MICROAEROPHILIC PHYSIOLOGY OF AQUASPIRILLUM MAGNETOTACTICUM

KEVIN ALLEN SHORT
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MICROAEROPHILIC PHYSIOLOGY OF AQUASPIRILLUM MAGNETOTACTICUM

Abstract
Little is known concerning the physiology of obligate microaerophiles. This thesis describes research aimed at furthering our understanding of the obligately microaerophilic nature of the magnetic diazotrophic denitrifier, Aquaspirillum magnetotacticum. Included are studies of iron respiration-driven proton translocation, of microaerobic denitrification and associated NO\textsuperscript{2-} toxicity, and of expression and cellular location of O\textsuperscript{2-} detoxifying enzymes in this organism.

Keywords
Biology, Microbiology
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Microaerophilic physiology of *Aquaspirillum magnetotacticum*

Short, Kevin Allen, Ph.D.

University of New Hampshire, 1987
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UMI
MICROAEROPHILIC PHYSIOLOGY OF
AQUASPIRILLUM MAGNETOTACTICUM

BY

KEVIN ALLEN SHORT
B.S. Botany, Oregon State University, 1980
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DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
in
Microbiology

September, 1987
This dissertation has been examined and approved.

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Date July 23, 1987
This dissertation is dedicated to my parents for always encouraging and nurturing my interests.
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ABSTRACT

MICROAEROPHILIC PHYSIOLOGY
OF AQUASPIRILLUM MAGNETOTACTICUM

by

Kevin Allen Short
University of New Hampshire, September, 1987

Little is known concerning the physiology of obligate microaerophiles. This thesis describes research aimed at furthering our understanding of the obligately microaerophilic nature of the magnetic diazotrophic denitrifier, Aquaspirillum magnetotacticum. Included are studies of iron respiration-driven proton translocation, of microaerobic denitrification and associated NO\textsuperscript{2} toxicity, and of expression and cellular location of O\textsubscript{2} detoxifying enzymes in this organism.
INTRODUCTION

Part One

Magnetic bacteria were first described just over a
decade ago (Blakemore, 1975). They are a ubiquitous and
morphologically diverse assemblage of Gram negative
eubacteria possessing magnetosomes (Balkwill et al.
1980) which orient them in the geomagnetic field
(Blakemore et al. 1980; Frankel et al. 1981; Moench and
Konetzka. 1978). There is but one species in pure
culture- the obligately microaerophilic diazotrophic
denitrifier Aquaspirillum magnetotacticum (Blakemore et
al. 1979). Frankel et al. (1979) demonstrated that
cells of A. magnetotacticum contain magnetite (Fe₃O₄).
At the 1980 Dahlem Conference on Mineral Deposit and the
Evolution of the Biosphere, Brock lamented the fact that
"there are no microbial structures that can be unequivoco-
cally associated with O₂ evolution". In chapter one of
this thesis- Microaerophilic Conditions are Required for
Magnetite Synthesis by Aquaspirillum magnetotacticum
(Blakemore, Short, Bazylnski, Rosenblatt, and
Frankel,1985. Geomicrobiol. J. 4:53-71) it is proposed
that biogenic magnetite particles may comprise a highly
sensitive indicator of the development of the Earth's
oxidizing atmosphere. Therefore, this introduction
includes a brief discussion of the development of
Earth's oxidizing atmosphere and the evolution of oxidative respiratory metabolism some 2-3 billion years before present (Ga; giga anni) as these events are currently thought to have occurred.

Geological evidence for the development of an oxidizing atmosphere is preserved in banded iron formations (BIF's); rocks consisting of alternating bands of oxidized and reduced Fe compounds. The ratio of oxidized Fe to total Fe in BIF's is 0.31-0.58 (Gole and Klein, 1981) with primary mineralogical components of quartz (SiO$_2$), magnetite (FeO Fe$_2$O$_3$) hematite (Fe$_2$O$_3$) and siderite (FeCO$_3$) (Walker et al. 1983). The most ancient BIF's were formed 3.8 Ga, but the vast majority were formed between 2.7 and 1.7 Ga, at which time it is believed that stable oceanic and atmospheric environments were established (Cloud, 1972; 1973). Cairns-Smith (1978) and others (Walker, 1980; Carver, 1981), raised the hypothesis that direct photo-dissociation of water vapor or seawater was a possible abiotic source of atmospheric O$_2$. It is widely believed, however, that ancient colonies of photosynthetic cyanobacteria, now preserved as stromatalites (Gurin, 1980; Nagy, 1983; Walter, 1983; Schopf and Walter, 1983) were almost solely responsible for the contribution of O$_2$ to the environment.
Oxidized Fe (Fe$^{3+}$), N (NO$_3^-$, NO$_2^-$), S (SO$_4^{2-}$, SO$_3^{2-}$), Mn (Mn$^{5+}$, Mn$^{3+}$) and O$_2$ became available simultaneously with, or just prior to the development of an oxidizing atmosphere (Chapman and Ragan, 1980). Bacteria with pre-existing proton-translocating ATPases, functioning perhaps to rid cells of fermentation acids, and proton-translocating electron-transport chains, perhaps functioning previously in fumarate reduction (Garland, 1981), evolved mechanisms for generating a proton motive force (Mitchell 1961, 1966) by reducing these available oxidized substrates as terminal electron acceptors. Bacteria have evolved respiratory chains branched (Smith, 1968; White and Sinclair, 1971) at the primary dehydrogenase (Jones, 1977), quinones (Mitchell, 1975) or at the chain terminus (Jones, 1982). A branched chain allowed bacteria to utilize one or more terminal electron acceptor species. Thus, it is likely that bacteria capable of respiring with Fe$^{3+}$, NO$_3^-$, NO$_2^-$, Mn$^{5+}$, Mn$^{3+}$, SO$_4^{2-}$, SO$_3^{2-}$ and O$_2$ first evolved during the same period in which BIF's were formed.

As proposed by Karlin et al. (1987), bacterial magnetite may be a metabolic by-product of cell's use of Fe redox transitions in energy transformation. Bacterial energy conservation (phosphate esterification) is accomplished either directly by substrate level phosphoryl-
lation or indirectly via chemiosmosis (Thauer et al. 1977). The chemiosmotic hypothesis, proposed by Mitchell in 1961 to describe oxidative and photosynthetic phosphorylation, has 3 primary requirements; (a) the presence of a proton-translocating redox system, (b) a proton-translocating ATP phosphohydrolase, and (c) a passive coupling membrane impermeable to ions, particularly to \( H^+ \) and \( OH^- \), except via specific exchange-diffusion systems including the \( H^+ \)-translocating ATPase (Jones, 1982). The transmembrane electrochemical potential difference or proton motive force (PMF), measured in mV, has both an electrical (\( \Delta \Psi \)) and chemical potential (\( \Delta pH \)) component.

\[
PMF = \Delta \Psi - 2.303 \frac{RT}{F} (pH_{out} - pH_{in})
\]

Bacterial respiration, therefore, involves substrate oxidation with electrons passing vectorially from redox carriers of high to low electronegativity. This electron "flow" ends with reduction of a terminal electron acceptor, and is coupled at discrete sites to translocation of protons to the cell exterior (Scholes and Mitchell, 1970). Proton translocation maintains the electrochemical gradient of \( H^+ \) ions. Proton translocation measurements (Scholes and Mitchell, 1970) have been widely used to assess bacterial respiration with diverse oxidants. In theory, terminal respiratory chains are composed of a series of usually 2 to 4 proton
translocating loops. The number of protons translocated per gram atom oxidant is therefore dependent on the number of loops proceeding the terminal electron accepting enzyme (an oxido-reductase) and on the number of electrons the oxidant species will consume. By collapsing the chemical component of the PMF (with a permeant ion) the rate of extracellular acidification by whole cells is extremely rapid and matches the rate of respiration (Scholes and Mitchell, 1970, Jones, 1982). Respiration with $O_2$, $Fe^{3+}$, or $NO_3^-$ involves a terminal respiratory chain which may differ in cytochrome composition, but which terminates with one or more specific reductases (e.g. $NO_3^-$ or $Fe^{3+}$ reductase) or cytochrome $c$ oxidases ($O_2$ reductase).

Cells capable of maintaining a proton motive force by disposal of electrons to ferric iron should, therefore, exhibit proton pulses when presented with this potential oxidant.

Chapter two of this thesis, *Iron respiration-driven proton translocation in aerobic bacteria* (Short and Blakemore, 1986, J. Bacteriol. 167:729-731) presents evidence for proton translocation by magnetic cells of *A. magnetotacticum* when provided with the oxidant iron ($Fe^{3+}$). Cells of a non-magnetic aerotolerant strain did not exhibit iron-driven proton translocation, although
cells of each strain translocated protons when provided
O₂ or NO₃⁻.

Fe respiration: Fe reduction by bacteria can be either
assimilatory or dissimilatory. Assimilatory uses
include synthesis of hemes (Dailey and Lascelles, 1974,
1977; Moody and Dailey, 1985), non-heme Fe proteins,
NO₃⁻ reductase, nitrogenase, and hydrogenases (Neilands,
1974).

Dissimilatory Fe reduction could serve as a means
of regenerating NAD, with Fe functioning as a H
sink (Jones et al. 1983), or as a direct means of energy
conservation, with Fe as the terminal electron
acceptor (Jones, 1982). Jones et al. (1983) found that
Fe reduction by a Vibrio sp. closely correlated with
growth. In cell-free extracts inhibitors of electron
transport. NO₃⁻ reductase, ATPase and ionophores
reduced the yield of Fe²⁺, although rotenone and
antimycin A did not affect the activity of whole cells.
The authors observed no increase in molar growth yield
in the presence of Fe³⁺ and proposed that cells used
Fe³⁺ as a H sink to allow regeneration of NAD.

As early as 1947, Roberts had reported that glucose
fermentation rate by Bacillus polymyxa was doubled (and
less H₂ produced) when insoluble FeOH was included in
the growth medium. Bromfield (1954) found cells of B.
circulans reduced Fe during growth and appeared to mediate reduction via a dehydrogenase since reduction was not inhibited by the respiratory inhibitors CN− or N3. Cells of Micrococcus lactilyticus consumed H2 while reducing Fe (Woolfolk and Whitely, 1962).

Balashova and Zavarzin (1980) isolated a facultatively anaerobic pseudomonad capable of reducing Fe with H2. The bacterium reduced Fe(OH)3, NO3− and NO2−. Both NO2− and Fe2+ inhibited growth.

Criticisms that Fe reduction is strictly a secondary result of fermentation or lowered redox potential, were refuted by Munch and Ottow (1982) in studies with Clostridium butyricum and B. polymyxa and later by Jones et al. (1983) in studies with a Vibrio sp. and by Tugel et al. (1986) in which it was demonstrated (by enclosing the Fe in a dialysis bag) that cell-Fe contact was necessary for Fe reduction to occur. However, there is considerable evidence that the rate and extent of bacterial Fe reduction depends on the form of the ferric iron available to cells. Amorphous ferric iron is more readily reduced than is crystalline ferric iron. For example iron phosphate (FePO4·4H2O) and iron hydroxide (Fe(OH)3) are reduced more easily than hematite (Fe2O3) (Troshanov, 1969; De Castro and Ehrlich, 1970; Munch and Ottow, 1982; Jones...

Bacteria reported to respire with Fe are usually NO$_3^-$ reducers, and many workers have reported NO$_3^-$ inhibition of Fe reduction (Ottow, 1968; 1970; Ottow and von Kloptopek, 1969, Obuekwe et al. 1981). Ottow (1970) isolated mutants unable to reduce NO$_3^-$ and found that cells of the mutant strain had decreased capacity to reduce Fe but this was not influenced by the presence of NO$_3^-$. Moreover, NO$_3^-$ reductaseless cells of *Thiobacillus ferrooxidans* and *T. thiooxidans* reduced Fe by coupling the reaction to oxidation of elemental sulfur via a heat-labile ferric iron-reducing system (Kino and Usami, 1982; Sugio et al. 1984). The mechanism of NO$_3^-$ inhibition of Fe$^{3+}$ reduction has been proposed to be diversion of electrons (Ottow, 1970), NO$_2^-$ inhibition of Fe$^{3+}$ reduction (Lascelles and Burke, 1978; Balashova and Zavazin, 1980) or chemical oxidation of Fe$^{2+}$ by NO$_2^-$ (Obuekwe et al. 1981).

There are few physiological studies addressing the respiratory components involved with Fe reduction; fewer document energy conservation via Fe respiration. Dailey and Lascelles (1977) working with membranes of *Aquaspirillum* (Spirillum) itersonii and respiratory inhibitors, suggested that the reduction of Fe
occurred at one or more sites on the respiratory chain before cytochrome c. Fe reduction was strongly inhibited by aeration, which may be analogous to the inhibition of NO$_3^-$ reduction by aeration. Lascelles and Burke (1978) worked with membranes of *Staphylococcus aureus* and based on the effect of respiratory inhibitors and NO$_3^-$ on Fe reduction, concluded that "reduced components of the electron transport chain that precede cytochrome b serve as the source of reductant for ferric iron and that these components are oxidized preferentially by a functional NO$_3^-$ reductase system".

Arnold et al. (1986) studied the components of the electron transport chain involved in O$_2$ and Fe$^{3+}$ reduction by cells of a *Pseudomonas* sp. and concluded that cells expressed a constitutive Fe$^{3+}$ reductase and an Fe$^{3+}$ reductase inducible by low O$_2$. They proposed that the constitutive reductase was part of a complete (dehydrogenase, quinones, cytochromes) proton translocating respiratory chain. In this chain, Fe was reduced prior to O$_2$ with a branch to Fe occurring between cytochromes b and c. The inducible Fe reductase was thought to receive electrons from the dehydrogenase, without the translocation of protons, and serve solely therefore as an electron sink.
The reduction of ferricyanide with NADH by membrane vesicles of *B. subtilis* (Bischof et al. 1976) or with formate by membrane vesicles of *Escherichia coli* (Boonstra et al. 1976) was linked to a functioning electron transport chain. Based on the reduction of chlorate (a structural analog of NO₃⁻), it was hypothesized that one of two sites of Fe reduction was also a NO₃⁻-reductase. However, chlorate mediates its effect by inhibiting Mn containing enzymes. Under anaerobic conditions, ferricyanide reduction generated sufficient proton motive force to drive active transport of proline. The redox potential of the Fe³⁺/Fe²⁺ couple ($E'_0 = +772$ mV) is high enough to drive electron transport phosphorylation with all known physiological electron donors (Thauer et al. 1977).

**Part Two**

As mentioned, *A. magnetotacticum* and apparently other magnetic bacteria, not yet in axenic culture, are obligate microaerophiles. What is the nature of this fastidious O₂ requirement? The optimal Pₒ₂ for growth of *A. magnetotacticum* varies with the N source. Denitrifying cells cultured with NO₃⁻ are more sensitive to O₂ than those cultured with NH₄⁺ (Blakemore et al. )
1985). These findings suggested that either:

1. Cells cultured with NH₄⁺ grow optimally at higher PO₂ than cells cultured with NO₃⁻ due to a limitation for terminal electron acceptor.

2. Cells cultured with NH₄⁺ express relatively higher activities of O₂ detoxifying enzymes (i.e., superoxide dismutase, catalase, and peroxidase) than do those cultured with NO₃⁻.

3. High O₂ concentration inhibited NO₂⁻ reduction to a greater degree than it did NO₃⁻ reduction, thereby allowing NO₂⁻ to accumulate in toxic quantities.

Chapter three of this thesis O₂ and NO₂⁻ toxicity in an obligately microaerophilic denitrifier (Short and Blakemore, submitted for publication, Can. J. Microbiol.) described efforts to understand more completely the microaerophilic nature of this organism. This research effort required that we explore the enhanced sensitivity to O₂ of the magnetic spirillum when cultured under denitrifying conditions.

Denitrification: Denitrifying bacteria dissipate 90% of available nitrogen oxides (NO₃⁻ and/or NO₂⁻) to gaseous oxides (NO, N₂O) and couple this reductive process to electron transport (Knowles, 1982; Bleakley and Tiedje, 1982). Most denitrifying bacteria use nitrogen oxides as terminal electron acceptors only in
the absence of $O_2$ (Knowles, 1982). Dissimilatory $NO_3^-$ reductase is anoxia derepressed and may or may not be induced by $NO_3^-$ (Payne, 1973). Several studies document tolerance of denitrifying bacteria to low levels of $O_2$ (Krul, 1975; Aida et al. 1984). Some denitrifying bacteria simultaneously respire with $O_2$ and $NO_3^-$ (Dunn et al. 1979; Bazylnski and Blakemore, 1983; Ottow and Fabig, 1983; Robertson and Kuenen, 1984a; 1984b).

Dissimilatory as opposed to assimilatory $NO_3^-$ reductase (which is encoded by different genes) is a molybdenum-iron-labile sulfide protein (Stouthamer, 1976; Payne, 1981). Except for one known exception (Gauthier et al. 1970), dissimilatory $NO_3^-$ reductase is a membrane bound protein located on the inner aspect of the cytoplasmic membrane (Jones, 1982; De Vries, 1984) or transcytoplasmic membrane (Weintjes et al. 1979). The enzyme is linked to the respiratory chain via b-type cytochromes (Lam and Nicholas, 1969; John and Whatley, 1970).

Dissimilatory $NO_2^-$ reductase (DNIR) has been described as a soluble protein and as a protein loosely associated with the outer surface of the cytoplasmic membrane (De Vries et al. 1982; Payne, 1981; Knowles, 1982). Lam and Nicholas (1969) found 70 % of total DNIR
activity in *P. denitrificans* in a soluble fraction. The enzyme was asserted to be on the cytoplasmic side of the cytoplasmic membrane in *P. denitrificans* (Kristjansson et al. 1978) and *P. aeruginosa* (Saraste and Kuronen, 1978), because of evidence obtained from stoichiometric proton translocation studies and by direct visualization using ferritin-conjugated antibodies. However, other studies with the same organisms, indicated that DNIR was located on the periplasmic side of the cytoplasmic membrane (Wood, 1978; Meijer et al. 1979; Aliefounder and Ferguson, 1980). Cells of *P. halodenitrificans* express an inner cytoplasmic membrane DNIR. Three known types of DNIR are classified on the basis of their prosthetic group (Henry and Bessieres, 1984): (a) a cytochrome cd$_1$ multiheme type, (b) a Cu protein type, and (c) a c heme-type. As a membrane associated protein tied to energy conservation (Koike and Hattori, 1975), cd$_1$ DNIR acts secondarily as an O$_2$-reductase whereas the Cu protein or the apoenzyme containing c-type alone has no O$_2$-reductase activity (Henry and Bessieres, 1984). DNIR activity was more strongly inhibited by (or sensitive to) O$_2$ than was NO$_3^-$ reductase activity (Lam and Nicholas, 1969; Krul and Veeningen, 1977; Meiberg et al. 1980). Therefore, (at elevated Fo$_2$) NO$_2^-$
could accumulate to toxic levels at elevated $P_{O_2}$.

Toxicity is thought to occur by at least two mechanisms. $NO_2^-$ is known to depress oxidase activity in cells of \textit{Pa. aeruginosa} (Rowe et al. 1979; Yang, 1985; Casella et al. 1986) by a uncompetitive mechanism of inhibition quite different from that of the oxidase inhibitors $CN^-$, $N_3^-$, and $CO$ (Yang, 1985). $NO_2^-$ also decreased the numbers of protons translocated per gram atom oxidant in \textit{Pa. putida}, \textit{Pa. aeruginosa} and \textit{P. denitrificans} (Rake and Eagon, 1980) and increased cytoplasmic membrane proton conductance in cells of \textit{P. denitrificans} (Meijer et al. 1979).

In chapter three of this thesis evidence is presented for the enhanced inhibitory role of $O_2$ in denitrifying cells as a consequence of its effect in inhibiting $NO_2^-$ reductase with consequent accumulation of toxic levels of $NO_2^-$. However, these findings did not explain the sensitivity of the magnetic strain to oxygen under non-denitrifying conditions (i.e. with $NH_4^+$). Nor did they explain the observed aerotolerance of a non-magnetic mutant of the magnetic strain. In chapters three and four of this thesis, evidence is presented that the differences in relative sensitivity of these strains to $O_2$ under non-denitrifying conditions is due to different specific activities of $O_2$ detoxifying
enzymes. As a prelude to these chapters, discussion is made next of O\textsubscript{2} respiration, O\textsubscript{2} toxicity and O\textsubscript{2} detoxification.

O\textsubscript{2}-respiration: Aerobic respiratory systems are similar to NO\textsubscript{3}\textsuperscript{-} and Fe\textsuperscript{3+} respiratory systems in that the basic redox carriers—flavoproteins, Fe-S proteins, quinones and cytochromes are present. Aerobic respiratory chains terminate with one or more O\textsubscript{2}-reducing cytochrome oxidases (Jones, 1978). Variations in the redox carriers can be induced by culturing cells under different growth conditions. Bacteria which synthesize cytochrome oxidase aa\textsubscript{3} (P. denitrificans, Alcaligenes eutrophus) completely or partially replace aa\textsubscript{3} with cytochrome oxidase o when cultured with low O\textsubscript{2} (Jones, 1978). Cells of E. coli, Klebsiella pneumoniae, Haemophilus parainfluenzae and Rahnella nitroreducens increase the synthesis of cytochrome oxidase d and cytochrome oxidase a\textsubscript{1} relative to cytochrome o, when cultured with low O\textsubscript{2} (Jones, 1978). Cells of A. magnetotacticum cultured at a dissolved O\textsubscript{2} tension (d.o.t.) of 1 % had cytochrome oxidases o and a\textsubscript{1} and cells cultured at > 5 % d.o.t. had a 12-fold decrease in cytochrome oxidase a\textsubscript{1}. In addition, cells cultured with NH\textsubscript{4}\textsuperscript{+} had decreased a-type, cd\textsubscript{1} (nitrite reductase) and b-type hemes as compared to those denitrifying with NO\textsubscript{3}\textsuperscript{-}.
(O'Brien et al. 1987).

**Oxygen Toxicity:** Bacterial cells respiring with \( \text{O}_2 \) produce \( \text{O}_2^- \) (McCord and Fridovich, 1969) and the hydroxyl radical (OH\(^-\)) both of which are capable of oxidizing proteins and lipids (Beaman and Beaman, 1984). Superoxide anion is an intermediate product in the univalent pathway of \( \text{O}_2 \) reduction to \( \text{H}_2\text{O} \), and is generated as an intermediate in autooxidation of ferredoxin, flavins, heme proteins and quinones (Fridovich, 1975; Hill, 1978). Superoxide toxicity is mediated by two distinct sets of reactions: the enzymatic dismutation of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) by superoxide dismutase (SOD), (McCord and Fridovich, 1969) or by conversion of \( \text{O}_2^- \) to OH\(^-\) via the Haber-Weiss (rxns 1 and 2) and Fenton (rxn 3) reactions (Minotti and Aust, 1987) as shown:

1. \( \text{Fe}^{3+} + \text{O}_2^- \rightarrow \text{Fe}^{2+} + \text{O}_2 \)
2. \( 2 \text{O}_2^- + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \)
3. \( \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \)

McCord and Day (1978) proposed that \( \text{Fe}^{3+} \), \( \text{Cu}^{2+} \) or \( \text{Mn}^{3+} \) react with \( \text{O}_2^- \) thereby generating \( \text{Fe}^{2+} \) which in turn reacts with \( \text{H}_2\text{O}_2 \) producing \( \text{OH}^- \) and \( \text{OH}^- \). Although it has been proposed that \( \text{O}_2^- \) destroys proteins, nucleic acids, carbohydrates, and membrane lipids (Fridovich, 1986), it
is currently believed that the OH\(^+\) radical generated from \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) in the presence of traces of transition metal ions is more deleterious than is \(\text{O}_2^-\) (Fee, 1980; Rao and Cammack, 1981; Fridovich, 1986). SOD serves to scavenge \(\text{O}_2^-\) and to minimize the initial reaction (Haber-Weiss) between \(\text{Fe}^{3+}\), (or analogous redox active metal in its high valence state) and \(\text{O}_2^-\). There are three distinct classes of SOD metalloenzymes: an Fe-type a Mn-type, and a Cu-Zn-type (Steinman, 1982). Cu-Zn SOD's are quite distinct from the Mn-type and Fe-type SOD, while Mn-type and Fe-type SOD are similar structurally (Rao and Cammack, 1981; Steinman, 1982) and the two families (Cu-Zn in contrast to Mn and Fe SOD's) apparently evolved from different ancestral proteins (Steinman, 1982a). General characteristic of the three classes of SOD metalloproteins are presented in Table 1.

There are a few exceptions to this classification scheme. \textit{E. coli}, cultured aerobically expresses a dimeric Mn-Fe hybrid SOD (Hassan and Fridovich, 1977). Two archaeobacteria, \textit{Thermoplasma} and \textit{Methanobacterium} spp. express tetrameric Fe-Zn SOD's (Morris et al. 1985). The molecular weight, amino acid composition, and catalytic site are similar to those of eubacterial dimeric Fe-SOD's (Jones et al. 1987). There is some evidence that the apoprotein of the Mn and Fe-SOD
<table>
<thead>
<tr>
<th>Type (cofactor)</th>
<th>Native Mol. Wt.</th>
<th>Other Characteristics</th>
<th>Sources</th>
</tr>
</thead>
</table>
| Cu-Zn (31,000-33,000) (Steinman, 1982a) | dimeric; 2 Cu, 2 Zn/mol | Eukaryotic (common) (Steinman, 1982a) | Eubacteria (uncommon) 
*Paracoccus denitrificans* (Vignais et al. 1982) 
*Photobacterium leiognathi* (Puget and Michelson, 1974; Dunlap and Steinman, 1986) 
*Caulobacter crescentus* (Steinman, 1982b) 
*Pseudomonas diminuta* (Steinman, 1985) 
*Pseudomonas maltophilia* (Steinman, 1985) |
| Mn (33,000) (Azo. chroococcum, Buchanan and Lees, 1980) 97,000 (Saccharomyces cerevisiae, Ravindranath and Fridovich, 1975) | dimeric, some multimeric; 1-4 Mn/mol | Mitochondrial matrix (Fridovich, 1975) (Jackson et al. 1978) | Eukaryotic algae (Rao and Cammack, 1981) 
Prokaryotic (common) (Steinman, 1982a) |
| Fe (36,000) (Plectonema boryanum, Misra and Keele, 1975) 88,000 (Mycobacterium tuberculosis, Kusunose et al. 1976) | dimeric, some multimeric; 0.5 - 1.0 Fe/mol | Vascular plants- 3 families 
Gingkoaceae, Nymphaceae, Brassicaceae (Bridges and Salin, 1981) 
Non-vascular plants (Asada et al. 1980) 
Protozoa (Asada et al. 1980) 
Prokaryotic (Steinman, 1982a) |
are similar enough that the metal cofactor can shift from a Fe to Mn. Cells of *Bacteroides fragilis* (Gregory and Dapper, 1983), *Propionibacterium shermanii* (Meier et al. 1982) and possibly *Streptococcus mutans* (Martin et al. 1984) produce either an Fe or Mn-SOD with apparently the same protein moiety depending on the availability of Fe and Mn and on the O$_2$ tension.

SOD activity is characteristically absent or low in obligate anaerobes (Fulghum and Worthington, 1984), and activity is increased in facultatively anaerobic and aerobic bacteria as the O$_2$ tension is increased (Yostin, et al. 1973; Gregory and Fridovich; Fridovich, 1986). There are a few notable exceptions to this observation. *Lactobacillus plantarum* does not express SOD, but instead sequesters intracellular manganese (up to 35 mM) and appears to scavenge O$_2^-$ with this pool of Mn (Archibald and Fridovich, 1981; Archibald and Duong, 1984). *Neisseria gonorrhoeae* does not express SOD, yet it is an obligate O$_2$ respirer which tolerates high extracellular O$_2^-$ and H$_2$O$_2$ due to very high constitutive levels of peroxidase and catalase activity combined with a cell envelope impervious to O$_2^-$ (Archibald and Duong, 1986).

Aerobic bacteria usually express SOD, and one or both of the heme proteins catalase and peroxidase.
Catalase and peroxidase function together to convert \( \text{H}_2\text{O}_2 \) formed by SOD to \( \text{H}_2\text{O} \). Catalase is most effective against high concentrations of \( \text{H}_2\text{O}_2 \), whereas peroxidases are most effective against low levels of \( \text{H}_2\text{O}_2 \) (Forman and Fisher, 1981). Bacterial cells express cytochrome c peroxidase and/or a NADH peroxidase (Forman and Fisher, 1981).

**Microaerophilism:** Microaerophilic bacteria and microaerophily were recently reviewed by Krieg and Hoffmann (1986). They defined a microaerophilic bacterium as one capable of \( \text{O}_2 \)-dependent growth (using \( \text{O}_2 \) as a terminal electron acceptor) but which the bacterium cannot grow, or can grow only poorly at atmospheric \( \text{O}_2 \) (21% v/v). In addition, some microaerophiles grow anaerobically using alternate terminal electron acceptors or by fermenting, yet "their preference for low \( \text{O}_2 \) levels when they use \( \text{O}_2 \) as a terminal electron acceptor is what distinguishes them from anaerobes that can tolerate low levels of \( \text{O}_2 \) and from aerobes or facultative anaerobes" (Krieg and Hoffmann, 1986).

Campylobacter sp. (Stouthamer et al. 1979), and under N-deficient conditions (N₂-fixing conditions) 


Various culture medium supplements may increase bacterial aerotolerance apparently by assisting or acting in place of protective enzymes. For example, addition of catalase, peroxidase, MnO₂, pyruvate, or dithiothreitol can destroy hydrogen peroxide. Addition of ferrated forms of norepinephrine, dihydroxy phenylalanine, or protocatuffyic acid can destroy O₂⁻. Mannitol or histidine added to media can destroy OH⁻ (Krieg and Hoffmann, 1986). Padgett et al. (1982) obtained an isolate of S. volutans that grew well with atmospheric O₂ by supplementing the medium with low levels of bisulfite. The addition of charcoal, ferrous sulfate, bisulfite, and pyruvate to the growth medium increased the O₂ tolerance of C. jejuni (Bolton and Coates, 1983). Bowdre et al. (1976) found that aerotolerance of S. volutans and C. jejuni was increased by the addition of ferric ion-binding dihydroxyphenyl compounds to the growth medium.

Various reasons have been suggested to explain differences between bacterial species in regard to O₂
sensitivity. Cole (1973) reported that certain tricarboxylic acid enzymes (fumarate hydratase and malate dehydrogenase) were primary sites for oxygen damage while Niskus et al. (1977) presented evidence that the principal site of oxygen damage in cells of C. amatorium subsp. bubulus was lactate dehydrogenase. Diazotrophic bacteria fix N₂ only in microaerobic conditions, presumably because of the O₂ labile nature of the nitrogenase complex (Ramos and Robson, 1985). Enzymes involved in denitrification, especially NO₂⁻-reductase, were sensitive to O₂ (Lam and Nicholas, 1969). The composition of the terminal respiratory chain influences O₂ sensitivity, and microaerophiles may have cytochromes, flavoproteins, or Fe-S proteins relatively sensitive to O₂, O₂⁻ and OH⁻ (Krieg and Hoffmann, 1986).

In conclusion, an organism may be sensitive to oxygen due to a dependence on oxygen-labile substrates, (e.g. Fe²⁺, manganese, and sulfide) the sensitivity of specific enzymes to O₂, or due to the action of deleterious O₂ respiratory intermediates. The expression of SOD, catalase, and peroxidase by a bacterial cell generally imparts increased aero-tolerance. The majority of microaerophilic bacteria expressed SOD and lack catalase (Krieg and Hoffmann, 1986). Proteins expressing catalase, peroxidase or
superoxide dismutase activity were usually found in the cytoplasm or associated with the cytoplasmic membrane. Chapter four of this thesis Characterization and periplasmic location of superoxide dismutase in cells of the microaerophile *A. magnetotacticum* (Short and Blakemore, manuscript in preparation) documents the presence of O₂ detoxifying enzymes in *A. magnetotacticum*, and describes research which establishes their cellular locations, and characterizes the SOD proteins of this organism. Appendix A Freezing and thawing cells of *Aquaspirillum magnetotacticum* selectively releases periplasmic proteins (Paoletti, Short, Blakemore and Blakemore, manuscript submitted) contains a description of a cell freezing and thawing method, developed for extracting periplasm which was used in the course of this research.
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CHAPTER ONE
CHAPTER ONE

MICROAEROBIC CONDITIONS ARE REQUIRED FOR MAGNETITE FORMATION WITHIN AQUASPIRILLUM MAGNETOTACTICUM

ABSTRACT

The amount of magnetite (Fe$_3$O$_4$) within magnetosomes of the microaerophilic bacterium *Aquaspirillum magnetotacticum* varies with oxygen and nitrogen supply. The development of optical methods for directly measuring cell magnetism in culture samples has enabled us to quantitate bacterial Fe$_3$O$_4$ yields. We measured final cell yields of growing cells. Cultures were grown with NO$_3^-$, NH$_4^+$, or both, in sealed, unshaken vials with initial headspace P$_{O_2}$ values ranging from 0 (trace) to 21 kPa.

More than 50% of cells had detectable magnetosomes only when grown in the range of 0.5 - 5.0 kPa O$_2$.

Optimum cell magnetism (and Fe$_3$O$_4$ formation) occurred under microaerobic conditions (initial headspace P$_{O_2}$ of 0.5 - 1.0 kPa) regardless of the N source. At optimal conditions for Fe$_3$O$_4$ formation, denitrifying cultures produced more of this mineral than those growing with O$_2$. 
as the sole terminal electron acceptor. This suggests that competition for O₂ exists between processes involving respiratory electron disposal and Fe₃O₄ formation. Oxygen may also be required for Fe₃O₄ formation by other species of magnetotactic bacteria.

Bacterial Fe₃O₄ appears to persist in sediments after death and lysis of cells. The presence of bacterial Fe₃O₄ in the fossil and paleomagnetic records may be of use as a retrospective indicator of sedimentation that has occurred in microaerobic waters.
INTRODUCTION

Magnetotactic bacteria contain intracellular, enveloped, magnetic particles or "magnetosomes" (Fig. 1). The magnetosomes of strains that have been studied consist of Fe$_3$O$_4$, the iron oxide magnetite (Frankel et al. 1983; Towe and Moench, 1981).

*Aquaspirillum magnetotacticum* strain MS-1, the most thoroughly studied magnetic bacterium, is an obligate microaerophile. Cells denitrify microaerobically and concomitantly consume O$_2$. However, unlike most other denitrifiers, they do not grow under strictly anaerobic conditions, even with NO$_3^-$ present (Blakemore et al. 1979; Bazyliński and Blakemore, 1983a). Rates of growth and intracellular Fe$_3$O$_4$ formation (Blakemore et al. 1979), denitrification (Bazyliński and Blakemore, 1983a) and nitrogen fixation (Bazyliński and Blakemore, 1983b) by this organism are noticeably depresssed when the initial O$_2$ tension in the culture headspace is greater than 6 kPa. Cultures growing with O$_2$ as the terminal electron acceptor (e.g., with NH$_4^+$ in lieu of NO$_3^-$ as the sole N source) contain fewer magnetic cells than do those which are denitrifying. However, the effect of O$_2$ and N source on Fe$_3$O$_4$ formation by cells has not previously been quantitated due to lack of a suitable method to measure cell magnetism.
We have previously estimated cell magnetism in liquid cultures by microscopically noting the extent to which suspended cells align with an applied magnetic field, or by qualitative estimates of their differential light scattering when cultures are held in a continuously rotating magnetic field (e.g. over a laboratory magnetic stirrer). The cells exhibit optical birefringence, however, and methods have been developed to quantitate cell magnetic moments (values of $\mu$) from measurements of magnetic field-dependent birefringence (Rosenblatt et al. 1982).

We undertook the present study to clarify the relationship between available $O_2$ and cell magnetism in cultures grown with $NO_3^-$ and/or $NH_4^+$ as the sole N source. Our findings are relevant to the biogeochemistry of iron, and, if representative of other species, indicate that bacteria produce $Fe_3O_4$ only with $O_2$ available but under microaerobic conditions.
Fig. 1. Transmission electron micrograph of negatively stained cells of *A. magnetotacticum*. Cells contain chains of magnetosomes which are often bisected by division planes. Bar = 1 µM.
MATERIALS AND METHODS

_A. magnetotacticum_ was cultured in a chemically defined medium (Blakemore et al. 1979) containing tartaric acid in lieu of succinic as a carbon source, and either 2 mM NaNO_3_, (NH_4)_2SO_4_, or both N sources, each at 1 mM. Cells were inoculated into 155-ml sealed vials, each containing 55 ml of culture medium under a gas atmosphere of known composition. Cultures were prepared in triplicate using seven different initial headspace gas compositions: 0.0 (trace), 0.5, 1.0, 2.5, 5.0, 10.0, and 20.9 \% (vol/vol) of O_2 in N_2. It is important to note that the culture system used lacked extensive redox buffering. Medium containing resazurin was colorless prior to inoculation but was faintly pink just afterward. Thus, trace amounts of O_2 added with the inoculum were sufficient to allow some cell growth. No sample in this study was strictly anaerobic prior to inoculation. In confirmation of earlier published findings, _A. magnetotacticum_, when tested under stringent conditions of anoxia, failed to grow even with NO_3^- present. Cultures were each inoculated to 1.3 x 10^6 cells/ml and incubated at 30°C without shaking. At the end of growth the following were evaluated:

1. cells/ml (by means of direct microscopic counts)
2. magnetosomes per cell (by means of direct transmission electron microscopic examination; magnetosomes within 100 cells from each culture were counted)

3. average cell magnetic moment (by measuring magnetic field-dependent culture birefringence)

The apparatus used to measure magnetic field-dependent culture birefringence has been described (Rosenblatt et al. 1982). Bacteria in 5 ml of culture fluid were fixed with a drop of 10% glutaraldehyde. Fixed cells in suspension were placed in a 3-ml cuvette (1-cm path length) situated within a Helmholtz coil pair that was used to vary the magnetic field applied to the sample. The entire assembly was placed within a Mumetal canister to cancel the ambient laboratory magnetic field on the cells. The optic axis of the sample was perpendicular to the applied magnetic field. Increases in the field strength over the range 0.1 - 25.0 Oe produced a corresponding increase in measured birefringence. Only magnetic cells contributed to the measured effect. The data were analyzed to yield the average value and distribution of $\mu$ for those cells.
RESULTS

We did not observe total inhibition of growth by high $O_2$ in this study. Cells exhibited a growth lag at high $O_2$ values but, despite their microaerophilic character, they eventually grew. We attribute this to the use of sealed, static cultures, because cells never grew from small inocula in a culture in free exchange with air. Additional studies using systems with constant $P_{O_2}$ are expected to define the precise $O_2$ tension for optimal cell growth and $Fe_3O_4$ formation.

Nevertheless, the results show that more than 50% of cells in cultures had magnetosomes only when the initial headspace $P_{O_2}$ was between 0.5 and 5 kPa (Tables 2-4; Figs. 2-4). Outside of this narrow range, $Fe_3O_4$ production diminished markedly regardless of the $N$ source, even though cells grew. The optimal $P_{O_2}$ for $Fe_3O_4$ formation was 1 kPa with $NO_3^-$ present (Tables 2, 4) or 0.5 kPa with $NH_4^+$ alone (Table 3). At the optimal $P_{O_2}$, the largest percentage of cells in the population contained magnetosomes (Tables 2-4) and the average number of magnetosomes per cell was also highest (Figs. 2-4). Figs. 2-5 also illustrate the effect of both low and high $O_2$ in inhibiting $Fe_3O_4$ formation by cells. Values of cell magnetic moment were also optimal at
approximately 1 kPa O₂ (Tables 2-4), illustrating the correlation between cell magnetosome content and intrinsic magnetic moment as determined from birefringence measurements. The average magnetic moments measured were those expected from Fe₃O₄ grains in the 400-500 Å range.

A somewhat higher value of μ was obtained for cells grown with trace amounts of O₂ and with NH₄⁺ as the sole N source (Table 3) than for those provided with NO₃⁻ (Tables 2 and 4). Since the cells did not grow well on NH₄⁺ with trace O₂, the relatively high value of μ (Table 3) was strongly influenced by that of the inoculum, which consisted of magnetic cells. The magnetic moment measured was not representative of Fe₃O₄ formation during incubation.

At the optimal P₀₂ for Fe₃O₄ formation, the average μ values for cells cultured with NO₃⁻ (Tables 2 and 4) were roughly comparable to that of cells grown on NH₄⁺ alone (Table 3). However, at the optimal P₀₂, 100 % of cultured cells grown with NO₃⁻ alone (Table 2; Fig. 2) possessed magnetosomes, whereas only 84 to 87 % of cultured cells grown with NH₄⁺ alone (Table 3; Fig. 3) contained magnetosomes. Furthermore, at their optimum P₀₂, cells grown with NH₄⁺ alone (Table 3) may have produced fewer magnetosomes than cells with NO₃⁻ alone.
(Table 2). The large standard deviations associated with these measurements reflect difficulty in our ability to differentiate between smaller magnetosomes and non-magnetosomes in electron micrographs of whole cells. In addition, during growth in the closed system used, cells may have exhibited variable magnetosome production as $O_2$ and Fe concentrations changed. The stimulatory effect of $NO_3^-$ was especially evident, however, when the total $Fe_3O_4$ yield of cultures was evaluated for each value of $O_2$ (Fig. 5). Since $NH_4^+$ at the concentration used does not repress denitrification (Bazylnski and Blakemore, 1983b), these results verify that $Fe_3O_4$ was produced in greatest quantity by denitrifying cells. This effect appeared to be the result of stimulation by $NO_3^-$ rather than inhibition by $NH_4^+$ because at the $P_{O_2}$ optimal for $Fe_3O_4$ formation, the combined effect of the two (Table 4, Fig. 4) was similar to that of $NO_3^-$ alone (Table 2; Fig. 2).
<table>
<thead>
<tr>
<th>Initial O₂ Concentration (kPa in headspace)</th>
<th>Final Cell Concentration (cells/ml)$^a$</th>
<th>Average No. of Magnetosomes (± SD$^b$/Cell)</th>
<th>Percent of Cells with Magnetosomes$^c$</th>
<th>Magnetic Moment (μ) ($\times 10^{-13}$ emu)$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trace</td>
<td>$1.8 \pm 0.0 \times 10^8$</td>
<td>$2.0 \pm 3.5$</td>
<td>41</td>
<td>0.6-0.7</td>
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<tr>
<td>0.5</td>
<td>$1.8 \pm 0.2 \times 10^8$</td>
<td>$10.0 \pm 10.0$</td>
<td>88</td>
<td>0.8-1.0</td>
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<tr>
<td>1.0</td>
<td>$2.5 \pm 0.3 \times 10^8$</td>
<td>$17.2 \pm 7.8$</td>
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<td>2.9-3.3</td>
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<tr>
<td>2.5</td>
<td>$2.4 \pm 0.0 \times 10^8$</td>
<td>$14.9 \pm 7.7$</td>
<td>94</td>
<td>2.8-3.1</td>
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<tr>
<td>5.0</td>
<td>$2.5 \pm 0.3 \times 10^8$</td>
<td>$13.0 \pm 15.9$</td>
<td>82</td>
<td>2.7-2.9</td>
</tr>
<tr>
<td>10.0</td>
<td>$1.1 \pm 0.0 \times 10^8$</td>
<td>$1.6 \pm 4.8$</td>
<td>14</td>
<td>1.0-1.2</td>
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<td>21.0</td>
<td>$8.7 \pm 0.3 \times 10^7$</td>
<td>$0.5 \pm 2.6$</td>
<td>4</td>
<td>ND$^e$</td>
</tr>
</tbody>
</table>

$^a$Values indicate means and standard deviations, respectively, obtained from triplicate cultures. Initial cell concentration was $1.3 \pm 0.2 \times 10^6$ cells/ml.

$^b$The average number of magnetosomes per cell ± the standard deviation. The magnetosomes from 100 cells cultured at each P₀₂ value were enumerated.

$^c$Values corrected for percent of cell yield as inoculum, assuming the inoculum consisted entirely of magnetic cells.

$^d$Measured by field-dependent birefringence. Values indicate ranges from duplicate cultures.

$^e$ND = None Detected.

as published in Geomicrobiol. J. 4:53-71
Table 3. Effect of O₂ on *A. magnetotacticum* growth and Fe₃O₄ synthesis with NH₄⁺

<table>
<thead>
<tr>
<th>Initial O₂ Concentration (kPa in headspace)</th>
<th>Final Cell Concentration (cells/ml)a</th>
<th>Average No. of Magnetosomes (± SD)b/Cell)</th>
<th>Percent of Cells with Magnetosomesc</th>
<th>Magnetic Moment (µ) (x 10⁻¹³ emu)d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trace</td>
<td>1.8 ± 0.3 x 10⁶</td>
<td>9.0 ± 9.1</td>
<td>19</td>
<td>3.3</td>
</tr>
<tr>
<td>0.5</td>
<td>2.6 ± 0.5 x 10⁷</td>
<td>11.5 ± 8.0</td>
<td>87</td>
<td>3.1-3.7</td>
</tr>
<tr>
<td>1.0</td>
<td>7.5 ± 3.1 x 10⁷</td>
<td>11.9 ± 9.7</td>
<td>84</td>
<td>3.2-3.6</td>
</tr>
<tr>
<td>2.5</td>
<td>9.7 ± 1.0 x 10⁷</td>
<td>6.0 ± 10.0</td>
<td>59</td>
<td>1.3-1.7</td>
</tr>
<tr>
<td>5.0</td>
<td>1.4 ± 0.1 x 10⁸</td>
<td>3.7 ± 4.8</td>
<td>54</td>
<td>1.7-2.3</td>
</tr>
<tr>
<td>10.0</td>
<td>1.8 ± 0.1 x 10⁸</td>
<td>3.1 ± 5.4</td>
<td>35</td>
<td>1.3</td>
</tr>
<tr>
<td>21.0</td>
<td>2.5 ± 0.1 x 10⁸</td>
<td>2.6 ± 4.4</td>
<td>48</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Values indicate means and standard deviations, respectively, obtained from triplicate cultures. Initial cell concentration was 1.3 ± 0.2 x 10⁶ cells/ml.

The average number of magnetosomes per cell ± the standard deviation. The magnetosomes from 100 cells cultured at each O₂ value were enumerated.

Values corrected for percent of cell yield as inoculum, assuming the inoculum consisted entirely of magnetic cells.

Measured by field-dependent birefringence. Values indicate ranges from duplicate cultures.

as published in Geomicrobiol. J. 4:53-71
**TABLE 4.**

Effect of O$_2$ on *A. magnetotacticum* growth and Fe$_3$O$_4$ synthesis with NO$_3^-$ and NH$_4^+$

<table>
<thead>
<tr>
<th>Initial O$_2$ Concentration (kPa in headspace)</th>
<th>Final Cell Concentration (cells/ml)$^a$</th>
<th>Average No. of Magnetosomes (± SD)$^i$/Cell</th>
<th>Percent of Cells with Magnetosomes$^c$</th>
<th>Magnetic Moment (µ) ($\times 10^{-15}$ emu)$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trace</td>
<td>$1.2 \pm 0.1 \times 10^8$</td>
<td>$1.2 \pm 3.2$</td>
<td>21</td>
<td>0.7-0.8</td>
</tr>
<tr>
<td>0.5</td>
<td>$2.7 \pm 0.1 \times 10^8$</td>
<td>$3.7 \pm 4.4$</td>
<td>55</td>
<td>1.0-1.1</td>
</tr>
<tr>
<td>1.0</td>
<td>$2.8 \pm 0.1 \times 10^8$</td>
<td>$14.4 \pm 8.3$</td>
<td>94</td>
<td>2.9-3.1</td>
</tr>
<tr>
<td>2.5</td>
<td>$2.2 \pm 0.3 \times 10^8$</td>
<td>$11.8 \pm 10.6$</td>
<td>89</td>
<td>2.1-2.6</td>
</tr>
<tr>
<td>5.0</td>
<td>$1.5 \pm 0.5 \times 10^8$</td>
<td>$10.6 \pm 10.0$</td>
<td>84</td>
<td>1.9-2.2</td>
</tr>
<tr>
<td>10.0</td>
<td>$1.1 \pm 0.2 \times 10^8$</td>
<td>$2.9 \pm 6.9$</td>
<td>36</td>
<td>1.0-2.6</td>
</tr>
<tr>
<td>21.0</td>
<td>$1.2 \pm 0.0 \times 10^8$</td>
<td>$0.8 \pm 2.7$</td>
<td>15</td>
<td>0.7-1.5</td>
</tr>
</tbody>
</table>

$^a$Values indicate means and standard deviations, respectively, obtained from triplicate cultures. Initial cell concentration was $1.3 \pm 0.2 \times 10^6$ cells/ml.

$^b$The average number of magnetosomes per cell ± the standard deviation. The magnetosomes from 100 cells cultured at each P$_0$ value were enumerated.

$^c$Values corrected from percent of cell yield as inoculum, assuming the inoculum consisted entirely of magnetic cells.

$^d$Measured by field-dependent birefringence. Values indicate ranges from duplicate cultures.

as published in *Geomicrobiol. J.* 4:53-71
FIGURE 2. Magnetosome distributions within A. magnetoii grown with NO$_3^-$ and (A) trace, (B) 1% and (C) 21% [kPa] O$_2$. Magnetosomes within 100 cells were counted by means of direct electron microscopy using cultures grown at each O$_2$ tension. Small numbers above the "zero" columns indicate the numbers of cells observed with no magnetosomes. Values of "30 +" in abscissas indicate numbers of cells observed that contained 30 or more magnetosomes.
FIGURE 3. Magnetosome distributions within *A. magnetotacticum* grown with NH$_4^+$ and (A) trace, (B) 1 %, and (C) 21 % [kPa] O$_2$. Magnetosomes within 100 cells were counted by means of direct electron microscopy using cultures grown at each O$_2$ tension (see also legend to Fig. 2)
FIGURE 4. Magnetosome distributions within "A. magnetofaciens" grown with NO₃⁻ plus NH₄⁺ and (A) trace, (B) 1 %, and (C) 21 % [kPa] O₂. Magnetosomes within 100 cells were counted by means of direct electron microscopy using cultures grown at each O₂ (see also legend to Fig. 2).
FIGURE 5. Total magnetite yield of cultures grown with various sole nitrogen sources at different initial headspace O$_2$ concentrations. Values were obtained by multiplying the average final cell yield by the corresponding average number of magnetosomes per cell. Values are normalized to the maximum yield obtained.
CULTURE MAGNETITE YIELD (% of max.)

[Graph showing various lines representing different conditions such as NH₄⁺, NO₃⁻, and NO₂⁻ with corresponding oxygen pressures (kPa).]
FIGURE 6. Transmission electron micrograph of sediment collected from a brackish environment containing magnetotactic bacteria. Thin section stained with uranyl acetate. Note the transverse sections through diatoms as well as through clumps of extracellular particles having a size and morphology of magnetosomes found with bacteria collected at the same site. Inset shows negatively stained magnetotactic bacteria collected from the sediment sample before it was prepared for thin sectioning. Bar = 1 μM.
DISCUSSION

Lower final growth yields at < 1 kPa O\(_2\), regardless of the N source, illustrate the specific requirement of this organism for O\(_2\). The nature of this requirement is unknown but may relate to a need for O\(_2\) in biosynthesis. With NH\(_4\)\(^+\) as sole N source, cells at < 1 kPa O\(_2\) were also limited by electron acceptor (O\(_2\)). They grew to higher cell yields at low Po\(_2\) when supplied with the alternate electron acceptor, NO\(_3^-\).

The reason for depressed growth of cells with NO\(_3^-\) present at high (10-21 kPa) O\(_2\) is unknown but may have been due to O\(_2\) inhibition of NO\(_2^-\) reduction. The resulting accumulation of NO\(_2^-\) would be toxic for cells (Bazylinski and Blakemore, 1983b).

The data indicate that bacterial Fe\(_3\)O\(_4\) was produced optimally under microaerobic conditions. More than 50% of cells had magnetosomes only when the initial head-space Po\(_2\) of a sealed culture was between 0.5 and 5 kPa. Thus, cells require O\(_2\) to produce Fe\(_3\)O\(_4\). Moreover, since atomic oxygen was always abundant in phosphate and nitrate of the culture medium, only dioxygen satisfied this requirement.

Lower magnetism at low Po\(_2\) of cells grown with NH\(_4\)\(^+\), compared to those grown with NO\(_3^-\), may indicate
competition between reactions employing $O_2$ as a terminal
electron acceptor and others requiring $O_2$ for formation
of the iron oxide, Fe$_3$O$_4$. Since the bulk of $O_2$ consumed
by growing cells is consumed in respiration (unpublished
results), the effect of such competition in decreasing
the Fe$_3$O$_4$ yield is most evident with cells grown on NH$_4^+$
under microaerobic conditions.

The $O_2$ requirement for the formation of Fe$_3$O$_4$ by
bacteria is of considerable physiological interest
because cells of this and of at least some other species
of magnetotactic bacteria (Spormann and Wolfe, 1984) are
aerotactic. As a result of their aerotactic response,
cells are directed to, and accumulate in, microaerobic
zones optimal for growth and for Fe$_3$O$_4$ formation.

We believe our findings may have broader implic-
tions in biogeochemistry and geology. Magnetotactic
bacteria produce Fe$_3$O$_4$ particles with a narrow size
range and with morphologies unique to various species
(Matsuda et al. 1983; Mann et al. 1984). Structures
with the unique morphology and size of bacterial Fe$_3$O$_4$
grains have been observed in sediments by several inves-
tigators (Blakemore, 1975; W. Ghiorse, personal commun-
ication; Kirschvink and Chang, 1984). They apparently
persist as magnetic microfossils in sediments after
their release from dead bacteria (Fig. 6). Because they
are single magnetic domains and have permanent remanent
moments, bacterial Fe$_3$O$_4$ grains might contribute a bio-
logical remanent magnetization to sediments containing
them. Kirschvink and Chang (1984) have determined that
the stable, natural remanent magnetization they detected
in marine calcareous oozes appeared to be, in large
part, due to single-domain sized Fe$_3$O$_4$ crystals. The
unique size and shape of these grains and their
similarity to Fe$_3$O$_4$ of bacterial origin led these
workers to suggest that the magnetotactic bacteria are a
prime source of an important component of the paleo-
magnetic record.

Studies of the occurrence and distribution of other
species of magnetotactic bacteria found in natural envi-
ronments and in enrichments (Blakemore, 1975, 1982;
Moench and Konetzka, 1978; Spormann and Wolfe, 1984)
lead us to believe that other types of magnetotactic
bacteria are also microaerophilic and require O$_2$ for
Fe$_3$O$_4$ formation. For instance, bacterial Fe$_3$O$_4$ is
formed from hydrous ferric oxide precursors produced, in
turn, by oxidation of ferrous ions (Frankel et al.
1983). The presence in the fossil record of biogenic
structures known to be produced only under microaerobic
conditions might provide a useful tool for examining the
occurrence of free O$_2$ in the early earth.
Our data, if applicable to other species, demonstrate that bacterial Fe$_3$O$_4$ formation (hence magnetotaxis) could have evolved only after free O$_2$ became available to cells. This may at first have been in microhabitats shared by oxygenic photoautotrophs, such as in algal mats. Do Precambrian stromatolites or Archean-Early Proterozoic banded iron formations, in particular, preserve evidence of magnetic bacteria? Current thinking suggests that from 1.7 to 2.3 billion years before the present, evolving O$_2$ apparently saturated its principal Archean sinks (Fe$^{2+}$ and reduced gases) and began to accumulate in the atmosphere (Walker et al. 1983). During this transition to an oxidizing global atmosphere, the earth's entire atmosphere became, by today's standards, microaerobic. The "microaerophiles", including magnetotactic bacteria, had a thermodynamic edge over cells unable to use O$_2$ as a terminal electron acceptor and may have become the most prevalent physiological group on earth during the Early Proterozoic. As the O$_2$ tension of the atmosphere increased towards its present level, extant species of microaerophiles apparently failed to evolve suitable means of detoxifying damaging products of O$_2$ metabolism. They appear to have once again become restricted in their distribution; this time, however, to microhabitats.
containing suitably low $P_2O_2$. The acquisition of magnetotaxis and aerotaxis would have helped ensure their localization in these microaerobic zones. It would be interesting if prokaryotic magnetosomes in the fossil and paleomagnetic records provided evidence to test these possibilities and to probe further the details of Earth's early evolution.
ACKNOWLEDGEMENTS

We gratefully acknowledge the technical assistance of Nancy Blakemore and Ali Geshnizgani. We are also grateful to S.-B. R. Chang, J. L. Kirschvink, A. M. Spormann, and R. S. Wolfe for sharing unpublished results. This work was supported by a National Science Foundation grant PCM 82-15900. The Francis Bitter National Magnet Laboratory is supported by the National Science Foundation. R. B. F., C. R., K. A. S., and R. P. B. were partially supported by the Office of Naval Research.
LITERATURE CITED


Washed cell suspensions of *Aquamirillum magnetotacticum* MS-1, *A. iersonii* E12639, *Bacillus subtilis* 6633, and *Escherichia coli* CSH27 translocated protons in response to the added oxidants \( \text{O}_2 \) or \( \text{NO}_3^- \), with triphenylmethylphosphonium bromide (TPMP\(^+\)) as permeant. Iron respiration-driven proton translocation was observed with *A. magnetotacticum* strain MS-1, *B. subtilis*, and *E. coli*, but not with a non-magnetite producing strain of *A. magnetotacticum* (strain NM-1A) or with *A. iersonii*. Proton translocation to \( \text{Fe}^{3+} \) was totally inhibited by 5 mM NaN\(_3\) or 0.5 \( \mu \text{M} \) carbonyl cyanide \( m \)-chlorophenylhydrazone (CCCP).
INTRODUCTION

Bacterial respirations with $O_2$, $NO_3^-$, $SO_4^{2-}$, $Mn^{5+}$, and other inorganic compounds as terminal oxidants have been subjects of numerous investigations. In contrast, physiological studies addressing dissimilatory $Fe^{3+}$ reduction by microbes are almost non-existent. Ferricyanide reduction with NADH by membrane vesicles of *Bacillus subtilis* (Bisschop et al. 1975) or with formate by *Escherichia coli* membrane vesicles (Boonstra et al. 1976) was linked to a functioning electron transport chain. It was suggested that there were two sites of ferricyanide reduction, one of which was the nitrate reductase. Under anaerobic conditions, ferricyanide reduction generated sufficient proton motive force to drive active transport of amino acids. Using membranes from cells of *Aquaspirillum itersonii*, Dailey and Lanceilles (1977) obtained evidence for $Fe^{3+}$ reduction with NADH or succinate as reductant. From studies with respiratory inhibitors, they concluded that the terminal $Fe^{3+}$ reductase accepted electrons from donors at one or more sites before cytochrome c in the electron transport chain. It is extremely interesting that aeration abolished the $Fe^{3+}$ reductase activity but not in the presence of antimycin A, suggesting the inhibition was
due to "diversion of reductant into the terminal oxidase system" (Dailey and Lascelles, 1977).

Lascelles and Burke (1978) examined Fe$^{3+}$ reduction by membranes of *Staphylococcus aureus* and from results of studies with respiratory inhibitors concluded that Fe$^{3+}$ interacted with a reduced component of the electron transport chain that preceded cytochrome b.

Bacterial substrate oxidation is accompanied by extrusion of protons to the cell exterior (Scholes and Mitchell, 1970). With substrate in excess, protons are translocated across the cytoplasmic membrane in direct proportion to the quantity of available suitable terminal electron acceptor. Proton translocation measurements (Scholes and Mitchell, 1970) have been widely used to assess bacterial respiration with diverse oxidants. In this paper we present evidence obtained with this technique for "iron respiration" in several species of aerobic bacteria.
MATERIALS AND METHODS

Bacteria and culture conditions. *A. magnetotacticum* strains were cultured microaerobically at 28°C in static bottles containing MSGM medium with 1.8 mM NaNO₃, 6 mM succinate and 20 uM ferric quinate as the nitrogen, carbon and Fe³⁺ sources, respectively (Blakemore et al. 1985). The non-magnetic strain used in this study was a sub-clone of strain NM-1A. Non-magnetic strain NM-1A was obtained from strain MS-1 by plating 0.1 ml (10⁷) cells on MSGM containing 0.005% sodium metabisulfite (1.0% agar). The plates were incubated in air for 14 day at 30°C. Two white, raised, circular colonies which appeared on one plate, consisted of spirochaetes similar in size and morphology to cells of *A. magnetotacticum*. As expected of cells grown at high O₂ (Blakemore et al. 1979; Blakemore et al. 1985), they lacked magnetosomes and although motile, were not magnetotactic. Unlike those of the parent strain, cells from each of these colonies, when inoculated into liquid culture medium and grown microaerobically, failed to synthesize magnetosomes after more than 20 passages. Cells of strain NM-1A have been shown to have similar mol % G+C (64-65) and outer membrane protein profile as those of MS-1. *A. iberiensis* E12639 (UNH culture collection), was
cultured with shaking (100 rpm) at 30° C in Fernbach flasks containing MSGM + 0.1% yeast extract. *B. subtilis* 6633 and *E. coli* CSH27 (UNH culture collection) were cultured with shaking (100 rpm) at 37° C in Fernbach flasks containing Difco trypticase soy medium. Cells were harvested at mid to late-exponential phase of growth, using a Pellicon filtration system (Millipore Corp., Bedford, MA.) and concentrated by centrifugation. They were washed twice in 150 mM KCl (pH 7.1) at 4° C and resuspended in 10 ml of the same to a density of more than 2 x 10^10 cells/ml.

**Oxidant Pulse Studies.** The apparatus, reagents and experimental techniques used for oxidant pulse studies were those of Kristjansson et al. (1978) and Castignetti and Hollocher (1983). The reaction vessel was a 5 cc vial containing a small magnetic stir bar and fitted with a rubber closure. A combination pH electrode (Value Mark Model H-445, Markson Science) and needles for gas inlet and outlet were inserted through the closure. A Beckman Altex Model 71 pH meter was used with a recorder set to 0.1 pH unit full scale. To 2 ml cell suspension (in KCl) in the vial were added TPMP⁺ (0.6 mM) and 7.2 units of carbonic anhydrase. The contents were then flushed with O₂-free N₂ and continuously stirred for 20 min to render them anaerobic. Subsequently, the pH of the
suspension was adjusted to ca. 7.1 with an anaerobic solution of 0.01 N NaOH.

Chemicals were prepared in gas-tight serum vials as dissolved gases or solids in 150 mM KCl and made anaerobic by first heating the contents to 45°C and cooling with repeated evacuation of the headspace and replacement with 1 atm O₂-free N₂ immediately prior to use. For tests with O₂, a known quantity of pure O₂ was equilibrated in 4 ml 150 mM KCl in a 9 cc serum vial prepared as above. Dissolved O₂ was calculated assuming 1.16 mM O₂-atm⁻¹ (Chappell, 1964). Nitrate was prepared as 2 mM NaNO₃ in 150 mM KCl. Iron was prepared as ferric quinate (2 mM) or ferric chloride (2 or 5 mM) in 150 mM KCl. The pH of chemicals tested as potential oxidants was adjusted to 7.10.

Iron reduction and nitrate reductase. Iron reduction by intact cells was determined with Ferrozine [3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,3-triazine; Sigma Chemical Co., St. Louis, Mo.] (Stookey, 1970). Nitrate reductase was assayed in cell-free extracts by coupling to NADH oxidation (Kristjansson and Hollocher, 1979).

Cells contained sufficient endogenous substrate for respiration as determined by O₂ consumption rates measured with a polarographic electrode (YSI, Model 53
Oxygen Monitor). They generated proton pulses repeatedly for up to several h with each addition of suitable oxidant. Pulse amplitudes were quantified using the graphical methods of Scholes and Mitchell (1970). The magnitude of the measured proton pulse was correlated with the molar concentration of extruded protons by injecting a known quantity of anaerobic 0.01 HCl. The effects of oxidants and inhibitors were tested by adding appropriate quantities as anaerobic solutions directly to the reaction vessel.
RESULTS AND DISCUSSION

Washed cell suspensions of *B. subtilis* 6633, *E. coli* CSH27 and *A. magnetotacticum* MS-1, but not those of the non-magnetic *A. magnetotacticum* strain or *A. itersonii* E12639 (Table 5) demonstrated proton translocation in response to added Fe$^{3+}$ with 0.6 mM TPMP$^+$ as the permeant ion. Washed cell suspensions of strain MS-1 similarly prepared actively reduced added Fe$^{3+}$ (2.5 ng of Fe$^{3+}$ reduced per µg of cell protein$^{-1}$·min$^{-1}$). *A. itersonii* cell suspensions equilibrated with 0.3, 0.6, or 1.2 mM TPMP$^+$, or with 0.6 mM valinomycin generated proton pulses with O$_2$ and NO$_3^-$ but not with Fe$^{3+}$. Cells of all species tested translocated protons in response to added O$_2$ and NO$_3^-$, as expected of these dissimilatory nitrate reducers. The specific activities of nitrate reductase in cell-free extracts of *A. itersonii*, *B. subtilis*, and *E. coli* were $8 \times 10^{-2}$, $2 \times 10^{-1}$, and $6 \times 10^{-1}$ µmol NO$_3^-$ reduced·mg of protein$^{-1}$·min$^{-1}$, respectively. Representative proton pulse curves for each bacterial species supplied with the oxidants examined in this study appear in Fig. 7. Proton translocation by each bacterial species tested was abolished by 0.5 µM carbonyl cyanide m-chlorophenylhydrazone, regardless of the acceptor.
some experiments (particularly with Fe$^{3+}$) the magnitude of the pulse was much greater than the mean value. Hence, the range and the mean for each oxidant are presented.

Proton translocation by *A. magnetotacticum* MS-1 with either NO$_3^-$ or Fe$^{3+}$ was completely inhibited by 250 µM NaN$_3$, while O$_2$-driven proton translocation was decreased by 40%. This concentration of NaN$_3$ decreased cell oxygen consumption by 70%. Proton translocation by *E. coli* and *B. subtilis* with Fe$^{3+}$ was completely inhibited by 500 and 250 µM NaN$_3$, respectively. These results are consistent with iron reduction at a terminal site in the electron transport chain.

We did not observe proton pulses when any of the bacteria were tested with fumarate, DMSO or manganese dioxide. However, proton pulses in response to fumarate were also not produced by *E. coli* cells from cultures grown with 6 mM sodium fumarate despite the use of 0.6 mM valinomycin, 0.6 mM nigericin, or 0.6 mM potassium thiocyanate as permeant ions. Similar difficulties in demonstrating proton translocation with fumarate by *E. coli* were reported by Bilous and Weiner (1985).

Our results demonstrate that three bacterial species cultured aerobically translocate protons in response to added Fe$^{3+}$ and presumably, therefore, are
capable of generating a proton motive force via the
dissimilatory reduction of Fe$^{3+}$. Our results also
suggest that differences between magnetic and non-
magnetic cells of *A. magneto*acticu*ma* may relate to the
ability of magnetic cells to reduce Fe$^{3+}$ in a dissimi-
latory manner. Bacterial magnetite formation involves
Fe$^{3+}$ reduction (Frankel et al. 1983). Microaerobic
aquatic habitats from which magnetic bacteria have been
collected, are typically low in O$_2$ and NO$_3^-$, but
relatively high (20 μM, or more) in Fe$^{3+}$. The ability
of this organism to respire with Fe$^{3+}$ could provide
competitive advantage in these habitats.
### TABLE 5: H⁺/g atom terminal acceptor for the strains tested

<table>
<thead>
<tr>
<th>Strain</th>
<th>Oxidant tested</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>O₂</td>
<td>NO₃⁻</td>
<td>Fe³⁺</td>
</tr>
<tr>
<td><em>A. magnetotactica</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>magnetic strain Ms-1</td>
<td>mean</td>
<td>6.0 (15)</td>
<td>4.5 (10)</td>
<td>2.6 (14)</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>2.2 - 11.7</td>
<td>0.8 - 9.6</td>
<td>0.2 - 7.8</td>
</tr>
<tr>
<td>non-magnetic strain</td>
<td>mean</td>
<td>7.7 (3)</td>
<td>5.1 (3)</td>
<td>N.D. (4)</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>7.0 - 8.2</td>
<td>4.6 - 5.9</td>
<td></td>
</tr>
<tr>
<td><em>A. l fresenii</em> E12639</td>
<td>mean</td>
<td>4.8 (5)</td>
<td>7.7 (4)</td>
<td>N.D. (5)</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>3.3 - 9.7</td>
<td>0.0 - 15.7</td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em> 6633</td>
<td>mean</td>
<td>4.5 (3)</td>
<td>2.8 (7)</td>
<td>8.0 (7)</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>1.6 - 9.1</td>
<td>0.2 - 5.8</td>
<td>0.5 - 24.8</td>
</tr>
<tr>
<td><em>E. coli</em> GSH27</td>
<td>mean</td>
<td>1.9 (3)</td>
<td>5.7 (3)</td>
<td>11.7 (3)</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>1.1 - 2.6</td>
<td>3.0 - 8.4</td>
<td>8.6 - 15.4</td>
</tr>
</tbody>
</table>

A. Numbers in parentheses indicate the number of cell preparations tested.

Each cell preparation was tested repeatedly a minimum of 4 times with the oxidant shown.

N.D. = proton translocation not detected
FIGURE 7. Proton pulse traces obtained with cells of \textit{A. magnetotacticum} (A. maq) (magnetic and nonmagnetic strains), \textit{A. itarsonii}, \textit{B. subtilis}, and \textit{E. coli} in the presence of 0.6 mM TPMP$^+$ and 7.2 U of carbonic anhydrase. Arrows indicate the addition of the following oxidants: a. 1 ng-atom of O$_2$; b. 1 ng-atom of NO$_3^-$; and c. 1 ng-atom of Fe$^{3+}$. Horizontal-scale bars equal 1 min. Vertical-scale bars equal 1 nmol of H$^+$ translocated to the cell exterior.
Amag. magnetic

A. mag. 1 min 1 nmol H^+ magnetic

non-magnetic

A. itersonii

B. subtilis

E. coli

82
ACKNOWLEDGEMENTS

We wish to thank Dr. Dennis Bazylinksi for advice on proton translocation. We thank Nancy Blakemore for isolating the non-magnetic mutant of *A. magnetotacticum*. This work was supported by NSF grants PCM-82-15900 and DMB-85-15540. KAS acknowledges support from a UNH CURF grant.


CHAPTER THREE
CHAPTER THREE

O₂ AND NO₂⁻ TOXICITY IN AN
OBLIGATELY MICROAEROPHILIC DENITRIFIER

ABSTRACT

Denitrifying cells of the obligate microaerophile Aquaspirillum magnetotacticum were more sensitive to O₂ than were non-denitrifying cells. Oxygen inactivated NO₂⁻ reductase with consequent NO₂⁻ accumulation to 10 μM in culture media. At this concentration, NO₂⁻ totally inhibited cell growth, and in similar concentrations inhibited O₂ respiration. Evidence was obtained that it also acted as a respiratory uncoupler by increasing the proton conductance of the cytoplasmic membrane.

Cells of an aerotolerant, non-magnetic mutant showed similar NO₂⁻ toxicity when denitrifying. However, unlike wild type (microaerophilic magnetic) cells, those of the mutant strain grew best under non-denitrifying conditions, at elevated oxygen. They differed from wild type cells by expressing catalase and elevated superoxide dismutase (SOD) activity.

Wild type magnetic cells of strain MS-1 concomitantly respired with O₂ and NO₃⁻. As such, they
experienced at least two harmful effects of elevated
$O_2$. They appeared to lack suitable means of removing
toxic $O_2$ reduction products and, when denitrifying,
also accumulated toxic quantities of $NO_2^-$ as a result
of decreased expression of $NO_2^-$ reductase activity.
INTRODUCTION

Denitrification can occur in both anaerobic (Payne, 1981; Knowles, 1982) and aerobic (Krul, 1976; Meiberg et al. 1980) conditions. The concomitant use of NO$_3^-$ and O$_2$ as electron acceptors was documented for cells of Klebsiella sp. (Dunn et al. 1979), Aquaspirillum magnetotacticum (Bazylinski and Blakemore, 1983) Moraxella sp. (Ottow and Fabig, 1983) and Thiophaera pantatropha (Robertson and Kuenen, 1984a; 1984b). Microaerobic denitrification, which has been observed in marine sediments (Jorgensen et al. 1984) and in soils (Trevors, 1985), could impart a selective advantage to organisms in environments with low or fluctuating NO$_3^-$ and O$_2$ but only if they were provided a means of ridding toxic reduction products.

A. magnetotacticum strain MS-1 is an obligate microaerophile which denitrifies (Blakemore et al. 1979; Escalante-Semerena et al. 1980). Cultured with 0.2 to 1.0 % (v/v) O$_2$ initially in the headspace, cells reduced NO$_3^-$ to N$_2$ while respiring with O$_2$. The cell yield was directly proportional to the NO$_3^-$ concentration (Bazylinski and Blakemore, 1983). Growth of denitrifying cells in sealed vials was depressed when the culture headspace initially contained either more or less than the optimal value of 1-5 % O$_2$ as expected of
an obligate microaerophile. However, at 10-20 % $O_2$, non-denitrifying cells (with $NH_4^+$) after a variable lag grew to even higher densities. Cells at the higher $O_2$ values, when provided with $NO_3^-$ together with $NH_4^+$, showed some growth inhibition. These results suggested that $O_2$ may have selectively inhibited $NO_2^-$ reduction leading to its accumulation to toxic concentrations.

Alternatively, the differences noted could result if non-denitrifying cells were less sensitive than denitrifying cells to $O_2$ or its reduction products; due to increased expression of catalase, superoxide dismutase (SOD) or peroxidase, for instance.

To discriminate between these and possibly other harmful effects of $O_2$ in this obligate microaerophile, we have employed a system for maintaining a constant culture $Po_2$ and compared magnetic cells of strain MS-1 with those of an aerotolerant, nonmagnetic mutant strain (NM-1Aa) under denitrifying and non-denitrifying conditions.
MATERIALS AND METHODS

Bacteria and growth conditions: *A. magnetotacticum* strains MS-1 and a nonmagnetic, aerotolerant mutant strain NM-1Aa (Short and Blakemore, 1985) were cultured microaerobically in chemically defined medium (Blakemore et al. 1985) containing either 5 mM NO₃⁻ or 4 mM NH₄⁺. The iron concentration was 20 μM, supplied as ferric quinate. Cultures, in 60 ml volumes, at an initial cell density of 2 x 10⁷ cells·ml⁻¹, were attached to a manifold and sparged continuously (20 cc·min⁻¹) with a mixture of compressed air and N₂. The O₂ content of the sparging gas was adjusted with rotometers and measured with a paramagnetic O₂ analyzer (Model E2 Oxygen Analyzer, Beckman Instruments, Inc. Fullerton, CA). The O₂ delivery rate was at least 10 times the maximum culture O₂ consumption rate. This prevented rate limitation by mass transfer of O₂ at any O₂ value used.

Cells were also grown in constantly stirred 15-L batch cultures maintained at the desired d.o.t. by varying the sparge rate. The d.o.t. was measured with a galvanic O₂ electrode immersed to a depth of 10 cm.

*Escherichia coli* strain B was cultured at 28°C.
in trypic soy broth (Difco Lab., Detroit, MI) either anaerobically (Florence flasks) or aerobically (2-L Fernbach flasks shaking at 220 rpm).

Cell preparation: Cells from 15-liter cultures were concentrated by filtration as described previously (Short and Blakemore, 1986). Concentrated cells were harvested by centrifugation (10,000 x g, 4° C). They were washed twice in 10 mM potassium phosphate buffer (KPB) at pH 6.8 by centrifugation and resuspended in KPB (pH 6.8). Cell numbers were estimated with a Petroff-Hausser counting chamber.

Chemical analyses: All biochemicals were from Sigma Chem Co. (St. Louis, MO). Nitrite was determined with Hach Nitriver 3 nitrite reagent (Hach, Ames, Iowa). Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Enzyme assays: Assays for SOD (Beauchamp and Fridovich, 1971), peroxidase (Clara and Knowles, 1984), catalase (Beers and Sizer, 1952) and nitrite reductase were performed with a DU-8 Beckman UV/vis spectrophotometer. The specific activities of SOD, peroxidase, and catalase were measured in soluble cell-free fractions prepared from washed cells disrupted by 2 passes at >10,000 p.s.i., 4° C, with a French Press. Unbroken cells and debris were removed by centrifugation at
Nitrite reductase activity determined was in a periplasmic cell fraction obtained by freezing and thawing cells (L. C. Paololetti, K. A. Short and R. P. Blakemore. 1986. Abstr. 86th Annu. Meet. Am. Soc. Microbiol. Washington, D. C. Abstr. No. 16). Enzyme activity was measured by coupling NO$_2^-$ reduction to oxidation of reduced phenazine methosulfate (PMSH$_2$) anerobically. Activity was expressed as change in absorbance at 388 nm (due to formation of oxidized PMS) per min per mg protein. The assay mixture in 2.5 ml consisted of: 20 mM potassium phosphate (pH 7.5), 60 mM phenazine methosulfate and 84 mM NADH. This mixture was placed in a gas tight spectrophotometer cell having a screw-top closure with a teflon/silicone septum (Spectrocell, Inc., Orland, PA). The contents of the cuvette were degassed by repeated evacuation and replacement of the headspace with O$_2$-free N$_2$. The absorbance at 388 nm was recorded until a constant baseline was established. Cell-free extract (10-50 μL) was then injected and a stable baseline was reestablished. Finally, 20 μL of 1 M anaerobic NaNO$_2$ was injected and the absorbance change at 388 nm was determined.

O$_2$ respiration rates: The O$_2$ respiration rates of washed whole cells of _A. magnetotacticum_, were
determined within 2 h of preparation by means of a polarographic O₂ electrode (Model 53 Yellow Springs Instruments, Yellow Springs, Ohio). Cells cultured with NO₃⁻ or NH₄⁺ at a d.o.t. < 1 % and in exponential growth (1-3 x 10⁸ cells.ml⁻¹) were harvested, washed twice in 10 mM KPB (pH 6.8) by centrifugation, resuspended to a density of 3 x 10¹¹ cells.ml⁻¹ and stored on ice. Cell endogenous respiration rates were constant for at least 2 h after preparation. The concentration of KCN, NaN₃, or NaN₃O₂ required for 50 % inhibition of the control endogenous rate was determined by successively adding 10-50 µl portions of inhibitor (10 µM KCN, 10 µM NaN₃ or 2 mM NaN₃O₂⁻) in 150 mM KCl (pH 7.1).

Oxidant pulse studies: The apparatus, reagents, and experimental techniques used for oxidant pulse studies were those of Kristjansson et al. (1979), and Castignetti and Hollocher (1983) as described previously (Short and Blakemore, 1986). KNO₂ (2 mM) was added in 150 mM KCl (pH 7.1) in the presence of 0.6 mM triphenylmethylphosphonium bromide (TPMP⁺) and 7.2 U of carbonic anhydrase. Chemicals were prepared immediately prior to use in gas-tight serum vials made anaerobic by heating to 50 °C with repeated evacuation and replacement of the headspace with O₂-free N₂, while cooling.
RESULTS and DISCUSSION

When cultured in medium equilibrated with gas containing greater than 2% (v/v) O₂, growth of wild type magnetic cells was depressed regardless of the N source (Fig. 8). This illustrated the obligately microaerophilic nature of this organism. However, the O₂ concentration optimal for growth of cells of either strain depended upon the nitrogen source (Figs. 8 and 9). Denitrifying magnetic cells grew optimally at 1% O₂ (Fig. 8, solid circles), whereas non-denitrifying magnetic cells (Fig. 8, solid squares) were inhibited to a lesser degree by higher O₂. Cells of the aerotolerant, nonmagnetic mutant also grew optimally at 1% O₂ when denitrifying (Fig. 9, solid circles) with decreased growth at higher O₂ values. However, unlike magnetic cells, non-denitrifying aerotolerant nonmagnetic cells (Fig. 9, solid squares) attained significantly higher densities at 3% O₂. Thus, cells grown at low O₂ with NH₄⁺ were electron acceptor limited, and cells grown with NO₃⁻ at high O₂ appeared inhibited by a toxic product associated with denitrification.

Previous results indicated that mM concentrations of NO₂⁻ were toxic for magnetic cells (Bazylnski and Blakemore, 1983). Thus, it seemed possible that one
effect of high $O_2$ might be to selectively inhibit $NO_2^-$ reductase allowing $NO_2^-$ to accumulate in toxic amounts. Nitrite did not accumulate in culture fluids of cells with $NH_4^+$ or those at a d.o.t. of $< 1\%$ regardless of the N source. However, at a d.o.t. of 8-10 $\%$, denitrifying cells accumulated $NO_2^-$ up to 10 $\mu$M (Table 6). This concentration of $NO_2^-$, added to non-denitrifying magnetic cells cultured near their optimal $O_2$ value, totally inhibited growth (Fig. 10). These results indicated that high $O_2$ caused $NO_2^-$ to accumulate in toxic quantity in denitrifying cultures. The $O_2$ was inhibitory to $NO_2^-$ reductase. The specific activity of $NO_2^-$ reductase measured in magnetic cells cultured at a d.o.t. of $< 1\%$ was 8 times that of cells cultured at a d.o.t. of 8-10 $\%$ (Table 7).

Nitrite appeared to have at least two toxic effects. It depressed cell $O_2$ respiration at a concentration similar to effective concentrations of other respiratory inhibitors (Table 8). These data were consistent with the known effect of $NO_2^-$ on oxidases (Rowe et al. 1979; Yang, 1985; Casella et al. 1986). Secondly, $NO_2^-$ enhanced the proton conductance of the cytoplasmic membrane of *A. magnetotacticum* cells, as evident from the atypical relaxation curve associated with the proton pulse (Fig. 11). As such it
would uncouple respiration-driven proton translocation. The ratio of protons ejected to gram atom added \( O_2 \), \( NO_3^- \), or \( Fe^{3+} \) as oxidant was subsequently depressed after adding \( NO_2^- \) (data not shown). Similar results have been reported for cells of *Paracoccus denitrificans* (Meijer et al. 1979). Nitrite reduction in the apparent absence of energy conservation by several microorganisms has been postulated as a mechanism of \( NO_2^- \) detoxification (Kaspar, 1982; Bazylniski et al. 1986; Casella et al. 1986).

These findings did not explain the observed differences in \( O_2 \) sensitivity between strains MS-1 (microaerophilic) and NM-1Aa (aerotolerant) when cultured with \( NH_4^+ \). Because denitrification was not involved, we investigated these differences by assaying enzymes normally involved in protecting cells against \( O_2 \) reduction products. Cells of strain MS-1 produced SOD and peroxidase, but failed to synthesize detectable quantities of catalase under any growth regime tested (Table 9). Activity of SOD, but not that of peroxidase, correlated with culture d.o.t. for strains MS-1 and NM-1Aa (Table 9). In contrast, cells of strain NM-1Aa cultured with \( NO_3^- \) at a d.o.t. of 8-10% had 8-fold higher SOD specific activity than did strain MS-1 cultured identically. Moreover, unlike magnetic cells,
those of the aerotolerant strain produced catalase when cultured at a d.o.t. of 8-10%. The activity measured was comparable to that measured in aerobically cultured cells of *E. coli* (Table 9). This mutant could be grown in culture media in free exchange with air.

Our data indicated two harmful effects of elevated $O_2$ on wild type magnetic spirilla. The relative sensitivity to $O_2$ of denitrifying (as compared to non-denitrifying) cells was due to $O_2$ repression or inhibition of $NO_2^-$ reductase allowing $NO_2^-$ to accumulate with harmful effects on cell respiration, membrane potential and growth. The aerotolerance of the mutant strain NM-1Aa, on the other hand, with corresponding higher specific activities of SOD and catalase suggested that the obligately microaerophilic wild type magnetic cells lacked suitable means of removing $O_2$ reduction products at high $O_2$. 

98
<table>
<thead>
<tr>
<th>Strain</th>
<th>N-source</th>
<th>d.o.t.</th>
<th>NO$_2^-$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-1</td>
<td>NH$_4^+$</td>
<td>&lt; 1 %</td>
<td>0</td>
</tr>
<tr>
<td>MS-1</td>
<td>NO$_3^-$</td>
<td>&lt; 1 %</td>
<td>0</td>
</tr>
<tr>
<td>NM-1Aa</td>
<td>NH$_4^+$</td>
<td>&lt; 1 %</td>
<td>0</td>
</tr>
<tr>
<td>NM-1Aa</td>
<td>NO$_3^-$</td>
<td>&lt; 1 %</td>
<td>0</td>
</tr>
<tr>
<td>MS-1</td>
<td>NH$_4^+$</td>
<td>8-10 %</td>
<td>0</td>
</tr>
<tr>
<td>MS-1</td>
<td>NO$_3^-$</td>
<td>8-10 %</td>
<td>10</td>
</tr>
<tr>
<td>NM-1Aa</td>
<td>NO$_3^-$</td>
<td>8-10 %</td>
<td>4</td>
</tr>
</tbody>
</table>

a. The d.o.t. was elevated to 8-10 % when the cell density reached $10^8$ cells.ml$^{-1}$. 
TABLE 7. Effect of culture d.o.t. and N source on NO\textsuperscript{-2} reductase activity\textsuperscript{a}

<table>
<thead>
<tr>
<th>Strain</th>
<th>N source</th>
<th>D.O.T.</th>
<th>Activity (\text{Abs/min/mg protein})</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-1</td>
<td>NH\textsubscript{4}\textsuperscript{+}</td>
<td>&lt; 1 %</td>
<td>ND\textsuperscript{c}</td>
</tr>
<tr>
<td>MS-1</td>
<td>NH\textsubscript{4}\textsuperscript{+}</td>
<td>8-10 %</td>
<td>ND</td>
</tr>
<tr>
<td>MS-1</td>
<td>NO\textsubscript{3}\textsuperscript{-}</td>
<td>&lt; 1 %</td>
<td>2.7</td>
</tr>
<tr>
<td>MS-1</td>
<td>NO\textsubscript{3}\textsuperscript{-}</td>
<td>1 %</td>
<td>3.6</td>
</tr>
<tr>
<td>MS-1</td>
<td>NO\textsubscript{3}\textsuperscript{-}</td>
<td>8-10 %</td>
<td>0.4</td>
</tr>
<tr>
<td>MS-1</td>
<td>NO\textsubscript{3}\textsuperscript{-} + NH\textsubscript{4}\textsuperscript{+}</td>
<td>&lt; 1 %</td>
<td>0.7</td>
</tr>
<tr>
<td>NM-1Aa</td>
<td>NH\textsubscript{4}\textsuperscript{+}</td>
<td>&lt; 1 %</td>
<td>ND</td>
</tr>
<tr>
<td>NM-1Aa</td>
<td>NH\textsubscript{4}\textsuperscript{+}</td>
<td>8-10 %</td>
<td>ND</td>
</tr>
<tr>
<td>NM-1Aa</td>
<td>NO\textsubscript{3}\textsuperscript{-}</td>
<td>&lt; 1 %</td>
<td>0.4</td>
</tr>
<tr>
<td>NM-1Aa</td>
<td>NO\textsubscript{3}\textsuperscript{-}</td>
<td>8-10 %</td>
<td>0.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Nitrite reductase activity was determined in periplasmic cell-free fraction obtained by freezing and thawing cells.
\textsuperscript{b} Mean values obtained from two or more cultures.
\textsuperscript{c} ND = not detected
TABLE 8. Effect of KCN, NaN₃ and NO₂⁻ on O₂ consumption by *Aquamarillum magnetotacticum*\(^a\)

Conc. required to reduce control rate by 50%

<table>
<thead>
<tr>
<th>Strain</th>
<th>KCN(μM)</th>
<th>NaN₃(μM)</th>
<th>NO₂⁻(μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-1 (NH₄⁺)</td>
<td>19</td>
<td>30</td>
<td>65</td>
</tr>
<tr>
<td>MS-1 (NO₃⁻)</td>
<td>50</td>
<td>123</td>
<td>49</td>
</tr>
<tr>
<td>NM1Aa (NO₃⁻)</td>
<td>46</td>
<td>190</td>
<td>160</td>
</tr>
<tr>
<td>MS-2 (NO₃⁻)</td>
<td>19</td>
<td>60</td>
<td>120</td>
</tr>
</tbody>
</table>

\(^a\) Cells were cultured at a d.o.t. < 1% with 4 mM NH₄⁺ or 5 mM NO₃⁻. Each test was carried out with 9 x 10¹¹ washed cells in 3 ml volume. The addition of 8 μM NO₂⁻ to a cell suspension of *A. magnetotacticum* NM-1Aa (4 x 10⁸ cells/ml) reduced the rate of O₂ consumption by 40%.
### TABLE 9. Superoxide dismutase, peroxidase, and catalase activity of cell-free extracts \(^a\)

<table>
<thead>
<tr>
<th>Species/strain</th>
<th>SOD (U/mg)</th>
<th>Peroxidase (ΔAbs./min/mg)</th>
<th>Catalase (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \times 10^{-2} )</td>
<td></td>
<td>( \times 10^{-2} )</td>
</tr>
<tr>
<td><strong>A. magnetotacticum MS-1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nitrate, &lt; 1 % d.o.t.</td>
<td>1.9</td>
<td>4.8</td>
<td>ND(^b)</td>
</tr>
<tr>
<td>nitrate. 8-10 % d.o.t.</td>
<td>2.5</td>
<td>7.8</td>
<td>ND</td>
</tr>
<tr>
<td>ammonium, &lt; 1 % d.o.t.</td>
<td>1.8</td>
<td>11.0</td>
<td>ND</td>
</tr>
<tr>
<td>ammonium. 8-10 % d.o.t.</td>
<td>2.6</td>
<td>4.8</td>
<td>ND</td>
</tr>
<tr>
<td><strong>A. magnetotacticum NM-1An</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nitrate, &lt; 1 % d.o.t.</td>
<td>3.9</td>
<td>2.1</td>
<td>ND</td>
</tr>
<tr>
<td>nitrate. 8-10 % d.o.t.</td>
<td>19.3</td>
<td>0.1</td>
<td>17</td>
</tr>
<tr>
<td>ammonium, &lt; 1 % d.o.t.</td>
<td>9.6</td>
<td>3.8</td>
<td>ND</td>
</tr>
<tr>
<td><strong>E. coli B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anaerobic</td>
<td>6.0</td>
<td>1.1</td>
<td>1</td>
</tr>
<tr>
<td>aerobic, shaking</td>
<td>25.1</td>
<td>0.5</td>
<td>14</td>
</tr>
</tbody>
</table>

\(^a\) Values are means of duplicate cultures.

\(^b\) ND = not detected
FIGURE 8. Effect of O₂ and N source on growth of *A. magnetotacticum* strain MS-1, cultured with (A) 1.0 %, (B) 2.0 % and (C) 3.0 % (v/v) O₂, in the sparging gas. Cell yields on NH₄⁺ (■) and NO₃⁻ (●) were determined from direct counts. Symbols and bars represent means and standard deviations, respectively, of triplicate cultures.
FIGURE 9. Effect of O$_2$ and N source on growth of *A. magnetotacticum* strain NM-1Aa, cultured with (A) 1.0 %, (B) 2.0 % and (C) 3.0 % (v/v) O$_2$, in the sparging gas. Cell yields on NH$_4^+$ (■) and NO$_3^-$ (●) were determined from direct counts. Symbols and bars represent means and standard deviations, respectively, of triplicate cultures.
FIGURE 10. Effect of NO$_2^-$ concentration on cell growth of *A. magnetotacticum* strain MS-1. Cells were cultured with 4 mM NH$_4^+$ and continuously poised at 2.4 % O$_2$ in 60-mL batch cultures. Symbols: O 0 µM, • 5 µM, ▲ 10 µM, ■ 20 µM NO$_2^-$; bars represent standard deviation of triplicate cultures.
FIGURE 11. Proton pulse traces obtained with cells of *A. magnetotacticum* in the presence of 0.6 mM TPMP⁺ and 7.2 U of carbonic anhydrase. Arrows indicate the addition of oxidant: O₂, NO₃⁻, Fe³⁺, or NO₂⁻. Horizontal-scale bars equal 1 min. Vertical-scale bars equal 1 nmol of H⁺ translocated to the cell exterior. The decay curve for NO₂⁻, unlike those for O₂, NO₃⁻, and Fe³⁺, indicates enhanced proton conductance of the cytoplasmic membrane.
$1 \text{ min} \quad \frac{1}{1 \text{ nmol H}^+}$

$O_2 \quad NO_3^-$

$Fe^{3+} \quad NO_2^-$
ACKNOWLEDGEMENTS

We thank N. Blakemore for providing *A. magnetotacticum* strains and R. Frankel for continued interest. We gratefully acknowledge J. Hoglen, D. Bazylnski and T. Hollocher of the Graduate Department of Biochemistry, Brandeis University for the nitrite reductase assay. This work was supported by ONR contract N00014-85-K-0502 and NSF grant DMB-85-15540. K. A. Short gratefully acknowledges support from a UNH CURF award.
LITERATURE CITED


CHAPTER FOUR
CHAPTER FOUR

CHARACTERIZATION AND PERIPLASMIC LOCATION
OF SUPEROXIDE DISMUTASE IN CELLS OF THE
MICROAEROPHILE AQUASPIRILLUM MAGNETOTACTION

ABSTRACT

Cells of the magnetic bacterium *Aqua spirillum magnetotacticum* and of an aerotolerant non-magnetic mutant, strain NM-1Aa, cultured with NO$_3^-$ or NH$_4^+$ at < 1 % or 8-10 % d.o.t. expressed 5 electrophoretically distinct periplasmic proteins with superoxide dismutase (SOD) activity. All 5 proteins were insensitive to inhibition by CN$^-$, and were therefore Fe or Mn-type SOD. Four of the SOD proteins, from cells cultured at < 1 % d.o.t., were sensitive to inhibition by H$_2$O$_2$ characteristic of Fe-type SOD. However, only 3 proteins from magnetic cells, and 2 proteins from aerotolerant non-magnetic cells, cultured at 8-10 % d.o.t., were sensitive to H$_2$O$_2$ inhibition. The Mn-SOD (CN$^-$ and H$_2$O$_2$ insensitive SOD) accounted for 12 % of the total SOD activity of cells cultured at < 1 % d.o.t., and increased to 46 % of total SOD activity detected in magnetic cells cultured at 8-10 % d.o.t. Based on SDS-
PAGE, the tentative molecular weight of the Mn-SOD and Fe-SOD was 35,000 and 23,000 daltons, respectively.

Cell periplasm contained 8-fold higher SOD specific activity than that detected in the pooled cytoplasmic + periplasmic fractions. In contrast, peroxidase activity was located predominantly in the cytoplasm and cytoplasmic membrane fraction. Total SOD activity in cells of the magnetic strain cultured at 8-10 % d.o.t. was 1.5 X that of cells cultured at < 1 % d.o.t. SOD activity in cells of the aerotolerant, non-magnetic strain cultured at 8-10 % d.o.t. was 5 X that of cells cultured at < 1 % d.o.t. SOD and peroxidase activities of cells of the magnetic strain cultured under denitrifying conditions with 20, 30, and 300 μM ferric quinate were identical.

The microaerophilic nature of magnetic cells of *A. magnetotacticum*, as compared with the non-magnetic mutant and cells of *A. iitroenii*, *A. bengal*, *E. coli* and *Azospirillum linoferum*, was associated with low SOD activity. Cells of the magnetic strain did not enhance SOD activity when cultured at elevated Po2.
INTRODUCTION

*Aquaspirillum magnetotacticum* strain MS-1 is a free-living diazotroph (Bazylinski and Blakemore, 1983b) capable of simultaneous use of O$_2$ and NO$_3^-$ (Bazylinski and Blakemore, 1983a) and probably Fe$^{3+}$ (Short and Blakemore, 1986) as terminal electron acceptors.

Magnetic cells do not express catalase activity (Blakemore et al. 1979) but do contain peroxidase (O'Brien et al. 1987) and superoxide dismutase (Short and Blakemore, 1987 Abstr. Annu. Meet. Am. Soc. Microbiol. Atlanta, GA I 123, p. 193). SOD mediates the dismutation of the superoxide radical (O$_2^-$) (McCord and Fridovich, 1969). McCord and Day (1978) proposed that SOD functions to limit the availability of O$_2^-$ for chemical conversion to OH$^-$ via the Haber-Weiss and Fenton reactions (Minotti and Aust, 1987). By these reactions O$_2^-$ reduces Fe$^{3+}$ to Fe$^{2+}$, which then reacts with H$_2$O$_2$ producing the hydroxyl anion (OH$^-$) and the hydroxyl radical (OH$'^-$. The presence of O$_2^-$ is injurious to cell proteins, nucleic acids, carbohydrates and membrane lipids (Halliwell, 1982; Fridovich, 1986). However, OH$^-$ is even more deleterious to cells than is O$_2^-$ (Rao and Cammack, 1981; Fridovich, 1986).

Magnetic bacteria contain intracellular magnetite
(Fe$_3$O$_4$), a low-density hydrous ferric oxide, a high-density hydrous ferric oxide (ferrihydrite) and ferrous iron (Frankel et al. 1983). Thus, cells of *A. magnetotacticum* could be particularly sensitive to O$_2$ due to rapid conversion of respiratory generated O$_2^-$ to OH$^-$ via the Haber-Weiss and Fenton reactions. Aerobic and facultatively anaerobic eubacteria generally produce Fe and/or Mn-type SOD's, although there are a few species with the Cu-Zn type more characteristic of eukaryotes (Puget and Michelson, 1974; Vignais et al. 1982; Martin and Fridovich, 1981; Steinman, 1982; 1985). In addition, the aerobic bacteria *Lactobacillus plantarum* (Archibald and Fridovich, 1981; Archibald and Duong, 1984) and *Neisseria gonorrhoeae* (Archibald and Duong, 1986) do not express SOD activity. The sensitivity to CN$^-$ of Cu-Zn SOD and of Fe-SOD to H$_2$O$_2$ has been widely used to discriminate between these forms of this enzyme on polyacrylamide gels.

Gregory et al. (1973) and Hassan and Fridovich (1977) reported that *Escherichia coli* B expressed a cytoplasmic Mn-SOD and a periplasmic Fe-SOD, but re-evaluated their findings (Britton and Fridovich, 1977) and concluded that both the Fe and Mn-type were cytoplasmic. Niekus et al. (1978) concluded that SOD activity in the microaerophile *Campylobacter jejuni* subspecies *bubulus* was localized in the cytoplasm.
Activity was present in a supernatant fraction (144,000 x g centrifugation for 1.5 h) of broken cells and therefore may have been cytoplasmic and/or periplasmic. Buchanan and Lees (1980) reported SOD activity of cytoplasmic origin in cells of the diazotroph *Azotobacter chroococcum*.

Buchanan (1977) demonstrated that exogenous $O_2^-$ inhibited *Azotobacter chroococcum* nitrogenase activity. Thus, SOD may be involved in the protection of nitrogenase (Robson and Postgate, 1980). These findings all suggest that in cells of *A. magnetotacticum* SOD could have multiple functions including dismutation of $O_2$-respiration generated $O_2^-$, limiting the production of iron generated $OH^-$ and protection of the nitrogenase complex.

We determined the locations of SOD and peroxidase activities in *A. magnetotacticum*, characterized the SOD's, and evaluated the effect of ferric iron on SOD activity in this organism grown under denitrifying conditions.
MATERIALS AND METHODS

Bacteria and growth conditions: Cells of *Aquaspirillum magnestotacticum* strains MS-1 and a nonmagnetic, aerotolerant mutant strain NM-1Aa were cultured as described previously (Short and Blakemore, 1986). The dissolved oxygen tension (d.o.t.) was measured with a galvanic electrode and was maintained within 10% of the desired value by varying the sparge rate.

*Escherichia coli* strain 12435 (University of New Hampshire culture collection) was cultured at 37°C in tryptic soy broth (Difco Lab, Detroit, MI) either anaerobically (Florence flask) or aerobically (2-L Fernbach flask shaking at 220 rpm). *A. itersonii* ATCC E12630, *A. bengal* and *Aquaspirillum limoferum* (all strains from the University of New Hampshire culture collection) were cultured as described previously (Short and Blakemore, 1986).

Cell preparation: Cells of *A. magnestotacticum* were harvested in exponential phase of growth with a Pellicon filtration system (Millipore Corp., Bedford, MA) and concentrated by centrifugation at 10,000 x g, 4°C, for 15 min. (Short and Blakemore, 1986). Cells of other strains were harvested in exponential phase of growth by centrifugation at 10,000 x g, 4°C, for 15 min. Concentrated cell pellets were washed once in 20 pellet
volumes 50 mM phosphate buffer, pH 6.8 (KPB), pelleted as before, and resuspended in 10-20 ml KPB. Cells were frozen at -70° C prior to fractionation.

Cell fractionation: Cells of *A. magnetotacticum* were fractionated into outer membrane proteins (OMP), cytoplasmic membrane proteins (CMP), periplasmic proteins (PP) and cytoplasmic + periplasmic protein (CP+PP) fractions. Periplasmic proteins were obtained by osmotic shock (Neu and Heppel, 1965), chloroform extraction (Ames et al. 1984) or freezing and thawing (Paoletti, Short and Blakemore, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, I6, p.166; submitted for publication) Fractions containing OMP, CMP, or CP+PP were obtained by the Schnaitman method (1981). Each fraction was dialyzed overnight against 10 mM Hepes buffer (pH 7.4).

Cell-free extracts were prepared from washed cells disrupted by 2 passes (> 10,000 p.s.i. at 4° C) through a French Press. Unbroken cells and debris were removed by centrifugation at 20,000 x g, 4° C, for 20 min. Soluble proteins were concentrated by membrane dialysis (Spectrapor, 6,000-8,000 mol wt cutoff. Spectrum Medical Industries, Inc., Los Angeles) in polyethylene glycol (solid flake. 20,000 mol wt. J. T. Baker Chemical Co., Phillipsburg, N. J.) at 4° C. Concentrated proteins were then dialyzed against KPB at 4° C, overnight.
Enzyme analysis: Superoxide dismutase (SOD) activity was determined spectrophotometrically as described by Beauchamp and Fridovich (1971). The amount of protein required to inhibit the rate of nitroblue tetrazolium reduction (Sigma Chemical Co., St. Louis, MO) by 50% was defined as 1 unit (U) of activity.

Peroxidase activity was determined spectrophotometrically as described by Clara and Knowles (1984) with 3,3'-diaminobenzidine (Aldrich Chemical Co. Inc., Milwaukee, WI) as the H+ donor. Specific activity was expressed as Δ A482·min⁻¹·mg protein⁻¹.

Polyacrylamide gel electrophoresis: Tube gel electrophoresis (10% polyacrylamide) was performed as described by Davis (1964). Slab gel electrophoresis (12% polyacrylamide) was performed using 1.5 mm gels. After electrophoresis, gels were removed and soaked for 1 h in 50 mM potassium phosphate buffer, pH 7.8 with 10⁻⁴ M ethylenediaminetetraacetic acid (KPB/EDTA) or in 5.0 mM H₂O₂ (Eastman Kodak Co., Rochester, NY) or in 1.0 mM CN⁻ (Eastman Kodak) in PB/EDTA. Gels were stained for SOD activity as described by Beauchamp and Fridovich (1971) and photographed or scanned by linear densitometry (Beckman Instruments, Fullerton, CA). SDS slab gel (1.5 mm thickness) electrophoresis was performed as described by Laemmli (1970). Prior to staining for SOD activity, gels were washed twice in KPB at 4°C overnight.
Molecular weight standards (Bio-Rad, Richmond, CA) and cell fractions solubilized by incubation at 24°C for 15 min in 150 mM tris-HCl (pH 6.8) buffer containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 1% bromophenol blue prior to electrophoresis.
RESULTS and DISCUSSION

The majority of SOD activity in cells of \textit{A. magnetotacticum} was located in the periplasm. Activity in each periplasmic fraction was 17-30 fold higher than in any membrane fraction tested (Table 10). Periplasm released by the freeze-thaw method (F/T) had 8-fold greater SOD specific activity than did the whole cell soluble fraction (cytoplasm + periplasm). A periplasmic location of SOD activity is unique, and distinct from the location of SOD in cells of \textit{E. coli} (Britton and Fridovich, 1977) and \textit{A. chroococcum} (Buchanan and Lees, 1980).

In contrast to SOD activity, peroxidase activity was detected predominantly in the cytoplasmic membrane and cytoplasm (Table 10). However, 6% of the peroxidase activity detected was periplasmic. O'Brien et al. (1987) had found that >60% of total c-type hemoproteins (which possess peroxidase activity) were released in the periplasmic fraction obtained by F/T. We conclude that c-type hemoproteins account for only a small fraction of the total peroxidase activity detected in our studies.

The periplasm from cells of \textit{A. magnetotacticum} strains MS-1 or NM-1Aa, cultured with NO$_3^-$ or NH$_4^+$, at a d.o.t. of <1% (of air saturation) contained 5 electo-
phoretically distinct proteins with SOD activity (Fig. 12). All 5 proteins were Mn- or Fe-type SOD based on their insensitivity to CN⁻ (Fig. 13). In both strains, 4 of these proteins were sensitive to inhibition by H₂O₂, hence were Fe-type SOD (Fig. 14). Cells expressed higher levels of the Mn-type SOD when cultured at increased culture Po₂. When magnetic cells were cultured at raised d.o.t. (8-10 %), only 3 of 5 proteins with SOD activity were of the Fe-type and 2 were Mn-type (Figs. 13 and 14). We estimated, from densitometric scans of tube gels, that Fe-type SOD accounted for 88 and 65 % of total SOD activity detected in magnetic cells cultured at < 1 % and 8-10 % d.o.t., respectively. Similar results were observed with cells of NM-1Aa, however, cells of the aerotolerant mutant expressed 2 Fe and 3 Mn-type SOD proteins when cultured at raised d.o.t. (8-10 %). Based on the results of SDS-PAGE, the molecular weight of the Mn-SOD was estimated to be at 35,000 daltons and that of the Fe-SOD to be 23,000 daltons (Fig. 13).

*E. coli* 12435 cultured aerobically expressed three electrophoretically distinct proteins (Rf values of 0.31, 0.46 and 0.60) with SOD activity. All were insensitive to inhibition by CN⁻ and 1 protein (Rf 0.60) was sensitive to H₂O₂. Similar results for aerobically
cultured *E. coli* have been reported by Hassan and Fridovich (1977) and Nettleton et al. (1984).

Cells of the microaerophilic wild-type strain (MS-1) cultured at a d.o.t. of < 1% d.o.t. had approximately the same SOD specific activity whether they were cultured with NO$_3^-$ or NH$_4^+$ (Table 11). Periplasmic SOD activity of strain MS-1 increased by less than 50% when the culture d.o.t. was increased from 1 to 8-10% d.o.t. Cells of strain NM-1Aa, on the other hand, showed a 5-fold increase in SOD activity over the same range of O$_2$ values.

In our study, varying the concentration of iron in culture medium did no affect a change in SOD or peroxidase specific activities. SOD and peroxidase activities of denitrifying *A. magnetotacticum* cells remained constant whether they were cultured with 20, 30, or 300 μM ferric quinate under denitrifying conditions.

Cells of *A. magnetotacticum* strain MS-1 express SOD activity at a level below that of other representative aerobes tested (Table 12). In our study the aerotolerant strain of *A. magnetotacticum* cultured at elevated d.o.t. (8-10% of saturation) expressed SOD activity at a level comparable to that of aerobically cultured *Azospirillum lipoferum*, or *Aquaspirillum*
cells but below that of aerobically cultured *E. coli* (Table 13). The microaerophilic nature of this bacterium is apparently due to its relatively low level of expression of SOD, its inability to significantly enhance expression of SOD when cultured at raised \( P_{O_2} \), its inability to express measureable levels of catalase, and may relate directly to the production of magnetite. Microaerobic conditions are required for bacterial magnetite formation (Blakemore et al. 1985). Although cells of the magnetic strain will grow aerobically in medium supplemented with exogenous catalase, cells do not retain their magnetic properties. In addition, cells of the aerotolerant non-magnetic strain NM-1Aa, compared to the microaerophilic strain, express elevated SOD and when cultured at 8-10 % d.o.t. express catalase (Short and Blakemore, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987. I 123, p. 193).

Interestingly, only 10 % of total peroxidase activity was present in the F/T periplasmic fraction. O'Brien et al. (1987) had found that > 60 % of total c-type cytochromes (which have peroxidase activity) were released in F/T fluids. Presumably, other cell proteins have much greater peroxidase specific activity.

The periplasmic location of SOD might be particularly advantageous to a microaerophile if the terminal
oxidases are oriented toward the periplasm. The deleterious effects of O\textsubscript{2} respiration generated O\textsubscript{2}\textsuperscript{-} radicals would be minimized by having reduction sites outside the cell cytoplasm.
TABLE 10. SOD and Peroxidase Specific Activity in *A.\_magneto\_tactica* Cell Fractions

<table>
<thead>
<tr>
<th>Cell Fraction (method of preparation)</th>
<th>SOD (U.mg protein(^{-1}))</th>
<th>Peroxidase ((\Delta AA\min(^{-1}).mg protein(^{-1}) x 10(^{-5}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM (Schnaitman, 1981)</td>
<td>0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>CM (Schnaitman, 1981)</td>
<td>0.3</td>
<td>2.1</td>
</tr>
<tr>
<td>CP+PP (Schnaitman, 1981)</td>
<td>2.8</td>
<td>2.1</td>
</tr>
<tr>
<td>Periplasm obtained by:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroform extraction</td>
<td>5.0</td>
<td>0.3</td>
</tr>
<tr>
<td>(Ames et al. 1984)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osmotic shock</td>
<td>5.8</td>
<td>0.2</td>
</tr>
<tr>
<td>(Neu and Heppel, 1965)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freeze/thaw</td>
<td>12.0</td>
<td>0.3</td>
</tr>
<tr>
<td>(Paoletti et al. 1985)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 11. Effect of N source and culture Po2 on SOD and Peroxidase Activities of Cell-free Periplasmic fractions\textsuperscript{a} of \textit{A. magnetotacticum}

<table>
<thead>
<tr>
<th></th>
<th>SOD</th>
<th>Peroxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(U.mg protein\textsuperscript{-1})</td>
<td>(\Delta A.min\textsuperscript{-1}.mg protein\textsuperscript{-1})</td>
</tr>
<tr>
<td><strong>strain MS-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO\textsubscript{3}\textsuperscript{-} &lt; 1 % d.o.t.</td>
<td>11</td>
<td>4.8 x 10\textsuperscript{-2}</td>
</tr>
<tr>
<td>8-10 % d.o.t.</td>
<td>14</td>
<td>7.5 x 10\textsuperscript{-2}</td>
</tr>
<tr>
<td>NH\textsubscript{4}\textsuperscript{+} &lt; 1 % d.o.t.</td>
<td>10</td>
<td>1.1 x 10\textsuperscript{-1}</td>
</tr>
<tr>
<td>8-10 % d.o.t.</td>
<td>15</td>
<td>4.8 x 10\textsuperscript{-2}</td>
</tr>
<tr>
<td><strong>strain HM-1Aa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO\textsubscript{3}\textsuperscript{-} &lt; 1 % d.o.t.</td>
<td>22</td>
<td>2.1 x 10\textsuperscript{-2}</td>
</tr>
<tr>
<td>8-10 % d.o.t.</td>
<td>110</td>
<td>1.0 x 10\textsuperscript{-3}</td>
</tr>
<tr>
<td>NH\textsubscript{4}\textsuperscript{+} &lt; 1 % d.o.t.</td>
<td>55</td>
<td>3.8 x 10\textsuperscript{-2}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Periplasmic fraction obtained by freezing and thawing.

\textsuperscript{b} The SOD specific activity in soluble PP obtained by freezing and thawing was 8 X that of PP + CP (obtained by the method of Schnaitman, 1980).
TABLE 12. SOD and Peroxidase Activities in Soluble (PP + CP) Protein Fractions of E. coli, A. bengal, A. itersonii and Azo. lipoforum.

<table>
<thead>
<tr>
<th></th>
<th>SOD (U·mg protein⁻¹)</th>
<th>Peroxidase (ΔA min⁻¹·mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli 12435</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anaerobic</td>
<td>8</td>
<td>3.4 x 10⁻²</td>
</tr>
<tr>
<td>aerobic</td>
<td>21</td>
<td>2.1 x 10⁻²</td>
</tr>
<tr>
<td><strong>A. bengal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aerobic, static</td>
<td>49</td>
<td>4.5 x 10⁻²</td>
</tr>
<tr>
<td><strong>A. itersonii E12639</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aerobic, static</td>
<td>5</td>
<td>8.7 x 10⁻²</td>
</tr>
<tr>
<td><strong>Azo. lipoforum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aerobic, static</td>
<td>15</td>
<td>4.1 x 10⁻²</td>
</tr>
</tbody>
</table>

a The CP + PP soluble fraction was the supernatant fluid from cells disrupted with a French Pressure Cell (>10,000 psi), and centrifuged (30,000 x g. 20 min.) and collection of the supernatant fluids. CP+PP (Schnaitman, 1981)
FIGURE 12. Non-reducing PAGE of _A. magnetotacticum_ periplasmic fractions. 6 U SOD applied per lane A, B, C, and D of Gel 1. 10 U SOD applied per lane E and F of Gel 1 and lanes A-D of Gels 2 and 3. Control Gel 1 was soaked in buffer and stained for SOD activity according to the method of Beauchamp and Fridovich (1971). Gel 2 was soaked in 1.0 mM CN for 1 h prior to staining. Gel 3 was soaked in 5.0 mM H$_2$O$_2$ for 1 h prior to staining.

Lanes: (A) & (E) strain MS-1, < 1 % d.o.t.; (B) & (F) strain MS-1, 8-10 % d.o.t.; (C) strain MS-2, < 1 % d.o.t.; (D) strain NM-1Aa approx. 10 % d.o.t.
FIGURE 13. Reducing (SDS) PAGE of cell-free *A. magnetotacticum* periplasmic fractions. Approximately 10 U SOD applied per lane. Samples were mixed with SDS, but not heated. The gel was washed for 8 h in 50 mM phosphate buffer (pH 6.8) and stained for SOD activity. The gel sliced and assayed for iron using the ferrozine assay (Stookey, 1970). Iron was detected only in the lower SOD band.
LITERATURE CITED


APPENDIX A
APPENDIX A

FREEZING AND THAWING CELLS OF
AQUASPIRILLUM MAGNETOTACTICUM
SELECTIVELY
RELEASES PERIPLASMIC PROTEINS.

ABSTRACT

Cells of the Gram-negative bacterium *Aqua spirillum magnetotacticum*, when suspended in buffer, frozen and thawed, produced pink-orange supernatant fluid. The fluid contained 2.0% or less of total extractable outer membrane component 2-keto-3-deoxyoctonate and of the cytoplasmic membrane marker succinic dehydrogenase. Electrophoretic banding patterns and difference spectra of proteins and hemoproteins released by freezing and thawing cells were distinct from those of membrane-associated and similar to those of periplasmic substances obtained by applying conventional fractionation methods to this organism.
INTRODUCTION

Freezing and thawing is known to have a profound effect on bacterial cells, and is often used as a pretreatment to cell disruption (Richardson and Parker, 1985; Schnaitman, 1981). Responses of Gram-negative cells depend upon the cell genotype (Calcott and Calcott, 1983), the menstruum they are suspended in and the freezing and thawing rates (Calcott and MacLeod, 1975). Outer sheath material from an oral spirochete has been isolated by freezing and thawing (10). Calcott and MacLeod (4) found that frozen and thawed lactose-limited Escherichia coli cells released considerable amounts of the periplasmic enzyme cyclic phosphodiesterase, but not the cytoplasmic enzyme glucose-6-phosphate dehydrogenase. A small, constant quantity (10 to 15% of total activity) of B-galactosidase (normally cytoplasmic) released was attributed to a possible periplasmic form of this enzyme.

Periplasmic substances of E. coli have been separated from other cellular components by means of osmotic shock or spheroplast formation (Neu and Heppel, 1965). Ames et al. (1984) demonstrated selective release of periplasmic proteins of E. coli cells treated with chloroform.

Freezing and thawing cell suspensions of
Aquaspirillum magnetotacticum strain MS-1 in 10 mM N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (Hepes) buffer (pH 7.4) or 10-50 mM potassium phosphate buffer (KPB) caused the release of soluble C551-type hemoproteins (W. O'Brien, M.S. thesis, University of New Hampshire, Durham, 1982; O'Brien et al. 1987). This freezing and thawing method (F/T) did not disrupt overall helical cell morphology. The objective of our study was to compare F/T with other cell fractionation methods applied to this organism to determine the cellular origin of the substances released including the soluble C551-type hemoprotein. This method also allowed us to partially purify this hemoprotein. Periplasmic soluble c-type hemoproteins of unknown function have been detected in Alcaligenes eutrophus (Probst and Schlegel, 1976). Aquaspirillum itersonii (Garrard, 1971), Paracoccus denitrificans (Husain and Davidson, 1986), and in Haemophilus parasuis (Niven, 1984). (Portions of this work have been reported [Paoletti. Short and Blakemore, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, Washington D.C.I6, p. 166]).
MATERIALS AND METHODS

Denitrifying cells of *Aquaspirillum magnetotacticum* MS-1 (ATCC 31632) or ammonium cultured cells of strain MS-1 and strain NM-1Aa (Short and Blakemore, 1986) were batch cultured in a chemically defined medium (Blakemore et al. 1979) microaerobically at a dissolved oxygen tension (d.o.t.) less than 1% of saturation. Cells of *A. itersonii* E12639 were cultured with the same medium (ammonium) supplemented with 0.05% yeast extract in 1-L stationary cultures. Cells of *A. magnetotacticum* were harvested by filtration (Short and Blakemore, 1986) when they reached a density of \(4 \times 10^8\) ml\(^{-1}\). Harvested cells and cells of *A. itersonii* E12639 were pelletted and washed once by centrifugation (8,000 x g, 30 min, 5°C) in at least 10 pellet volumes of 50 mM KPB (pH 6.8) or 10 mM Hepes buffer (pH 7.4). Cells from a single 40 L culture of denitrifying MS-1 were suspended in 100 ml of KPB or Hepes buffer and equal portions were fractionated by the procedures described below.

Cells of ammonium cultured MS-1, NM-1Aa, and *A. itersonii* were fractionated according to Schnaitman (1981). The outer membrane protein fractions obtained by this procedure were applied to SDS-PAGE, and the gel was subsequently stained with silver (Oakley et al. 1980).
The F/T technique consisted of storing washed, resuspended cells at -20°C overnight. The freezing rate was 0.7°C·min⁻¹. The sample was thawed at room temperature and cells were pelleted by centrifugation (10,000 x g, 15 min, 5°C). The pink-orange supernatant fluid was clarified by ultracentrifugation (100,000 x g, 1 h, 5°C) and concentrated by membrane dialysis (Spectrapor, 6,000-8,000 mol wt cutoff. Spectrum Medical Industries, Inc., Los Angeles) on a bed of polyethylene glycol (solid flake. 20,000 mol wt. J. T. Baker Chemical Co., Phillipsburg, New Jersey) at 4°C.

Periplasmic proteins were obtained by two methods: osmotic shock (Neu and Heppel), or chloroform extraction (Ames et al. 1984). The Schnaitman method (1981) was also used to separate outer membrane proteins, cytoplasmic membrane proteins and soluble (cytoplasm and periplasm) proteins. Cells were disrupted in a French press (10,000 p.s.i.) prior to treatment with 2% (v/v) Triton X-100 and 10 mM MgCl₂ in 10 mM Hepes buffer (pH 7.4). Each fraction was dialyzed overnight at 4°C against Hepes buffer prior to analysis.

The relative activity of succinic dehydrogenase (SDH), an integral enzyme of the cytoplasmic membrane (Dobrozogosz, 1981; Hederstedt and Rutberg, 1981), and the concentration of 2-keto-3-deoxyoctonate (KDO), a
constituent of outer membrane lipopolysaccharide were used as indices of the purity of cell fractions (Karkhanis). Proteins and molecular weight standards (Bio-Rad, Richmond, California) were solubilized and separated by SDS-PAGE (Paoletti and Blakemore, 1986) and stained with silver (Oakley et al. 1980). Room temperature dithionite-reduced minus air-oxidized difference spectra were performed with the soluble protein fractions as previously described (15). The ability of cells to survive F/T was evaluated by a standard plate assay. Thawed cells were quantitatively diluted and aliquots prepared as pour plates in semi-solid medium in triplicate. Plates were incubated one week at room temperature microaerobically. Colony counts were compared to those of control (non-F/T) cells plated similarly.
RESULTS AND DISCUSSION

Supernatant fluids obtained by F/T contained 1.3 % of the total SDH activity detected and 2.0 % of the total KDO recovered (Table 13). These results suggest that F/T did not markedly disrupt either the outer or inner cell membranes with attendant release of these markers. Soluble fractions obtained by chemical treatment (chloroform or lysozyme-EDTA) or mechanical disruption (French press) of strain MS-1 cells had comparable proportions of total detectable SDH activity and KDO (Table 13). Most (87 %) of the total SDH activity and 90 % of the total KDO recovered were in the cytoplasmic and outer membrane fractions of strain MS-1 cells, respectively (Table 13).

Electrophoretograms of each soluble fraction (Fig. 14, lanes 4-7) exhibited similar protein banding patterns. In each of these fractions, more than 60 proteins were evident including several major proteins with molecular weights ranging between 28,000 and 85,000 daltons. Four proteins with apparent molecular weights of 29,000, 41,000, 44,500 and 45,000 dalton were unique to the periplasm (Fig. 14, lanes 4-7). The cytoplasmic membrane (Fig. 14, lane 3) contained three major proteins (16,500, 56,000 and 85,000 daltons) also present in the periplasmic fraction. The outer membrane (Fig. 14, lane
and periplasmic fractions (Fig. 14, 4-7) contained few proteins in common.

Soluble fractions obtained by F/T, chloroform treatment, osmotic shock or French press disruption of strain MS-1 cells contained substances with absorption spectra (Fig. 15) typical of c551-type hemes (maxima at 419, 522 and 551 nm). Spent growth medium and cell wash fluids of *A. magnetotacticum* concentrated 100-fold did not contain detectable quantities of protein or c-type hemoproteins.

In a comparison of outer membrane protein (OMP) profiles from ammonium cultured cells of *A. magnetotacticum* strains MS-1 with cells of *A. magnetotacticum* strain NM-1Aa it is readily apparent that the profiles of MS-1 and NM-1Aa are very similar (Fig. 16). Moreover, the OMP profile from *A. ibersonii* is quite distinct from either MS-1 or NM-1Aa OMP profiles (Fig. 16).

The effects of F/T on strain MS-1 cells were evaluated by the plate assay and electron microscopy. Only 1-7% of the number of control (non-F/T) cells were recovered as colony forming units following F/T. Survivors were magnetotactic. F/T treated cells when negatively stained with uranyl acetate and observed by electron microscopy lacked flagella but appeared
otherwise structurally intact as compared to control cells. They retained their helical morphology and did not form spheroplasts or show blebbing.

Our results indicate F/T provides a rapid, simple, reproducible method of selectively releasing periplasmic substances including the soluble c551-type hemoproteins from *A. magnetotacticum* without recourse to chemical treatments.

We have applied F/T to cells of *A. iitrogenii* and *Azospirillum lipoferum* and obtained spectral evidence for release of c-type hemoproteins from these organisms as well (data not shown). Recently, F/T was applied to cells of nine genera of Gram-negative bacteria. The method was comparable to the chloroform method (Ames et al. 1984) for the release of periplasm (B. E. Eribo, S. D. Lall and J. M. Jay, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, I152, p. 197).

In conclusion, F/T is a selective method for obtaining periplasm from cells of *A. magnetotacticum*. This method had several advantages over conventional techniques used to obtain periplasm from *A. magnetotacticum*. These include (i) the absence of chemical treatment such as with lysozyme, chloroform, toluene or EDTA, (ii) the rapid and selective recovery of periplasmic substances including enzymes, and (iii) lack of apparent gross cell damage.
FIGURE 14. Silver stained SDS-PAGE of *Aquaspirillum magnetoaerophilum* cell proteins. Lane 1, molecular weight standards in kdal. Lane 2, outer membrane fraction. Lane 3, inner membrane fraction. Lane 4, periplasm/cytoplasm fraction. Lane 5, proteins obtained by the freeze/thaw method. Lane 6, proteins released by osmotic shock. Lane 7, proteins obtained with lysozyme-EDTA treatment. Each lane contained 3.5 µg protein. Dashes indicate proteins restricted to the periplasm.
FIGURE 15. Reduced minus oxidized difference spectra of soluble proteins of *Aquaspirillum magnetoautotrophicum* obtained by fractionation using: A. Freezing/thawing method (0.7 mg protein; absorbance divisions = 0.045). B. Schnaitman procedure (0.9 mg protein; absorbance divisions = 0.260). C. Chloroform treatment (0.04 mg protein; absorbance divisions = 0.045). D. Osmotic shock (0.04 mg protein; absorbance divisions = 0.010).
FIGURE 16. SDS-PAGE of OMPs from cells of *A. magnetotacticum* strains MS-1 and NM-1Aa and *A. iersonii*. Lane 1 = MS-1, Lane 2 = NM-1Aa, Lane 3 = *A. iersonii*. Approximately 15 µg protein was applied per lane. The gel was subsequently stained with silver (Oakley et al. 1980).
<table>
<thead>
<tr>
<th>Fractionation Method</th>
<th>Total KDO (mg)</th>
<th>Total SDH[^a] (units x 10^-2)</th>
</tr>
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<tbody>
<tr>
<td>Triton X-100/MgCl₂</td>
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<td></td>
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<tr>
<td>OMP</td>
<td>1330</td>
<td>30</td>
</tr>
<tr>
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<td>6</td>
<td>0.9</td>
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<tr>
<td>CMP</td>
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<td>200</td>
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<tr>
<td>Chloroform</td>
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<td></td>
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<tr>
<td>(Ames et al. 1984)</td>
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<td></td>
</tr>
<tr>
<td>Periplasm</td>
<td>11</td>
<td>0.9</td>
</tr>
<tr>
<td>Osmotic shock</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Neu and Heppel, 1965)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periplasm</td>
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<td>2.0</td>
</tr>
<tr>
<td>Freeze/Thaw</td>
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<td></td>
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<td>(this study)</td>
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</tr>
<tr>
<td>Periplasm</td>
<td>20</td>
<td>5.0</td>
</tr>
</tbody>
</table>

[^a]: µmoles cytochrome c reduced mg⁻¹ protein min⁻¹.
ACKNOWLEDGEMENTS

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LITERATURE CITED


APPENDIX B
PROTON TRANSLOCATION METHODS

The method for conducting proton translocation experiments described is that of Scholes and Mitchell (1970) as modified by Kristjansson et al. (1978), Leibowitz et al. (1982), Castignetti and Hollocher (1983). There are two distinctly different approaches to conducting proton translocation experiments. One approach is to work with cells depleted of endogenous reductant and to add various exogenous substrates (de Vries et al. 1982, Lawford and Haddock, 1973). The other is to use washed cells harvested during the exponential phase of growth and containing adequate endogenous substrate (Scholes and Mitchell, 1970; Castignetti and Hollocher, 1983; Bazylinski et al. 1986) but limited for oxidant.

Before initiating studies on proton translocation I recommend reading Scholes and Mitchell's 1970 article as well as Jones' book Bacterial Respiration and Photosynthesis (1982), in particular chapter 2.

Materials:
- pH meter: Beckman Altex pH meter Model 71 Fullerton, CA
- pH probe: Markson pencil (semi-Micro) size Model K-445 Value Mark: 15 cm length, 6 mM circumference (Markson 7815 S. 46th Street, Phoenix, AZ 85044-5399)

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- chart recorder: Houston Instruments OmniScribe Chart Recorder 8500 Cameron Rd., Austin, Texas 78753
- syringes: Supelco, Inc. Supelco Park, Bellefonte, PA 16823-0048
Unimetrics Universal Corporation syringes
<table>
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<th>size</th>
<th>model</th>
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<tr>
<td>5 μl</td>
<td>5005R</td>
</tr>
<tr>
<td>10 μl</td>
<td>TP5010TLC</td>
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<tr>
<td>25 μl</td>
<td>TP5025TLC</td>
</tr>
<tr>
<td>50 μl</td>
<td>TP5050</td>
</tr>
</tbody>
</table>
- serum vials 2-10 ml: Pierce Chemical Co. P.O. Box 117 Rockford, IL 61105
- stir bar of size appropriate for a 3-ml reaction chamber
- butyl rubber septum stopper for reaction vessel: Pierce Chem. Co.
- O₂ free N₂ gas (Wesco Gas Co. Portsmouth, NH)
- appropriately sized needles for injection and gas escape ports of the reaction vessel

**Chemicals:** all from Sigma Chemical Co. St. Louis, MO unless indicated otherwise
- 150 mM KCl, pH 7.10
- hydrochloric acid J.T. Baker Chemical Co. Phillipsburg, NJ analytical grade diluted to 100 mM in 150 mM KCl
- NaNO₃, NaNO₂, FeCl₃, ferric quinate, MnO₂, (all 2 mM) in 150 mM KCl.
- O₂: Supelco, Inc. Scott Specialty Gases solubility of oxygen in 150 mM KCl at 23 °C is 1.16 mM per atmosphere (Chappell, 1984; Hollocher et al. 1982)
- NaOH (100 mM) in 150 mM KCl
- carbonic anhydrase, triphenylmethy1 phosphonium (TPMP⁺) (each 10 mM) in 150 mM KCl.
The final concentration of TPMP⁺ that was optimal for
experiments with *A. magnetotacticum* was 0.6 mM. 7.2 U carbonic anhydrase was adequate to prevent acidification by carbonic acid. For each cell type the optimal concentration of protonophore or permeant ion must first be determined (by measuring the minimum quantity of protonophore giving the maximum H⁺/g atom oxidant) since above a certain concentration they can act as uncouplers of the membrane potential (e.g. Leibowitz et al. 1982)

- carbonyl cyanide-m- chlorophenyl hydrazone (CCCP) (2 mM) in 95% Ethanol

**Methods:**

*Preparation of chemicals:* As described in chapter two of this thesis and by Short and Blakemore (1986), all chemicals to be tested as oxidants, a solution of 150 mM KCl, 100 mM NaOH, and 100 mM HCl are prepared in gas-tight serum vials as dissolved gasses or solids in 150 mM KCl and made anaerobic by first heating the contents to 45-55°C and then cooling them with repeated evacuation of the headspace, followed by replacement with 1 atm (101.29 kPa) of O₂-free N₂ immediately prior to use. For tests with O₂, a known quantity of pure O₂ is equilibrated in 4 ml of 150 mM KCl in a 9-ml serum vial prepared as described above. TPMP⁺ carbonic anhydrase, and CCCP are evacuated several times and replaced with O₂-free N₂. All chemicals were prepared in the same manner each time just prior to use.

*Preparation of cells:* Cells are harvested in the exponential phase of growth as described (Short and Blakemore, 1986) and kept on ice for immediate use.

*Pulse experiments:* The reaction vessel is a 5-ml vial containing a small magnetic stir bar and fitted with a rubber closure. A combination pH electrode and needles for gas inlet, gas outlet, and oxidant sample injection are inserted through the closure (Fig. 17). A pH meter is used with a recorder set to 0.1 pH unit, full scale. To a 2-ml cell suspension (in KCl) in the vial are added 0.6 mM TPMP⁺ and 7.2 U carbonic anhydrase. The contents are then flushed with O₂-free N₂ and continuously stirred for 15-20 min to render them anaerobic and to allow the pH baseline to stabilize. Subsequently, the pH of the suspension is adjusted to ca. 7.1 with an
anaerobic solution of 0.01 N NaOH. The solution is continuously stirred and sparged for the duration of the experiment.

Check the buffering capacity of the cells suspension, by measuring the pH deflection upon adding a known quantity of protons (as HCl). By determining the number of protons which will alter the pH by a certain number of units (H⁺/pH unit) you will later be able to quantify the pulse curves, by measuring the deflection (acidification) and determine the quantity of H⁺ translocated per g atom oxidant. Repeat this determination a few times and average.

Next, inject a control solution of KCl prepared as were all oxidant species and observe whether you've successfully prepared anaerobic solutions. Whenever, the pH falls below 7.00 readjust to ca. 7.1 with base. With the buffering capacity of the cell suspension quantified and your first control check complete you're ready to continue with the pulse experiments.

Add oxidant species in various concentrations in replicate. If a particular oxidant does not produce a pulse of protons, vary the concentration of protonophore or try other ionophores or permeant ions. Periodically and calculate the mean value of H⁺ translocated per g atom oxidant to ensure that endogenous substrates are sufficient (i.e. not depressing H⁺/oxidant ratios).

Test several cell suspensions, with numerous injections of each oxidant, add CCCP (0.5 μM final concentration) and test whether proton translocation in response to each oxidant species is abolished. Increase the concentration of CCCP as necessary.

As a final control inject the various oxidant species with killed cells and into an anaerobic solution of 150 mM KCl + TPMP⁺ + carbonic anhydrase and ensure that true proton translocation "pulses" were observed.

Calculate the number of protons translocated per g atom of oxidant. Pulse amplitudes are quantified by using the graphic methods of Scholes and Mitchell (1970).
FIGURE 17. Schematic of the reaction vessel for proton translocation experiments.
2 ml washed cell suspension + TPMP™ and carbonic anhydrase

magnetic stir bar

Injection port

N₂ gas
LITERATURE CITED


APPENDIX C
APPENDIX C

KINETIC SPECTROPHOTOMETRIC ASSAYS FOR
PEROXIDASE AND SUPEROXIDE DISMUTASE

All bacteria which respire with oxygen, or grow in the presence of oxygen, experience toxicity to oxygen or to oxygen-generated reactive species. Superoxide dismutase (SOD), catalase, and peroxidase, are enzymes variably expressed by bacterial cells, which protect the cell from these reactive species. Aerobic bacteria usually express all 3 enzymes. Anaerobic bacteria may or may not express SOD and peroxidase, but do not express catalase. This appendix describes assays for the determination of peroxidase and SOD activity in cell-free soluble preparations. Peroxidase and SOD proteins have distinct functions and in tandem convert superoxide radical (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) to water and molecular oxygen. These reactions are summarized below:

\[
\text{SOD: } 2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2
\]

Peroxidase: catalyze the oxidation of organic compounds, by hydrogen peroxide, which is in turn reduced to water. The reaction is a 2-step process with regeneration of the enzyme occurring in the second reaction.

peroxidase + H$_2$O$_2$ \(\rightarrow\) compound

compound + AH$_2$ \(\rightarrow\) peroxidase + 2H$_2$O + A
Spectrophotometric assays for SOD and peroxidase activity have been developed. All employ cell-free extracts. Prior to analysis, extracts should be dialyzed against 50 mM potassium phosphate buffer, pH 6.8 at 4°C for 8-16 h. The concentration of protein for each fraction after dialysis is determined using the procedure of Lowry et al. (1951).

**SOD spectrophotometric kinetic assay:** Because of the instability of the superoxide radical (the substrate for SOD) the assay is indirect and depends on the ability of SOD to scavenge superoxide from the reaction mixture and thus to competitively inhibit reactions caused by O_2^-. The assay described below was developed by Beauchamp and Fridovich (1971). In this assay a flux of O_2^- is generated by the aerobic action of xanthine oxidase on xanthine. Nitro blue tetrazolium (NBT) reacts with the O_2^- radical which upon reduction forms blue formazan (A_{max} = 560 nm). Therefore, SOD activity is measured by observing the degree of inhibition of the formation of blue formazan. Beauchamp and Fridovich (1971) defined one unit of SOD as the amount of enzyme giving 50% inhibition of a defined rate (ΔA_{560} min^-1 = 0.0165) of reduction of NBT. It is important not to exceed approximately 50% inhibition since inhibition is not linear beyond this value (see Beauchamp and Fridovich, 1971).

**Protocol for Kinetic Assay of SOD specific activity:**

(a) Reagents- Sigma Chemical Co. St. Louis, MO

- Reaction mixture (per 100 ml reagent grade H_2O)
  - 0.42 g NaHCO_3 (5.0 x 10^-2 M)
  - 2.0 mg nitro blue tetrazolium (2.5 x 10^-4 M)
  - 1.52 mg xanthine (1.0 x 10^-4 M)
  - 3.8 mg EDTA (1.0 x 10^-4 M)
  - Adjust pH to 10.2 with NaOH.

- Xanthine oxidase (from buttermilk: Sigma No. x-4500)
(b) Procedure- Set the spectrophotometer to 560 nm. The assay should be performed in subdued light. Add 3.0 ml of reaction mixture to a cuvette, and let this incubate for 3 min to allow equilibration. First, determine the quantity of xanthine oxidase to add to the reaction mixture to give a rate of increase in absorbance (a result of blue formazan) of 0.0165/min in the absence of SOD. This quantity of xanthine oxidase is added to each cuvette during the enzyme assay. Then, determine the quantity of cell-free extract which inhibits the rate of increase in absorbance by 50 % or less. Specific activity is equal to the number of units per mg protein.

(c) An example- It is determined that 5 µl xanthine oxidase added to 3.0 ml reaction mixture results in A

\[ \Delta A_{560} \cdot \text{min}^{-1} = 0.0165 \]

Next, 20 µl of cell-free extract is added to 3.0 ml reaction mixture. a baseline established, and then 5 µl xanthine oxidase is added. If the rate of inhibition exceeds 50 %, the quantity of cell-free extract added is decreased. However, in this case, 20 µl of cell-free extract inhibited the reaction rate by 40 % (\(\Delta A \text{ min}^{-1} = 0.0095\)). Therefore, there are 0.8 units SOD per 20 µl extract. Express this as units/mg protein.

Peroxidase spectrophotometric kinetic assay: In the presence of peroxidase and hydrogen peroxide, 3,3'-diaminobenzidine (DAB) acts as a electron donor yielding a stable polymer, red in color, at the reaction site. The reduced DAB has an absorbance maximum at 482 nm. With substrate in excess, the rate of increase in absorbance at 482 nm is directly proportional to peroxidase activity. The assay described was used by Clara and Knowles (1984).

Protocol for Kinetic Assay of Peroxidase specific activity:

(a) Reagents- Sigma Chemical Co. St.Louis. MO

- Reaction mixture (per 100 ml 50 mM potassium phosphate buffer, pH 7.2)
  19.8 mg DAB (0.5 mM)(potential carcinogen)
  22.6 µl \(\text{H}_2\text{O}_2\) (2.0 mM)
(b) Procedure- Set the spectrophotometer to 462 nm. Place 2.9 ml of reaction mixture in a cuvette. Place the cuvette in the spectrophotometer and incubate 3 min. Then add 0.10 ml of cell-free extract to the cuvette and record Δ A/min. Specific activity is expressed as Δ A/min/mg protein.
LITERATURE CITED

