclusions of cells grown with 20 µM nickel. Note the reduction in sodium and phosphorus peaks.
FIG. 26. Nickel grown cell of *A. magnetotacticum* exhibiting aberrant magnetosome chain morphology.
FIG. 27. *A. magnetotacticum* grown in the presence of 20 \( \mu M \) added lead. Note the duel chain morphology which is abnormal in this species.
FIG. 28. EDAX spectrum of magnetosomes within a cell cultured with 20 μM added nickel. Note the absence of a nickel peak. The peak which is adjacent to the region expected for nickel is a copper response generated from the grid.
FIG. 29. EDAX spectrum of cytoplasm which is adjacent to a magnetosome within a cell grown with 20 \( \mu \text{M} \) added nickel.
APPENDIX A

Procedure for Low Temperature Cytochrome Spectra

Cross section of SPECAC 21.000 variable temperature cell.

Equipment Installation

- Remove cover plate (6 screws) from chamber door
- Remove chamber door (2 screws)
- Remove sample carrier and patch cord
- Remove both pieces of chamber floor
- Install variable temperature cell holder (2 thumbscrews)
- Slide variable temperature cell vacuum jacket into the cell carrier
- Install chamber door

Attach the window heater lead to the receptacle located on one half of the cover plate. Secure both halves of the cover plate with six screws and make the chamber light tight with electrical tape. Attach window heater patch cord to the power supply. Turn on the power and the window heater switches.
Create the following program:

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<th>Parameter</th>
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</thead>
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<tr>
<td>10</td>
<td>Interval = 0 min.</td>
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<td>l speed = 100</td>
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<td>24</td>
<td>End l = 370</td>
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<td>25</td>
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<tr>
<td>52</td>
<td>Plot scale: Yes</td>
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</table>

**Sample Preparation**

- Adjust protein concentration of whole or fractionated cells to about 10 mg/ml in 10 mM HEPES buffer (pH 7.4).
- Dilute 2 parts sample with 1 part ethylene glycol.
- Thoroughly bubble preparation with air by means of a 1000 µl pipetman.
- Using a 1 ml syringe fitted with a threaded adapter, fill sample cell through the bottom port while allowing all bubbles to escape through the top port.
- Seal both ports using hex screws.

**Recording Absorbance of Oxidized Sample**

- Turn off most of the room lights.
- Place sample holder in vacuum jacket.
- Push Gainset
- After the machine makes a noticeable Click, quickly remove sample cell holder and plug top of vacuum jacket with sponge.
- After the plot axes are drawn, remove sponge and insert sample cell holder into vacuum jacket.
- Attach vacuum line and turn on pump.
- Pour liquid nitrogen into cooling core of sample cell holder.
- Add more as liquid boils away.
- Monitor temperature and absorbance. When both are stable, push Run.
- Absorbance of oxidized sample is now being recorded into memory.
Dithionite Reduction of Sample

- Thaw oxidized sample by turning on cell heater.
- Turn cell heater OFF when sample temperature is just below room temperature.
- In subdued light, release vacuum, remove sample cell holder, and plug top of vacuum jacket with sponge.
- Remove sample and mix with unused sample.
- Add a few grains of sodium dithionite and mix well.
- Inject reduced sample into sample cell.
- Remove Sponge and place sample cell holder in vacuum jacket.
- Turn on vacuum pump.
- Cool to liquid nitrogen temperature as before.
- Push run to plot spectrum.

Changing Scale

- If peaks are small, you may wish to expand the scale.
- Push Stop before the scan reaches 370 nm.
- Push Modify.
- If baseline is near 0.000, enter 46 in response to Question #?
- Enter 0.2 in response to Suppress?
- Enter 0.2 in response to Span?
- Enter default values for any other questions.
- To begin modified plot, push Run.