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

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NITROGEN METABOLISM AND IRON REDUCTION IN *AQUASPIRILLUM MAGNETOTACTICUM*

By

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B.S., Northeastern University, 1976
M.S., Northeastern University, 1980

DISSERTATION

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

Doctor of Philosophy
in
Microbiology

May, 1984

This thesis has been examined and approved.

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ABSTRACT

NITROGEN METABOLISM AND IRON REDUCTION IN <u>AQUASPIRILLUM MAGNETOTACTICUM</u>

by

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University of New Hampshire, May, 1984

<u>Aquaspirillum magnetotacticum</u> 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_2O, NO, and N_2 but not NO_2^- or NH_2OH. The inclusion of NH_4^+ in growth medium prevented NO_3^- reduction to NH_4^+ but not to N_2O 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 <u>A. magnetotacticum</u> 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 5×10^{-13} emu per cell. Magnetosomes of A. magnetotacticum consist of magnetite (Fe_3O_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 NO_3^- or NH_4^+ as a sole nitrogen source. Nevertheless, cell yields varied directly with NO_3^- concentration under microaerobic conditions. Products of NO_3^- reduction included NH_4^+, N_2O, NO, and N_2. NO_2^- and NH_2OH, each toxic to cells at 0.2 mM, were not detected as products of cells growing on NO_3^-. NO_3^- reduction to NH_4^+ was completely repressed by the addition of 2mM NH_4^+ to the growth medium whereas NO_3^- reduction to N_2O or to N_2 was not. C_2H_2 completely inhibited N_2O reduction to N_2 by growing cells. These results indicate that *A. magnetotacticum* is a microaerophilic denitrifier that is versatile in its nitrogen metabolism, concomitantly reducing NO_3^- by assimilatory and dissimilatory means. This bacterium appears to be the first described denitrifier with an absolute requirement for O_2. The process of NO_3^- 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_3O_4). Magnetosomes impart to each cell a permanent magnetic dipole moment (Balkwill et al., 1980; Frankel and Blakemore, 1980). Cells synthesizing Fe_3O_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_2O 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_2O) 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_2O 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_2O 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, KH_2PO_4 0.69, $NaNO_3$ 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 $Na_2MoO_4 \cdot 2H_2O$ per l. Ammonium ion was added to the medium as required, either as $(NH_4)_2SO_4$ or NH_4Cl as indicated. $NaNO_2$ or $NH_2OH \cdot HCl$ 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. O_2-free N_2 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 N_2 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 O_2

was added to the bottles to obtain an initial headspace concentration of 0.2 to 1.0 % (v/v) O_2 (200-1000 Pa O_2). 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-Hausser 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 N_2O Reduction

We used established methods to inhibit N_2O reduction with acetylene (C_2H_2) (Federova et al. 1973; Yoshinari et al., 1977). C_2H_2 was generated from distilled water and CaC_2 (granular, Fisher Scientific Co.). All cultures grown with C_2H_2 were incubated on a shaker at 30°C.

Chemical Analyses

NO_3^- was determined with a Beckman SelectIon 2000 Ion Analyzer (Beckman Instruments, Irvine, CA.). NO_2^- was analyzed using sulfanilamide-N-1-naphthylethylene-diamine dihydrochloride (American Public Health Association, 1980). NH_4^+ was determined by the reductive amination of α-ketoglutarate (Sigma Technical Bulletin No. 170-UV, Sigma Chemical Co., St. Louis, MO.). Bound and free NH_2OH was assayed by the Csaki (1948) procedure and by the method of Magee and Burris (1954).

N_2O, NO, and O_2 were measured by gas chromatography on a Varian Series 2400 gas chromatograph equipped with a ^{63}Ni electron capture detector (ECD) (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. O_2-free 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 H_2, He, O_2, NO, CO_2, N_2O, C_2H_2, and H_2O were separated.

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. O_2-free helium at a flow rate of 30ml/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 O_2-free N_2 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 10^7 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 $CaSO_4$ 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 Na_2EDTA to remove iron. Amino acids and intracellular NH_4^+ 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

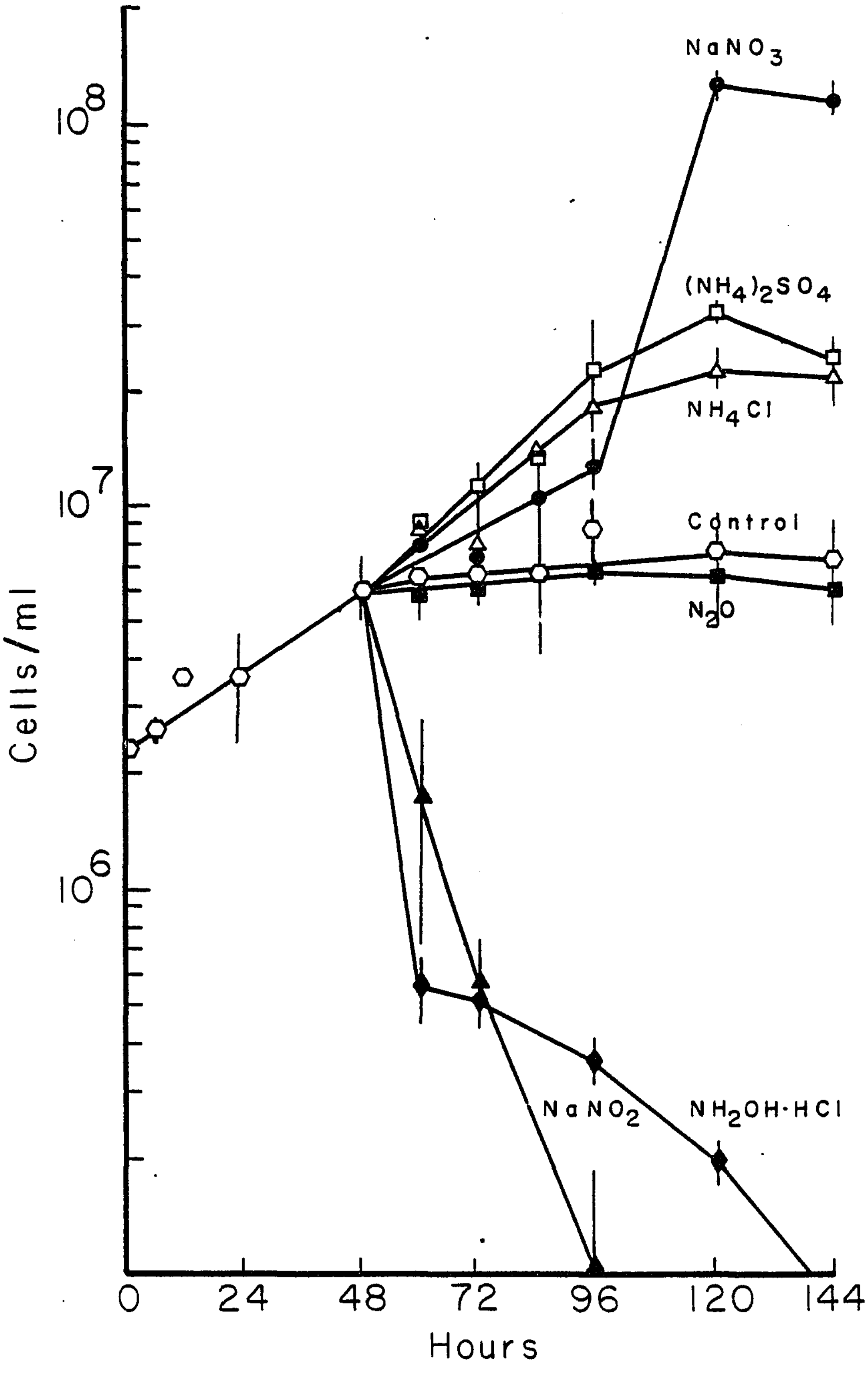
Effect of Nitrogenous Compounds on Growth and Magnetite Synthesis

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 NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, N_2O, $NaNO_2$, or $NH_2OH{\cdot}HCl$, 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.


NaNO$_3$
(NH$_4$)$_2$SO$_4$
NH$_4$Cl
Control
N$_2$O
NaNO$_2$
NH$_2$OH·HCl
Cells/ml
10^8
10^7
10^6
0
24
48
72
96
120
144
Hours

(Appendix 1: Table 10).

Cultures grown with NO_3^- under microaerobic conditions frequently showed a biphasic growth pattern (Figs. 1 and 2). Figure 3 shows the utilization of NO_3^- and O_2 as well as the production of NO_2^-, NO, and N_2O by growing cells. O_2 steadily disappeared throughout growth. NO_3^-, on the other hand, was utilized most extensively after 40 h. The accumulation of N_2O correlated with the extent of 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 NO_2^- and/or NO were occasionally detected during growth of A. magnetotacticum but neither of these accumulated in significant amounts. Of note, cells actively using NO_3^- also continued to consume O_2.

Effect of Oxygen on Growth of A. magnetotacticum

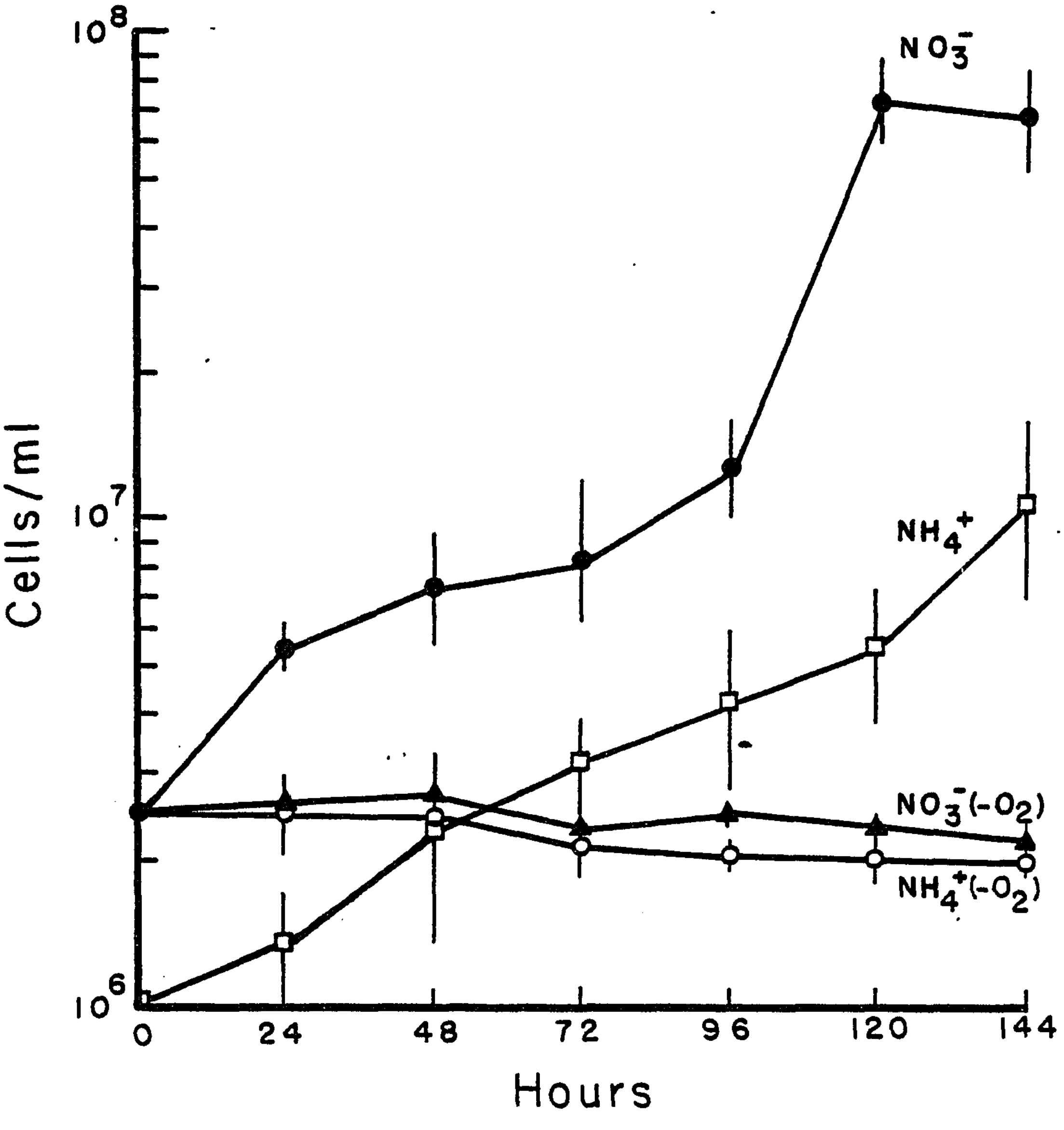
In confirmation of previous results (Blakemore et al., 1979), cells did not grow anaerobically (resazurin colorless) either with NO_3^- or NH_4^+ as the sole nitrogen source (Fig. 2). Under anaerobic conditions cells eventually became nonmotile, an effect that was reversible for at least several hours. Cells retained their magnetism under anaerobic conditions.

Effect of C_2H_2 on Growth and N_2O Reduction

At a concentration of 10 kPa, C_2H_2 inhibited growth and resulted in aberrant non-motile and coccoid cells (Appendix 1: Fig. 10). C_2H_2 at a final headspace concentration of 1 kPa did not adversely affect cell growth (Appendix 1: Fig. 10) or morphology but completely inhibited N_2O

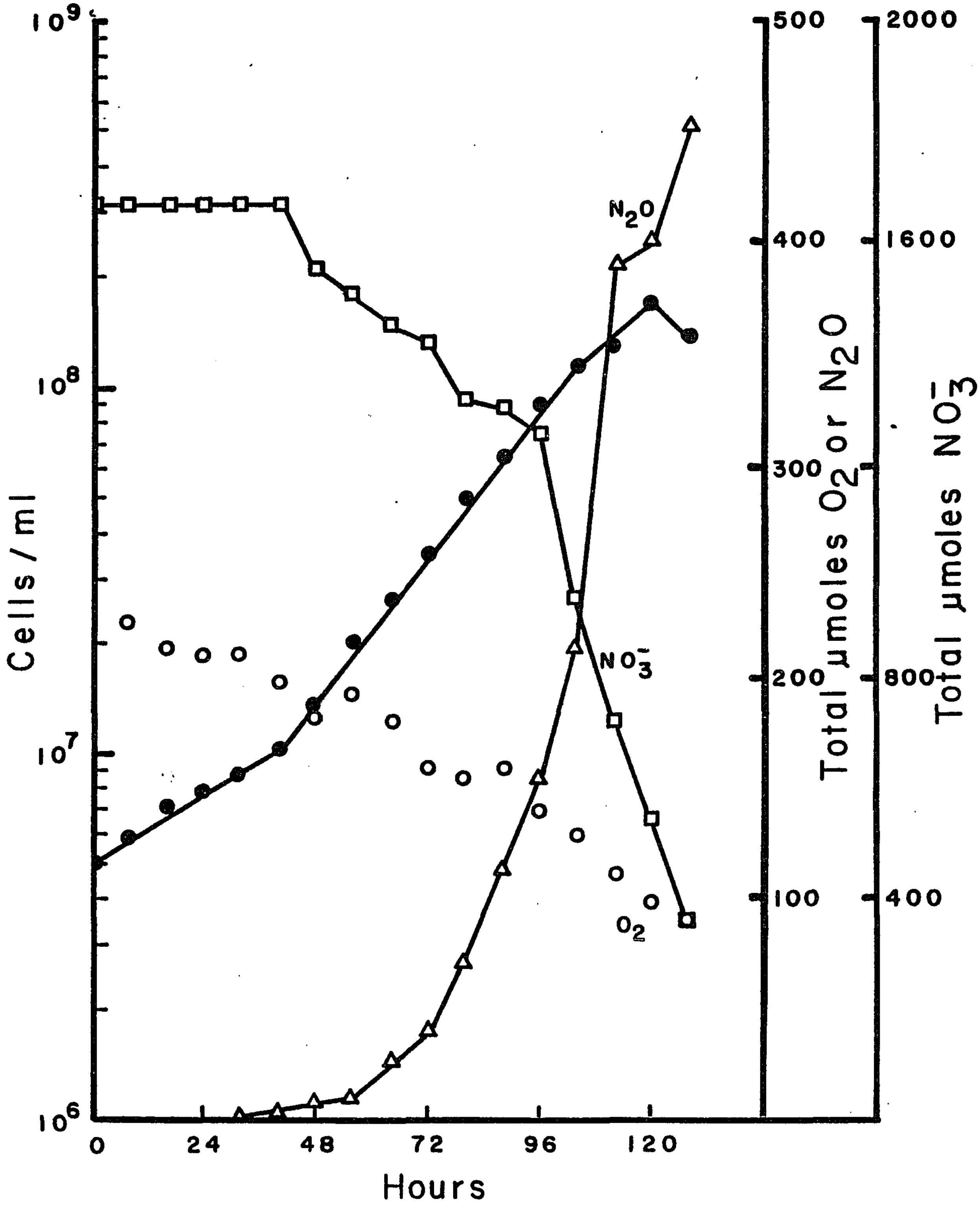
FIGURE 2. Effect of O_2 on the growth of A. magnetotacticum with or without NH_4^+ or NO_3^-. To limit the introduction of O_2, 1% inocula (vol/vol) were from cultures grown until O_2 had completely disappeared. Cells used as inocula came from culture medium similar to that used in the experiment. Data points and bars represent means and standard deviations, respectively, obtained with triplicate cultures.

Symbols: ●, NO_3^- (2 mM), microaerobic conditions (initial Po_2, 0.2 kPa)
▲, NO_3^- (2 mM), anaerobic conditions (resazurin colorless)
□, NH_4^+ (2 mM), microaerobic conditions (initial Po_2, 0.2 kPa)
○, NH_4^+ (2 mM), anaerobic conditions (resazurin colorless).



Cells/ml
10^8
10^7
10^6
0
24
48
72
96
120
144
Hours
NO_3^-
NH_4^+
$NO_3^-(-O_2)$
$NH_4^+(-O_2)$

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.



10^9
10^8
10^7
10^6
Cells / ml
0
24
48
72
96
120
Hours
N_2O
NO_3^-
O_2
500
400
300
200
100
Total µmoles O_2 or N_2O
2000
1600
800
400
Total µmoles NO_3^-

reduction (Table 1). With NH_4^+ present together with C_2H_2 (assimilatory NO_3^- reduction to NH_4^+ repressed), 96.4% of the N supplied as NO_3^- was recovered as N_2O (Table 1).

Products and Mass Balance of NO_3^- Reduction

Cells growing microaerobically reduced NO_3^- to NH_4^+, N_2O, and N_2 (Table 1). Only trace amounts of NO were ever detected. Free (or bound) NH_2OH 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_2O, 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_2O (Table 1). The remainder was recovered in cell material.

Growing cells supplied with NH_4^+ and NO_3^- in the presence of C_2H_2, produced N_2O stoichiometrically equivalent to the amount of NO_3^- utilized (Table 1). In the absence of C_2H_2, and with NO_3^- initially at 0.66 mM or less, N_2O 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_2O or NO_3^- remained. When the initial NO_3^- concentration was raised to 2 mM, N_2O 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.

Culture Medium Supplement	Products[a] (% NO_3^--N recovered as)		
	N_2O	N_2	NH_4^+
NH_4^+ (2 mM) + NO_3^- (0.66 mM) + C_2H_2	96.4 ± 4.1%	0%	N.A.[b]
NH_4^+ (2mM) + NO_3^- (0.66 mM)	0%[c]	101.9 ± 1.3%	N.A.
NO_3^- (0.66 mM) + C_2H_2	80.0 ± 2.1%	0%	3.2 ± 0.0%[d]

---

[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.

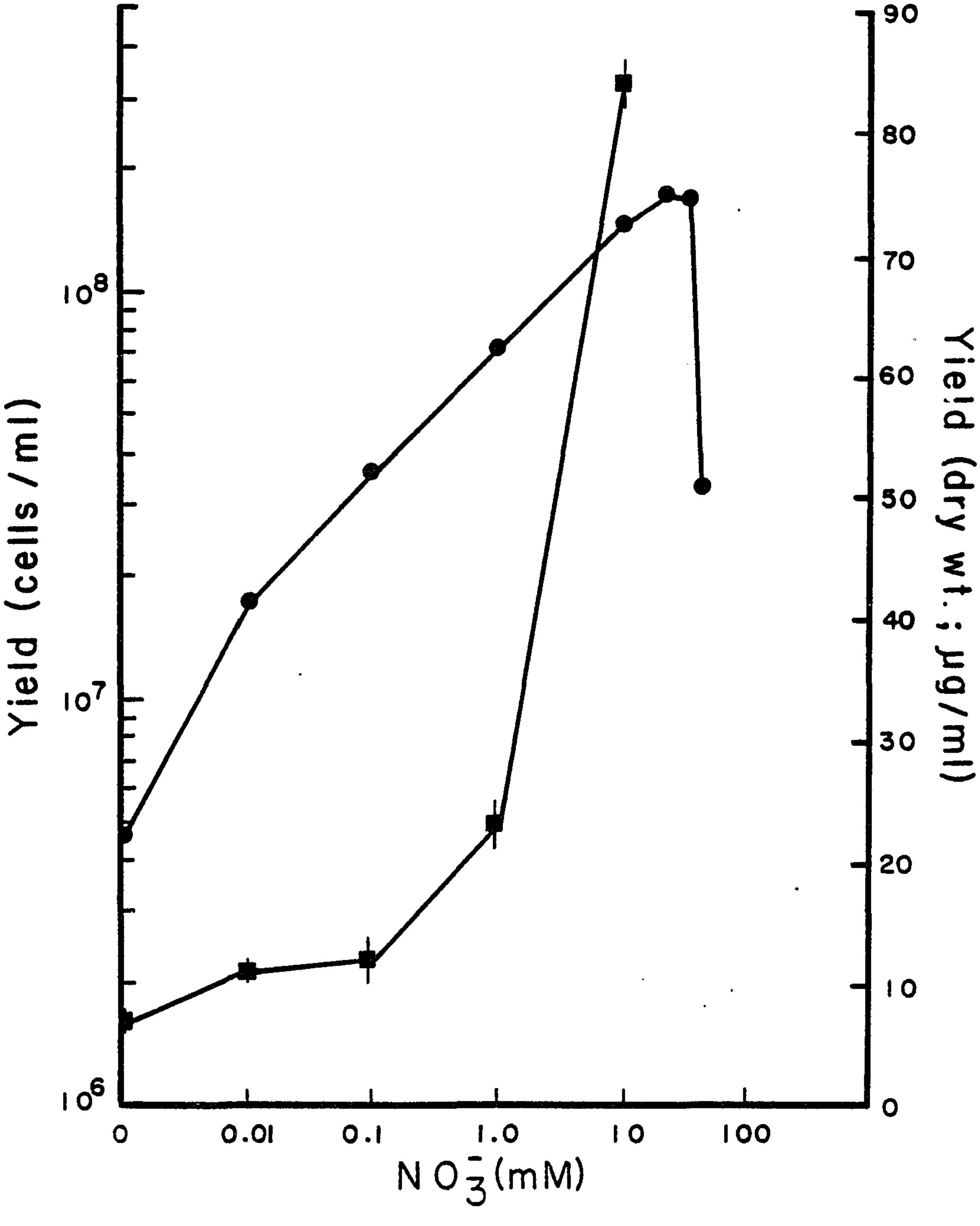
TABLE 2. Amino acid composition of whole cells of <u>A. magnetotacticum</u> grown microaerobically with NO_3^- (2 mM) as the sole N source.

<u>Amino acid</u>	
Alanine	560.4[a]
Glycine	475.9
Aspartic Acid	421.9
Leucine	405.7
Glutamic Acid	403.7
Valine	337.5
Lysine	291.3
Threonine	247.8
Serine	236.9
Arginine	216.6
Isoleucine	209.8
Proline	198.9
Phenylalanine	177.0
Methionine	112.9
Tyrosine	101.1
Histidine	97.6
% 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^- concentrationon final cell yields of <u>A. magnetotacticum</u>. 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.


Yield (cells / ml)
Yield (dry wt.; µg/ml)
NO$_3^-$(mM)
10^6
10^7
10^8
0
0.01
0.1
1.0
10
100
0
10
20
30
40
50
60
70
80
90

DISCUSSION

Aquaspirillum magnetotacticum strain MS-1 cells synthesize all their required nitrogenous compounds de novo from NH_4^+ or NO_3^- ions. Because they grow with NO_3^- as a sole nitrogen source, thereby producing NH_4^+, this organism is capable of assimilatory 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. delicatum are the only other members known to grow with NO_3^- as a sole nitrogen source (Hylemon et al., 1973). Of course, the inability of some species to grow with NO_3^- may reflect requirements for peptides or other constituents of complex media used in culturing them. Although it is uncertain whether free NH_2OH is produced during bacterial assimilatory NO_3^- reduction (Kemp and Atkinson, 1966; Payne, 1973; Prakash and Sadana, 1972; Yordy and Delwiche, 1979), 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 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 NO_3^- reduction to 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 NO_2^- to NH_3 in Escherichia coli

(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_2O in the presence of C_2H_2; N_2 in its absence) under these conditions. Moreover, neither N_2O 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_2O while reducing NO_3^-. These appeared as transient intermediates and were subsequently reduced to N_2.

With sufficient NH_4^+ present to repress assimilatory NO_3^- reduction, increased concentrations of NO_3^- resulted in increased final growth yields. This suggests that NO_3^- 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 A. magnetotacticum is indeed a denitrifier. Of the N supplied to cells as NO_3^- alone, 80% was recovered as N-gas. The remainder was recovered in cell material and excreted nitrogenous products including NH_4^+. Thus, under conditions where NO_3^- is the sole N source, cells of A. magnetotacticum concomitantly carry out denitrification and assimilatory NO_3^- reduction to NH_4^+.

Cell growth with NO_3^- in batch culture is biphasic. The onset of rapid cell growth appeared to correlate with the onset of N_2O production from NO_3^-. The data also suggest that NO_3^- dissimilation commenced when the dissolved O_2 reached approximately 4.1 μmols per liter.

Denitrification is associated generally with anoxic conditions because O_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_2 while denitrifying (Payne, 1973). Cells of A. magnetotacticum are obligately microaerophilic and do not grow, even with NO_3^-, 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_2H_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.

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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_2H_2 microaerobically but not anaerobically even with NO_3^-. This organism apparently is not capable of NO_3^--dependent nitrogen fixation. Cells of *A. magnetotacticum* reduced C_2H_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_3^- did not reduce C_2H_2 until O_2 was introduced. Optimum rates of C_2H_2 reduction by *A. magnetotacticum* were obtained at 200 Pa O_2. C_2H_2 reduction was inhibited by more than 1 kPa O_2 or 0.2 mM NO_3^- or NH_4^+. These results suggest that *A. magnetotacticum* fixes N_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 NO_3^- to gaseous products including N_2O and 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 NO_3^- in the medium (Blakemore et al., 1979). Cells also possess an NH_3-repressible NO_3^- reductase activity (assimilatory NO_3^- reduction). Thus, while denitrifying they concomitantly reduce NO_3^- to NH_4^+ (Bazylinski and Blakemore, 1983). Under microaerobic conditions, cells utilize NO_3^- or NH_4^+ (but not NO_2^- nor, apparently, N_2O) 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 N_2. The use of the acetylene (C_2H_2) reduction assay to assess N_2 fixation is widely accepted and well documented (Child, 1981; Dilworth, 1974). Subsequent studies of C_2H_2 reduction confirmed that this strain fixes 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 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_2H_2 optimally under microaerobic conditions (Okon et al., 1976; 1977) but is also capable of NO_3^--dependent anaerobic nitrogen fixation (C_2H_2 reduction) (Neyra et al., 1977; Neyra and Van Berkum, 1977; Scott et al., 1979). Thus, some bacteria capable of denitrification also fix N_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_2-fixing heterotrophic spirilla. Since strain MS-1 is one of few known denitrifying N_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 <u>A. magnetotacticum</u> and MS-2 were routinely cultured microaerobically in liquid medium contained in 160 ml serum vials as previously described (Bazylinski and Blakemore, 1983). In cultures in which C_2H_2 reduction was measured, the source of combined nitrogen, $NaNO_3$ or $(NH_4)_2SO_4$, was eliminated unless stated otherwise. Red sleeve-type rubber stoppers were used (VWR Scientific Inc.) which did not release C_2H_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 O_2 or air was added to the culture vials to obtain initial headspace concentrations of 0.2 to 5.0 % (v/v) O_2 (200-5000 Pa 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 C_2H_2 Reduction

C_2H_2, generated from distilled H_2O and CaC_2 (granular, Fisher Scientific Co.), was added to cultures at a headspace concentration of 0.1 atm. All cultures containing C_2H_2 were incubated in a shaking water bath (20 oscillations/min) at 30°C.

Ethylene (C_2H_4) was determined using a Varian Series 2400 gas chromatograph equipped with a H_2 flame ionization detector (FID). C_2H_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

C_2H_2 Reduction

Cells in growing cultures of A. magnetotacticum strain MS-1 actively reduced C_2H_2 for 18 to 24 h after inoculation. The production of C_2H_4 then ceased, cells became nonmotile, and coccoid bodies appeared. Consistent with previous observations using the "acetylene block" technique to study denitrification (Bazylinski and Blakemore, 1983), C_2H_2 at a concentration of 0.1 atm, was toxic to growing cells of this organism. Rates of C_2H_2 reduction by A. magnetotacticum and other nitrogen-fixing species are shown in Table 3. Generally, the rates of C_2H_2 reduction were quite variable from experiment to experiment. Although the highest rate observed by strain MS-1 was 0.70 nmols C_2H_4 produced 10^6 cells^{-1} h^{-1} (0.2 kPa O_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 A. magnetotacticum reduced C_2H_2 at rates comparable to or slightly higher than those obtained with Azospirillum lipoferum but much lower than that of Azotobacter vinelandii.

Effect of O_2 on C_2H_2 Reduction

The effect of O_2 on the rate of C_2H_2 reduction is shown in Table 4. Cells never reduced C_2H_2 in the absence of O_2. However,

TABLE 3. C_2H_2 reduction rates by various N_2-fixing bacteria.

Bacterium	nmols $C_2H_4/10^6$ cells/h	
	Mean ± SD	Maximum Rate Observed
Azotobacter vinelandii[a]	2.29 ± 0.01	2.40
Azospirillum lipoferum[b]	0.09 ± 0.04	0.13
Strain MS-2[c]	0.04 ± 0.05	0.10
Aquaspirillum magnetotacticum[c]		
Strain MS-1	0.14 ± 0.12	0.31
Strain NM-1A	0.43 ± 0.28	0.74
Strain NM-1B	0.34 ± 0.16	0.52

[a] Initial Po_2 = 21 kPa.

[b] Initial Po_2 = 0.1 kPa.

[c] Initial Po_2 = 0.2 kPa.

TABLE 4. The effect of O_2 on C_2H_2 reduction by *Aquaspirillum magnetotacticum* strain MS-1.

Initial O_2 Concentration (kPa in headspace)	Rate of C_2H_2 Reduction (nmols $C_2H_4/10^6$ cells/h)
0	0
0.1	0.15
0.2	0.18
0.4	0.014
1.0	0.012
5.0	0

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_2H_2 reduction.

Effect of NO_3^- and NH_4^+ on C_2H_2 Reduction

The effect of NO_3^- and NH_4^+ on C_2H_2 reduction is shown in Figure 6. Cells growing under microaerobic conditions did not reduce C_2H_2 when NO_3^- or NH_4^+ (either at 0.2 mM) were included in the culture medium. Growing cells did not reduce C_2H_2 anaerobically with 0.2 mM NO_3^-.

FIGURE 5. Effect of O_2 on C_2H_2 reduction during growth of A. magnetotacticum strain MS-1. At 0 h, 10% of the culture headspace volume was replaced with C_2H_2. At 12 h, 1 cc of sterile air (●) (final headspace concentration of O_2 = 0.2 kPa) or N_2 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.

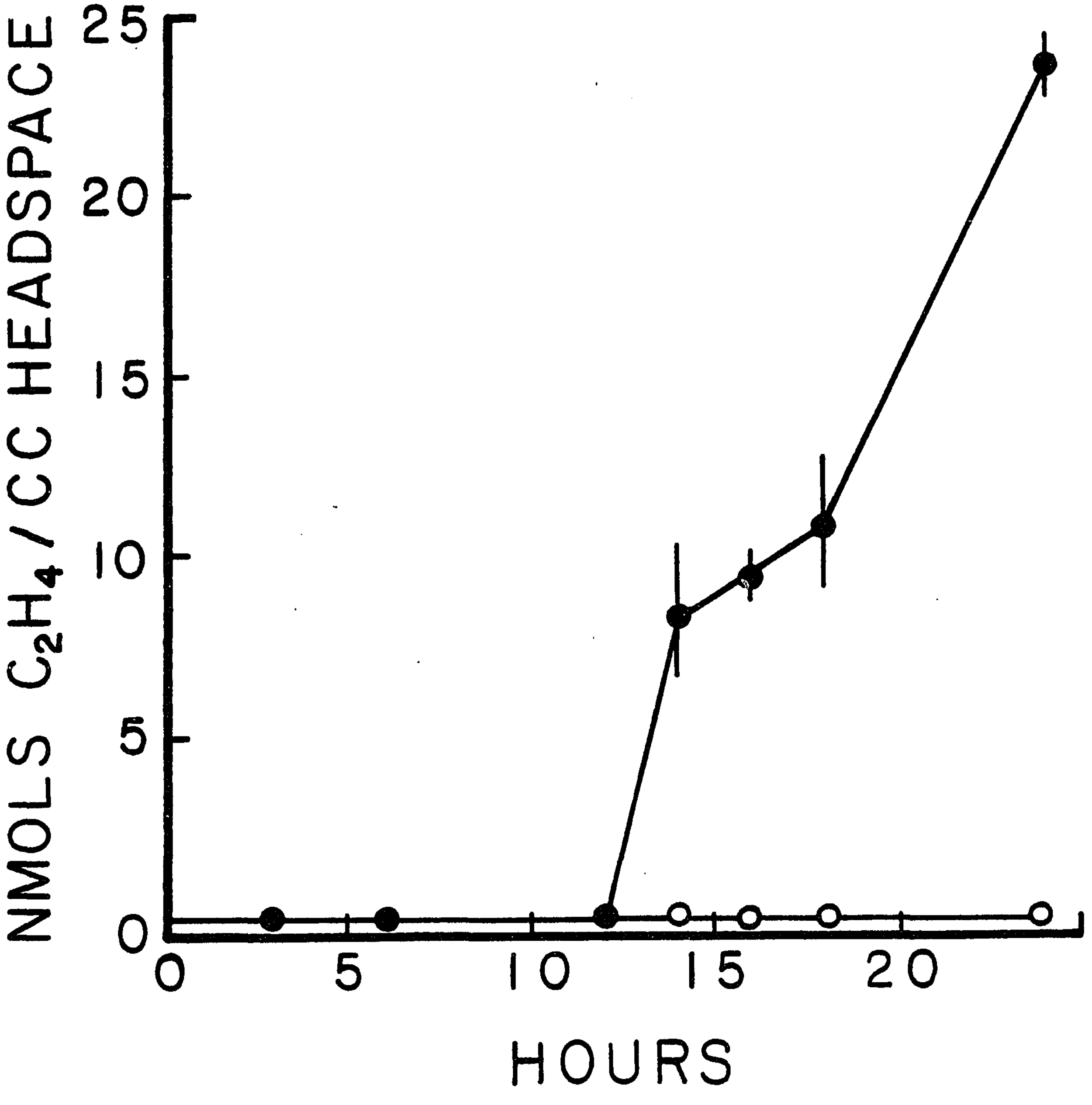
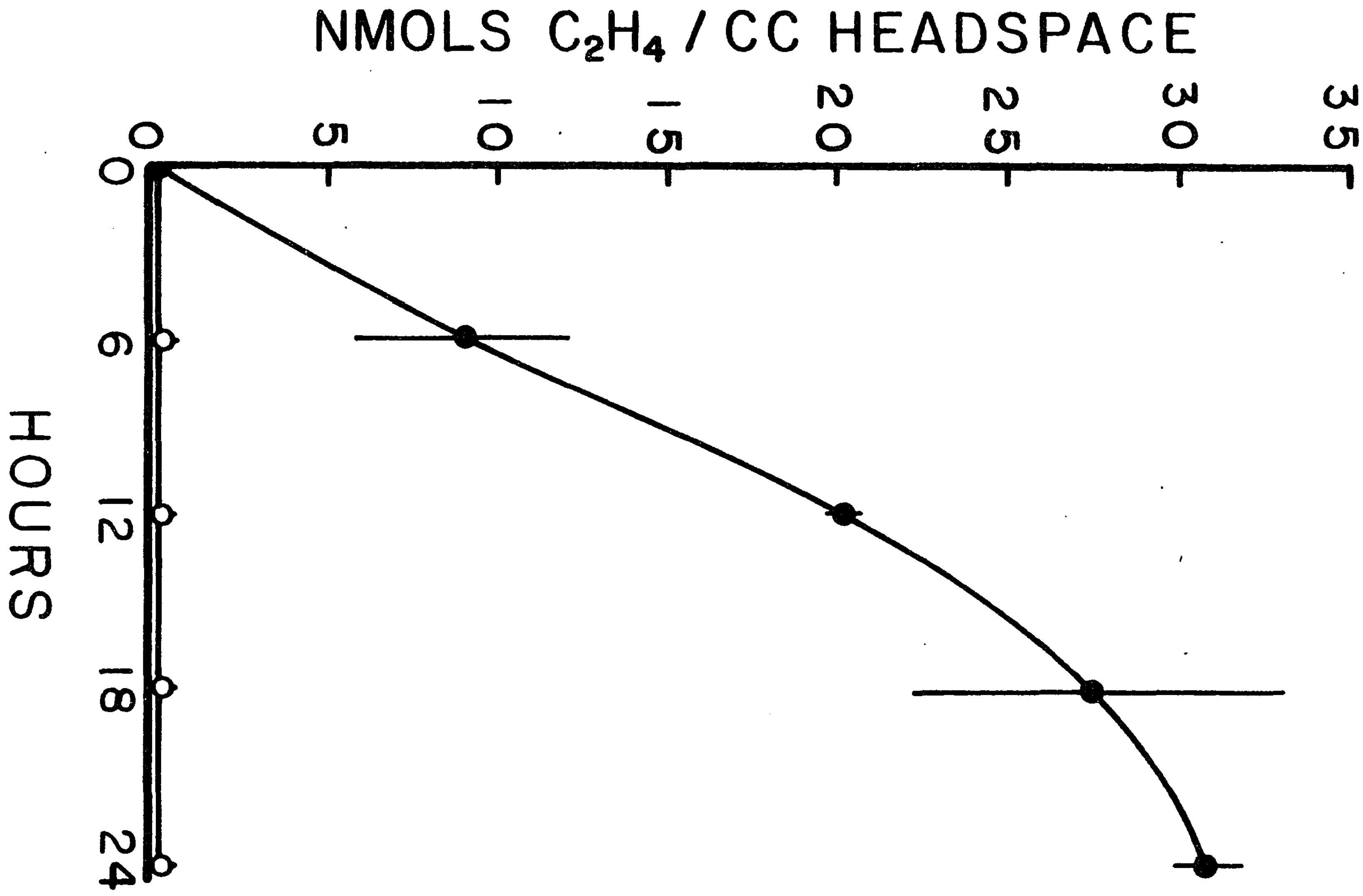

NMOLS C_2H_4 / CC HEADSPACE
0
5
10
15
20
25
0
5
10
15
20
HOURS

FIGURE 6. Effect of NO_3^- and NH_4^+ on C_2H_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_2H_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_4Cl (0.2 mM) added at inoculation.


NMOLS C_2H_4 / CC HEADSPACE
0
5
10
15
20
25
30
35
HOURS
0
6
12
18
24

DISCUSSION

Growing cells of A. magnetotacticum reduced C_2H_2 only under microaerobic conditions but not when 0.2 mM NO_3^- or NH_4^+ was added to the culture medium. They neither grew nor reduced C_2H_2 under anaerobic conditions even in the presence of 0.2 mM NO_3^-. This suggests that cells can fix N_2 only under N-limiting conditions. Moreover, introduction of O_2 into anaerobic cultures initiated C_2H_2 reduction. Thus, cells of this microaerophilic organism meet their energy requirement for nitrogenase activity only with O_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_3^- did not support C_2H_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_3^- as a terminal electron acceptor. We showed previously, however, that increased amounts of NO_3^- 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_2^- is reduced. Unfortunately, results of growth experiments do not enable us to test this because NO_2^- does not accumulate during NO_3^- reduction by this organism. Moreover, NO_2^- is toxic when provided exogenously to cells (Bazylinski and Blakemore, 1983).

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.

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CHAPTER THREE

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

ABSTRACT

Possible relationships between the reduction of NO_3^-, NO_2^-, and Fe^{+3} by cell-free extracts of *Aquaspirillum magnetotacticum* were investigated using gas chromatography and spectrophotometry. NO_3^- and Fe^{+3} reductase activities were associated with the soluble cell fraction. NO_2^- reduction occurred only with the cell membrane fraction. The optimum rate of NO_3^--N reduced to NO_2^- (specific activity = 6.5 μmol N mg cell extract protein^{-1} h^{-1}) was comparable to that of NO_2^--N reduced to N_2O (sp. act. = 6.7). NO was not detected as a product of NO_2^- reduction. Extracts of cells grown on NH_4^+ as a sole N source reduced Fe^{+3} (sp. act. = 107 nmol Fe^{+2} h^{-1} mg cell extract protein^{-1}) nearly as rapidly as those cultured with NO_3^- as a sole N source (sp. act. = 67). Extracts of non-magnetic mutants reduced Fe^{+3} at rates (sp. acts. 62-77) comparable to that of the wild-type magnetic strain MS-1. NO_3^- as high as 10^{-2} *M*, had no effect on Fe^{+3} reduction. NO_3^- reduction was completely inhibited by 10^{-1} *M* NaN_3 but not by 10^{-1} NaCN. NO_2^- reduction was completely inhibited by 10^{-4} *M* NaCN or by 10^{-2} *M* NaN_3. Fe^{+3} reduction was insensitive to 10^{-3} *M* NaCN or NaN_3, or 4 μ*M* Antimycin A, rotenone, or HQNO. Fe^{+3} reduction was inhibited by aeration. These results suggest that overall Fe^{+3} 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_3O_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_3O_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_3O_4 precursors and precipitation of Fe_3O_4 in this species occurs in a sequence in which iron reduction is involved in formation of ferrihydrite ($5Fe_2O_3 \cdot 9H_2O$) as well as its transformation to Fe_3O_4 (Frankel et al., 1983). Thus, bacterial Fe_3O_4 synthesis appears to parallel that of the chiton, a marine mollusc, in which Fe_3O_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). A. magnetotacticum is a microaerophilic denitrifier capable of concomittant assimilatory NO_3^- reduction to NH_4^+ (Bazylinski and Blakemore, 1983). Nitrous oxide (N_2O), 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_3O_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_3O_4 synthesis.

(Portions of this work were presented at the 6th 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 $(NH_4)_2SO_4$ or $NaNO_3$ (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 (1×10^8 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/m^2. 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

NO_3^- reductase activity was assayed by colorimetrically determining the production of NO_2^- (Pichinoty et al., 1971). Dissimilatory NO_2^- reductase activity was measured in a similar reaction mixture with methyl viologen as the electron donor, $NaNO_2$ (1 umol) was used in place of $NaNO_3$ as the substrate. The reaction mixture contained (μmols in a final volume of 6 ml): potassium phosphate buffer, 462 (pH = 6.9); $NaNO_3$, 100 or $NaNO_2$, 1; $Na_2S_2O_4 \cdot 2H_2O$, 3.8; methyl viologen, 0.6. NO and N_2O were measured by gas chromatography as previously described (Bazylinski and Blakemore, 1983). NH_4^+ is not detected as a product of dissimilatory NO_2^- 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 O_2-free N_2. The reactions were started by the addition of $Na_2S_2O_4$. NO_2^- and N_2O production proceeded linearly for at least 30 min under the conditions assayed (Appendix 1: Figures 11 and 12).

Fe^{+3} 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 (μmols 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_2O (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^-. NO_2^- reduction was totally inhibited by 10^{-4} M CN^- and 10^{-2} 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^{+2}-ferrozine complex proceeded

TABLE 5. NO_3^- and NO_2^- reductase activities of cell fractions of *A. magnetotacticum* strain MS-1.

Cell Fraction	Reductase Activity	
	NO_3^- [a]	NO_2^- [b]
Soluble	6.5	0.0
Membrane	4.4	3.4
Washed membrane (3x in phosphate buffer)	0.7	2.1
Boiled crude cell extract (Placed in 100 C water bath for 10 min)	0.0	0.0

[a] measured as µmol NO_2^- formed/mg protein/h.

[b] measured as µmol N_2O 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^+.

	Specific Activity	
	Fe^{+3} Reductase[a]	
Substrate	NH_4^+-grown	NO_3^--grown
Ferric citrate	107	67
Ferric quinate	23	19
$FeCl_3$	43	32
	NO_3^- Reductase[b]	
NO_3^-	7.3	6.5

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

[b] µmol NO_2^-/mg protein/h.

TABLE 7. Fe^{+3} and NO_3^- reductase activity by magnetic and non-magnetic strains of *A. magnetotacticum*.

	Specific Activity	
Strain	Fe^{+3} Reductase[a]	NO_3^- Reductase[b]
MS-1	67	6.5
NM-1A	77	8.5
NM-1B	62	12.8

[a] nmol Fe^{+2}/mg protein/h

[b] μmol NO_2^-/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.

Compound	Concentration	% Activity
Rotenone	4 µM	100
Antimycin A	4 µM	100
HQNO	4 µM	100
NaCN	1 mM	93
NaN_3	1 mM	93
$NaNO_3$	1 mM	99
$NaNO_3$	10 mM	100

TABLE 9. Fe^{+3} reductase activity in cell-free extracts of various bacteria.

Organism	Cell Fraction	Specific Activity[a]
Aquaspirillum magnetotacticum strain MS-1	Cytoplasmic	107
	Membrane	NDA[b]
Bacillus subtilis	Cytoplasmic	144
	Membrane	NDA
Escherichia coli	Cytoplasmic	22
	Membrane	NDA
Pseudomonas aeruginosa	Cytoplasmic	11
	Membrane	12

[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 electon 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_3O_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.

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APPENDIX ONE

TABLE 10. Effect of O_2 on growth and Fe_3O_4 synthesis with various nitrogen sources.

Initial O_2 Concentration (kPa in headspace)	Final Cell Yield (cells/ml)[a]	Ave. # of Magnetosomes + SD[b]	% Of Cells Without Magnetosomes	Magnetic Moment (μ) ($\times 10^{-13}$ emu)[c]	% of Yield as Inoculum[d]
		Cells grown with 2 mM NO_3^-			
Trace	$1.8 \pm 0.0 \times 10^8$	2.04 ± 3.54	59	0.59-0.69	0.7
0.5	$1.8 \pm 0.2 \times 10^8$	9.99 ± 10.04	11	0.80-0.97	0.7
1.0	$2.5 \pm 0.3 \times 10^8$	17.22 ± 7.78	0	2.92-3.30	0.5
2.5	$2.4 \pm 0.0 \times 10^8$	14.86 ± 7.69	6	2.82-3.13	0.5
5.0	$2.5 \pm 0.3 \times 10^8$	13.00 ± 15.86	18	2.73-2.94	0.5
10.0	$1.1 \pm 0.0 \times 10^8$	1.59 ± 4.82	86	0.96-1.21	1.1
21.0	$8.7 \pm 0.3 \times 10^7$	0.49 ± 2.62	96	ND[e]	1.4
		Cells grown with 2 mM NH_4^+			
Trace	$1.8 \pm 0.3 \times 10^6$	8.97 ± 9.12	36	3.30	69.4
0.5	$2.6 \pm 0.5 \times 10^7$	11.50 ± 7.99	9	3.08-3.68	4.8
1.0	$7.5 \pm 3.1 \times 10^7$	11.86 ± 9.67	14	3.17-3.60	1.7
2.5	$9.7 \pm 1.0 \times 10^7$	5.96 ± 9.97	40	1.25-1.70	1.3
5.0	$1.4 \pm 0.1 \times 10^8$	3.71 ± 4.77	45	1.66-2.29	0.9
10.0	$1.8 \pm 0.1 \times 10^8$	3.08 ± 5.40	65	1.28	0.7
21.0	$2.5 \pm 0.1 \times 10^8$	2.57 ± 4.35	52	0.61	0.5
		Cells grown with 1 mM NO_3^- and 1 mM NH_4^+			
Trace	$1.2 \pm 0.1 \times 10^8$	1.24 ± 3.17	79	0.69-0.77	1.1
0.5	$2.7 \pm 0.1 \times 10^8$	3.71 ± 4.38	45	0.97-1.07	0.5
1.0	$2.8 \pm 0.1 \times 10^8$	14.41 ± 8.32	6	2.87-3.05	0.4
2.5	$2.2 \pm 0.3 \times 10^8$	11.84 ± 10.60	11	2.13-2.60	0.6
5.0	$1.5 \pm 0.5 \times 10^8$	10.58 ± 9.97	15	1.93-2.23	0.9
10.0	$1.1 \pm 0.2 \times 10^8$	2.91 ± 6.86	64	1.02-2.57	1.1
21.0	$1.2 \pm 0.0 \times 10^8$	0.79 ± 2.72	85	0.68-1.50	1.0

[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 Po_2 value were enumerated.

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

[d] Initial cell concentration was 1.25×10^6 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.

Amino acid	
Alanine	556.0[a]
Glycine	471.5
Aspartic Acid	387.9
Leucine	387.2
Glutamic Acid	451.9
Valine	374.5
Lysine	268.3
Threonine	229.8
Serine	218.6
Arginine	228.3
Isoleucine	193.2
Proline	186.7
Phenylalanine	141.4
Methionine	113.6
Tyrosine	96.0
Histidine	80.3
% recovery of protein[b]	91.4
Ammonia	1079.4

[a] nmols detected per mg cell dry weight.

[b] Protein = 59.4% of cell dry weight.

FIGURE 7. Growth response of *A. magnetotacticum* to added $NaNO_2$. At 18 h, cultures previously grown without a source of fixed nitrogen were provided with various concentrations of NO_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 $NaNO_2$
○, 0.2 mM $NaNO_2$
●, Control

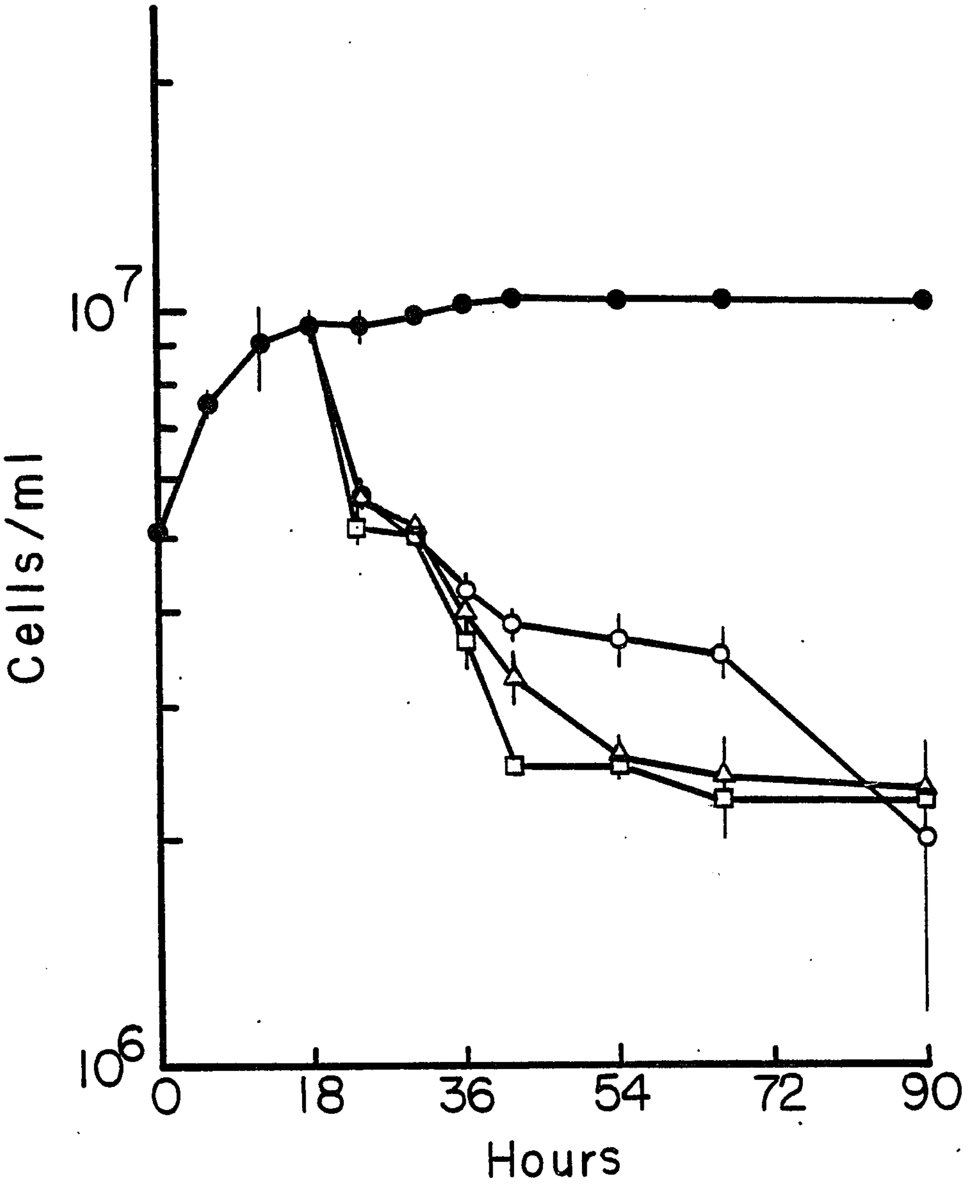

Cells/ml
10^7
10^6
0
18
36
54
72
90
Hours

FIGURE 8. Growth response of <u>A. magnetotacticum</u> to added NH_2OH HCl. At 48 h, cultures previously grown without a source of fixed nitrogen were provided with various concentrations of $NH_2OH \cdot HCl$. 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: △ , 1 mM $NH_2OH \cdot HCl$
□ , 0.5 mM $NH_2OH \cdot HCl$
○ , 0.2 mM $NH_2OH \cdot HCl$
● , Control

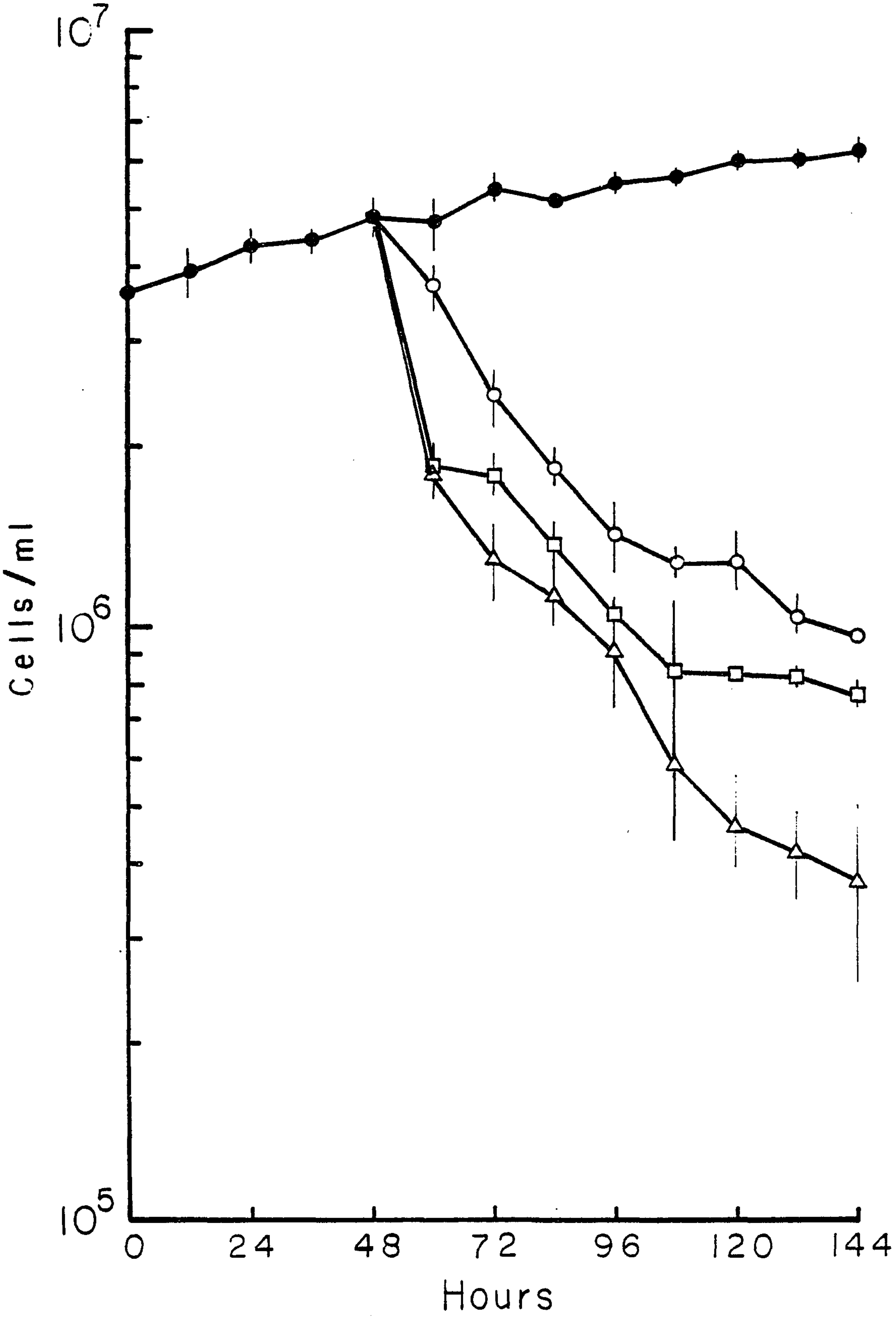

10^7
10^6
10^5
Cells / ml
0
24
48
72
96
120
144
Hours

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

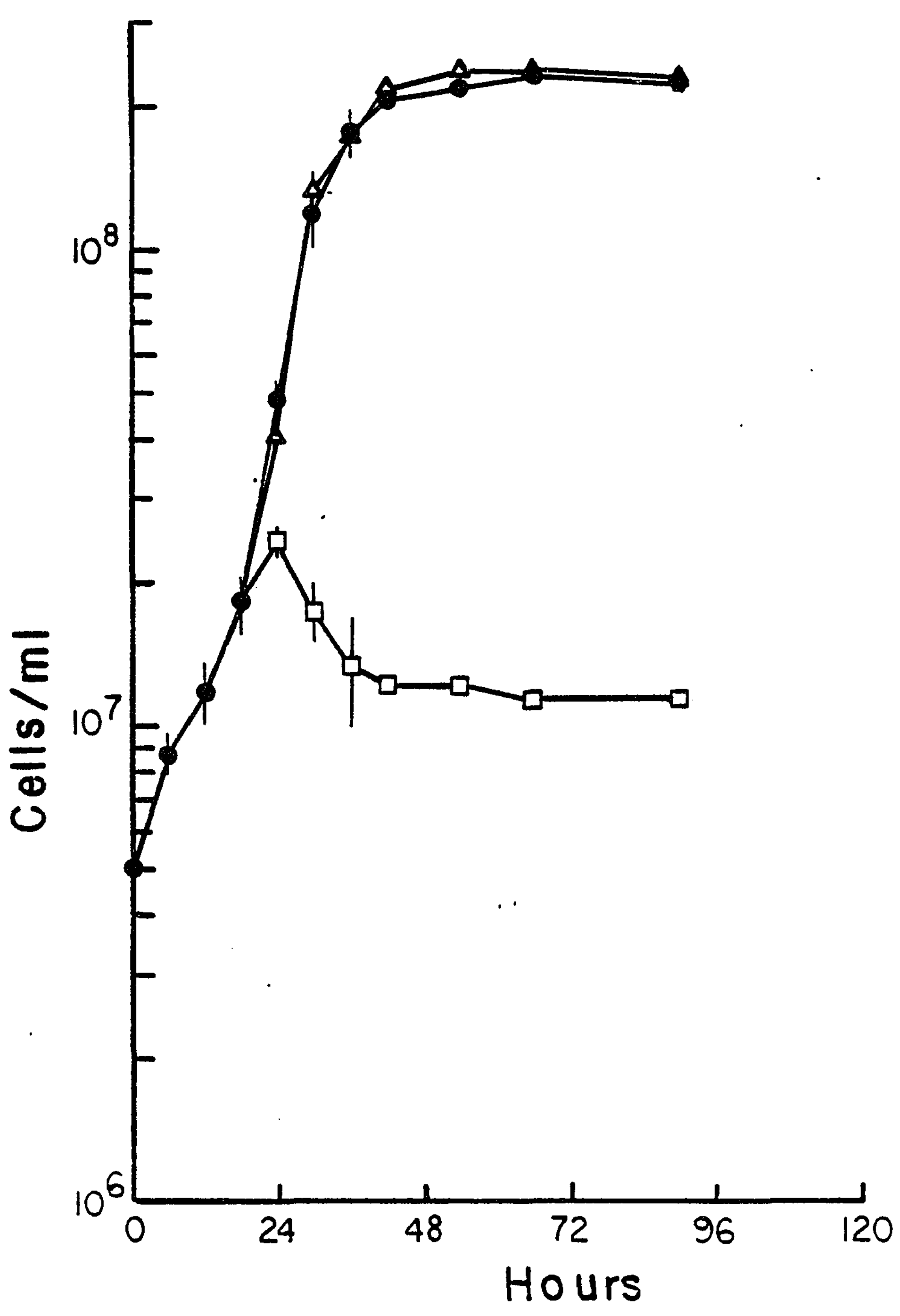

Cells/ml
10^6
10^7
10^8
0
24
48
72
96
120
Hours

FIGURE 10. Effect of C_2H_2 on growth of *A. magnetotacticum* with 2 mM NO_3^-. Cultures initially contained 1 kPa O_2 in the headspace and various concentrations of C_2H_2. Cultures were incubated on a shaker at 30°C. Symbols and bars represent means and standard deviations, respectively, obtained with triplicate cultures.

Symbols: □ , 10 kPa C_2H_2 (0.10 atm)
○ , 1 kPa C_2H_2 (0.01 atm)
● , Control

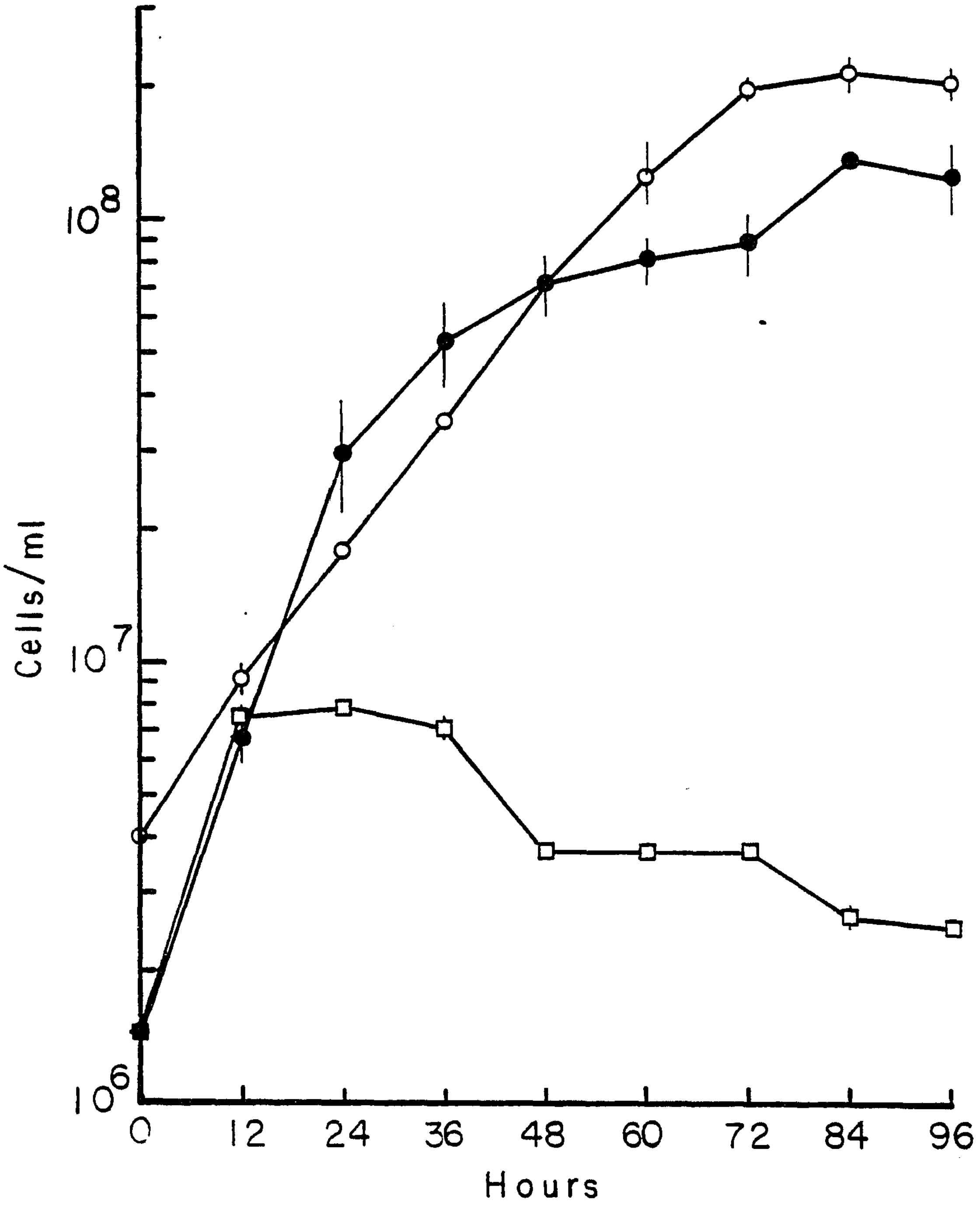
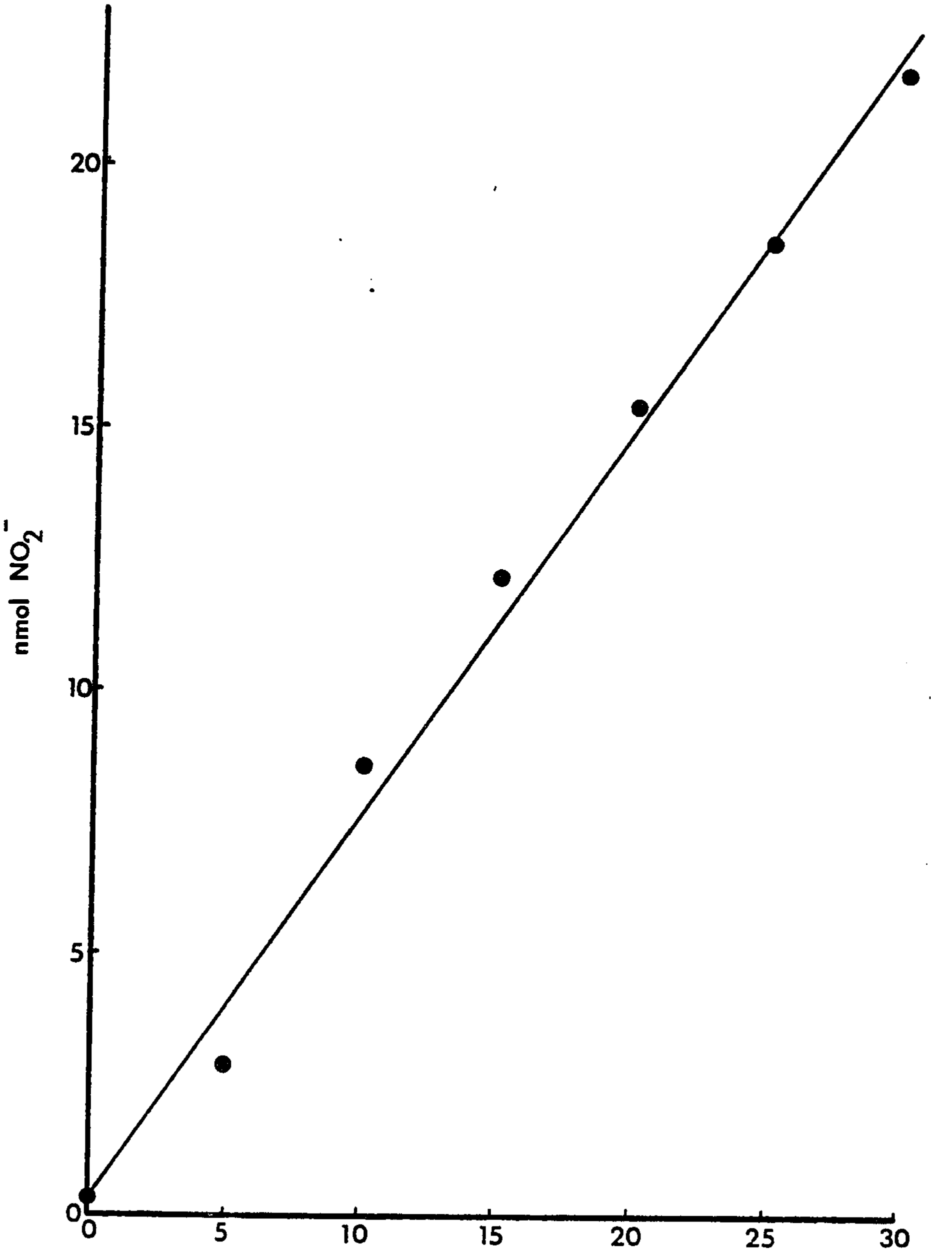
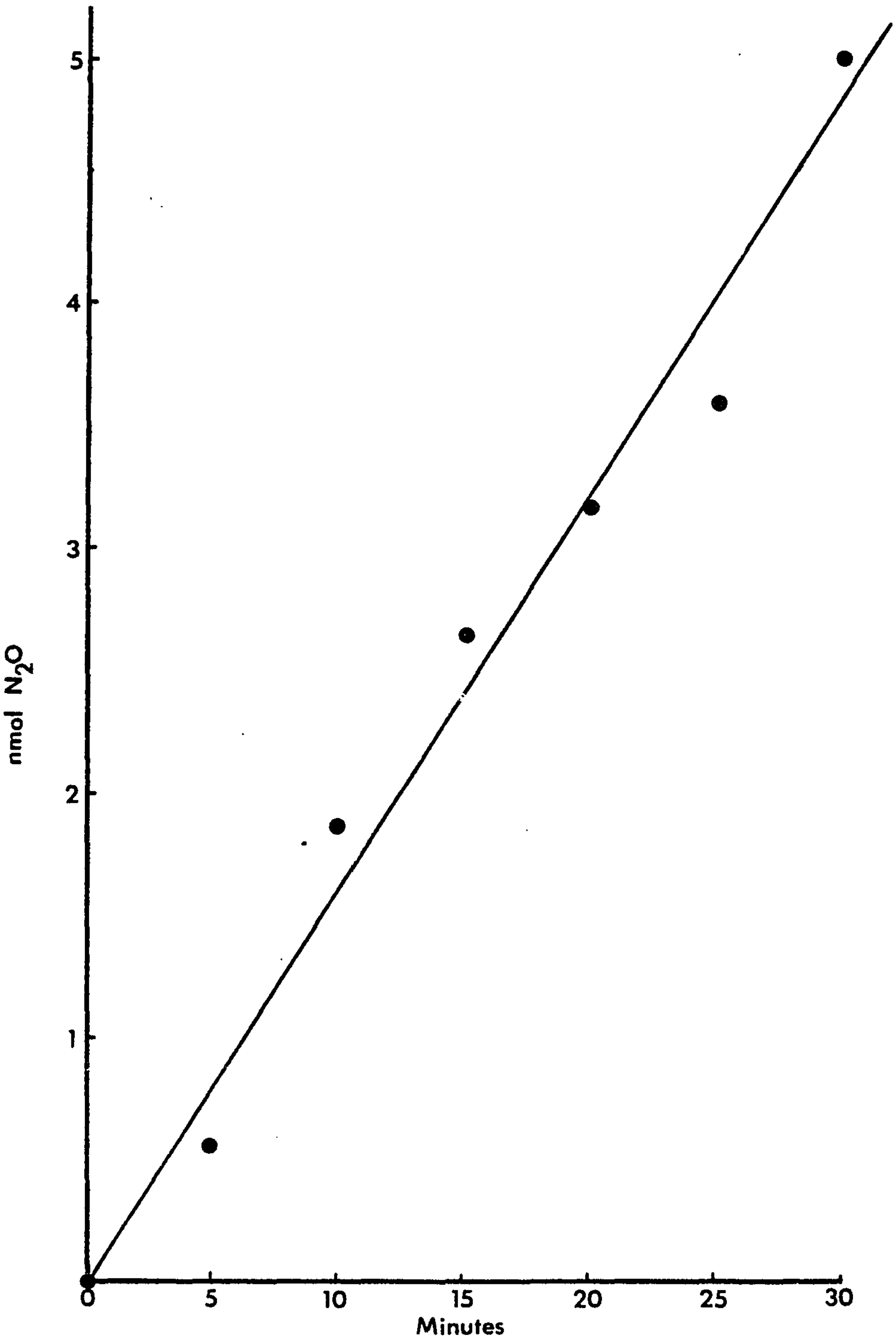

Cells/ml
10^8
10^7
10^6
0
12
24
36
48
60
72
84
96
Hours

FIGURE 11. NO_3^- reductase activity in a cytoplasmic fraction of *A. magnetotacticum* strain MS-1. The reaction mixture contained 8.5 μg of cell extract protein. NO_2^- production proceeded linearly for at least 30 minutes.



nmol NO_2^-
20
15
10
5
0
0
5
10
15
20
25
30
Minutes

FIGURE 12. NO_2^- reductase activity in a membrane fraction of <u>A. magnetotacticum</u> strain MS-1. The reaction mixture contained 5.0 µg cell extract protein. N_2O production proceeded linearly for at least 30 minutes. NO was not detected as a product of NO_2^- reduction by membrane fractions of <u>A. magnetotacticum</u>.



nmol N_2O
1
2
3
4
5
0
5
10
15
20
25
30
Minutes

FIGURE 13. Effect of aeration on Fe^{+3} reduction by a cytoplasmic fraction of <u>A. magnetotacticum</u> 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.

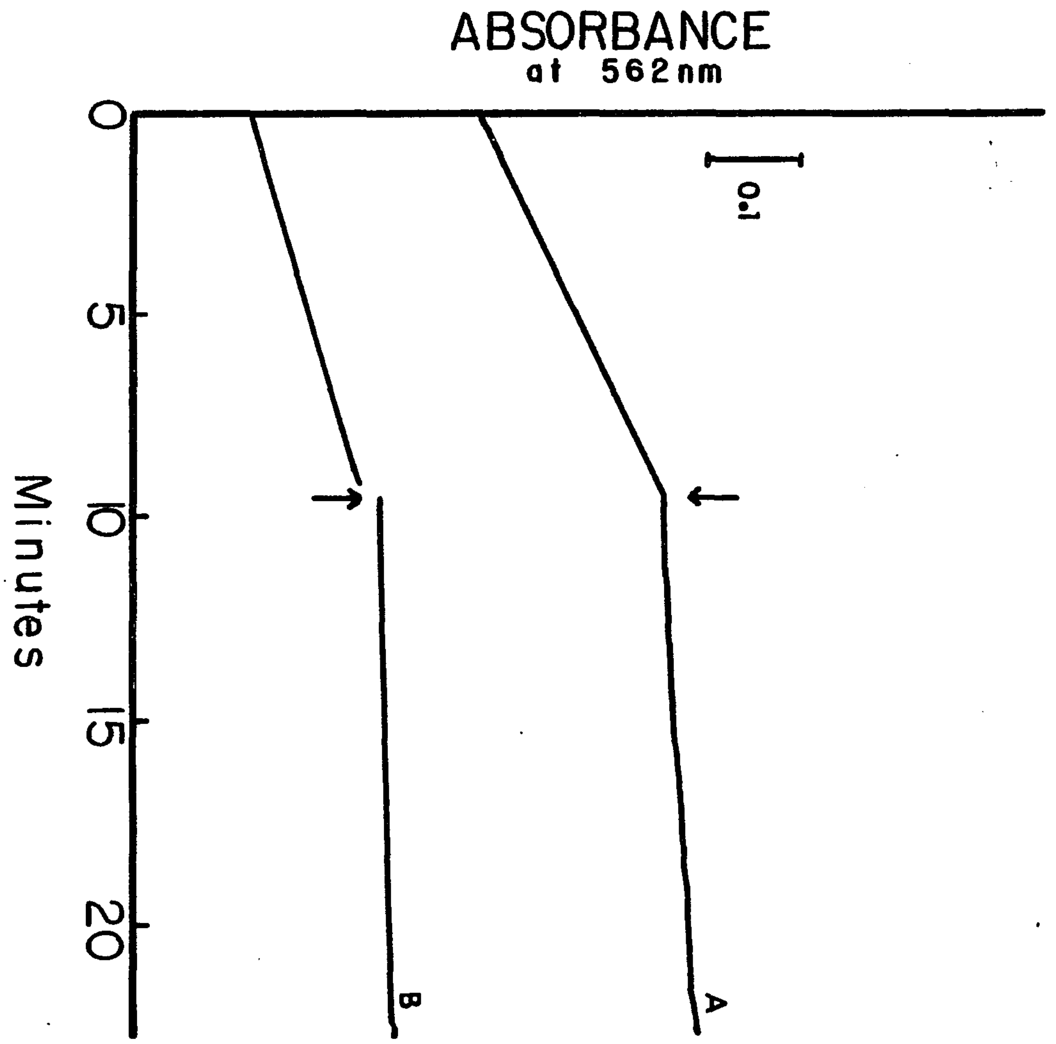
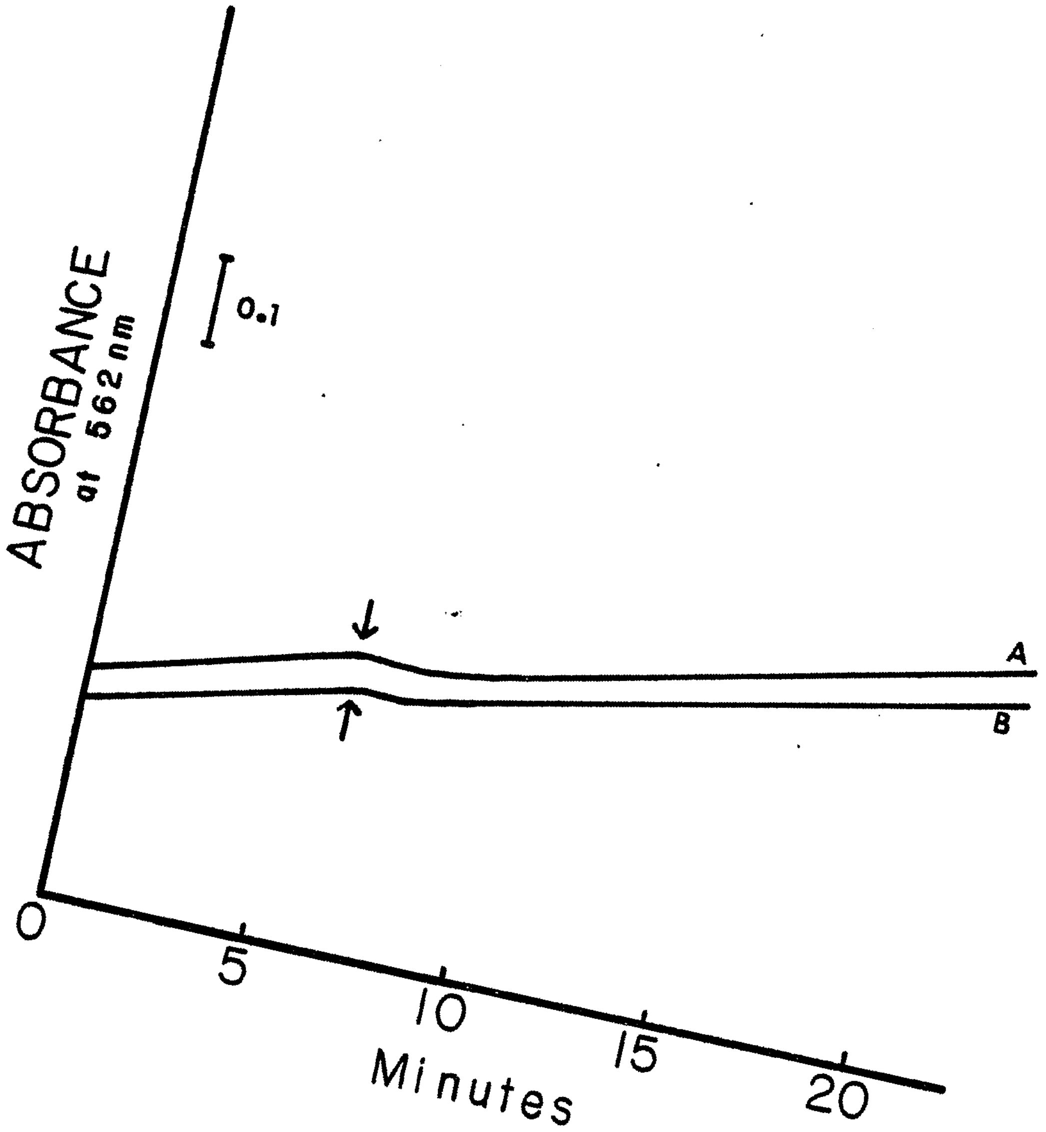

ABSORBANCE
at 562nm
0.1
0
5
10
15
20
Minutes
A
B

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.


ABSORBANCE at 562 nm
0.1
A
B
0
5
10
15
20
Minutes

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 O_2 and NO_3^- (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 NO_3^- or NO_2^- 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 NO_3^- (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 NO_3^- or NO_2^-.

Aquaspirillum magnetotacticum strain MS-1, a magnetic bacterium, is a microaerophilic denitrifier which reduces NO_3^- to N_2, transiently accumulating N_2O but not NO_2^- (Escalante-Semerena et al., 1980; Bazylinski 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 (Bazylinski and Blakemore, 1984). Cell extracts prepared from NO_3^--

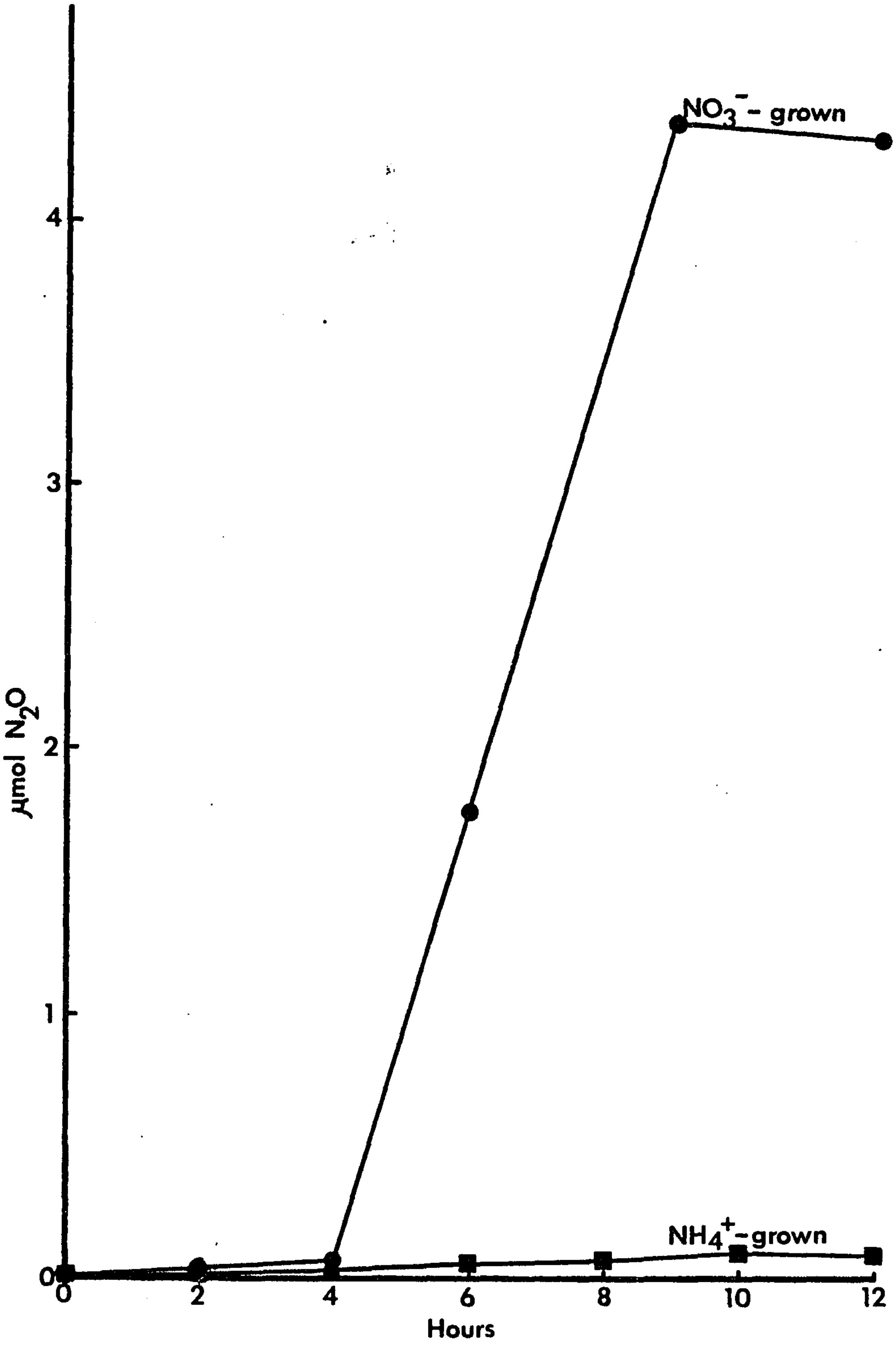
grown cells have nitrate reductase activity similar to that of NH_4^+-grown cells (Bazylinski and Blakemore, 1984). This report describes aspects of denitrification by cell suspensions of A. magnetotacticum.

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_4Cl 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_2O 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_2O 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_2O 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.


NO3⁻- grown
NH4⁺-grown
μmol N2O
0
1
2
3
4
0
2
4
6
8
10
12
Hours

NO_2^- (Table 12), whereas NH_4^+-grown cells produced only small amounts of N_2O (2% of that produced by NO_3^--grown cells) and accumulated NO_2^-. The apparent lag observed with NO_3^--grown cells (Fig. 15) may be due to inhibition by O_2 which may have affected the cells during harvesting. These data indicate that nitrite reduction by A. magnetotacticum requires protein synthesis and the presence of NO_3^-. It is possible that NO_2^- induces nitrite reductase synthesis but this seems unlikely since this toxic intermediate never accumulates to detectable amounts during growth of A. magnetotacticum (Bazylinski and Blakemore, 1983). Dissimilatory nitrate reductase may be a constitutive enzyme in A. magnetotacticum since nitrate reductase acivities measured in extracts of cells grown with NO_3^- or NH_4^+ as the sole nitrogen source were similar (Bazylinski and Blakemore, 1984). Alternately this activity may be derepressed at low Po_2. Unfortunately this latter possibility this is difficult to test with growing cells because this obligate microaerophile will not grow aerobically nor anaerobically even with NO_3^- in the medium (Blakemore et al., 1979; Bazylinski and Blakemore, 1983).

Cyanide or azide at 1 mM inhibited production of N_2O in washed suspensions of NO_3^--grown cells (Table 12). Cells treated with cyanide, however, accumulated NO_2^-. 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_2^- does not accumulate in growing cultures of A. magnetotacticum due to differences in rates of nitrite reductase activity compared to nitrate reductase

TABLE 12. Products of NO_3^- reduction by cell suspensions[a] of *A. magnetotacticum* in the presence of chloramphenicol.

Cells and Treatment	NO_2^-	N_2O
	Total µmol after 12 hours	
NO_3^--grown cells	0	4.4
NH_4^--grown cells	1.2	0.1
NO_3^--grown cells with 1 mM NaCN	0.2	0
NO_3^--grown cells with 1 mM NaN_3	0	0.2
Boiled cells	0	0

[a] Cell concentration was 2.0 x 10^9 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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AFTERWORD

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_2O and thus possess a N_2O 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.