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Nitrogen Fixation (Acetylene Reduction) in *Aquaspirillum magnetotacticum*

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Abstract. *Aquaspirillum magnetotacticum* strain MS-1 and two nonmagnetic 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 m*M* NO_3^- or NH_4^+. These results suggest that *A. magnetotacticum* fixes N_2 only under microaerobic, N-limited conditions.

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 [1a, 7]. This organism is an obligately microaerophilic denitrifier, however, and will not grow anaerobically, even with NO_3^- in the medium [3]. 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^+ [1b]. Under microaerobic conditions, cells utilize NO_3^- or NH_4^+ (but not NO_2^- nor, apparently, N_2O) for growth [1b, 3].

In studies of its nitrogen nutrition, strain MS-1 grew after three sequential passages in semisolid medium lacking a combined N source [3, 10]. 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 [5, 6]. Subsequent studies of C_2H_2 reduction confirmed that this strain fixes N_2 [1a].

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 [20]. Other *Aquaspirillum* species may also, although a comprehensive survey of the genus in this regard is yet to be made [8]. *Azospirillum lipoferum* [21] is the most widely recognized nitrogen-fixing spirillum. It reduces C_2H_2 optimally under microaerobic conditions [15, 16], but is also capable of NO_3^--dependent anaerobic nitrogen fixation (C_2H_2 reduction) [13, 14, 19]. Thus, some bacteria capable of denitrification also fix N_2. These include, in addition to *Azospirillum* [13], certain strains of *Rhizobium* [17, 22], and possibly *Rhodopseudomonas sphaeroides* forma sp. *denitrificans* [18].

We undertook this study to better understand the nitrogen-fixing ability of *A. magnetotacticum* and to compare it with 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 nonmagnetotactic mutants (strains NM-1A and NM-1B) derived from it. Strains NM-1A and NM-1B were subcultures of two aerotolerant colonies that 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

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Table 1. C_2H_2 reduction rates by various N_2-fixing bacteria

Bacterium	nmol $C_2H_4/10^6$ 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.

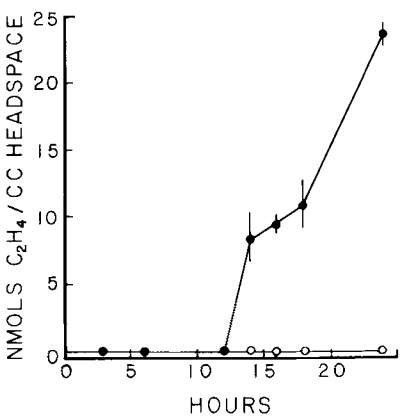

Fig. 1. Effect of O_2 on C_2H_2 reduction during growth of *Aquaspirillum magnetotacticum* strain MS-1. At 0 h, 10% of the culture headspace volume was replaced with C_2H_2. At 12 h, 1 cc of sterile air (●) (final headspace concentration of O_2 = 0.2 kPa) or N_2 gas (○) was added to the culture headspace. Cultures were incubated at 30°C on a rotary shaker. Points and bars represent means and ranges, respectively, of values obtained from duplicate cultures.

Table 2. 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 (nmol $C_2H_4/10^6$ cells/h)
0	0
0.1	0.15
0.2	0.18
0.4	0.014
1.0	0.012
5.0	0

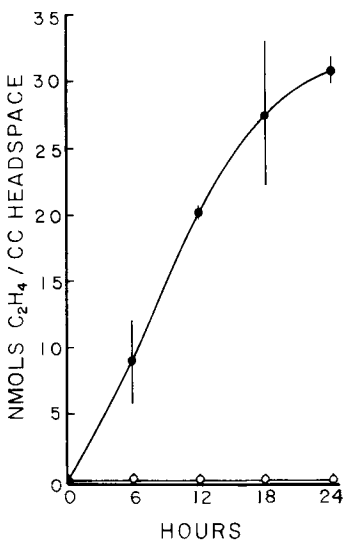

Fig. 2. Effect of NH_4^+ and NO_3^- on C_2H_2 reduction during growth of *Aquaspirillum magnetotacticum* strain MS-1 in culture medium lacking a combined nitrogen source. At 0 h, 10% of the culture headspace volume was replaced with C_2H_2. Cultures were incubated at 30°C on a rotary shaker. Points and bars represent means and ranges, respectively, of values obtained from duplicate cultures. ● = no N source added; ○ = $NaNO_3$ or NH_4Cl (0.2 m*M*) added at inoculation.

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 [3] 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 [14]. *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 [9].

All strains of *A. magnetotacticum* and MS-2 were routinely cultured microaerobically in liquid medium contained in 160-ml serum vials as previously described [1b]. 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.). These 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%–5.0% (vol/vol) O_2 (200–5,000 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 × 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 18–24 h after inoculation. The production of C_2H_4 then ceased, cells became nonmotile, and coccoid bodies appeared. This is consistent with our previous observations, using the "acetylene block" technique to study denitrification, that C_2H_2 at a concentration of 0.1 atm is toxic to growing cells of this organism [1b]. Rates of C_2H_2 reduction by *A. magnetotacticum* and other nitrogen-fixing species are shown in Table 1. 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 nmol C_2H_4 produced 10^6 cells^{-1} h^{-1} (0.2 kPa O_2 in the headspace), the rates shown in Table 1 are more representative and reflect this variability. Similar results were obtained with the nonmagnetotactic 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 2. Cells never reduced C_2H_2 in the absence of O_2, but nitrogenase activity was not observed when the PO_2 was higher than 1 kPa in the headspace. Moreover, as shown in Fig. 1, the introduction of O_2 (PO_2 = 0.2 kPa) into nonfixing 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 Fig. 2. Cells growing under microaerobic conditions did not reduce C_2H_2 when NO_3^- or NH_4^+ (either at 0.2 m*M*) were included in the culture medium. Growing cells did not reduce C_2H_2 anaerobically with 0.2 m*M* NO_3^-.

Discussion

Growing cells of *A. magnetotacticum* reduced C_2H_2 only under microaerobic conditions, but not when 0.2 m*M* 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 m*M* 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, it appears that 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 [5, 6].

We find it interesting that low concentrations (0.2 m*M*) of NO_3^- did not support C_2H_2 reduction. In fact, it was inhibitory at the concentrations used successfully by others [14, 19]. This suggests that sufficient energy may not be conserved to supply the requirement for nitrogenase activity with NO_3^- as a terminal electron acceptor. We previously showed, however, that increased amounts of NO_3^- resulted in higher final growth yields of this organism [1b]. 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 [1b].

Denitrifying strains of *Azospirillum lipoferum* couple to N_2 fixation the reduction of NO_3^- to

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) [19]. Moreover, these strains all accumulate NO_2^- transiently during denitrification [13, 14, 19]. 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 m*M* NH_4^+ was included in the growth medium. Repression of nitrogenase by NH_4^+ or other reduced nitrogenous compounds is well recognized [5, 6]. 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 m*M* NO_3^-, or 0.2 m*M* 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 [14, 17, 19, 22]. Diverse types of magnetotactic bacteria live in microaerobic and N-limited habitats [2, 4, 12]. 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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