

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-01881a REPORT SECURITY CLASSIFICATION
(U)1b RESTRICTIVE MARKINGS
NA

2a SECURITY CLASSIFICATION AUTHORITY

NA
DECLASSIFICATION/DOWNGRADING SCHEDULE
NA3 DISTRIBUTION/AVAILABILITY OF REPORT
Distribution Unlimited

FORMING ORGANIZATION REPORT NUMBER(S)

5 MONITORING ORGANIZATION REPORT NUMBER(S)
NA

NAME OF PERFORMING ORGANIZATION

Cornell University

6b OFFICE SYMBOL
(If applicable)
NA7a. NAME OF MONITORING ORGANIZATION
Office of Naval Research

ADDRESS (City, State, and ZIP Code)

Dept of Microbiology, Stocking Hall
Ithaca NY 14853

7b ADDRESS (City, State, and ZIP Code)

800 N. Quincy St.
Arlington VA 22217-5000NAME OF FUNDING/SPONSORING
ORGANIZATION

Office of Naval Research

6b OFFICE SYMBOL
(If applicable)
ONR

9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER

N00014-86-U-0781

ADDRESS (City, State, and ZIP Code)

800 N. Quincy Street
Arlington VA 22217-5000

10 SOURCE OF FUNDING NUMBERS

PROGRAM
ELEMENT NOPROJECT
NOTASK
NOWORK UNIT
ACCESSION NO

NR441d009

11. TITLE (Include Security Classification)

The Nitrogenase in a Methanogenic Archaeobacterium and its Regulation

12. PERSONAL AUTHOR(S)

Zinder, Stephen H.

13a. TYPE OF REPORT

Final

13b TIME COVERED

FROM 9/86 TO 3/89

14 DATE OF REPORT (Year, Month, Day)

February 2, 1990

15 PAGE COUNT

7

16 SUPPLEMENTARY NOTATION

17 COSATI CODES

FIELD

GROUP

SUB-GROUP

18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)

Archaeobacteria, nitrogen fixation, regulation,
nutrition, ENZYMES, BACTERIOLOGY, Microbiology,
DIAZOTROPHIC GROWTH

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

The objectives of this project are to gain an understanding of nitrogen fixation in the archaeobacterium *Methanosarcina barkeri* strain 227, and to compare the process with that in eubacteria. Nitrogen fixation is energetically costly to strain 227, as it is to eubacteria. Diazotrophic growth was stimulated by Mo and inhibited by W , suggesting that the nitrogenase is a molybdoenzyme. We have purified the nitrogenase 30-40-fold, and have found that it is a two-component enzyme, as in eubacteria. Crude extracts and purified enzyme preparations show very low acetylene reducing activity and N_2 is used preferentially to acetylene, similar to certain alternative nitrogenases in eubacteria. The component 2-analogue in strain 227 is absent in Western blots from ammonia-grown cells, consistent with ammonia repression of nitrogenase. Cells also switched-off acetylene reducing activity when exposed to ammonia at concentrations as low as $10 \mu\text{M}$. No evidence for ADP-ribosylation or other modification of the iron component analogue was obtained in Western blot analysis of switched-off cells. The regulation of synthesis and activity of nitrogenase in strain 227 makes it one of the most highly regulated enzyme systems known in the methanogenic archaeobacteria.

molybdenum

tungsten

nitrogen

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT

☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION

U

22a. NAME OF RESPONSIBLE INDIVIDUAL

M. Marron

22b. TELEPHONE (Include Area Code)

202/696-4760

22c. OFFICE SYMBOL

ONR

AD-A218 346

PROGRESS REPORT ON CONTRACT N00014-86-K-0781

R&T CODE: 441d009--02

PRINCIPAL INVESTIGATOR: Stephen H. Zinder

CONTRACTOR: Cornell University

CONTRACT TITLE: The Nitrogenase in a Methanogenic Archaeobacterium and its Regulation.

START DATE: 1 September 1986

RESEARCH OBJECTIVE: We were one of the laboratories to discover nitrogen fixation in methanogenic archaeobacteria (1). Our goals were to characterize nitrogen fixation and nitrogenase in *Methanosarcina barkeri* strain 227 and regulation of nitrogenase synthesis and activity, and to compare results obtained with those from eubacteria.

ACCOMPLISHMENTS.

1. Growth physiology. The initial phases of the project included studying the growth physiology of diazotrophy by *Methanosarcina barkeri* strain 227 and demonstration and characterization of the properties of the nitrogenase system in crude cell-free extracts. Much of this work was published in 1988.

We demonstrated that fixing nitrogen was as energetically costly to strain 227 as it is to eubacteria, causing a halving of growth yield and rates. Molybdate at levels as low as 10 nM stimulated diazotrophic growth while tungstate inhibited it, consistent with the nitrogenase being a molybdo-enzyme. No evidence was obtained for growth stimulation by vanadium or chromium. Whole cells reduced acetylene, but at an extremely low rate, ca. $1 \text{ nmol min}^{-1} [\text{mg protein}]^{-1}$, two to three orders of magnitude lower than the rate found in typical eubacterial diazotrophs. Acetylene reducing activity was only found in N_2 -grown cells.

2. Enzymology.

One of the primary goals of this project was to purify and characterize the nitrogenase from strain 227. A clear need was to have enough cell material to work with. Yields of methanol grown cells



Library Codes	
Dist	Avail and/or Special
A-1	

were low when grown under strictly diazotrophic conditions in mineral medium. We found that adding 0.05 g/l Difco yeast extract to the growth medium allowed rapid initial growth, and by the time the cells had produced 50 mmol/liter CH_4 , they actively reduced acetylene. From a 10 liter fermenter, we could obtain several grams of cells within 10 days. Other problems included low nitrogenase activities, and nitrogenase being one of the less abundant iron sulfur proteins, in contrast with most diazotrophs where one can simply "catch the brown bands" coming off a column.

We hypothesized that cell-free extracts should show much higher acetylene reducing activity than whole cells since ATP and reducing power would be directly supplied to the enzymes. However, the activity we initially found in crude extracts (2) was only marginally higher than that in whole cells (ca. $5 \text{ nmol min}^{-1} [\text{mg protein}]^{-1}$), while extracts from *Klebsiella* prepared using the same procedures showed 100-fold higher activity. This activity was only found in extracts from N_2 -grown cells, and showed marked inhibition by addition of ADP, a hallmark of eubacterial nitrogenases, which are strongly regulated by the energy status of the cell. We have since achieved activities as high as $130 \text{ nmol min}^{-1} [\text{mg protein}]^{-1}$ when using extracts from freshly harvested cells with protein concentrations exceeding 20 mg/ml, although extracts usually had less than half this activity. This is still 5-10 fold lower than that expected to support diazotrophic growth.

Our initial studies (2) using SDS polyacrylamide gel electrophoresis revealed a band in dinitrogen-grown cells not found in ammonia grown cells which had a much lower molecular weight (26K) than the conventional iron protein (35K). Another band was seen at 55K. A third band near 60K would be expected but may have been obscured by other bands. A more faintly staining band at 31 kdal was also detected. Subsequent analysis (Lobo and Zinder, in preparation) has shown that the actual component 2 analogue in strain 227 is represented by the band near 31 kilodaltons. This is supported by "Western" immunoblotting using antibodies to *Rhodospirillum rubrum* component 2 (anti-Rr2, kindly provided by P. Ludden), which stained the band at 31 kilodaltons in lanes from N_2 -grown cells while no reaction was obtained in lanes from ammonium-grown cells (Figure 1, lanes 1 and A). This band also co-purified with nitrogenase activity (see below). The identity of the heavily staining band at 26 kilodaltons is not known. It may represent a proteolytic cleavage fragment of the 31 kilodalton protein, which may also explain the low acetylene reducing activity seen in extracts. If so, this fragment has lost the ability to react with antibodies to Rr2.

We have recently obtained a 20-30-fold purification of nitrogenase from *M. barkeri* strain 227 using FPLC chromatography inside an anaerobic glove-box (Figure 2). We have found that the enzyme is binary and shows subunit properties similar to those of eubacterial nitrogenases. One

component has molecular weight near 240 kilodaltons, as determined by Superose 12 gel permeation chromatography, and shows two heavily staining bands near 57 and 62 kilodaltons in SDS gels, making it similar to eubacterial component 1. The other protein has a subunit molecular weight near 31 kilodaltons, but shows an anomalously high molecular weight of 120 kilodaltons from Superose 12 chromatography, suggesting that it is a tetramer rather than a dimer. Molecular weight determinations using other methods should be used before a firm conclusion can be drawn. The highest specific activity we have obtained in a purified enzyme preparation is approximately 1000 nmol ethylene formed h^{-1} [mg protein] $^{-1}$. This value should be compared to the values of 60,000-150,000 nmol ethylene formed h^{-1} [mg protein] $^{-1}$ typically found for purified eubacterial nitrogenases.

We have used partially purified nitrogenase proteins to examine whether the low rates of acetylene reduction are due to acetylene being a poor substrate for the enzyme. We have found that the nitrogenase from strain 227 reduces N_2 at least 1.7 fold faster per electron than acetylene. Control experiments with *K. pneumoniae* extracts showed a ratio of 1.0. Of interest are the results of Chisnell et al. (3) on the non-Mo non-V containing alternative nitrogenase from *Azotobacter vinelandii* which had a ratio near 1.9. Many of the characteristics of the nitrogenase from strain 227 resemble those of this alternative nitrogenases, including low specific activity, poor stability, and a preference for N_2 over acetylene. The recent finding that the deduced amino acid sequence of a *nifH*-like gene from *Methanococcus thermolithotrophicus* (4) shows the highest similarity with this alternative nitrogenase makes attractive the possibility that methanogen nitrogenases are of this class. This hypothesis has to be reconciled with the results from ours and other laboratories indicating that diazotrophic growth in methanogens is stimulated by Mo, suggesting that nitrogenase is a molybdoenzyme. Determination of the metal content in methanogen nitrogenases will resolve this issue.

3. Regulation of nitrogenase synthesis and activity.

Since nitrogen fixation is an energetically costly process, nearly all free-living eubacterial diazotrophs repress synthesis of nitrogenase proteins in when a preferred combined N source such as ammonium is added. Furthermore, many diazotrophs also rapidly "switch-off" the activity of their nitrogenases in response to addition of combined N. The best studied system is that in *Rhodospirillum rubrum* in which one subunit of the component 2 is ADP-ribosylated (5) leading to a splitting of the band from that protein to two bands in SDS gels. This pattern has not been seen in all eubacteria exhibiting ammonium switch-off.

We have examined strain 227 for similar patterns of regulation. We have found that the 31K band reacting with anti-Rr2 in Western immunoblots is only present in N₂-grown cells (Figure 1), consistent with ammonium repression of nitrogenase in methanogens. We had already reported that addition of 5 mM ammonium caused a rapid switch-off of acetylene reduction in strain 227. We have since shown that the system is exquisitely sensitive to ammonium, with 10 μ M causing transient switch-off (Figure 3). We have examined Western blots from switched off cells (Figure 1) and have found no sign of covalent modification of the component 2 analogue. Extracts from switched-off cells have identical activity to those from non-switched-off cells. This suggests that ADP ribosylation is not involved in switch-off, although it is possible that such modification is lost during harvesting and processing the cells for analysis. Also, modification by a smaller group, such as methylation, may not have been detected. This represents one of the most tightly regulated metabolic systems in the methanogenic archaeobacteria.

4. References

1. Murray, P. A. and S. Zinder. 1984. Nitrogen fixation by a methanogenic archaeobacterium. *Nature* (London) 312: 284-286.
2. Lobo, A. L., and S. H. Zinder. 1988. Diazotrophy and nitrogenase activity in the archaeobacterium *Methanosarcina barkeri* 227. *Appl. Environ. Microbiol.* 54:1656-1661.
3. Chisnell, J. R., R. Premakumar, and P. E. Bishop. 1988. Purification of a second alternative nitrogenase from a *nifHDK* deletion strain of *Azotobacter vinelandii*. *J. Bacteriol.* 170: 27-33.
4. Souillard, N., and L. Sibold. 1989. Primary structure, functional organization, and expression of nitrogenase structural genes of the thermophilic archaeobacterium *Methanococcus thermolithotrophicus*. *Molec. Microbiol.* 3: 541-551.
5. Pope, M. P., S. A. Murrel, and P. W. Ludden. 1985. Covalent modification of the iron protein of the nitrogenase from *Rhodospirillum rubrum* by adenosine diphosphoribosylation of a specific arginine residue. *Proc. Natl. Acad. Sci. USA* 82: 3173-3177.

INVENTIONS:

None.

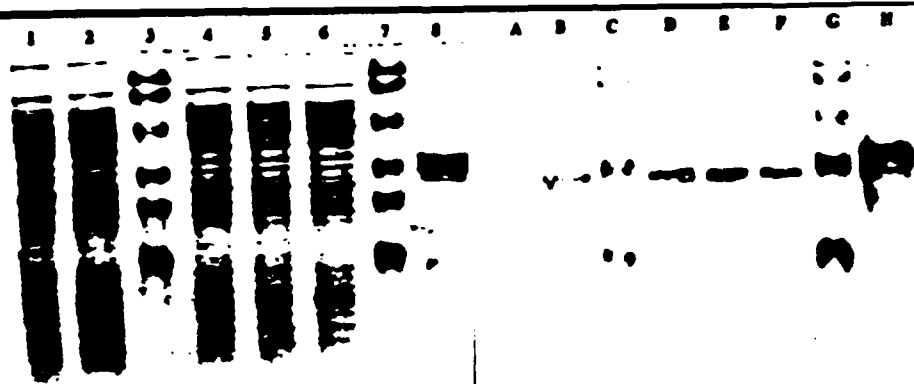


Figure 1 SDS gel and corresponding immunoblot (lanes A-H) of *M. barkeri* 227 cell extracts before and after switch-off by 5mM NH_4Cl . Lanes 1 and A, ammonia grown extract; lanes 2 and B, N_2 grown extract before addition of NH_4^+ ; lanes 4 and D, N_2 grown extract 10 minutes after addition of NH_4^+ ; lanes 5 and E, N_2 grown extract 1 hour after addition of NH_4^+ ; lanes 6 and F, N_2 grown extract 3 hours after addition of NH_4^+ ; lanes 8 and H, purified inactivated R2; lanes 3, 7, C and G, molecular weight standards (from top [in kilodaltons]: 110, 84, 47, 33, 24, and 16)

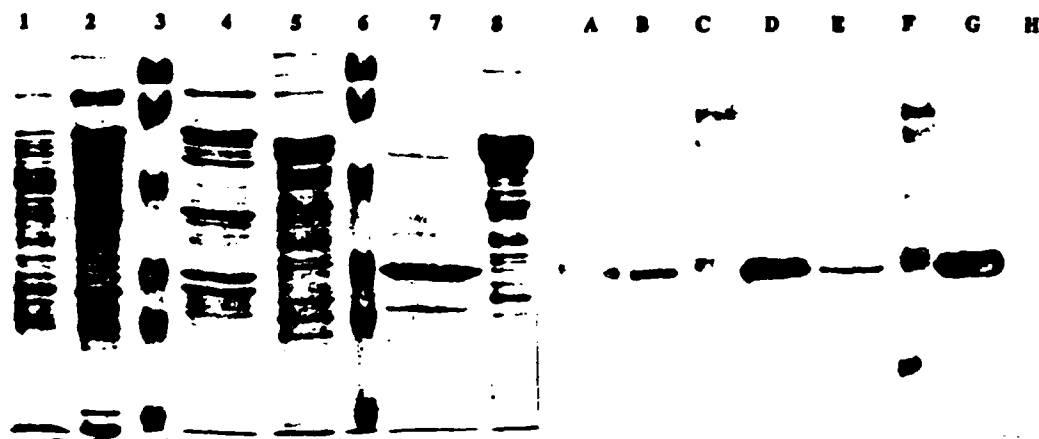


Figure 2 SDS gel and corresponding immunoblot of steps of *M. barkeri* 227 nitrogenase purification. Lanes 1 and A, N_2 -grown crude extract; lanes 2 and B, protamine precipitate; lanes 4 and D, Mono Q 0.42 M NaCl fraction; lanes 5 and E, Mono Q 0.46 M NaCl fraction; lanes 7 and G, 12.4 ml Superose 12 fraction; lanes 8 and H, 10.7 ml Superose 12 fraction; lanes 3, 6, C and F, molecular weight standards (from top [in kilodaltons]: 110, 84, 47, 33, 24, and 16)

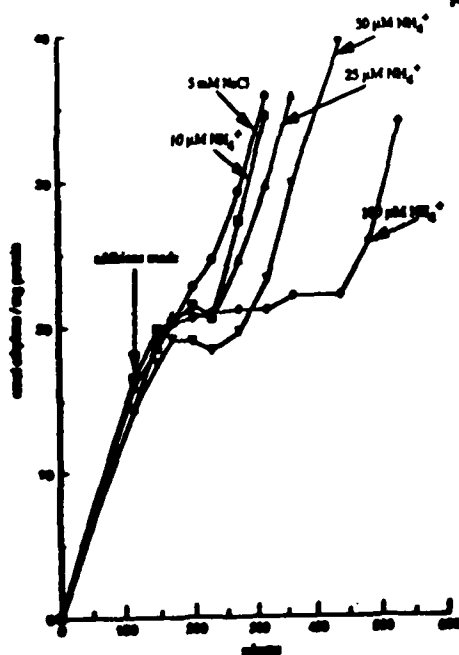


Figure 3 Duration of switch-off by 10µM, 25µM, 50µM, and 100 µM NH_4Cl compared with control addition of 5mM NaCl. The additions were made at the time indicated.

PUBLICATIONS AND REPORTS:

Journal Articles:

Lobo, A. L., and S. H. Zinder. 1988. Diazotrophy and nitrogenase activity in the archaeobacterium *Methanosarcina barkeri* 227. Appl. Environ. Microbiol. 54:1656-1661.

Lobo, A. L., and S. H. Zinder. 1990. Partial purification and characterization of nitrogenase from *Methanosarcina barkeri* 227. To be submitted to J. Bacteriol.

Book Chapters:

Lobo, A. L. and S. H. Zinder. 1990. Nitrogen fixation in methanogens. In H. Evans, G. Stacey, and R. Burris, Eds., Biological Nitrogen Fixation. Chapman Hall, NY. In Press.

Zinder, S. H. 1990. Nitrogen fixation in *Methanosarcina barkeri* strain 227. Proc. 8th Int. Symp. Nitrogen Fixation. In preparation.

Abstracts and Presentations:

Lobo, A. L., and S. H. Zinder. 1987. Nitrogenase activity in the archaeobacterium *Methanosarcina barkeri* strain 227. Abst. 87 Ann. Mtg. Amer. Soc. Microbiol.

Lobo, A. L. and S. H. Zinder. 1989. Partial purification and characterization of nitrogenase from *Methanosarcina barkeri* 227. Abst. 89 Ann. Mtg. Amer. Soc. Microbiol.

Lobo, A. L., and S. H. Zinder. 1990. Purification of Nitrogenase from *Methanosarcina barkeri* strain 227 and further characterization of ammonia switch-off. Abst. 90 Ann. Mtg. Amer. Soc. Microbiol.

Lobo, A. L., and S. H. Zinder. 1987. Nitrogenase activity in the archaeobacterium *Methanosarcina barkeri* strain 227. Northeast Microbiology, Physiology, Ecology and Taxonomy (NEMPET) Meeting, June 8-11, 1987, Amherst, Mass.

Lobo, A. L., and S. H. Zinder. 1987. Nitrogenase activity in the archaebacterium *Methanosarcina barkeri* strain 227. Gordon Conference on Molecular Biology of Methanogenesis, June 28-July 3, 1987.

S. Zinder. 1988. Nitrogen fixation in *Methanosarcina barkeri* strain 227. Seminar presented at the Swiss Federal Institute of Technology (ETH).

S. Zinder. 1990. Nitrogen fixation in *Methanosarcina barkeri* strain 227. To be presented to the Eighth International Symposium on Nitrogen Fixation.

TRAINING ACTIVITIES:

Research Assistantship for Graduate Student Anthony L. Lobo, PhD, received 12/89.

Women, minorities or non US citizens: 0