Diazotrophy and Nitrogenase Activity in the Archaebacterium

* Methanosarcina barkeri 227 *

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Nitrogen fixation (diazotrophy) has recently been demonstrated in several methanogenic archaebacteria. To compare the process in an archaebacterium with that in eubacteria, we examined the properties of diazotrophic growth and nitrogenase activity in *Methanosarcina barkeri* 227. Growth yields with methanol or acetate as a growth substrate were significantly lower in N₂-grown cultures than in NH₄⁺-grown cultures, and the culture doubling times were increased, indicating that diazotrophy was energetically costly, as it is in eubacteria. Growth of nitrogen-fixing cells was inhibited when molybdenum was omitted from the medium; addition of 10 nM molybdate stimulated growth, while 1 μM molybdate restored maximum diazotrophic growth. Omission of molybdenum did not inhibit growth of ammonia-grown cells. Tungstate (100 μM) strongly inhibited growth of molybdenum-deficient diazotrophic cells, while ammonia-grown cells were unaffected. The addition of 100 nM vanadate or chromate did not stimulate diazotrophic growth of molybdenum-starved cells. These results are consistent with the presence of a molybdenum-containing nitrogenase in *M. barkeri*. Acetylene, the usual substrate for assaying nitrogenase activity, inhibited methanogenesis by *M. barkeri* and consequently needed to be used at a low partial pressure (0.3% of the headspace) when acetylene reduction by whole cells was assayed. Whole cells reduced 0.3% acetylene to ethylene at a very low rate (1 to 2 nmol h⁻¹ mg of protein⁻¹), and they “switched off” acetylene reduction in response to added ammonia or glutamine. Crude extracts from diazotrophic cells reduced 10% acetylene at a rate of 4 to 5 nmol of C₂H₂ formed h⁻¹ mg of protein⁻¹ when supplied with ATP and reducing power, while extracts of *Klebsiella pneumoniae* prepared by the same procedures had rates 100-fold higher. Acetylene reduction by extracts required ATP and was completely inhibited by 1 mM ADP in the presence of 5 mM ATP. The low rates of C₂H₂ reduction could be due to improper assay conditions, to switched-off enzyme, or to the nitrogenase’s having lower activity towards acetylene than towards dinitrogen.

Until recently, the property of biological nitrogen fixation (diazotrophy) was thought to be the exclusive domain of the eubacteria (18). The discovery of diazotrophy among methanogens (1, 4, 15, 16, 20), members of the Urkingdom Archaeabacteria, raises questions about the origin and evolution of the nitrogen-fixing system: did diazotrophy originate before or after the divergence of the archaeabacteria and eubacteria, or was it transferred from one Urkingdom to the other?

Nitrogen fixation among the eubacteria is widespread, yet the process of diazotrophy is highly conserved for molybdenum nitrogenases (18). In all eubacterial diazotrophs studied so far, the nitrogenase reaction (the reduction of dinitrogen to ammonia) requires the two components of the nitrogenase enzyme complex (dinitrogenase and dinitrogenase reductase), ATP, and a reductant. Besides dinitrogen, nitrogenases can reduce other triple-bonded substrates, such as acetylene, azide, nitrous oxide, and cyanide and other nitriles. Nitrogenase can also reduce protons to dihydrogen (9).

Since molybdenum is a component of the nitrogenase enzyme, diazotrophs typically have a higher requirement for this trace element (18). Tungsten is antagonistic to diazotrophic growth because it is an analog of molybdenum function in vivo. The exception to these characteristics is the alternative vanadium nitrogenase purified from *Azotobacter chroococcum*, in which the dinitrogenase component contains vanadium in place of molybdenum (19). A third *Azoto-

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concentrations: NaHCO₃, 1 g/liter; CaCl₂ · 2H₂O, 0.06 g/liter; Na₂S · 9H₂O, 0.4g/liter; methanol, 100 mM, or sodium acetate, 40 mM (as carbon and energy sources); and NH₄Cl (for experiments requiring ammonia-growing cells), 10 mM. A fresh mid-log-phase (40 to 50 mM methane produced from 100 mM methanol) culture was used as the inoculum (0.5% [vol/vol]). Substrate consumption was followed by measuring methane production by cultures. Typically, a second addition of 100 mM methanol was made, and cells were harvested when they had produced 70 to 100 mmol of CH₄ per liter of culture medium.

For the trace metal requirement experiments, the medium was as described above except that molybdenum was omitted from the trace metal solution and ultrapure water was used. The purified water was measured to have a resistance of 18 MΩ by the conductivity meter of the Water I System (Gelman Sciences, Inc., Ann Arbor, Mich.). All glassware used was rinsed with concentrated sulfuric acid and ultrapure water. The molybdenum-free medium was dispensed in 10-ml amounts into 27-ml tubes with butyl rubber aluminum-crimp seals (Belco Biotechnology, Vineland, N.J.) inside an anaerobic glove box. Sodium molybdate, sodium tungstate, sodium vanadate, vanadyl sulfate, and potassium chromate were added as sterile anaerobic stock solutions when required. Growth in tubes was measured as optical density at 600 nm with a Sequouia-Turner model 340 spectrophotometer (Sequouia-Turner Corp., Mountain View, Calif.). As a result of the precautions taken to minimize metal contamination, the results obtained were repeatable over three separate experiments.

Klebsiella pneumoniae M5a1 was obtained from J. Noti and grown under nitrogen-fixing conditions in the medium described by Yoch and Pengra (24). The cells were grown at 37°C in 1-liter bottles containing 500 ml of medium under a headspace of N₂.

Gas chromatography. Methane was determined by using a Gow-Mac 550 thermal conductivity gas chromatograph (Gow-Mac Instrument Co., Bound Brook, N.J.), operated under the following conditions: carrier gas, He; flow rate, 50 ml/min; column, copper (1 m by 2 mm [inner diameter]) packed with 120/140 Carbosieve S (Supelco, Inc., Bellefonte, Pa.); column temperature, 100°C. Acetylene and ethylene were quantitated by using a Varian 2400 series gas chromatograph (Varian Instrument Group, Walnut Creek, Calif.) with an H₂ flame ionization detector operated as follows: detector temperature, 110°C; N₂ carrier gas, 30 ml/min; column, Teflon (2 m by 3 mm [inner diameter]) packed with 80/100 Porapak R (Supelco); column temperature, 40°C.

Cell yield determinations. Final cell dry weight was measured by filtering 10 ml of uniformly suspended cells from a culture which had produced 25 to 30 mM methane from 50 mM methanol or 40 mM acetate through a polycarbonate membrane filter (0.4-μm pore size; Nucleopore Corp., Pleasanton, Calif.). The filters were dried for about 48 h in a desiccator at 50°C with silica gel (6-16 mesh; Fisher Scientific Co., Rochester, N.Y.) as a desiccant. The filters were then weighed in a Mettler analytical balance (Mettler Instrument Co., Hightstown, N.J.). The yield was calculated as the dry weight of cells (in grams) per mole of methane produced.

Acetylene reduction assays. In vivo acetylene reduction experiments were done with 10 ml of a mid-logarithmic-phase culture incubated in 37-ml serum vials sealed with butyl rubber stoppers. The vials were first flushed with argon, and then enough anaerobic NaHCO₃, 10% on and HCl were added by syringe to generate about 5 r of CO₂ into the headspace. Additional sulfide and methanol were added as anaerobic solutions to final concentrations of 0.5 g/liter and 100 mM, respectively. The cells were added, and the overpressure inside the vials was released by piercing the stopper for 1 to 2 s with a needle. Acetylene, generated by the hydration of calcium carbide in an evacuated 125-mi serum vial, was then added to the percentage of the headspace indicated. For the switch-off experiments, other additions were made from anaerobic stock solutions. The vials were incubated at 37°C. The glutamine solution used in the switch-off experiments was assayed for contaminating ammonia by collecting the ammonia in a microdiffusion apparatus and quantifying it colorimetrically by the indophenol method, as described by Burris (5). With this procedure, as little as 10 nmol of ammonia present in the sample could be detected accurately.

In vitro acetylene reduction assays were carried out by a procedure based on those developed for other nitorgenase systems (5, 6). One liter of cells from mid-logarithmic-phase cultures was harvested by centrifugation at 6,000 × g inside an anaerobic glove box. The cells were washed once and suspended in Ar-sparged 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (Sigma Chemical Co., St. Louis, Mo.), pH 7.5, containing 1 mM sodium dithionite (Sigma) and 0.1 g of dithiothreitol (Bio-Rad Laboratories, Richmond, Calif.) per liter. The cell suspension (10 ml) was loaded into a French pressure cell inside the glove box. The cells were broken at 20,000 lb/in², and the extract was collected in an argon-flushed vial. Cell debris was removed by centrifugation at 6,000 × g inside the glove box. Extracts prepared in this manner generally contained 3 to 5 mg of protein per ml as estimated by the Coomassie brilliant blue method with reagents purchased from Bio-Rad, with lysozyme (Sigma) as a standard. The assay was done in 11-ml serum vials flushed with argon and sealed with butyl rubber stoppers. The reaction mixture (1 ml total volume) consisted of 5 mM ATP, 12.5 mM MgCl₂ · 2H₂O, 40 mM creatine phosphate (Sigma), 0.1 mg of creatine kinase (Sigma), and 2.5 or 20 mM sodium dithionite, in 25 mM HEPES (pH 7.5) (6). Acetylene was added to the headspace in the desired partial pressures, and approximately 1 mg of protein was added to start the reaction. The vials were incubated at 37°C.

During incubation of the assays, gas samples were taken by using a 1-ml Perfektum tuberculin syringes (Popper & Sons, Inc., New Hyde Park, N.Y.) fitted with Mininert syringe valves (Supelco) to maintain gas pressure. Teflon tape was wrapped around the Luer tip of each syringe to ensure a tight fit between the syringe and the valve.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was done by the method of Laemmli (13), with 10% (wt/vol) acrylamide and 0.26% (wt/vol) bisacrylamide in 1.5-mm-thick slabs.

### TABLE 1. Effect of diazotrophy on growth rates and yields of M. barkeri grown in batch culture on methanol or acetate

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>N source</th>
<th>(g/mol)</th>
<th>Doubbling time (h)</th>
</tr>
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<tbody>
<tr>
<td>Methanol</td>
<td>NH₄⁺</td>
<td>3.7 ± 0.6</td>
<td>14</td>
</tr>
<tr>
<td>Acetate</td>
<td>NH₄⁺</td>
<td>2.8 ± 0.4</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>N₂</td>
<td>1.2 ± 0.5</td>
<td>72</td>
</tr>
</tbody>
</table>

*Y(CH₄)* is defined as grams (dry weight) of cells per mole of CH₄ produced. Values are the average of three separate experiments ± standard deviation.
RESULTS

Effect of diazotrophy on growth yields and rates. The effect of nitrogen fixation on the growth of *M. barkeri* was examined by measuring growth rates and yields of batch cultures with \( \text{N}_2 \) or \( \text{NH}_4^+ \) as a nitrogen source and with methanol or acetate as the growth substrate (Table 1). A significant decrease of growth yield accompanied by an increase in the culture doubling time was seen under nitrogen-fixing conditions with either methanol or acetate.

Trace metal requirements for diazotrophy. The effect of adding molybdate, tungstate, or vanadate to the culture medium was investigated for growth of *M. barkeri* with \( \text{N}_2 \) or \( \text{NH}_4^+ \) as a nitrogen source (Fig. 1). The inoculum for these experiments was grown under nitrogen-fixing conditions with no added molybdate to eliminate carryover. Diazotrophic growth was inhibited by molybdenum starvation, while growth with ammonia was unaffected (Fig. 1A). Diazotrophic growth in medium with no molybdenum added was considerably less than that in medium with added molybdenum and was most likely due to trace contamination with molybdenum. Adding 10 nM sodium molybdate stimulated growth, and 1 \( \mu \text{M} \) sodium molybdate restored maximum growth. Tungstate (100 \( \mu \text{M} \)) severely inhibited growth of Mo-starved *M. barkeri* with \( \text{N}_2 \) but had no effect on growth with \( \text{NH}_4^+ \) (Fig. 1B). Diazotrophic growth was not affected in the presence of 1 \( \mu \text{M} \) tungstate, and the effect of 10 \( \mu \text{M} \) tungstate varied (data not presented). Vanadate could not replace molybdate in restoring maximum diazotrophic growth of Mo-starved *M. barkeri* (Fig. 1C), while growth with ammonia was not stimulated or inhibited by vanadate. Neither vanadyl sulfate nor potassium chromate at 100 nM or 1 \( \mu \text{M} \) stimulated diazotrophic growth (data not presented).

Acetylene reduction by whole cells. Nitrogenase activity in...
whole cells is typically assayed by measuring acetylene reduction to ethylene (18). However, acetylene is a well-known inhibitor of methanogenesis (1, 17, 21). Similar to other investigators (1, 17, 21), we found that acetylene partial pressures greater than 1% of the headspace (0.01 atm [ca. 1 kPa] partial pressure) completely inhibited methanogenesis (data not presented) so that the partial pressure of 10% acetylene which is usually prescribed for the acetylene reduction assay (6) could not be used. M. barkeri was able to reduce acetylene to ethylene at lower partial pressures in vivo despite the inhibition of methanogenesis by acetylene. Ethylene production from various partial pressures of acetylene by dinitrogen-grown M. barkeri is shown in Fig. 2. It was determined that 0.3% acetylene in the headspace was optimal for ethylene formation. From these experiments it was determined that dinitrogen-grown M. barkeri cells reduced acetylene at a rate typically near 1.4 nmol of C$_2$H$_4$ produced h$^{-1}$ mg of protein$^{-1}$. Under 0.3% acetylene, the increase in acetylene reduction paralleled an increase in methane formation, indicating that the cells grew during the course of the assay (data not presented). This may account for the apparent increase in acetylene reduction rate that occurred during extended incubation. Ammonia-grown cells did not reduce acetylene (data not presented).

**Switch-off of acetylene reduction by whole cells.** The in vivo acetylene-reducing activity of M. barkeri was used to examine its ability to switch off nitrogenase in response to the availability of fixed nitrogen. The production of ethylene from acetylene by M. barkeri cell suspensions was halted within 2 h (the minimum time we could measure a significant effect) by the addition of 5 mM ammonium chloride or 5 mM glutamine, compared with those which received no added fixed nitrogen (Fig. 3). We could not detect free ammonia in the glutamine solution used in this experiment. The presence of 1 mM methionine sulfoximine had no effect on acetylene-reducing activity or on the switch-off by ammonia (data not presented).

**Acetylene reduction by cell extracts.** The ability of cell extracts of M. barkeri, prepared by standard procedures for the nitrogenase assay (6), to reduce acetylene was tested. The extracts reduced acetylene to ethylene at a constant rate at both 10% and 0.3% acetylene, with a greater rate at 10% acetylene (Table 2). Extracts from ammonia-grown cells did not reduce acetylene at any concentration tested (data not presented). When the ATP-regenerating system was omitted from the assay mixture (Fig. 4), the rate of acetylene reduction began to level off. Ethylene production was completely inhibited by 1 mM ADP in the presence of 5 mM ATP.

Table 2 shows a comparison of acetylene reduction rates by extracts of M. barkeri with those of Klebsiella pneumoniae prepared and measured under identical conditions. M. barkeri extracts reduced acetylene at rates comparable to those in whole cells. Lowering the dithionite concentration of the assay mixture increased activity somewhat, but it remained approximately 100-fold lower than rates measured for extracts of K. pneumoniae. Adding different amounts of methanogen extract to the assay mixture had no effect on the rate of acetylene reduction by K. pneumoniae extracts (data not presented).

**SDS-polyacrylamide gel electrophoresis.** SDS-polyacrylamide gel electrophoresis was used to detect polypeptides specific to dinitrogen-grown M. barkeri. The dinitrogen-grown cell extracts showed major polypeptide bands at 55

![FIG. 2. Ethylene production from various partial pressures of acetylene by whole cells of dinitrogen-grown M. barkeri.](image)

**TABLE 2. Rates of acetylene reduction by crude cell extracts of M. barkeri and K. pneumoniae.**

<table>
<thead>
<tr>
<th>Extract and relevant reaction conditions</th>
<th>Reduction rate$^a$ (nmol/h)</th>
</tr>
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<tbody>
<tr>
<td>M. barkeri</td>
<td></td>
</tr>
<tr>
<td>20 mM dithionite, 10% C$_2$H$_4$</td>
<td>1.4–1.7</td>
</tr>
<tr>
<td>20 mM dithionite, 0.3% C$_2$H$_4$</td>
<td>0.4–0.7</td>
</tr>
<tr>
<td>2.5 mM dithionite, 10% C$_2$H$_4$</td>
<td>4.9–5.5</td>
</tr>
<tr>
<td>K. pneumoniae, 20 mM dithionite, 10% C$_2$H$_4$</td>
<td>450</td>
</tr>
</tbody>
</table>

$^a$ Nanomoles of C$_2$H$_4$ produced per hour per milligram of protein.
and 26 kilodaltons on 10% acrylamide gels which were not found in ammonia-grown extracts (Fig. 5). A fainter band near 32 kilodaltons was seen in the lane from diazotrophically grown cells in Fig. 5, but was not seen in several other preparations. No bands unique to diazotrophy other than the 55-kilodalton band were seen in the 50- to 60-kilodalton region of gels from dinitrogen-grown extracts.

**DISCUSSION**

Certain aspects of diazotrophy in *M. barkeri* were similar to those in its eubacterial counterparts. The stimulation of growth on dinitrogen by molybdenum and the inhibition by tungsten in *M. barkeri* were characteristics typical of conventional diazotrophs, indicating the participation of a molybdenoprotein in the nitrogenase of *M. barkeri*. The lack of acetylene-reducing activity in ammonia-grown cells and the disappearance of putative nitrogenase bands in SDS gels of crude extracts of NH₄⁺-grown cells is consistent with repression of nitrogenase in *M. barkeri*, as is generally seen in free-living eubacterial diazotrophs (18). A switch-off phenomenon similar to that observed in other diazotrophs was indicated by the rapid cessation of acetylene reduction by cells after the addition of ammonia or glutamine. Unlike other diazotrophs which can switch off nitrogenase activity (22), *M. barkeri* could not be made to override the switch-off by adding the glutamine synthetase inhibitor methionine sulfoximine. However, the cells may not have been able to take up this compound. Another similarity with the eubacterial system was inhibition of acetylene reduction by extracts by ADP, even in the presence of ATP (18).

This evidence of tight regulation of nitrogenase along with the significant reduction of growth rates and yields by diazotrophy indicates that nitrogen fixation is an energetically costly process for *M. barkeri*, as is the case for eubacterial diazotrophs. We were unable to estimate from the growth yield data in batch cultures the number of moles of ATP required to fix 1 mol of N₂ (12), since the number of moles of ATP per mole of CH₄ for methanol or acetate is not known, and such determinations are better made on chemostat cultures growing at identical rates to eliminate the effects of maintenance energy (12). Diazotrophic growth on acetate was especially slow, perhaps because acetate provides less energy than methanol and is a poorer reductant (25).

*M. barkeri* is similar to other diazotrophic methanogens in the low rates at which acetylene is reduced by both whole cells and crude extracts (1, 4, 15). Since acetylene has been shown to inhibit methanogenesis and to lower ATP pools in methanogens (21), we hypothesized that the acetylene reduction rates in crude extracts might be much higher than in whole cells, since this inhibition by acetylene would be obviated by providing ATP and reducing power in vitro. Also, acetylene could be used at 10% rather than at 0.3%, as it was for whole-cell assays. Even under those conditions, however, the rates of acetylene reduction remained low, and the rate at 0.3% acetylene in whole cells and cell extracts was quite similar.

This low acetylene reduction rate suggests at least two possibilities: (i) the assay conditions were unfavorable (including the possibility that the extracts contained inactive or switched-off enzyme); and (ii) acetylene is a poor substrate for the *M. barkeri* nitrogenase. The vanadium nitrogenase purified from *A. chroococcum* reduces acetylene at a low rate compared with dinitrogen or protons (19), as do *A. chroococcum* and *A. vinelandii* grown under conditions for expression of the alternative nitrogenase (2, 7). This is in contrast with eubacterial molybdenum nitrogenases, which typically reduce acetylene and dinitrogen at nearly the same rate per electron (9). Another hallmark of the alternative nitrogenases is their higher ATP-dependent hydrogen-evolving hydrogenase activity (19). Our attempts at assaying this activity were hampered by the very high background.

**FIG. 4.** Effect of omission of ATP or the ATP-regenerating system from the assay mixture or of the addition of ADP on ethylene production from 10% acetylene by cell extracts of *M. barkeri*. The dithionite concentration of the assay mixture was 2.5 mM.

**FIG. 5.** SDS-polyacrylamide gel electrophoresis protein profiles of *M. barkeri* extracts from cells grown with dinitrogen (N₂) and ammonia (NH₄⁺). Numbers indicate locations of molecular size standards (in kilodaltons), and large arrows indicate major bands unique to dinitrogen-grown extracts.
hydrogenase activity in \textit{M. barkeri} crude extracts (data not presented). The measured rate for acetylene reduction by crude cell extracts, 5 nmol of \( \text{C}_2\text{H}_2 \) produced h\(^{-1}\) mg of protein\(^{-1}\), is less than the rate we estimated would be required to provide the cells with fixed nitrogen during growth on methanol, the equivalent of ca. 1,000 nmol of \( \text{C}_2\text{H}_2 \) produced h\(^{-1}\) mg of protein\(^{-1}\).

SDS-polyacrylamide gel electrophoresis consistently revealed two bands which were unique to diazotrophy in \textit{M. barkeri}. If these bands represent polypeptides which are analogous to subunits of the nitrogenase proteins from eubacterial diazotrophs, then some differences exist between the two in band patterns. The dinitrogen component of the eubacterial nitrogenase shows two bands on SDS gels at about 55 and 60 kilodaltons. Only one diazotroph-specific band could be seen in this region from \textit{M. barkeri} extracts, but the presence of other heavily staining bands may have obscured a second band in this region. It is also possible that there is only one subunit type for this component or that the two subunits have about the same molecular weight in \textit{M. barkeri}. Regarding this possibility, there is evidence that the genes which code for the \( \alpha \) and \( \beta \) subunits of dinitrogenase (nifD and nifK) from \textit{Rhizobium japonicum} originated from the same gene (23). The band at 26 kilodaltons in the \textit{M. barkeri} preparation may represent a subunit of the dinitrogen reductase component of nitrogenase. If so, it is significantly smaller than the 35-kilodalton polypeptide characteristic of eubacterial diazotrophs (18). A similar pattern of polypeptide bands was seen in extracts of nitrogen-fixing \textit{Methanococcus thermolithothrophicus} (15). Two bands, at 29 and 55 kilodaltons, were seen in extracts of dinitrogen-grown cells, and the 29-kilodalton band reacted with antidinitrogenase reductase antibodies in Western blots (immunoblots).

Examining the differences between diazotrophy in methanogens and in eubacteria may lead to a greater understanding of the process. The nitrogen-fixing system in \textit{M. barkeri} resembles typical eubacterial molybdenum nitrogenases in trace metal requirements, energy consumption, and regulation of activity and synthesis. With regard to activity, however, it may share characteristics with the alternative vanadium-based nitrogenases of \textit{Azobacter} spp. We are presently examining this possibility.

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**LITERATURE CITED**