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NITROGEN METABOLISM AND IRON REDUCTION IN AQUASPIRILLUM MAGNETOTACTICUM (NITRATE, FIXATION, MAGNETIC BACTERIUM)

DENNIS ALFRED BAZYLINKSI

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NITROGEN METABOLISM AND IRON REDUCTION IN
AQUASPIRILLUM MAGNETOTACTICUM

By

Dennis A. Bazylinski
B.S., Northeastern University, 1976
M.S., Northeastern University, 1980

DISSERTATION

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ABSTRACT

NITROGEN METABOLISM AND IRON REDUCTION IN AQUASPIRILLUM MAGNETOTACTICUM

by

DENNIS A. BAZYLINSKI

University of New Hampshire, May, 1984

Aquaspirillum magnetotacticum strain MS-1 grew microaerobically but not anaerobically with NO$_3^-$ or NH$_4^+$ as the sole nitrogen source. Cell yields varied directly with NO$_3^-$ concentration under microaerobic conditions. Products of NO$_3^-$ reduction by growing cells included NH$_4^+$, N$_2$O, NO, and N$_2$ but not NO$_2^-$ or NH$_2$OH. The inclusion of NH$_4^+$ in growth medium prevented NO$_3^-$ reduction to NH$_4^+$ but not to N$_2$O or N$_2$. Cells consumed O$_2$ while denitrifying and this appears to be the first described species with an absolute requirement for O$_2$ while denitrifying.

Cultures grown with NO$_3^-$, in contrast to NH$_4^+$, contained fewer cells without magnetosomes. Moreover, among the cells with these intracellular magnetic particles, a higher average number per cell and a higher average cell magnetic moment was obtained with NO$_3^-$.

This effect of cell nitrogen source on culture magnetism was investigated further with growing cells and cell-free extracts. The results indicated that Fe$^{+3}$ reduction by cell-free extracts of A. magnetotacticum was independent of electron transport chain components.
and suggested that Fe$^{3+}$ and NO$_3^-$ reduction proceeded independently in the cell.

*A. magnetotacticum* strain MS-1 and several non-magnetic mutants derived from it, fixed N$_2$ (reduced acetylene) microaerobically but not anaerobically even with NO$_3^-$. Cells of *A. magnetotacticum* reduced acetylene at rates comparable to those of *Azospirillum lipoferum* under similar conditions but at a much lower rate than that of *Azotobacter vinelandii* grown aerobically.
INTRODUCTION

*Aquaspirillum magnetotacticum* is a microaerophilic, bipolarly flagellated magnetic spirillum species. Cells each synthesize "magnetosomes" (intracellular, enveloped iron-rich crystals) which impart a permanent magnetic moment of about $5 \times 10^{-13}$ emu per cell. Magnetosomes of *A. magnetotacticum* consist of magnetite ($\text{Fe}_3\text{O}_4$). Bacterial magnetite is produced in a sequence of steps including iron reduction. Iron reduction and nitrate reduction in various biological systems often appear to be intimately related, although biochemical details remain unclear. *A. magnetotacticum* is a nitrate reducer with a versatile nitrogen metabolism. It grows with nitrate or ammonium as the sole nitrogen source and also fixes atmospheric dinitrogen. Cells grown with nitrate (denitrifying) are more magnetic than those grown with ammonium as the sole nitrogen source. However, the role of cell nitrogen metabolism in magnetite synthesis has not been investigated.

This study was undertaken to clarify the biochemistry of iron and nitrate reduction in *A. magnetotacticum*. A major goal was to identify products of nitrate and ammonia metabolism in this organism, to establish, for instance, whether it is a true denitrifier by currently accepted criteria, and also to establish whether cells can fix dinitrogen. A second goal was to examine the possibility that nitrate reduction and iron reduction are linked in this organism and thereby providing an explanation for the observed influence of cell nitrogen source on cell magnetite synthesis.
CHAPTER ONE
CHAPTER ONE

DENITRIFICATION AND ASSIMILATORY NITRATE REDUCTION IN

AQUASPIRILLUM MAGNETOTACTICUM

ABSTRACT

_Aquaspirillum magnetotacticum_ strain MS-1 grew microaerobically but not anaerobically with NO₃⁻ or NH₄⁺ as a sole nitrogen source. Nevertheless, cell yields varied directly with NO₃⁻ concentration under microaerobic conditions. Products of NO₃⁻ reduction included NH₄⁺, N₂O, NO, and N₂. NO₂⁻ and NH₂OH, each toxic to cells at 0.2 mM, were not detected as products of cells growing on NO₃⁻. NO₃⁻ reduction to NH₄⁺ was completely repressed by the addition of 2mM NH₄⁺ to the growth medium whereas NO₃⁻ reduction to N₂O or to N₂ was not. C₂H₂ completely inhibited N₂O reduction to N₂ by growing cells. These results indicate that _A. magnetotacticum_ is a microaerophilic denitrifier that is versatile in its nitrogen metabolism, concomitantly reducing NO₃⁻ by assimilatory and dissimilatory means. This bacterium appears to be the first described denitrifier with an absolute requirement for O₂. The process of NO₃⁻ reduction appears well adapted for avoiding accumulation of several nitrogenous intermediates toxic to cells.
INTRODUCTION

Motile bacteria whose principal swimming directions are influenced by magnetic fields, including the geomagnetic field, are common in sediments of diverse aquatic habitats (Blakemore, 1975; Moench and Konetzka, 1978). Cells of the bipolarly flagellated, obligate microaerophile, *Aquaspirillum magnetotacticum* (Blakemore et al., 1979; Frankel et al. 1979; Maratea and Blakemore, 1981) synthesize magnetosomes (intracellular, enveloped, iron-rich crystals) consisting of magnetite (Fe$_3$O$_4$). Magnetosomes impart to each cell a permanent magnetic dipole moment (Balkwill et al., 1980; Frankel and Blakemore, 1980). Cells synthesizing Fe$_3$O$_4$ from soluble (chelated) iron accumulate the hydrous ferric oxide, ferrihydrite (Frankel et al., 1983). Thus, bacterial magnetite synthesis appears to parallel the process of magnetite biomineralization in chitons (class Mollusca), involving iron reduction and dehydration of a ferrihydrite precursor (Lowenstam, 1981). Iron reduction by nitrate reductase has been suggested for soil microorganisms (Ottow, 1969, 1970). Moreover, Sørensen (1982) obtained evidence that oxidized iron may replace NO$_3^-$ as a terminal electron acceptor in microorganisms found in surface sediments.

This study was undertaken to clarify the biochemistry of NO$_3^-$ reduction in *A. magnetotacticum* as a prelude to establishing whether enzymes of NO$_3^-$ reduction are involved in its ability to synthesize magnetite via iron reduction under microaerobic conditions.
NO$_3^-$ reduction in *Aquaspirillum* species is poorly understood. Within this genus, *A. itersonii* and *A. psychrophilum* also reduce NO$_3^-$ beyond the NO$_2^-$ stage but only the latter species forms visible gas (Gauthier et al., 1970; Krieg, 1976). N$_2$O is the terminal product of NO$_3^-$ reduction in *A. itersonii* (Bryan, 1981). *A. fasciculus*, *A. gracile*, and *A. polymorphum* appear to reduce NO$_3^-$ to NO$_2^-$ only (Hylemon et al., 1973; Krieg, 1976). *A. dispar* (ATCC 27510 and 27650) was found to grow anaerobically with NO$_3^-$, reducing it beyond the NO$_2^-$ stage (Krieg and Hylemon, 1976).

Cells of *A. magnetotacticum* grow microaerobically with NO$_3^-$ or NH$_4^+$ as a sole N source. NO$_3^-$ is reduced forming NH$_3$ and nitrous oxide (N$_2$O) but no detectable NO$_2^-$ (Blakemore et al., 1979; Escalante-Semerena et al., 1980; Bazylinski and Blakemore, Abstr. 82nd Annu. Meet. Am. Soc. Microbiol., 1982, I53, p. 103). Thus, this bacterium appears to assimilate products of NO$_3^-$ reduction while denitrifying.

True denitrifiers typically reduce 90% or more of the available N-oxide (NO$_3^-$ or NO$_2^-$) to N-gas and couple this reduction to electron transport phosphorylation (Bleakley and Tiedje, 1982; Bryan, 1981). Certain non-denitrifying NO$_3^-$ reducers including strains of *Escherichia coli* produce N$_2$O in amounts less than 30% of the N-oxide (Bleakley and Tiedje, 1982; J. M. Tiedje, personal communication). Because the gaseous products of NO$_3^-$ reduction in *Aquaspirillum* species have not been quantified, the role of these organisms in denitrification is still unclear. Moreover, some non-denitrifying bacteria produce N$_2$O during NO$_3^-$ reduction to NH$_4^+$ (Bleakley and Tiedje, 1982; Smith, 1982; Smith and Zimmerman, 1981). Thus, another goal of this study was to
establish whether *A. magnetotacticum* can be considered a denitrifier by currently accepted criteria despite its absolute requirement for $O_2$. 
MATERIALS AND METHODS

Bacteria and Growth Conditions

The organism used in this study was *Aquaspirillum magnetotacticum* strain MS-1. Magnetotactic cells of this strain and those of a non-magnetotactic variant (see below) were cultured routinely in a growth medium containing the following (g/l): tartaric acid 0.75, KH₂PO₄ 0.69, NaNO₃ 0.17, and sodium thioglycolate 0.06. To each liter of this medium were added 2.0 ml of 10 mM ferric quinate (Blakemore et al., 1979), 10 ml of vitamin mixture (Wolin et al., 1963), 5 ml of mineral solution (Wolin et al., 1963), and 0.1 ml of 1% (w/v) aqueous resazurin. The mineral solution was modified by the addition of 0.4 g of Na₂MoO₄·2H₂O per l. Ammonium ion was added to the medium as required, either as (NH₄)₂SO₄ or NH₄Cl as indicated. NaNO₂ or NH₂OH·HCl was added to the medium as indicated. The pH of the medium was adjusted to 6.75 with NaOH prior to sterilization.

Experiments were carried out with cells cultured microaerobically at 30°C in stoppered 160 ml serum vials each containing 60 ml culture medium. O₂-free N₂ or He was bubbled through the medium (approx. 500 cc/min) for 15 min at room temperature prior to sealing each vial. The headspace gas of each was then replaced with either N₂ or He after repeatedly evacuating the vials with the use of a vacuum manifold as described by Balch and Wolfe (1976). After autoclaving, the medium was anaerobic (colorless). Just prior to inoculation, sterile air or O₂
was added to the bottles to obtain an initial headspace concentration of 0.2 to 1.0 % (v/v) O₂ (200-1000 Pa O₂). Cells were also mass cultured in 10-15 l batch cultures as previously described (Blakemore et al., 1979).

**Estimation of Cell Yield**

Cell numbers were determined by means of direct cell counts using a Petroff-Hauser cell counting chamber. Dry cell weights were determined by filtering culture samples through 0.2 μm polycarbonate filters (Nucleopore Corp., Pleasanton, CA.) which were then dried to constant weight at 60°C.

**Cell Magnetism**

 Cultures were assessed for their magnetism by microscopically noting the fraction of cells, living or dead, that reversed direction when a small magnetic stirring bar 5-10 cm away from the microscope stage was rotated 180° from its initial position. Occasionally cells were negatively stained with 0.5% uranyl acetate (w/v, pH 4.2) and examined by electron microscopy for the presence of magnetosomes.

**Use of Acetylene to Block N₂O Reduction**

We used established methods to inhibit N₂O reduction with acetylene (C₂H₂) (Federova et al. 1973; Yoshinari et al., 1977). C₂H₂ was generated from distilled water and CaC₂ (granular, Fisher Scientific Co.). All cultures grown with C₂H₂ were incubated on a shaker at 30°C.
Chemical Analyses

\( \text{NO}_3^- \) was determined with a Beckman SelectIon 2000 Ion Analyzer (Beckman Instruments, Irvine, CA.). \( \text{NO}_2^- \) was analyzed using sulfanilamide-N-1-naphthylethylene-diamine dihydrochloride (American Public Health Association, 1980). \( \text{NH}_4^+ \) was determined by the reductive amination of \( \alpha \)-ketoglutarate (Sigma Technical Bulletin No. 170-UV, Sigma Chemical Co., St. Louis, MO.). Bound and free \( \text{NH}_2\text{OH} \) was assayed by the Csaki (1948) procedure and by the method of Magee and Burris (1954).

\( \text{N}_2\text{O}, \text{NO}, \text{and } \text{O}_2 \) were measured by gas chromatography on a Varian Series 2400 gas chromatograph equipped with a \(^{63}\text{Ni} \) electron capture detector (Varian Instruments, Walnut Creek, CA.). Two Porapak Q columns (3 mm x 1.8 m) were arranged in series, the meshes being 80/100 and 60/80 respectively. \( \text{O}_2\)-free \( \text{N}_2 \) at a flow rate of 25 ml/min was the carrier gas. The operating temperatures were as follows (°C): detector, 300; column oven, 55; injector, 70. Under these conditions \( \text{H}_2, \text{He, O}_2, \text{NO, CO}_2, \text{N}_2\text{O, C}_2\text{H}_2, \) and \( \text{H}_2\text{O} \) were separated.

\( \text{N}_2 \) was determined using a Perkin-Elmer model 3920A gas chromatograph equipped with a thermal conductivity (HW) detector. A Molecular Sieve 5A column (60/80 mesh, 3 mm x 1.8 m) was the stationary phase. \( \text{O}_2\)-free helium at a flow rate of 30 ml/min was the carrier gas. The bridge current was 225 mA and the operating temperatures were as follows (°C): detector, 130; injector, 120; column oven, 40.

Peak areas were determined with a Hewlett-Packard model 3390A computing integrator. For each analysis standard curves were prepared.
using pure gases (Scott Environmental Technology, Inc.).

Samples of the culture headspace gas were removed with a gas-tight syringe (Series A-2, Precision Scientific Co.) previously flushed at least three times with O₂-free N₂ or He and were immediately injected into the gas chromatograph.

To determine the total concentration of a gaseous product, the amount present in solution was calculated using Henry's Law and published values of solubility coefficients (Linke, 1965).

Cells grown to late exponential phase (10 l; 8-9 x 10⁷ cells/ml) were harvested by continuous flow centrifugation in a CEPA-model LE electrically driven centrifuge equipped with water cooling. Cells were washed several times with 50 mM potassium phosphate buffer (pH 6.90) by centrifugation (11,000 x g; 15 min at 5°C) and dried to constant weight in vacuo over CaSO₄ at 110°C. Dried cells were analyzed for total protein, amino acids and their elemental composition.

For amino acid analysis samples of whole cells were hydrolysed with HCl and treated with 10.74 mM aqueous Na₂EDTA to remove iron. Amino acids and intracellular NH₄⁺ were determined using single column acid-hydrolysate methodology (Spinco Application Note AN-001, 4/77, Beckman Instruments, Spinco Division) with a Beckman model 118CL amino acid analyzer equipped with a Varian model CDS-111C peak integrator. The analyzer/integrator system was calibrated using a Beckman standard reference mixture (Beckman Instruments).

Total cell protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard.
Cell elemental composition was determined with a Perkin-Elmer model 240B elemental analyzer using acetanilide as the standard.
RESULTS

Effect of Nitrogenous Compounds on Growth
and Magnetite Synthesis

$NH_4^+$ and $NO_3^-$ are utilized as sole sources of nitrogen by $A$. magnetotacticum (Maratea and Blakemore, 1981; Fig. 1). After an initial lag period, the growth rate observed with $NO_3^-$ was much higher than that with $NH_4^+$ (Fig. 1). A lag period was not observed with either $(NH_4)_2SO_4$ or $NH_4Cl$. Higher cell yields were obtained with $NaNO_3$ (1.2 x $10^8$ cells/ml) than with $(NH_4)_2SO_4$ (2.9 x $10^7$ cells/ml) or $NH_4Cl$ (2.1 x $10^7$ cells/ml). Cell growth ceased 72 h after addition of $NO_3^-$ or $NH_4^+$ to the culture medium (Fig. 1).

The effect of known intermediates of $NO_3^-$ reduction by other bacteria was determined. Free $NH_2OH$ or $NO_2^-$ (2 mM) were toxic to cells and produced lysis (Fig. 1). When added to growth medium containing no fixed nitrogen source each compound exhibited toxicity even at a concentration of 0.2 mM (Appendix 1: Figures 7 and 8). However, $NO_2^-$ at less than 1 mM was not toxic for cells actively growing on $NO_3^-$ (Appendix 1: Fig. 9). $N_2O$ (120 μmols added to the headspace of shaking cultures) had no detectable effect on growth of cells in the absence of a combined nitrogen source (Fig. 1).

Although cultures grown with $NH_4^+$ or $NO_3^-$ each contained magnetotactic cells, those grown with $NH_4^+$ frequently contained a higher proportion of nonmagnetotactic cells than those grown with $NO_3^-$.
FIGURE 1. Growth response of *A. magnetotacticum* to added nitrogen compounds. At 48 h, cultures previously grown without a source of fixed nitrogen were provided with NH₄Cl, (NH₄)₂SO₄, NaN₃, N₂O, NaN₂O₃, or NH₂OH·HCl, each at a final concentration of 2 mM N. The controls received an equal volume of anaerobic growth medium minus an N source. Symbols and bars represent means and standard deviations, respectively, obtained with triplicate cultures.
Cultures grown with $\text{NO}_3^-$ under microaerobic conditions frequently showed a biphasic growth pattern (Figs. 1 and 2). Figure 3 shows the utilization of $\text{NO}_3^-$ and $\text{O}_2$ as well as the production of $\text{NO}_2^-$, NO, and $\text{N}_20$ by growing cells. $\text{O}_2$ steadily disappeared throughout growth. $\text{NO}_3^-$, on the other hand, was utilized most extensively after 40 h. The accumulation of $\text{N}_20$ correlated with the extent of $\text{NO}_3^-$ utilization. At about 40 h the cell growth rate increased from a culture doubling time of about 40 to 16 h. Traces of $\text{NO}_2^-$ and/or NO were occasionally detected during growth of $\text{A. magnetotacticum}$ but neither of these accumulated in significant amounts. Of note, cells actively using $\text{NO}_3^-$ also continued to consume $\text{O}_2$.

**Effect of Oxygen on Growth of $\text{A. magnetotacticum}$**

In confirmation of previous results (Blakemore et al., 1979), cells did not grow anaerobically (resazurin colorless) either with $\text{NO}_3^-$ or $\text{NH}_4^+$ as the sole nitrogen source (Fig. 2). Under anaerobic conditions cells eventually became nonmotile, an effect that was reversible for at least several hours. Cells retained their magnetism under anaerobic conditions.

**Effect of $\text{C}_2\text{H}_2$ on Growth and $\text{N}_2\text{O}$ Reduction**

At a concentration of 10 kPa, $\text{C}_2\text{H}_2$ inhibited growth and resulted in aberrant non-motile and coccoid cells (Appendix 1: Fig. 10). $\text{C}_2\text{H}_2$ at a final headspace concentration of 1 kPa did not adversely affect cell growth (Appendix 1: Fig. 10) or morphology but completely inhibited $\text{N}_2\text{O}$
FIGURE 2. Effect of $O_2$ on the growth of *A. magnetotacticum* with or without $NH_4^+$ or $NO_3^-$. To limit the introduction of $O_2$, 1% inocula (vol/vol) were from cultures grown until $O_2$ had completely disappeared. Cells used as inocula came from culture medium similar to that used in the experiment. Data points and bars represent means and standard deviations, respectively, obtained with triplicate cultures.

Symbols:
- $\bullet$, $NO_3^-$ (2 mM), microaerobic conditions (initial $Po_2$, 0.2 kPa)
- $\triangle$, $NO_3^-$ (2 mM), anaerobic conditions (resazurin colorless)
- $\Box$, $NH_4^+$ (2 mM), microaerobic conditions (initial $Po_2$, 0.2 kPa)
- $\circ$, $NH_4^+$ (2 mM), anaerobic conditions (resazurin colorless).
FIGURE 3. Growth of *A. magnetotacticum* with NO$_3^-$.

Cells were grown in a 2-liter serum-stoppered culture vessel containing 1 liter of growth medium with 2 mM NO$_3^-$ and 2 mM NH$_4^+$. The inoculum (1%) came from a culture grown in similar medium. ● = cells per milliliter.
reduction (Table 1). With NH$_4^+$ present together with C$_2$H$_2$
(assimilatory NO$_3^-$ reduction to NH$_4^+$ repressed), 96.4% of the N
supplied as NO$_3^-$ was recovered as N$_2$O (Table 1).

**Products and Mass Balance of NO$_3^-$ Reduction**

Cells growing microaerobically reduced NO$_3^-$ to NH$_4^+$, N$_2$O, and N$_2$
(Table 1). Only trace amounts of NO were ever detected. Free (or
bound) NH$_2$OH and NO$_2^-$ were never detected in growing cultures supplied
with NO$_3^-$ or NH$_4^+$ as a nitrogen source. Cells grown with NH$_4^+$ did not
produce NO, N$_2$O, or N$_2$.

When NO$_3^-$ was the sole N source and the acetylene block was used,
80% of the N supplied as NO$_3^-$ was recovered as N$_2$O (Table 1). The
remainder was recovered in cell material.

Growing cells supplied with NH$_4^+$ and NO$_3^-$ in the presence of C$_2$H$_2$,
produced N$_2$O stoichiometrically equivalent to the amount of NO$_3^-$
utilized (Table 1). In the absence of C$_2$H$_2$, and with NO$_3^-$ initially at
0.66 mM or less, N$_2$O accumulated transiently. At the end of growth
under these conditions the amount of N$_2$ detected corresponded to the
amount of NO$_3^-$ consumed and no N$_2$O or NO$_3^-$ remained. When the initial
NO$_3^-$ concentration was raised to 2 mM, N$_2$O accumulated through the end
of growth and some NO$_3^-$ remained in the culture medium (Fig. 3).

**Chemical Analysis of Whole Cells**

Whole cells consisted of (% dry weight ± 0.1): nitrogen, 10.1;
carbon, 48.2; and hydrogen, 7.1. Cells harvested in exponential growth
consisted of 59.4 ± 0.7% protein.
### TABLE 1. Recovery of $N_2O$, $NH_4^+$, and $N_2$ by cells grown on $NO_3^-$ and $O_2$.

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>Products $^a$ (% $NO_3^-$-N recovered as)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$N_2O$</td>
</tr>
<tr>
<td>$NH_4^+$ (2 mM) + $NO_3^-$ (0.66 mM) + $C_2H_2$</td>
<td>96.4 ± 4.1%</td>
</tr>
<tr>
<td>$NH_4^+$ (2 mM) + $NO_3^-$ (0.66 mM)</td>
<td>0% $^c$</td>
</tr>
<tr>
<td>$NO_3^-$ (0.66 mM) + $C_2H_2$</td>
<td>80.0 ± 2.1%</td>
</tr>
</tbody>
</table>

$^a$ Values represent means and standard deviations, respectively, obtained using triplicate cultures. Values corrected for amounts detected in inoculum.

$^b$ N.A. = Not applicable.

$^c$ $N_2O$ appeared transiently as described in results.

$^d$ Includes intra- and extracellular $NH_3$. In this experiment cell-N accounted for $19.4 ± 2.8$% of $NO_3^-$-N. Excreted N was estimated at 2.3%. Total recovery of $NO_3^-$-N = $101.7 ± 4.9$%. 
Amino acid analyses of cells grown with NO$_3^-$ were determined (Table 2). The composition of cells grown on NH$_4^+$ was similar (Appendix 1; Table 11). Cells accumulated a large amount of NH$_4^+$ intracellularly, particularly when grown with NO$_3^-$ (Table 2). Intracellular NH$_4^+$ accounted for 87% of the total NH$_3$ detected in cultures grown with NO$_3^-$. 

**Effect of NO$_3^-$ on Final Cell Yield**

Final cell yields in cultures with 2 mM NH$_4^+$ sufficient to repress assimilatory NO$_3^-$ reduction (Table 1) were higher with increased amounts of NO$_3^-$ present (Fig. 4). A substantial change in cell mass occurred when NO$_3^-$ was raised from 1 to 10 mM. No corresponding increase in cell numbers over this range of NO$_3^-$ values was detected. Cells grown with 10 mM NO$_3^-$ or more were abnormally long (20-50 um) and poorly motile. At lower NO$_3^-$ concentrations cultures contained cells that were smaller (2-10 um), actively motile, and magnetotactic. Cell growth was inhibited at NO$_3^-$ values above 40 mM.
TABLE 2. Amino acid composition of whole cells of *A. magnetotacticum* grown microaerobically with NO$_3^-$ (2 mM) as the sole N source.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>560.4$^a$</td>
</tr>
<tr>
<td>Glycine</td>
<td>475.9</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>421.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>405.7</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>403.7</td>
</tr>
<tr>
<td>Valine</td>
<td>337.5</td>
</tr>
<tr>
<td>Lysine</td>
<td>291.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>247.8</td>
</tr>
<tr>
<td>Serine</td>
<td>236.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>216.6</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>209.8</td>
</tr>
<tr>
<td>Proline</td>
<td>198.9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>177.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>112.9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>101.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>97.6</td>
</tr>
</tbody>
</table>

% recovery of protein$^b$ 93.9%

Ammonia 1761.2

$^a$ nmols detected per mg cell dry weight.

$^b$ Protein= 59.4% of cell dry weight.
FIGURE 4. Effect of initial NO$_3^-$ concentration on final cell yields of *A. magnetotacticum*. Cells were grown microaerobically (initial Po$_2$, 1 kPa) in 500-ml batch cultures. Final cell yields are reported as direct cell counts (○) and dry cell weights (●). Symbols and bars represent means and standard deviations, respectively, of triplicate analysis.
DISCUSSION

_Aquaspirillum magnetotacticum_ strain MS-1 cells synthesize all their required nitrogenous compounds de novo from \( \text{NH}_4^+ \) or \( \text{NO}_3^- \) ions. Because they grow with \( \text{NO}_3^- \) as a sole nitrogen source, thereby producing \( \text{NH}_4^+ \), this organism is capable of assimilatory \( \text{NO}_3^- \) reduction. This capability is widespread among bacteria and fungi (Payne, 1973) but apparently not among members of the genus _Aquaspirillum_. _A. itersonii_ and _A. delicaturn_ are the only other members known to grow with \( \text{NO}_3^- \) as a sole nitrogen source (Hylemon et al., 1973). Of course, the inability of some species to grow with \( \text{NO}_3^- \) may reflect requirements for peptides or other constituents of complex media used in culturing them. Although it is uncertain whether free \( \text{NH}_2\text{OH} \) is produced during bacterial assimilatory \( \text{NO}_3^- \) reduction (Kemp and Atkinson, 1966; Payne, 1973; Prakash and Sadana, 1972; Yordy and Delwiche, 1979), \( \text{NO}_2^- \) has definitely been observed as a free intermediate in other species (Knowles, 1981; Payne, 1973). We did not detect either of these compounds during growth of _A. magnetotacticum_ with \( \text{NO}_3^- \), nor did they support growth of this organism. In fact, at concentrations similar to those used by others in culturing bacteria (Yordy and Delwiche, 1979) each was toxic, producing cell lysis. Thus, the eight electron transfer occurring during \( \text{NO}_3^- \) reduction to \( \text{NH}_3 \) in _A. magnetotacticum_ may occur without the production of free intermediates. This possibility is supported by data suggesting a six electron transfer involved in reducing \( \text{NO}_2^- \) to \( \text{NH}_3 \) in _Escherichia coli_.

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(Kemp and Atkinson, 1966), Achromobacter fischeri (Prakash and Sadana, 1972), and Veillonella alcalescens (Yordy and Delwiche, 1979). A similar process in A. magnetotacticum might preclude the accumulation of NO$_2^-$ or possibly other toxic intermediates of assimilatory NO$_3^-$ reduction.

Assimilatory reduction of NO$_3^-$ to NH$_3$ was repressed by 2 mM NH$_4^+$ in the culture medium as evidenced by the conversion of nitrogen supplied as NO$_3^-$ to an N-gas (N$_2$O in the presence of C$_2$H$_2$; N$_2$ in its absence) under these conditions. Moreover, neither N$_2$O nor N$_2$ was detected when NO$_3^-$ was omitted. This is consistent with the well recognized repression of assimilatory NO$_3^-$ reductase by NH$_4^+$ and by other reduced nitrogenous compounds (Payne, 1973). These findings also suggest that a dissimilatory pathway of NO$_3^-$ reduction to NH$_4^+$, of significance in the production of NH$_4^+$ in soils (Caskey and Tiedje, 1979; Smith and Zimmerman, 1981) and in the bovine rumen (Kaspar and Tiedje, 1981), is not present in cells of A. magnetotacticum strain MS-1.

The similar amino acid composition of cells grown either with NH$_4^+$ or with NO$_3^-$ suggests a similar mechanism of NH$_3$ assimilation by each cell type.

Production of free NO$_2^-$, which characterizes dissimilatory NO$_3^-$ reduction by many organisms (Knowles, 1981; Payne, 1973) was never apparent during denitrification by A. magnetotacticum. Thus, cells of strain MS-1 possess an efficient means of reducing toxic NO$_2^-$ . It seems likely that the rate of NO$_2^-$ reduction may be higher than the rate of NO$_3^-$ reduction. Cells of A. magnetotacticum produced only trace amounts of NO and accumulated N$_2$O while reducing NO$_3^-$ . These
appeared as transient intermediates and were subsequently reduced to N\textsubscript{2}.

With sufficient NH\textsubscript{4}\textsuperscript{+} present to repress assimilatory NO\textsubscript{3}\textsuperscript{-} reduction, increased concentrations of NO\textsubscript{3}\textsuperscript{-} resulted in increased final growth yields. This suggests that NO\textsubscript{3}\textsuperscript{-} reduction under microaerobic conditions is coupled to energy conservation in this organism.

True denitrifiers typically reduce 90% or more of the N-oxide to N-gas and couple this reduction to electron transport phosphorylation (Bleakley and Tiedje, 1982; Bryan, 1981). By these criteria, our data confirm that \textit{A. magnetotacticum} is indeed a denitrifier. Of the N supplied to cells as NO\textsubscript{3}\textsuperscript{-} alone, 80% was recovered as N-gas. The remainder was recovered in cell material and excreted nitrogenous products including NH\textsubscript{4}\textsuperscript{+}. Thus, under conditions where NO\textsubscript{3}\textsuperscript{-} is the sole N source, cells of \textit{A. magnetotacticum} concomitantly carry out denitrification and assimilatory NO\textsubscript{3}\textsuperscript{-} reduction to NH\textsubscript{4}\textsuperscript{+}.

Cell growth with NO\textsubscript{3}\textsuperscript{-} in batch culture is biphasic. The onset of rapid cell growth appeared to correlate with the onset of N\textsubscript{2}O production from NO\textsubscript{3}\textsuperscript{-}. The data also suggest that NO\textsubscript{3}\textsuperscript{-} dissimilation commenced when the dissolved O\textsubscript{2} reached approximately 4.1 μmols per liter.

Denitrification is associated generally with anoxic conditions because O\textsubscript{2} not only inhibits denitrifying enzyme activity, but represses synthesis of new denitrifying enzymes as well (Knowles, 1981). However, some organisms tolerate limited quantities of O\textsubscript{2} while denitrifying (Payne, 1973). Cells of \textit{A. magnetotacticum} are obligately microaerophilic and do not grow, even with NO\textsubscript{3}\textsuperscript{-}, anaerobically
(Blakemore et al., 1979). Moreover, they consume $O_2$ while denitrifying. Thus, this bacterium appears to be the first described denitrifier which actually requires, rather than tolerates $O_2$. This may reflect a specific requirement for $O_2$ as a substrate for oxygenases participating in cell biosynthesis (e.g. heme or lipid synthesis). We have been unable to relieve this $O_2$ requirement by growing cells in complex media or by adding hemin, however. We lack evidence that $O_2$ is specifically required for $NO_3^-$ reduction. Moreover, our data do not enable us to determine whether, under microaerobic conditions, respiration involving $O_2$ and $NO_3^-$ as terminal electron acceptors occurs simultaneously.

Cultures grown microaerobically with $NH_4^+$ or $NO_3^-$ as the sole nitrogen source contain some non-magnetotactic cells. However, we have frequently observed that cultures grown with $NH_4^+$ in contrast to those with $NO_3^-$ contain a larger proportion of cells that are not magnetotactic and do not contain magnetosomes (Appendix 1: Table 10). This explanation is consistent with the possible involvement of $NO_3^-$ reducing enzymes in magnetite synthesis. Dissimilatory $NO_3^-$ reductase is an induced enzyme in most bacteria which synthesize it (Payne, 1973) and therefore would not be synthesized by cells growing with $NH_4^+$ as the sole N source unless a suitable inducer (perhaps even $Fe^{+3}$) was present. It is not yet known whether any of the enzymes involved in denitrification in *A. magnetotacticum* can reduce ferric iron, however.

Alternately, the formation of bacterial magnetite might result from the oxidation of ferrous hydroxide [$Fe(OH)_2$] coupled with a reduction of $NO_3^-$ or $N_2O$. This reaction can occur non-biologically at pH 8 (Buresh and Moraghan, 1976; Moraghan and Buresh, 1977) although it
has not yet been shown to occur enzymatically.

Our results which confirm and extend those of Escalante-Semerena et al (1980), clearly establish that *A. magnetotacticum* is a microaerophilic denitrifier. The possibility that denitrification is a characteristic shared by other magnetotactic bacteria is an interesting one. Knowledge of this process in strain MS-1 can be expected to lead to more information concerning the ecological niche of these interesting organisms. We showed that growing cells of *A. magnetotacticum* reduce C$_2$H$_2$ microaerobically (Bazylinski and Blakemore, 1983). Thus, in addition to its capacity for assimilatory and dissimilatory NO$_3^-$ reduction, this species also fixes atmospheric N$_2$. Its versatility with respect to nitrogen metabolism may play a significant role in magnetite synthesis and can be expected to favor its survival in microaerobic aquatic habitats.
ACKNOWLEDGEMENTS

We gratefully acknowledge valuable communications, comments, and encouragement from J. M. Tiedje and P. Cornell of Michigan State University and R. B. Frankel of the Massachusetts Institute of Technology. We are grateful to N. Blakemore and A. Geshnizgani for valuable technical assistance. Amino acid and elemental analyses were performed through the Instrumentation Center of the University of New Hampshire.

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LITERATURE CITED


CHAPTER TWO
CHAPTER TWO

NITROGEN FIXATION (ACETYLENE REDUCTION) IN

AQUASPIRILLUM MAGNETOTACTICUM

ABSTRACT

Aquaspirillum magnetotacticum strain MS-1 and two non-magnetic mutants derived from it, reduced C\textsubscript{2}H\textsubscript{2} microaerobically but not anaerobically even with NO\textsubscript{3}\textsuperscript{-}. This organism apparently is not capable of NO\textsubscript{3}\textsuperscript{-}-dependent nitrogen fixation. Cells of A. magnetotacticum reduced C\textsubscript{2}H\textsubscript{2} at rates comparable to those of Azospirillum lipoferum grown under similar conditions but much lower than that of Azotobacter vinelandii grown aerobically. Cells of A. magnetotacticum in anaerobic cultures lacking NO\textsubscript{3}\textsuperscript{-} did not reduce C\textsubscript{2}H\textsubscript{2} until O\textsubscript{2} was introduced. Optimum rates of C\textsubscript{2}H\textsubscript{2} reduction by A. magnetotacticum were obtained at 200 Pa O\textsubscript{2}. C\textsubscript{2}H\textsubscript{2} reduction was inhibited by more than 1 kPa O\textsubscript{2} or 0.2 mM NO\textsubscript{3}\textsuperscript{-} or NH\textsubscript{4}\textsuperscript{+}. These results suggest that A. magnetotacticum fixes N\textsubscript{2} only under microaerobic, N-limited conditions.
INTRODUCTION

*Aquaspirillum magnetotacticum* strain MS-1 carries out a number of nitrogen transformations important in aquatic ecosystems. Growing cells denitrify, thereby reducing $\text{NO}_3^-$ to gaseous products including $\text{N}_2\text{O}$ and $\text{N}_2$ (Bazylinski and Blakemore, 1982; Escalante-Semerena et al., 1980). This organism is an obligately microaerophilic denitrifier, however, and will not grow anaerobically, even with $\text{NO}_3^-$ in the medium (Blakemore et al., 1979). Cells also possess an $\text{NH}_3^-$-repressible $\text{NO}_3^-$ reductase activity (assimilatory $\text{NO}_3^-$ reduction). Thus, while denitrifying they concomitantly reduce $\text{NO}_3^-$ to $\text{NH}_4^+$ (Bazylinski and Blakemore, 1983). Under microaerobic conditions, cells utilize $\text{NO}_3^-$ or $\text{NH}_4^+$ (but not $\text{NO}_2^-$ nor, apparently, $\text{N}_2\text{O}$) for growth (Blakemore et al., 1979; Bazylinski and Blakemore, 1983).

In studies of its nitrogen nutrition, strain MS-1 grew after three sequential passages in semisolid medium lacking a combined N source (Blakemore et al., 1979; Maratea, 1979). This suggested that it might be capable of fixing atmospheric $\text{N}_2$. The use of the acetylene ($\text{C}_2\text{H}_2$) reduction assay to assess $\text{N}_2$ fixation is widely accepted and well documented (Child, 1981; Dilworth, 1974). Subsequent studies of $\text{C}_2\text{H}_2$ reduction confirmed that this strain fixes $\text{N}_2$ (Bazylinski and Blakemore, 1982).

Nitrogen fixation is common among aquatic bacteria, particularly at low values of dissolved oxygen. Within the genus *Aquaspirillum*, *A. peregrinum* and *A. fasciculus* have been shown to fix $\text{N}_2$ under
microaerobic conditions (Strength et al., 1976). Other *Aquaspirillum* species may also, although a comprehensive survey of the genus in this regard is yet to be made (Krieg, 1976). *Azospirillum lipoferum* (Tarrand et al., 1978) is the most widely recognized nitrogen-fixing spirillum. It reduces C\textsubscript{2}H\textsubscript{2} optimally under microaerobic conditions (Okon et al., 1976; 1977) but is also capable of NO\textsubscript{3}^- -dependent anaerobic nitrogen fixation (C\textsubscript{2}H\textsubscript{2} reduction) (Neyra et al., 1977; Neyra and Van Berkum, 1977; Scott et al., 1979). Thus, some bacteria capable of denitrification also fix N\textsubscript{2}. These include in addition to *Azospirillum* (Neyra et al., 1977), certain strains of *Rhizobium* (Rigaud, et al., 1973; Zablotowicz and Focht, 1979), and possibly *Rhodopseudomonas sphaeroides* forma sp. *denitrificans* (Satoh et al., 1974).

We undertook this study to better understand the nitrogen-fixing ability of *A. magnetotacticum* and to compare it to that of other N\textsubscript{2}-fixing heterotrophic spirilla. Since strain MS-1 is one of few known denitrifying N\textsubscript{2}-fixers, we also hoped to gain a better understanding of relationships between these two processes in the overall physiology of the cell.
MATERIALS AND METHODS

Bacteria and Growth Conditions

The principal organisms used were *Aquaspirillum magnetotacticum* strain MS-1 and two non-magnetotactic mutants (strains NM-1A and NM-1B) derived from it. Strains NM-1A and NM-1B were subcultures of two aerotolerant colonies which appeared on plates of growth medium containing 0.005% sodium metabisulfite (Sigma Chemical Co., St. Louis, MO) and 1% agar. The plates had each been seeded with $10^7$ cells from a magnetic, microaerophilic culture. They were incubated at 30° C aerobically. The two colonies selected were the only ones appearing out of 15 such plates (N. Blakemore, personal communication). Strain MS-2 is an uncharacterized, microaerophilic, magnetic heterotrophic spirillum species isolated from the water treatment plant, Durham, New Hampshire. Isolation and culture methods were similar to those described previously (Blakemore et al., 1979) except that standard streaking methods on solid media were used in cloning procedures (N. Blakemore, personal communication). *Azospirillum lipoferum* was obtained from Dr. Noel R. Krieg at the Virginia Polytechnic Institute and State University. It was cultured microaerobically as previously described (Neyra and Van Berkum, 1977). *Azotobacter vinelandii* was provided by Dr. G. Watt at the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio. It was cultured aerobically by the method of Jones and Redfearn (1966).
All strains of *A. magnetotacticum* and MS-2 were routinely cultured microaerobically in liquid medium contained in 160 ml serum vials as previously described (Bazylniński and Blakemore, 1983). In cultures in which \( \text{C}_2\text{H}_2 \) reduction was measured, the source of combined nitrogen, \( \text{NaNO}_3 \) or \( (\text{NH}_4)_2\text{SO}_4 \), was eliminated unless stated otherwise. Red sleeve-type rubber stoppers were used (VWR Scientific Inc.) which did not release \( \text{C}_2\text{H}_4 \) before or after autoclaving. They did not leak air into cultures provided that the vials with stoppers inserted were autoclaved and allowed to cool while clamped in a Hungate type tube press from which the upper rubber pad was removed. Prior to inoculation, sterile \( \text{O}_2 \) or air was added to the culture vials to obtain initial headspace concentrations of 0.2 to 5.0 % (v/v) \( \text{O}_2 \) (200–5000 Pa \( \text{O}_2 \)).

**Estimation of Cell Yield**

Cell numbers were estimated by means of direct cell counts using a Petroff-Hausser cell counting chamber with a Zeiss standard research phase-contrast microscope. Samples were diluted into an equal volume of 0.1 % formalin to arrest cell motility prior to counting.

**Assessing \( \text{C}_2\text{H}_2 \) Reduction**

\( \text{C}_2\text{H}_2 \), generated from distilled \( \text{H}_2\text{O} \) and \( \text{CaC}_2 \) (granular, Fisher Scientific Co.), was added to cultures at a headspace concentration of 0.1 atm. All cultures containing \( \text{C}_2\text{H}_2 \) were incubated in a shaking water bath (20 oscillations/min) at 30°C.

Ethylene (\( \text{C}_2\text{H}_4 \)) was determined using a Varian Series 2400 gas
chromatograph equipped with a H$_2$ flame ionization detector (FID). C$_2$H$_4$
concentrations were determined from measurements of peak heights.
Standard curves using purified gases (Scott Environmental Technology,
Inc.) were prepared at the time of each experiment. O$_2$-free N$_2$ at a
flow rate of 25 ml/min was the carrier gas. The stationary phase was
Porapak N (80/100 mesh, 3 mm x 1.8 m column) at 110°C. The detector
and injector temperatures were each 175°C.
RESULTS

\textbf{C}_2\text{H}_2 \text{ Reduction}

Cells in growing cultures of \textit{A. magnetotacticum} strain MS-1 actively reduced C\textsubscript{2}H\textsubscript{2} for 18 to 24 h after inoculation. The production of C\textsubscript{2}H\textsubscript{4} then ceased, cells became nonmotile, and coccoid bodies appeared. Consistent with previous observations using the "acetylene block" technique to study denitrification (Bazylnski and Blakemore, 1983), C\textsubscript{2}H\textsubscript{2} at a concentration of 0.1 atm, was toxic to growing cells of this organism. Rates of C\textsubscript{2}H\textsubscript{2} reduction by \textit{A. magnetotacticum} and other nitrogen-fixing species are shown in Table 3. Generally, the rates of C\textsubscript{2}H\textsubscript{2} reduction were quite variable from experiment to experiment. Although the highest rate observed by strain MS-1 was 0.70 nmols C\textsubscript{2}H\textsubscript{4} produced 10\textsuperscript{6} cells\textsuperscript{-1} h\textsuperscript{-1} (0.2 kPa O\textsubscript{2} in the headspace), the rates shown in Table 3 are more representative and reflect this variability. Similar results were obtained with the non-magnetotactic strains NM-1A and NM-1B. Strains of \textit{A. magnetotacticum} reduced C\textsubscript{2}H\textsubscript{2} at rates comparable to or slightly higher than those obtained with \textit{Azospirillum lipoferum} but much lower than that of \textit{Azotobacter vinelandii}.

\textbf{Effect of O\textsubscript{2} on C\textsubscript{2}H\textsubscript{2} Reduction}

The effect of O\textsubscript{2} on the rate of C\textsubscript{2}H\textsubscript{2} reduction is shown in Table 4. Cells never reduced C\textsubscript{2}H\textsubscript{2} in the absence of O\textsubscript{2}. However,
TABLE 3. \( \text{C}_2\text{H}_2 \) reduction rates by various \( \text{N}_2 \)-fixing bacteria.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Mean ± SD</th>
<th>Maximum Rate Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Azotobacter vinelandii} \textsuperscript{a}</td>
<td>2.29 ± 0.01</td>
<td>2.40</td>
</tr>
<tr>
<td>\textit{Azospirillum lipoferum} \textsuperscript{b}</td>
<td>0.09 ± 0.04</td>
<td>0.13</td>
</tr>
<tr>
<td>Strain MS-2 \textsuperscript{c}</td>
<td>0.04 ± 0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>\textit{Aquaspirillum magnetotacticum} \textsuperscript{c}</td>
<td>0.14 ± 0.12</td>
<td>0.31</td>
</tr>
<tr>
<td>Strain MS-1</td>
<td>0.43 ± 0.28</td>
<td>0.74</td>
</tr>
<tr>
<td>Strain NM-1A</td>
<td>0.34 ± 0.16</td>
<td>0.52</td>
</tr>
<tr>
<td>Strain NM-1B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Initial \( \text{Po}_2 \) = 21 kPa.

\textsuperscript{b} Initial \( \text{Po}_2 \) = 0.1 kPa.

\textsuperscript{c} Initial \( \text{Po}_2 \) = 0.2 kPa.
TABLE 4. The effect of $O_2$ on $C_2H_2$ reduction by *Aquaspirillum magnetotacticum* strain MS-1.

<table>
<thead>
<tr>
<th>Initial $O_2$ Concentration (kPa in headspace)</th>
<th>Rate of $C_2H_2$ Reduction (nmols $C_2H_2/10^6$ cells/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.15</td>
</tr>
<tr>
<td>0.2</td>
<td>0.18</td>
</tr>
<tr>
<td>0.4</td>
<td>0.014</td>
</tr>
<tr>
<td>1.0</td>
<td>0.012</td>
</tr>
<tr>
<td>5.0</td>
<td>0</td>
</tr>
</tbody>
</table>
nitrogenase activity was not observed when the Po$_2$ was higher than 1 kPa in the headspace. Moreover, as shown in Figure 5, the introduction of O$_2$ (Po$_2$ = 0.2 kPa) into non-fixing anaerobic cultures initiated C$_2$H$_2$ reduction.

Effect of NO$_2^-$ and NH$_4^+$ on C$_2$H$_2$ Reduction

The effect of NO$_3^-$ and NH$_4^+$ on C$_2$H$_2$ reduction is shown in Figure 6. Cells growing under microaerobic conditions did not reduce C$_2$H$_2$ when NO$_3^-$ or NH$_4^+$ (either at 0.2 mM) were included in the culture medium. Growing cells did not reduce C$_2$H$_2$ anaerobically with 0.2 mM NO$_3^-$. 
FIGURE 5. Effect of O₂ on C₂H₂ reduction during growth of _A. magnetotacticum_ strain MS-1. At 0 h, 10% of the culture headspace volume was replaced with C₂H₂. At 12 h, 1 cc of sterile air (●) (final headspace concentration of O₂ = 0.2 kPa) or N₂ gas (■) was added to the culture headspace. Cultures were incubated at 30°C on a rotary shaker. Points and bars represent means and ranges, respectively, of values obtained from duplicate cultures.
FIGURE 6. Effect of NO$_3^-$ and NH$_4^+$ on C$_2$H$_2$ reduction during growth of 
*A. magnetotacticum* strain MS-1 in culture medium lacking a 
combined nitrogen source. At 0 h, 10% of the culture 
headspace volume was replaced with C$_2$H$_2$. Cultures were 
incubated at 30° on a rotary shaker. Points and bars 
represent means and ranges, respectively, of values obtained 
from duplicate cultures. 
Symbols: •, No N source added

○, NaNO$_3$ or NH$_4$Cl (0.2 mM) added at inoculation.
NMOLS $\text{C}_2\text{H}_4 / \text{CC HEADSPACE}$

HOURS

0  6  12  18  24  30  35

0  6  12  18  24  30  35
DISCUSSION

Growing cells of *A. magnetotacticum* reduced C\textsubscript{2}H\textsubscript{2} only under microaerobic conditions but not when 0.2 mM NO\textsubscript{3}\textsuperscript{-} or NH\textsubscript{4}\textsuperscript{+} was added to the culture medium. They neither grew nor reduced C\textsubscript{2}H\textsubscript{2} under anaerobic conditions even in the presence of 0.2 mM NO\textsubscript{3}\textsuperscript{-}. This suggests that cells can fix N\textsubscript{2} only under N-limiting conditions. Moreover, introduction of O\textsubscript{2} into anaerobic cultures initiated C\textsubscript{2}H\textsubscript{2} reduction. Thus, cells of this microaerophilic organism meet their energy requirement for nitrogenase activity only with O\textsubscript{2} as a terminal electron acceptor. All nitrogenases studied to date have a specific requirement for ATP (Child, 1981; Dilworth, 1974).

We find it interesting that low concentrations (0.2 mM) of NO\textsubscript{3}\textsuperscript{-} did not support C\textsubscript{2}H\textsubscript{2} reduction. At the concentrations used successfully by others (Neyra and Van Berkum, 1977; Scott et al., 1979) it was inhibitory. This fact suggests that sufficient energy may not be conserved to supply the requirement for nitrogenase activity with NO\textsubscript{3}\textsuperscript{-} as a terminal electron acceptor. We showed previously, however, that increased amounts of NO\textsubscript{3}\textsuperscript{-} resulted in higher final growth yields of this organism (Bazylinski and Blakemore, 1983). This may indicate that energy is conserved in phosphorylation during denitrification but only when NO\textsubscript{2}\textsuperscript{-} is reduced. Unfortunately, results of growth experiments do not enable us to test this because NO\textsubscript{2}\textsuperscript{-} does not accumulate during NO\textsubscript{3}\textsuperscript{-} reduction by this organism. Moreover, NO\textsubscript{2}\textsuperscript{-} is toxic when provided exogenously to cells (Bazylinski and Blakemore,
Denitrifying strains of *Azospirillum lipoferum* couple to $N_2$ fixation the reduction of $NO_3^-$ to $NO_2^-$ (nitrate respiration) but not the further reduction of $NO_2^-$ to gaseous products (denitrification) (Scott et al., 1979). Moreover these strains all accumulate $NO_2^-$ transiently during denitrification (Neyra et al., 1977; Neyra and Van Berkum, 1977; Scott et al., 1979). Thus, unlike *A. magnetotacticum*, this species appears to obtain sufficient ATP for nitrogenase activity by reducing $NO_3^-$ to $NO_2^-$ under anaerobic conditions but not from the further reduction of $NO_2^-$. Cells of *A. magnetotacticum* did not reduce $C_2H_2$ when 0.2 mM $NH_4^+$ was included in the growth medium. Repression of nitrogenase by $NH_4^+$ or other reduced nitrogenous compounds is well-established (Child, 1981; Dilworth, 1974). Since, as mentioned, *A. magnetotacticum* carries out assimilatory $NO_3^-$ reduction to $NH_4^+$, it is possible that one or more products of this pathway, rather than $NO_3^-$ itself, represses nitrogenase activity. By this means, cells might be prevented from wasting energy by fixing $N_2$ when alternative nitrogen sources are available.

The data show that *A. magnetotacticum* strains MS-1, NM-1A, NM-1B, and a recently isolated magnetotactic spirillum strain MS-2 all fix $N_2$ under microaerobic, $N$-limited conditions. Moreover, $N_2$-fixation is inhibited by more than 1 kPa $O_2$, 0.2 mM $NO_3^-$, or 0.2 mM $NH_4^+$. The apparent lack of coupling between $NO_3^-$ reduction (denitrification) and $N_2$-fixation by *A. magnetotacticum* contrasts with results obtained by others using other species of denitrifying $N_2$-fixers (Neyra and Van
Berkum, 1977; Rigaud et al., 1973; Scott et al. 1979; Zablotowicz and Focht, 1979). Diverse types of magnetotactic bacteria live in microaerobic and N-limited habitats (Blakemore, 1975; Blakemore, 1982; Moench and Konetzka, 1978). Our findings, if they also apply to other species, could provide a greater understanding of the biology of these peculiar bacteria and of enrichment conditions applicable to their isolation from natural habitats. More importantly, this organism is one of the few presently recognized denitrifying N$_2$-fixing species. This versatility in its N metabolism affords it at least two important biogeochemical positions in the N cycling of aquatic habitats.
ACKNOWLEDGEMENTS

Strains MS-2, NM-1A, and NM-1B were isolated by N. Blakemore, whose valuable technical assistance we also gratefully acknowledge. This work was supported by National Science Foundation grant PCM 79-22224 and Office of Naval Research contract N0014-80-C-0029.
LITERATURE CITED


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CHAPTER THREE
CHAPTER THREE

NITRATE, NITRITE, AND IRON REDUCTION IN CELL-FREE EXTRACTS OF AQUASPIRILLUM MAGNETOTACTICUM

ABSTRACT

Possible relationships between the reduction of NO₃⁻, NO₂⁻, and Fe³⁺ by cell-free extracts of *Aquaspirillum magnetotacticum* were investigated using gas chromatography and spectrophotometry. NO₃⁻ and Fe³⁺ reductase activities were associated with the soluble cell fraction. NO₂⁻ reduction occurred only with the cell membrane fraction. The optimum rate of NO₃⁻-N reduced to NO₂⁻ (specific activity = 6.5 μmol N mg cell extract protein⁻¹ h⁻¹) was comparable to that of NO₂⁻-N reduced to N₂O (sp. act. = 6.7). NO was not detected as a product of NO₂⁻ reduction. Extracts of cells grown on NH₄⁺ as a sole N source reduced Fe³⁺ (sp. act. = 107 nmol Fe²⁺ h⁻¹ mg cell extract protein⁻¹) nearly as rapidly as those cultured with NO₃⁻ as a sole N source (sp. act. = 67). Extracts of non-magnetic mutants reduced Fe³⁺ at rates (sp. acts. 62-77) comparable to that of the wild-type magnetic strain MS-1. NO₃⁻ as high as 10⁻² M, had no effect on Fe³⁺ reduction. NO₃⁻ reduction was completely inhibited by 10⁻¹ M NaN₃ but not by 10⁻¹ NaCN. NO₂⁻ reduction was completely inhibited by 10⁻⁴ M NaCN or by 10⁻² M NaN₃. Fe³⁺ reduction was insensitive to 10⁻³ M NaCN or NaN₃, or 4 μM Antimycin A, rotenone, or HQNO. Fe³⁺ reduction was inhibited by aeration. These results suggest that overall Fe³⁺ reduction by this
species is independent of electron transport chain components and is not mediated by the dissimilatory NO$_3^-$ reductase activity detected. Thus, the results obtained do not enable us to establish, using cell-free extracts, a link between NO$_3^-$ reduction, Fe$^{3+}$ reduction and Fe$_3$O$_4$ synthesis in magnetic bacteria.
INTRODUCTION

Magnetotactic bacteria, which are ubiquitous in freshwater and marine sediments (Blakemore, 1975, 1982; Moench and Konetzka, 1978), contain enveloped, magnetite (Fe$_3$O$_4$) crystals, called magnetosomes (Frankel et al., 1979; Balkwill et al., 1980). Cells of *Aquaspirillum magnetotacticum* strain MS-1 also contain a low density hydrous ferric oxide, a high density ferric oxide (ferrihydrite), and ferrous iron (Frankel et al., 1983). These compounds are believed to be Fe$_3$O$_4$ precursors and precipitation of Fe$_3$O$_4$ in this species occurs in a sequence in which iron reduction is involved in formation of ferrihydrite (5Fe$_2$O$_3$·9H$_2$O) as well as its transformation to Fe$_3$O$_4$ (Frankel et al., 1983). Thus, bacterial Fe$_3$O$_4$ synthesis appears to parallel that of the chiton, a marine mollusc, in which Fe$_3$O$_4$ in radular denticles also results from the reduction of iron and the dehydration of a ferrihydrite precursor (Lowenstam, 1962, 1981; Towe and Lowenstam, 1967).

Fe$^{+3}$ reduction by microorganisms is important biogeochemically in diverse habitats (Brock and Gustafson, 1976; Cox, 1980; Dailey and Lascelles, 1977; Gaines et al., 1981; Jones et al., 1983; Lascelles and Burke, 1978; Lodge et al., 1982; Obuekwe et al., 1981; Ottow, 1968; Ottow and Klopotek, 1969; Sørensen, 1982). Several membrane-associated (Dailey and Lascelles, 1977; Lascelles and Burke, 1978) and cytoplasmic (Arceneaux and Byers, 1980; Cox, 1980; Lodge et al., 1982) Fe$^{+3}$ reducing systems have been identified in microbial cells. Furthermore,
Fe\(^{+3}\) reduction is often encountered under conditions where NO\(_3^-\) reduction is taking place (Lascelles and Burke, 1978; Ottow, 1968, 1969; Sørensen, 1982). \textit{A. magnetotacticum} is a microaerophilic denitrifier capable of concomittant assimilatory NO\(_3^-\) reduction to NH\(_4^+\) (Bazylinski and Blakemore, 1983). Nitrous oxide (N\(_2\)O), but not NO\(_2^-\), accumulates transiently and N\(_2\) is produced by denitrifying cells of this organism (Escalante-Semerena et al., 1980; Bazylinski and Blakemore, 1983).

Since Fe\(_3\)O\(_4\) synthesis proceeds through steps involving Fe\(^{+3}\) reduction, and cultures grown with NO\(_3^-\) rather than NH\(_4^+\) as the sole N source consistently have a larger percentage of magnetic cells (Appendix: Table 11), a study was undertaken to establish whether NO\(_3^-\) reducing enzymes are involved in Fe\(^{+3}\) reduction and/or Fe\(_3\)O\(_4\) synthesis.

(Portions of this work were presented at the 6\(^{th}\) International Symposium for Environmental Biogeochemistry, Santa Fe, NM, 1983.)
MATERIALS AND METHODS

Bacteria and Growth Conditions

The principal organisms used were *Aquaspirillum magnetotacticum* strain MS-1 (Blakemore et al., 1979) and two aerotolerant non-magnetic mutants derived from it designated strains NM-1A and NM-1B (Frankel et al., 1983; Bazylinski and Blakemore, 1983). Cells were mass cultured at 30°C in 10 l batch cultures in growth medium containing (NH₄)₂SO₄ or NaNO₃ (2 mM N) as the sole N source (Balkwill et al., 1980).

Strains of *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Escherichia coli* were obtained from the culture collection in the Microbiology Department, University of New Hampshire. These organisms were grown aerobically in glutamate-glycine-succinate medium with 0.1% yeast extract (Clark-Walker et al., 1967). Cultures (1 l) were incubated in 2 l flasks placed in a shaking waterbath (20 oscillations/min) at 37°C.

Preparation of Cell Fractions

Cells grown to late exponential phase (1 x 10⁸ cells/ml) were harvested by continuous flow centrifugation in a CEPA-model LE electrically driven centrifuge equipped with water cooling. They were washed twice with 50 mM potassium phosphate buffer (pH = 6.9) and resuspended in this buffer. Crude cell extracts were prepared with a French pressure cell at 286,000 kg/m². The crude extract was
centrifuged at 4°C for 10 min at 11,000 x g to remove cell debris. The supernatant fraction was centrifuged for 1.5 h at 105,000 x g (4°C). The pellet (cell membranes) was suspended in 50 mM potassium phosphate buffer (pH = 6.9) to a concentration of 4-6 mg extract protein ml⁻¹.

**Assay of Enzyme Activities**

NO₃⁻ reductase activity was assayed by colorimetrically determining the production of NO₂⁻ (Pichinoty et al., 1971). Dissimilatory NO₂⁻ reductase activity was measured in a similar reaction mixture with methyl viologen as the electron donor, NaNO₂ (1 umol) was used in place of NaNO₃ as the substrate. The reaction mixture contained (μmol in a final volume of 6 ml): potassium phosphate buffer, 462 (pH = 6.9); NaNO₃, 100 or NaNO₂, 1; Na₂S₂O₇·2H₂O, 3.8; methyl viologen, 0.6. NO and N₂O were measured by gas chromatography as previously described (Bazylinski and Blakemore, 1983). NH₄⁺ is not detected as a product of dissimilatory NO₂⁻ reduction (Bazylinski and Blakemore, 1983). Both assays were carried out at 28°C in 10 ml sealed serum vials in which the headspace was replaced with O₂-free N₂. The reactions were started by the addition of Na₂S₂O₇. NO₂⁻ and N₂O production proceeded linearly for at least 30 min under the conditions assayed (Appendix 1: Figures 11 and 12).

Fe³⁺ reduction was determined spectrophotometrically in anaerobic Thunberg cuvettes using ferrozine which specifically binds ferrous iron (Stookey, 1970). The method was essentially that of Dailey and Lascelles (1977) except that a potassium phosphate buffer (pH = 6.9) system was used. The reaction mixture contained (μmol in a final
volume of 2.4 ml): potassium phosphate, 185; ferrozine, 2.0; reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1.0; Fe$^{3+}$ ion (usually as ferric citrate), 0.4. Prior to the start of the reaction the atmosphere in the cuvettes was replaced with O$_2$-free N$_2$. The reaction was started by the addition of cell extract (0.2 ml, 0.5-1.0 mg cell extract protein) and the increase in absorbance at 562 nm (25°C) was measured with a Beckman DU-8 spectrophotometer programmed for enzyme kinetics. Concentrations of Fe$^{2+}$ were determined using a molar extinction coefficient of 28,600 (Gibbs, 1976).

Antimycin A, 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO), and rotenone (Sigma Chemical Co., St. Louis, MO) were dissolved in ethanol before addition to the reaction mixture. Cyanide and azide were added as NaCN and NaN$_3$, respectively. All inhibitors were preincubated with the enzyme preparation for at least 15 min. NADH, NADPH, ferric citrate, and ferrozine were purchased from Sigma. Ferric quinate was prepared as described previously (Blakemore et al., 1979).

**Protein Analysis**

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.
RESULTS

NO$_3^-$ and NO$_2^-$ Reducing Activity in Cell-free Extracts of *A. magnetotacticum* strain MS-1.

Cytoplasmic and membrane fractions each showed NO$_3^-$ reductase activity although this activity nearly disappeared from membranes washed with 50 mM phosphate buffer (Table 5). Membrane but not cytoplasmic fractions reduced NO$_2^-$ to N$_2$O (Table 5). NO was never detected. Cell extracts did not reduce NO$_3^-$ or NO$_2^-$ in the absence of the artificial electron donor, reduced methyl viologen, or when boiled prior to the assay.

Extracts of NH$_4^+$-grown cells reduced NO$_3^-$ at rates comparable to those of NO$_3^-$-grown cells (Table 6). Extracts of strains NM-1A and NM-1B also reduced NO$_3^-$ but at rates somewhat higher than those of strain MS-1 (Table 7).

NO$_3^-$ reduction was relatively insensitive to CN$^-$ (58 % inhibition at 10$^{-1}$ M) but was totally inhibited by 10$^{-1}$ M N$_3^-$.

Fe$^{3+}$ Reductase Activity in *A. magnetotacticum*

Cytoplasmic but not membrane fractions of *A. magnetotacticum* strain MS-1 showed Fe$^{3+}$ reductase activity under anaerobic conditions (Table 6). This activity was inhibited by aeration (Appendix 1: Figure 13). The rate of formation of the Fe$^{3+}$-ferrozine complex proceeded
TABLE 5. \( \text{NO}_3^- \) and \( \text{NO}_2^- \) reductase activities of cell fractions of \textit{A. magnetotacticum} strain MS-1.

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>Reductase Activity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{NO}_3^- ) a</td>
<td>( \text{NO}_2^- ) b</td>
</tr>
<tr>
<td>Soluble</td>
<td>6.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Membrane</td>
<td>4.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Washed membrane</td>
<td>0.7</td>
<td>2.1</td>
</tr>
<tr>
<td>(3x in phosphate buffer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boiled crude cell extract</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>(Placed in 100 °C water bath for 10 min)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( a \) measured as \( \mu\text{mol NO}_2^- \) formed/mg protein/h.

\( b \) measured as \( \mu\text{mol N}_2\text{O} \) formed/mg protein/h.
TABLE 6. Fe$^{3+}$ and NO$_3^-$ reductase activity in the cytoplasmic fraction of _A. magnetotacticum_ strain MS-1 grown with NO$_3^-$ or NH$_4^+$.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Fe$^{3+}$ Reductase$^a$</th>
<th>NO$_3^-$ Reductase$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH$_4^+$-grown</td>
<td>NO$_3^-$-grown</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>107</td>
<td>67</td>
</tr>
<tr>
<td>Ferric quinate</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>FeCl$_3$</td>
<td>43</td>
<td>32</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td></td>
<td>7.3</td>
</tr>
</tbody>
</table>

$^a$ nmol Fe$^{2+}$/mg protein/h.

$^b$ μmol NO$_2^-$/mg protein/h.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Fe$^{+2}$ Reductase$^a$</th>
<th>NO$_3^-$ Reductase$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-1</td>
<td>67</td>
<td>6.5</td>
</tr>
<tr>
<td>NM-1A</td>
<td>77</td>
<td>8.5</td>
</tr>
<tr>
<td>NM-1B</td>
<td>62</td>
<td>12.8</td>
</tr>
</tbody>
</table>

$^a$ nmol Fe$^{+2}$/mg protein/h  
$^b$ umol NO$_3^-$/mg protein/h
linearly for at least 30 min. Cell extracts did not show activity in the absence of reductant or when boiled. NADPH provided only 19% of the activity observed with NADH as electron donors. Succinate (2 mM) was ineffective as an electron donor. Cytoplasmic fractions reduced Fe$^{3+}$ maximally when it was supplied as ferric citrate rather than as ferric quinate or when uncomplexed (Table 6).

The rate of Fe$^{3+}$ reduction was consistently higher with extracts of NH$_4^+$-grown cells than with those of cells grown on NO$_3^-$ (Table 6). Extracts of the non-magnetic strains NM-1A and NM-1B reduced Fe$^{3+}$ at rates comparable to that of strain MS-1 (Table 7) although, unlike results obtained with MS-1, lags of up to 15 min prior to establishment of a linear rate were observed with these strains.

**Effect of Respiratory Inhibitors and NO$_3^-$ on Fe$^{3+}$ Reduction**

Antimycin A, rotenone, or HQNO (each at 4 μM) had no effect on Fe$^{3+}$ reduction by cytoplasmic cell fractions of _A. magnetotacticum_ (Table 8). CN$^-$ or N$_3^-$, each at 1 mM, were slightly inhibitory (Table 8).

Fe$^{3+}$ reduction was unaffected by the addition of 1 or 10 mM NO$_3^-$ to the reaction mixture regardless of whether it was preincubated with cell extract (Table 8) or added during the assay (Appendix 1: Figure 14).

**Fe$^{3+}$ Reduction by Extracts of Other Bacteria**

Cytoplasmic fractions from all bacterial species tested reduced Fe$^{3+}$ with NADH as reductant and ferric citrate as substrate (Table 9). Membrane fractions except for that of _Pseudomonas aeruginosa_ were inactive.
TABLE 8. Effect of respiratory inhibitors and NO$_3^-$ on Fe$^{3+}$ reduction by cytoplasmic fractions of A. magnetotacticum strain MS-1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotenone</td>
<td>4 µM</td>
<td>100</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>4 µM</td>
<td>100</td>
</tr>
<tr>
<td>HQNO</td>
<td>4 µM</td>
<td>100</td>
</tr>
<tr>
<td>NaCN</td>
<td>1 mM</td>
<td>93</td>
</tr>
<tr>
<td>NaN$_3$</td>
<td>1 mM</td>
<td>93</td>
</tr>
<tr>
<td>NaNO$_3$</td>
<td>1 mM</td>
<td>99</td>
</tr>
<tr>
<td>NaNO$_3$</td>
<td>10 mM</td>
<td>100</td>
</tr>
</tbody>
</table>
TABLE 9. Fe$^{+3}$ reductase activity in cell-free extracts of various bacteria.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Cell Fraction</th>
<th>Specific Activity$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aquaspirillum magnetotacticum</em> strain MS-1</td>
<td>Cytoplasmic</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>Membrane</td>
<td>NDA$^b$</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Cytoplasmic</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>Membrane</td>
<td>NDA</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Cytoplasmic</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Membrane</td>
<td>NDA</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Cytoplasmic</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Membrane</td>
<td>12</td>
</tr>
</tbody>
</table>

$^a$ nmol Fe$^{2+}$/mg protein/h.

$^b$ NDA = No detectable activity.
DISCUSSION

Cell-free extracts of *A. magnetotacticum* reduce NO$_3^-$, NO$_2^-$, and Fe$^{3+}$. NO$_3^-$ reductase in this species is a soluble or a readily solubilized enzyme weakly associated with the cell membrane. Dissimilatory NO$_3^-$ reductases in other bacteria are membrane bound (Payne, 1973) except for that of *Spirillum itersonii* (Gauthier et al., 1970). In contrast, NO$_2^-$ reductase appeared to be strongly membrane-bound in *A. magnetotacticum*. Dissimilatory NO$_2^-$ reductase is a soluble or a readily solubilized enzyme in most other denitrifiers although it is membrane-bound in *Thiobacillus denitrificans* (Bryan, 1981).

Fe$^{3+}$ reductase activity was also confined to the cytoplasmic fraction from *A. magnetotacticum*. The rates of Fe$^{3+}$ reduction by magnetic spirilla were comparable to those of other bacteria tested in this study and to those of another spirillum species, *Spirillum itersonii* (Dailey and Lascelles, 1977). This correlation suggests that despite the fact that magnetic bacteria accumulate so much intracellular iron, their enzyme systems are not especially facile in reducing Fe$^{3+}$ when compared to those of common bacterial species.

Fe$^{3+}$ reductase activity in *A. magnetotacticum* was insensitive to respiratory inhibitors used successfully by others to inhibit Fe$^{3+}$ reducing systems (Dailey and Lascelles, 1977; Lascelles and Burke, 1978). Probably the Fe$^{3+}$ reduction we measured is independent of electron transport chain components and therefore not associated with cell energy conservation.
Extracts of cells grown with NH$_4^+$ had similar NO$_3^-$ reductase but higher Fe$^{+3}$ reductase than those from cells grown with NO$_3^-$ (Table 6). NO$_3^-$, even at 10 mM, had no discernable effect on Fe$^{+3}$ reduction by cell extracts. We conclude that there was no competition between NO$_3^-$ and Fe$^{+3}$ for a common reducing enzyme.

Our results with cell-free extracts do not establish a close relationship between NO$_3^-$ reduction and overall cell Fe$^{+3}$ reduction. Moreover, since comparable rates of Fe$^{+3}$ reduction were measured in extracts both of magnetotactic cells and non-magnetotactic mutants, our data suggest that the intracellular rate of Fe$^{+3}$ reduction is not the factor limiting Fe$_3$O$_4$ synthesis in non-magnetic mutant strains NM-1A and NM-1B.

Cells of *Aquaspirillum magnetotacticum* participate significantly in Fe$^{+3}$ and NO$_3^-$ reduction, thereby illustrating how bacteria may couple the biogeochemical cycling of several key elements in aquatic environments.
ACKNOWLEDGEMENTS

Strains NM-1A and NM-1B were isolated by N. Blakemore whose technical assistance is gratefully acknowledged. We are also indebted to R. B. Frankel of the Massachusetts Institute of Technology for valuable comments, suggestions, and continual encouragement in this work.

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APPENDIX ONE
TABLE 10. Effect of $O_2$ on growth and Fe$_3$O$_4$ synthesis with various nitrogen sources.

<table>
<thead>
<tr>
<th>Initial $O_2$ Concentration (kPa in headspace)</th>
<th>Final Cell Yield (cells/ml)</th>
<th>Ave. # of Magnetosomes + SD$^b$</th>
<th>% Of Cells Without Magnetosomes</th>
<th>Magnetic Moment (μemu)</th>
<th>% of Yield as Inoculum$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cells grown with 2 mM $NO_3^-$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trace</td>
<td>$1.8 \pm 0.0 \times 10^8$</td>
<td>$2.04 \pm 3.54$</td>
<td>$59$</td>
<td>$0.59-0.69$</td>
<td>$0.7$</td>
</tr>
<tr>
<td>0.5</td>
<td>$1.8 \pm 0.2 \times 10^8$</td>
<td>$9.99 \pm 10.04$</td>
<td>$11$</td>
<td>$0.80-0.97$</td>
<td>$0.7$</td>
</tr>
<tr>
<td>1.0</td>
<td>$2.5 \pm 0.3 \times 10^8$</td>
<td>$17.22 \pm 7.78$</td>
<td>$0$</td>
<td>$2.92-3.30$</td>
<td>$0.5$</td>
</tr>
<tr>
<td>2.5</td>
<td>$2.4 \pm 0.0 \times 10^8$</td>
<td>$14.86 \pm 7.69$</td>
<td>$6$</td>
<td>$2.82-3.13$</td>
<td>$0.5$</td>
</tr>
<tr>
<td>5.0</td>
<td>$2.5 \pm 0.3 \times 10^8$</td>
<td>$13.00 \pm 15.86$</td>
<td>$18$</td>
<td>$2.73-2.94$</td>
<td>$0.5$</td>
</tr>
<tr>
<td>10.0</td>
<td>$1.7 \pm 0.0 \times 10^8$</td>
<td>$1.59 \pm 4.82$</td>
<td>$86$</td>
<td>$0.96-1.21$</td>
<td>$1.1$</td>
</tr>
<tr>
<td>21.0</td>
<td>$0.7 \pm 0.3 \times 10^7$</td>
<td>$0.49 \pm 2.62$</td>
<td>$96$</td>
<td>ND$^e$</td>
<td></td>
</tr>
<tr>
<td><strong>Cells grown with 2 mM $NH_4^+$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trace</td>
<td>$1.8 \pm 0.3 \times 10^8$</td>
<td>$8.97 \pm 9.12$</td>
<td>$36$</td>
<td>$3.30$</td>
<td>$69.4$</td>
</tr>
<tr>
<td>0.5</td>
<td>$2.6 \pm 0.5 \times 10^7$</td>
<td>$11.50 \pm 7.99$</td>
<td>$9$</td>
<td>$3.08-3.68$</td>
<td>$4.8$</td>
</tr>
<tr>
<td>1.0</td>
<td>$7.3 \pm 3.1 \times 10^7$</td>
<td>$11.86 \pm 9.67$</td>
<td>$14$</td>
<td>$3.17-3.60$</td>
<td>$1.7$</td>
</tr>
<tr>
<td>2.5</td>
<td>$9.7 \pm 1.0 \times 10^7$</td>
<td>$5.96 \pm 9.97$</td>
<td>$40$</td>
<td>$1.25-1.70$</td>
<td>$1.3$</td>
</tr>
<tr>
<td>5.0</td>
<td>$1.4 \pm 0.1 \times 10^8$</td>
<td>$3.71 \pm 4.77$</td>
<td>$45$</td>
<td>$1.66-2.29$</td>
<td>$0.9$</td>
</tr>
<tr>
<td>10.0</td>
<td>$1.8 \pm 0.1 \times 10^8$</td>
<td>$3.08 \pm 5.40$</td>
<td>$65$</td>
<td>$1.28$</td>
<td>$0.7$</td>
</tr>
<tr>
<td>21.0</td>
<td>$2.5 \pm 0.1 \times 10^8$</td>
<td>$2.57 \pm 4.35$</td>
<td>$52$</td>
<td>$0.61$</td>
<td>$0.5$</td>
</tr>
<tr>
<td><strong>Cells grown with 1 mM $NO_3^-$ and 1 mM $NH_4^+$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trace</td>
<td>$1.2 \pm 0.1 \times 10^8$</td>
<td>$1.24 \pm 3.17$</td>
<td>$79$</td>
<td>$0.69-0.77$</td>
<td>$1.1$</td>
</tr>
<tr>
<td>0.5</td>
<td>$2.7 \pm 0.1 \times 10^8$</td>
<td>$3.71 \pm 4.38$</td>
<td>$45$</td>
<td>$0.97-1.07$</td>
<td>$0.5$</td>
</tr>
<tr>
<td>1.0</td>
<td>$2.8 \pm 0.1 \times 10^8$</td>
<td>$14.41 \pm 8.32$</td>
<td>$6$</td>
<td>$2.87-3.05$</td>
<td>$0.4$</td>
</tr>
<tr>
<td>2.5</td>
<td>$2.2 \pm 0.3 \times 10^8$</td>
<td>$11.84 \pm 10.60$</td>
<td>$11$</td>
<td>$2.13-2.60$</td>
<td>$0.6$</td>
</tr>
<tr>
<td>5.0</td>
<td>$1.5 \pm 0.5 \times 10^8$</td>
<td>$10.58 \pm 9.97$</td>
<td>$15$</td>
<td>$1.95-2.23$</td>
<td>$0.9$</td>
</tr>
<tr>
<td>10.0</td>
<td>$1.1 \pm 0.2 \times 10^8$</td>
<td>$2.91 \pm 6.86$</td>
<td>$64$</td>
<td>$1.02-2.57$</td>
<td>$1.1$</td>
</tr>
<tr>
<td>21.0</td>
<td>$1.2 \pm 0.0 \times 10^8$</td>
<td>$0.79 \pm 2.72$</td>
<td>$85$</td>
<td>$0.68-1.50$</td>
<td>$1.0$</td>
</tr>
</tbody>
</table>

$^a$ Values indicate means and standard deviations, respectively, obtained from triplicate cultures.

$^b$ The average number of magnetosomes per cell ± the standard deviation. The magnetosomes from 100 cells cultured at each $P_{O_2}$ value were enumerated.

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

$^d$ Initial cell concentration was $1.25 \times 10^6$ cells/ml.

$^e$ ND = None Detected.
TABLE 11. Amino acid composition of whole cells of *A. magnetotacticum* grown microaerobically with NH$_4^+$ (2 mM) as the sole N source.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>recovery of protein$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>556.0$^a$</td>
</tr>
<tr>
<td>Glycine</td>
<td>471.5</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>387.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>387.2</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>451.9</td>
</tr>
<tr>
<td>Valine</td>
<td>374.5</td>
</tr>
<tr>
<td>Lysine</td>
<td>268.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>229.8</td>
</tr>
<tr>
<td>Serine</td>
<td>218.6</td>
</tr>
<tr>
<td>Arginine</td>
<td>228.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>193.2</td>
</tr>
<tr>
<td>Proline</td>
<td>186.7</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>141.4</td>
</tr>
<tr>
<td>Methionine</td>
<td>113.6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>96.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>80.3</td>
</tr>
<tr>
<td></td>
<td>91.4</td>
</tr>
</tbody>
</table>

**Ammonia** 1079.4

$^a$ nmols detected per mg cell dry weight.

$^b$ Protein = 59.4% of cell dry weight.
FIGURE 7. Growth response of \textit{A. magnetotacticum} to added NaNO₂. At 18 h, cultures previously grown without a source of fixed nitrogen were provided with various concentrations of NO₂⁻. The controls received an equal volume of anaerobic growth medium minus an N source. Symbols and bars represent means and standard deviations, respectively, obtained with triplicate cultures.

Symbols: \(\Delta\), 1 mM NaNO₂
\(\square\), 0.5 mM NaNO₂
\(\circ\), 0.2 mM NaNO₂
\(\bullet\), Control
FIGURE 8. Growth response of *A. magnetotacticum* to added $\text{NH}_2\text{OH \cdot HCl}$.

At 48 h, cultures previously grown without a source of fixed nitrogen were provided with various concentrations of $\text{NH}_2\text{OH \cdot HCl}$. The controls received an equal volume of anaerobic growth medium minus an N source. Symbols and bars represent means and standard deviations, respectively, obtained with triplicate cultures.

Symbols: 

- $\bigtriangleup$, 1 mM $\text{NH}_2\text{OH \cdot HCl}$
- $\square$, 0.5 mM $\text{NH}_2\text{OH \cdot HCl}$
- $\circ$, 0.2 mM $\text{NH}_2\text{OH \cdot HCl}$
- $\bullet$, Control
FIGURE 9. Effect of NO$_2^-$ on *A. magnetotacticum* with NO$_3^-$. At 18 h, cultures growing with 2 mM NO$_3^-$ were provided with various concentrations of NaNO$_2$. The controls received an equal volume of anaerobic growth medium minus an N source. Symbols and bars represent means and standard deviations, respectively, obtained with triplicate cultures.

Symbols: □, 1 mM NaNO$_2$

△, 0.5 mM or less NaNO$_2$

•, Control
[Description or analysis of the diagram here, if necessary]
FIGURE 10. Effect of $\text{C}_2\text{H}_2$ on growth of *A. magnetotacticum* with 2 mM $\text{NO}_3^-$. Cultures initially contained 1 kPa $\text{O}_2$ in the headspace and various concentrations of $\text{C}_2\text{H}_2$. Cultures were incubated on a shaker at 30°C. Symbols and bars represent means and standard deviations, respectively, obtained with triplicate cultures.

Symbols: $\square$, 10 kPa $\text{C}_2\text{H}_2$ (0.10 atm)

$\circ$, 1 kPa $\text{C}_2\text{H}_2$ (0.01 atm)

$, \text{Control}$
FIGURE 11. $\text{NO}_3^-$ reductase activity in a cytoplasmic fraction of *A. magnetotacticum* strain MS-1. The reaction mixture contained 8.5 $\mu$g of cell extract protein. $\text{NO}_2^-$ production proceeded linearly for at least 30 minutes.
FIGURE 12. NO$_2^-$ reductase activity in a membrane fraction of *A. magnetotacticum* strain MS-1. The reaction mixture contained 5.0 µg cell extract protein. N$_2$O production proceeded linearly for at least 30 minutes. NO was not detected as a product of NO$_2^-$ reduction by membrane fractions of *A. magnetotacticum*. 
FIGURE 13. Effect of aeration on Fe$^{3+}$ reduction by a cytoplasmic fraction of *A. magnetotacticum* cells grown with NO$_3^-$ (A) or NH$_4^+$ (B) as the sole nitrogen source. At 10 minutes, air was bubbled into the reaction mixture for 60 seconds. Fe$^{3+}$ reduction was measured by the increase in absorbance at 562 nm. Cuvettes A and B contained 633 and 822 µg cell extract protein, respectively.
ABSORBANCE at 562 nm
FIGURE 14. Effect of NO$_3^-$ on Fe$^{3+}$ reduction by a cytoplasmic fraction of *A. magnetotacticum* cells grown with NO$_3^-$ as the sole nitrogen source. At the arrow, anaerobic NO$_3^-$ in 77 mM potassium phosphate buffer pH 6.9 was added to cuvette A. Final concentration of NO$_3^-$ was 10 mM. Cuvette B received an equal volume of anaerobic 77 mM potassium phosphate buffer pH 6.9. Each cuvette contained 820 µg of cell extract protein. Similar results were obtained when the final NO$_3^-$ concentration was 1 mM and when cell extracts from NH$_4^+$ grown cells were used.
APPENDIX TWO
APPENDIX TWO

REGULATION OF DENITRIFICATION (NITRITE REDUCTION)

IN AQUASPIRILLUM MAGNETOTACTICUM

The regulation of nitrate and nitrite reduction has been studied in a number of bacterial species. The synthesis of nitrate reductase is controlled by O₂ and NO₃⁻ (Payne, 1973; Knowles, 1982). Production of this enzyme in *Proteus mirabilis*, *Escherichia coli*, and *Bacillus licheniformis* is anoxia derepressed although more nitrate reductase is synthesized by *P. mirabilis* when NO₃⁻ or NO₂⁻ is present during anaerobiosis (de Groot and Stouthamer, 1970; Showe and DeMoss, 1968; Schulp and Stouthamer, 1970). Nitrate reductase in *Bacillus stearothermophilus* is induced by NO₃⁻ (Downey and Nuner, 1967).

Nitrite reductase, on the other hand, whether it is the cytochrome cd (Iwasaki and Matsubara, 1971; Newton, 1969; van Verseveld et al., 1977) or the copper-containing enzyme (Pichinoty et al., 1969), is usually induced in the presence of NO₃⁻ or NO₂⁻.

*Aqua spirillum magnetotacticum* strain MS-1, a magnetic bacterium, is a microaerophilic denitrifier which reduces NO₃⁻ to N₂, transiently accumulating N₂O but not NO₂⁻ (Escalante-Semerena et al., 1980; Bazyllinski and Blakemore, 1983). Nitrate reductase activity in *A. magnetotacticum* is associated with the soluble cell fraction while the nitrite reductase activity is confined to the cell membrane fraction (Bazyllinski and Blakemore, 1984). Cell extracts prepared from NO₃⁻-
grown cells have nitrate reductase activity similar to that of NH$_4^+$-grown cells (Bazylinski and Blakemore, 1984). This report describes aspects of denitrification by cell suspensions of _A. magnetotacticum_.

Cells of _A. magnetotacticum_ strain MS-1 were cultured as previously described (Blakemore et al., 1979) except that the growth medium contained tartaric acid (0.75 g/l) as the sole carbon source. The growth medium contained 2 mM NaNO$_3$ or NH$_4$Cl as indicated.

Cells from 10 l batch cultures were collected at late exponential phase (0.8-1.0 x 10$^{-8}$ cells/ml) by centrifugation at 10,000 x g for 10 min at 4°C. They were resuspended in anaerobic 50 mM potassium phosphate buffer (pH 6.9) containing 0.5 mM sodium thioglycolate and 100 μg chloramphenicol ml$^{-1}$ and recentrifuged. Washed cells were resuspended in this buffer and injected into 36 ml capacity serum vials each containing 2 mM NaNO$_3$, 0.5 mM sodium thioglycolate, and 100 μg chloramphenicol ml$^{-1}$ in 20 ml of 50 mM potassium phosphate buffer (pH 6.9). Chloramphenicol inhibits growth of _A. magnetotacticum_ at 25 μg/ml (N. Blakemore, personal communication). The headspace gas within the vials contained 0.2 kPa O$_2$ and 1 kPa acetylene. Acetylene was added to inhibit N$_2$O reduction (Yoshinari and Knowles, 1976; Yoshinari et al., 1977). The final cell concentration in each vial was 2.0 x 10$^9$ cells/ml. N$_2$O was determined by gas chromatography using an electron capture detector (Bazylinski and Blakemore, 1983) and NO$_2^-$ was measured with sulfanilamide-N-1-naphthylethylene-diamine dihydrochloride (Standard Methods, 1980).

Suspensions of cells grown with NO$_3^-$ or NH$_4^+$ reduced NO$_3^-$ actively (Fig. 15). NO$_3^-$-grown cells reduced NO$_3^-$ to N$_2$O without accumulating
FIGURE 15. Production of $N_2O$ by cell suspensions of *A. magnetotacticum* in the presence of chloramphenicol. Cells were grown microaerobically with 2 mM $NO_3^-$ (●) or $NH_4^+$ (■) as the sole nitrogen source.
NO\textsubscript{2}\textsuperscript{-} (Table 12), whereas NH\textsubscript{4}\textsuperscript{+}-grown cells produced only small amounts of N\textsubscript{2}O (2% of that produced by NO\textsubscript{3}\textsuperscript{-}-grown cells) and accumulated NO\textsubscript{2}\textsuperscript{-}.

The apparent lag observed with NO\textsubscript{3}\textsuperscript{-}-grown cells (Fig. 15) may be due to inhibition by O\textsubscript{2} which may have affected the cells during harvesting. These data indicate that nitrite reduction by \textit{A. magnetotacticum} requires protein synthesis and the presence of NO\textsubscript{3}\textsuperscript{-}. It is possible that NO\textsubscript{2}\textsuperscript{-} induces nitrite reductase synthesis but this seems unlikely since this toxic intermediate never accumulates to detectable amounts during growth of \textit{A. magnetotacticum} (Bazylinski and Blakemore, 1983). Dissimilatory nitrate reductase may be a constitutive enzyme in \textit{A. magnetotacticum} since nitrate reductase activities measured in extracts of cells grown with NO\textsubscript{3}\textsuperscript{-} or NH\textsubscript{4}\textsuperscript{+} as the sole nitrogen source were similar (Bazylinski and Blakemore, 1984). Alternately this activity may be derepressed at low P\textsubscript{O}_{2}. Unfortunately this latter possibility is difficult to test with growing cells because this obligate microaerophile will not grow aerobically nor anaerobically even with NO\textsubscript{3}\textsuperscript{-} in the medium (Blakemore et al., 1979; Bazylinski and Blakemore, 1983).

Cyanide or azide at 1 mM inhibited production of N\textsubscript{2}O in washed suspensions of NO\textsubscript{3}\textsuperscript{-}-grown cells (Table 12). Cells treated with cyanide, however, accumulated NO\textsubscript{2}\textsuperscript{-}. This is consistent with previous findings in which cyanide strongly inhibited nitrite reductase activity but not nitrate reductase activity in cell-free extracts (Bazylinski and Blakemore, 1984). Moreover, this suggests that NO\textsubscript{2}\textsuperscript{-} does not accumulate in growing cultures of \textit{A. magnetotacticum} due to differences in rates of nitrite reductase activity compared to nitrate reductase
TABLE 12. Products of NO₃⁻ reduction by cell suspensions of *A. magnetotacticum* in the presence of chloramphenicol.

<table>
<thead>
<tr>
<th>Cells and Treatment</th>
<th>NO₂⁻ Total μmol after 12 hours</th>
<th>N₂O Total μmol after 12 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₃⁻-grown cells</td>
<td>0</td>
<td>4.4</td>
</tr>
<tr>
<td>NH₄⁺-grown cells</td>
<td>1.2</td>
<td>0.1</td>
</tr>
<tr>
<td>NO₃⁻-grown cells with 1 mM NaCN</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>NO₃⁻-grown cells with 1 mM NaN₃</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>Boiled cells</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Cell concentration was 2.0 x 10⁹ cells/ml.
activity. This kinetic hypothesis has been used to explain the accumulation of intermediates during denitrification in other microorganisms (Betlach and Tiedje, 1971). This is also supported by data obtained using artificial electron donors indicating that the observed rate of nitrite reductase activity was comparable to the observed nitrate reductase activity (Bazylinski and Blakemore, 1984). It is possible, however, that the rates observed with cell-free extracts do not reflect those of growing cells.

In summary, the data obtained using intact, washed, resting cells of *A. magnetotacticum* indicate that synthesis of nitrite reductase (the enzyme activity which defines denitrification) in this bacterium, is induced under microaerobic conditions in the presence of NO$_3^-$ . Oxygen may play a role in the regulation of this enzyme because low activity was detected in cells grown without NO$_3^-$ . The effect of O$_2$ remains unclear at present, however.
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of nitrous oxide reduction and measurement of denitrification and

Commun. 69:705-710.
AFTERWORD
This study has shown important progress as to the physiology of *Aquaspirillum magnetotacticum* and perhaps magnetic bacteria in general. However, in doing so it has exposed more questions and subsequently, other interesting areas of research.

*A. magnetotacticum* is the first described oxygen requiring denitrifier. The role of oxygen in this process, however, is unclear and demands further attention. It would be very interesting if this organism can use O$_2$ and NO$_3^-$ (or NO$_2^-$) simultaneously as terminal electron acceptors. This exciting possibility is being explored in several laboratories and recent reports suggest that denitrification and O$_2$ respiration may occur simultaneously in some other bacteria. Moreover, recent experiments suggest that O$_2$ is required for magnetite synthesis. Thus, O$_2$ apparently plays several significant roles in the physiology of *A. magnetotacticum*.

Although a link between NO$_3^-$ and magnetite synthesis was not established using a biochemical approach and cell-free extracts, cells of *A. magnetotacticum* grown with NO$_3^-$ are more magnetic than those grown with NH$_4^+$. Perhaps it is a difference in the redox potential of the medium due to the presence of NO$_3^-$ that is responsible for this increase in magnetism rather than an enzymatic interaction.

The enzymes of denitrification have been studied in detail in a number of bacteria. These enzymes have not been closely examined in spirilla, however (with the exception of nitrate reductase in
Aquaspirillum itersonii). Like A. itersonii, A. magnetotacticum appears to have a soluble nitrate reductase. Most respiratory nitrate reductases are membrane bound and interact with the electron transport chain. It is unclear how a soluble enzyme is able to do this. Moreover, the actual sites of phosphorylation during denitrification by A. magnetotacticum are not known. Although final cell yield increased with an increase in NO$_3^-$ concentration, it is possible that cell energy is conserved during the reduction of NO$_2^-$ rather than NO$_3^-$. Cells apparently can reduce N$_2$O and thus possess a N$_2$O reductase activity. Nothing is known about this activity in A. magnetotacticum.

Magnetic bacteria are found in widely diverse aquatic habitats. They are more specifically located at the water-sediment interface rather than in the sediments or the water column. This area is a location of intense microbiological and biogeochemical activity. Many metal ions (such as iron) are continually being oxidized and reduced through chemical and microbiological activity. Microbiological processes such as denitrification, oxygen respiration, sulfate reduction and even nitrification have been shown to occur at this narrow, usually microaerobic zone although not necessarily simultaneously. The ability of this organism to use alternate terminal electron acceptors in a habitat where O$_2$ is limiting or occasionally depleted and to fix N$_2$ when nitrogen is limited is advantageous to its survival in these habitats. Cells of A. magnetotacticum participate in the cycling of two important elements, nitrogen and iron, but more importantly these findings may give important clues as to the physiology of magnetic bacteria. Thus, this bacterium may represent a
model from which studies of its nitrogen metabolism may lead to the isolation of new strains which is of obvious importance to the overall knowledge of magnetic bacteria.