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SECURITY CLASSIFICATION OF THIS PAGE U

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

AD-A210 399

DTIC ELECTE JUL 10 1989 D

1b RESTRICTIVE MARKINGS: NA	
2b DECLASSIFICATION/DOWNGRADING SCHEDULE: NA	3 DISTRIBUTION/AVAILABILITY OF REPORT: Distribution Unlimited
4 PERFORMING ORGANIZATION REPORT NUMBER(S): University of Oklahoma 89-00014-01	5 MONITORING ORGANIZATION REPORT NUMBER(S): NA
6a NAME OF PERFORMING ORGANIZATION: University of Oklahoma / 6b OFFICE SYMBOL (If applicable): NA	7a NAME OF MONITORING ORGANIZATION: Office of Naval Research
6c ADDRESS (City, State, and ZIP Code): Department of Botany and Microbiology, University of Oklahoma, 770 Van Vleet Oval, Norman, Oklahoma 73019-0245	7b ADDRESS (City, State, and ZIP Code): 800 N. Quincy St., Arlington, VA 22217-5000
8a NAME OF FUNDING/SPONSORING ORGANIZATION: Office of Naval Research / 8b OFFICE SYMBOL (If applicable): ONR	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: N00014-86-K-0222
8c ADDRESS (City, State, and ZIP Code): 800 N. Quincy St., Arlington, VA 22217-5000	10 SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO 61153N; PROJECT NO RR04106; TASK NO 441d006; WORK UNIT ACCESSION NO

11 TITLE (Include Security Classification)

(U) Molecular biology and physiology of methanogenic archaebacteria

12 PERSONAL AUTHOR(S)

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13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Annual	FROM July 1988 TO June 1989	1989 June 27	16

16 SUPPLEMENTARY NOTATION

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Methanogens, archaebacteria, DNA, formate
08			

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

Methane-producing archaebacteria are worthy of note for their novel biology and potential in anaerobic bioprocessing. This work continues to study the biochemistry, genetics, and molecular biology of the thermophilic autotroph Methanobacterium thermoautotrophicum. DNA from antimetabolite-resistant mutant strains was used to transform sensitive recipient cells to resistance, and DNA was cloned into Escherichia coli plasmids. This DNA will be mutated with transposons in the E. coli host, then isolated and used to transform methanogen cells to selectable mutant phenotypes. Mutant strains resistant to purine analogs were used to determine that wild type cells of M. thermoautotrophicum possess an almost complete set of enzymes for uptake, activation, and interconversion of purine bases and nucleosides. These mutants and the information about the pathways will be the basis for generating a genetic map. Metabolic studies of a unique formate auxotroph revealed a new role for this one carbon compound in the anabolic metabolism of this methanogen.

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	(U)
22a NAME OF RESPONSIBLE INDIVIDUAL: Dr. Michael Marron Code 1141MB	22b TELEPHONE (Include Area Code): (202)-696-4760 / 22c OFFICE SYMBOL: ONR

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE U U

S/N 0102-LF-014-6603

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ANNUAL REPORT

Office of Naval Research Grant N00014-86-0222
"Molecular Biology and Physiology of Methanogenic Archaebacteria."
1 July 1988 to 30 June, 1989

The University of Oklahoma Department of Botany and Microbiology
Norman, Oklahoma
David P. Nagle, Jr., David R. McCarthy and Ralph S. Tanner.

I. Background and Objectives.

Methanogens are members of the group known as archaebacteria. These organisms are biochemically novel and ecologically important. By virtue of their position at the terminus of anaerobic food chains, the methanogens contribute to the mineralization of large amounts of organic matter. The end product of their metabolism, methane, contains a large percentage of the free energy of formation of the growth substrates (e.g. acetate, methanol, hydrogen and carbon dioxide) and therefore is a useful fuel substance.

It is now possible to culture and manipulate these strict anaerobes routinely. Using eubacterial host-vector systems, significant progress has been made in the isolation and sequencing of genes, defining of structurally important regions, and study of regulation [2, 3, 12]. Genetic exchange systems are not developed to a similar extent; at this writing, transformation methods have been published for Methanococcus voltae and M. thermoautotrophicum [1, 11]. Two recent meeting reports appear to be significant advances in this area. Bertani reported a transduction-like transfer of genes in Methanococcus voltae strain PS (G. Bertani. 1989. Abstr. Ann. Mtg. Amer. Soc. Microbiol. I-30, p. 222), and Leisinger reported a phage (ψM-1) which mediated transduction in M. thermoautotrophicum (T. Leisinger, 1989. "Gene transfer systems", Seminar on structure and expression of genes in archaebacteria, Ann. Mtg. Amer. Soc. Microbiol.). Plasmid pME2001 from M. thermoautotrophicum strain Marburg is of particular interest to this study; it has been cloned, and an in vivo transcript of unknown function has been identified [7].

Major objectives of this work include:

A. To optimize the transformation system in Methanobacterium thermoautotrophicum

B. Obtain additional mutants, especially those whose phenotypes permit direct selection

C. Generate a simple genetic map.

D. Characterize mutants in 1) purine and pyrimidine metabolism, and 2) formate metabolism

E. Develop a cloning vector for M. thermoautotrophicum.

F. In the long term, to understand aspects of cellular metabolism and mechanisms for adaptation to growth on different substrates, and to set the stage for engineering of methanogen strains with altered properties, such as the ability to use new growth substrates or electron donors.

II. Experimental Approach.

The organism under investigation is the thermophilic autotroph, Methanobacterium thermoautotrophicum strain Marburg. We have selected mutants that are resistant to purine and pyrimidine analogues and to kanamycin. These strains were obtained from single colony isolates that arose spontaneously or after chemical mutagenesis. The analog-resistant mutants were characterized for the ability to take up purine and pyrimidine bases. Salvage enzyme activities were assayed in crude extracts by spectrophotometry or by monitoring the conversion of radiolabelled substrate to product [8; Worrell and Nagle, in preparation].

Strain RT103, a formate auxotroph was isolated from the kanamycin-resistant strain after hydroxylamine mutagenesis and enrichment in mineral medium in the presence of bacitracin. This mutant was characterized for its ability to metabolize labelled formate in vivo, and anoxic cell-free extracts were prepared. Formate dehydrogenase activity was assayed with selected electron acceptors.

Transformation with high molecular weight chromosomal DNA from M. thermoautotrophicum strains is carried out as described [11]. Plasmid libraries of M. thermoautotrophicum PstI fragments of chromosomal DNA have been prepared in plasmid pBR322 and pUC8, and maintained in E. coli. We are testing the possibility that transposon mutagenesis of M. thermoautotrophicum genes can be carried out indirectly by additive transformation. Cloned M. thermoautotrophicum DNAs will be mutagenized with miniTn10km in an E. coli background. The nonhomologous transposons will enter the chromosome by recombination involving the chromosomal sequences that flank each cloned transposon insertion. Although the km^r marker will not express phenotypically in M. thermoautotrophicum, the defective phenotypes that result from the disruption of the chromosomal genes (such as auxotrophy or base analog-resistance) can be detected in selection or screening assays.

III. Results.

A. Isolation of mutants with defects in DNA precursor metabolism.

We obtained mutants in Methanobacterium thermoautotrophicum strain Marburg which were resistant to nucleic acid base analogues, and determined that most of these compounds were bacteriocidal to wild type cells [8, 11; in preparation] (Table 1). The spontaneous mutation frequencies for some of the phenotypes were as follows: 8-azahypoxanthine, 6-methylmercaptopurine riboside, and 5-fluorouridine, $1 - 5 \times 10^{-6}$; for 5-fluorouracil and 5-fluorodeoxyuridine, 5.3×10^{-8} and 3.5×10^{-9}, respectively; and for 6-mercapto purine at 0.3 mg/ml, 1.5×10^{-6}, and at 1 mg/ml, $<3 \times 10^{-9}$ (Worrell et al., unpublished results). The fluorouracil-resistant strain was deficient in the enzyme uracil phosphoribosyltransferase, which activates the base to

Table 1. Inhibition of M. thermoautotrophicum by purine analogs.

	Analog	CFU/ml ($*10^8$)
Controls	none (H_2/CO_2)	6.8
	none (N_2/CO_2)	0.13
	none (bacitracin)	0.00009
Noninhibitory[a]	2-amino-6-methylmercaptopurine	6.4
Inhibitory[b]	2,6-diaminopurine	0.35
Bacteriostatic[c]	6-methylaminopurine	0.09
	6-methylmercaptopurine riboside	0.16
Bacteriocidal[d]	8-aza-2,6-diaminopurine	0.0011
	6-thioguanine	0.0004
	8-azaguanine	0.0004
	6-mercaptopurine	0
	8-azahypoxanthine	0

Analogs were added to concentrations of 500 ug/ml mid-log phase cultures which were then incubated for 18 hr. The number of viable cells present was determined. The compounds were classified as noninhibitory, bacteriostatic or bacteriocidal according to viable counts.

a. same as H_2/CO_2 control. This class of compounds also includes 8-azaxanthine, 6-chloroxanthine, 6-chloropurine, 6-dimethylaminopurine, and isopropylidine guanoside

b. less than H_2/CO_2 control, but greater than N_2/CO_2 control

c. same as N_2/CO_2 control

d. less than N_2/CO_2 control

the nucleotide. This deficiency would suffice to explain the phenotype of resistance, since the mutant could not produce nucleotides containing fluorouracil, which are known to be toxic in other bacteria [8].

The purine analog-resistant strains have been used as a tool to determine the complex network of reactions of purine base, nucleoside, and nucleotide interconversion activities found in M. thermoautotrophicum. Table 2 presents the activities of enzymes of purine nucleobase, nucleoside, and nucleotide metabolism we detected in wild type cells. Taken together with data from analog-resistant strains, the results are summarized in Figure 1. The figure was based on patterns of susceptibility to other analogs, labelling of cells with authentic bases, and in vitro enzyme activities (Worrell and Nagle, 1989. Abstr. Ann. Mtg. Amer. Soc. Microbiol. I-22, p. 221; in preparation). The arrows indicate enzyme activities which have been demonstrated in extracts of wild type cells. One activity found in eubacteria and eucaryotes which was not detected in extracts of M. thermoautotrophicum was GMP reductase, which converts GMP to XMP.

Strain VW-102, selected for resistance to 8-azahypoxanthine, failed to accumulate authentic [^{14}C]-labelled hypoxanthine or guanine, but did take up adenine. Extracts from cells of this strain were deficient in hypoxanthine and guanine phosphoribosyltransferase activities, but did contain adenine phosphoribosyltransferase activity. Similar data were obtained for strain VW-109, which was selected for resistance to thioguanine. These results suggest that hypoxanthine and guanine may be substrates for a single hypoxanthine/guanine phosphoribosyl transferase activity, which is similar to the case in eubacteria and eucaryotes. The set of reactions of purine base, nucleoside, and nucleotide interconversion in this autotrophic methanogen is surprisingly similar to those of enteric bacteria [9] and pathogenic protozoa [5].

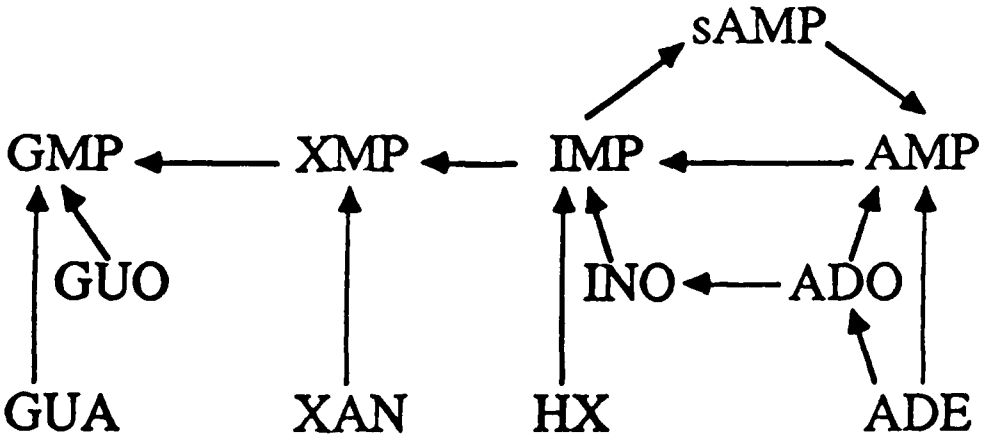


Figure 1. Purine Interconversion Pathway of Methanobacterium thermoautotrophicum strain Marburg.

Table 2. Purine interconversion enzyme activities in extracts of *M. thermoautotrophicum* strain Marburg.

Enzyme	Enzyme activity (nmol product/min-mg protein)[a]
Phoshoribosyltransferases	
ADE	0.14
GUA	3.70
HX	1.37
XAN	0.06
Nucleoside phosphorylases	
ADE	0.20
GUA	0.04
HX	0.02
XAN	<0.001
Nucleoside kinase	
ADO	7.50
GUO	37.5
INO	38.5
XUO	<0.001
Deaminases	
ADE	0.02
ADO	26.5
AMP	3.16
GUA	<0.001
XAN oxidase	0.014
IMP dehydrogenase	1.99
GMP synthetase	3.84
GMP reductase	<0.001
Succinyl-AMP synthetase	28.3
Succinyl-AMP lyase	30.3

[a] Enzyme activities are the average of duplicate or triplicate determinations of 2 different batches of extract.

Our collection of about a dozen discrete base analog resistant mutants will be useful in development of a genetic map by transformation. It may be the basis for dominant selectable markers based upon the wild-type property, analogous to systems based on hypoxanthine-guanine phosphoribosyltransferase in eucaryotic cells [4].

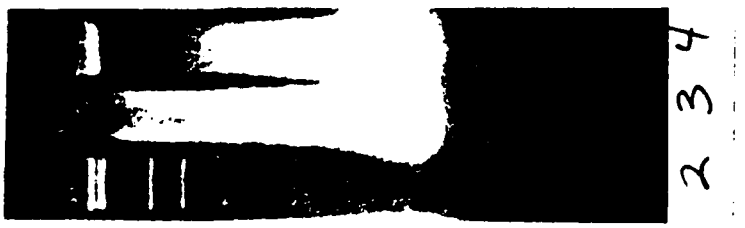
B. Genetic transformation.

The phenotypes of 5-fluorouracil-resistance and 6-mercaptopurine resistance have been transferred into wild-type cells with chromosomal DNA from the resistant strains RTAE-1 and RTVW-1 [11; unpublished results]. Those variables which permit cells to become transformed in our system remain unknown. Recently, we found that many of our preparations of methanogen DNA which appeared to contain a high percentage of high molecular weight fragments (> 20 kb) as demonstrated by agarose electrophoresis actually contained numerous single-stranded breaks. Thus, the DNA was damaged during preparation. These preparations of DNA did not support transformation. We have now demonstrated that these preparations can be partially repaired by means of DNA ligase treatment, as shown in Figure 2 (J. Bradley, unpublished). A preliminary result suggested that the ligase repaired DNA supported transformation of fluorouracil-resistance to wild type cells. This result must be confirmed with additional DNA preparations and other markers from our collection of mutants. We will pursue the implemention of new isolation methods for DNA which minimize damage. These may include the use of pseudolysozyme from Methanobacterium wolfei, and solvent lysis methods. If DNA damage cannot be prevented with the use of new lysis techniques, then we will purify repaired DNA from the damaged DNA by benzoylated-napthoylated DEAE cellulose chromatography. The repaired DNA should be suitable for cloning and transformation.

C. Construction of M. thermoautotrophicum chromosomal DNA libraries.

Our attempts to construct an M. thermoautotrophicum chromosomal library have met with limited success. We were able to construct libraries in both low-copy number and high-copy number plasmid cloning vectors. However, in each case, most of the recombinant plasmids contained inserts of 1000 base pairs or less. The bias in favor of small inserts was not reflected in the original restriction digest of the chromosomal DNA. In order to improve the efficiency with which we clone large fragments, we are focusing on the effect of frequent strand breaks in the chromosomal DNA. A large fraction of the strand breaks can be repaired by DNA ligase (see Figure 2).

Figure 2. Denaturing agarose gel electrophoresis of M. thermoautotrophicum strain RTAE-1 DNA.
Lane 2: lambda HindIII fragments; 3: DNA as isolated; 4: DNA after repair with DNA ligase.



We are now cloning fragments from a chromosomal DNA sample that was treated with DNA polymerase and DNA ligase. If the strand breaks interfere with the cloning of large chromosomal fragments then we should obtain a larger yield of high-molecular-weight fragments from the repaired DNA.

D. Construction of new shuttle vectors for transposon mutagenesis and additive transformation.

Our transposon mutagenesis procedure requires that cloned M. thermoautotrophicum DNA fragments be passed through an E. coli strain that contains a transposon mutator plasmid. The mutated plasmids should be separated from the mutant plasmids prior to the introduction of the mutated inserts into M. thermoautotrophicum. To accomplish this we constructed a series of Haemophilus influenzae/E. coli shuttle plasmid vectors. M. thermoautotrophicum chromosomal fragments will be cloned into a shuttle vector and then the resulting library will be mutagenized in E. coli. The mixture of mutated and mutator plasmids will be transformed into H. influenzae to remove the mutator plasmids since these plasmids cannot transform H. influenzae. A preparation containing only mutated plasmids then will be transformed into M. thermoautotrophicum to construct auxotrophic mutants.

E. Isolation and characterization of a novel formate auxotroph.

We isolated a formate auxotroph of M. thermoautrophicum. The formate requirement appears to be specific; known nutrients alone or in combination, yeast extract, or casamino acids all failed to support the growth of the mutant strain. In medium supplemented with at least 3 mM formate the mutant grows at the same rate and to the same extent as does the wild type. Neither wild type nor mutant was capable of using formate for methanogenesis in the absence of hydrogen, or in the absence carbon dioxide. Only about 10% (0.2-0.3 mM) of the total mass of formate present was removed by mutant cells: the wild type and strain ΔH cells removed formate to the same extent. The low level of formate utilized suggested that formate was used biosynthetically for some component such as an amino acid or vitamin, which would be a novel role for this compound in methanogens. Previous labelling studies suggested that formate might be incorporated into purines and histidine [10]. However, purines or casamino acids did not replace the requirement for formate in the auxotroph, although adenine, guanine, and hypoxanthine were taken up and incorporated into macromolecules by wild type cells (Worrell and Nagle, unpublished).

Preliminary results showed that [^{14}C]-formate was incorporated into soluble, lipid, RNA, DNA, and protein pools in cells of both the formate auxotroph and the wild type (Table 3) (Tanner et al., submitted for publication). The fate of label from formate in cultures of four strains of M. thermoautotrophicum is shown in Table 4. Between 3.5 and 4% of the radioactivity added was taken up by the cells of all four strains. It is noteable that strains Marburg and ΔH behaved similarly, although they are quite different from each other. After incubation, most of the radioactivity present was contained in methane. This was unexpected, as the organism cannot utilize formate to synthesize methane. These and other data (not shown) suggested that radiolabel was exchanged between formate and CO_2.

Table 3. Labelling of cellular pools and macromolecules by [^{14}C]-formate in Methanobacterium thermoautotrophicum.

CELL FRACTION[a]	dpm x 10^4 per 10^{10} cells of strain:	
	K^r	RT103
Soluble pool	3.8	7.4
Lipid	6.9	6.8
RNA	1.9	2.8
DNA	0.9	1.0
Protein	2.2	1.7

a. [^{14}C]-formate (1.0 x 10^8 dpm per umol) at 3.7 mM in 20 ml cultures which were pressurized to 220 kPa overpressure with hydrogen:carbon dioxide (80:20) twice daily for 48 hr. Strain K^r is a kanamycin-resistant derivative of strain Marburg and parent of the formate auxotroph RT103.

Cell-free extracts of strains RT-100 (kanamycin-resistant wild type) and strain RT-103 were screened for the presence of formate dehydrogenase activity. This enzyme had not been described previously in M. thermoautotrophicum. Although no activity was detected with the artificial electron acceptor benzyl viologen, extracts of the wild type carried out formate-dependent reduction of both NAD and NADP (Table 5). The negative value for strain RT-103 is due to suppression of the formate-independent background activity in undialyzed extracts. These two strains can be distinguished biochemically on the basis of their ability to reduce NAD with formate. The M. thermoautotrophicum formate dehydrogenase activity was noteable for its low pH optimum, pH 6.5, which is almost 2 pH units below that reported for some catabolic FDH enzymes in methanogens. The activity was oxygen-sensitive, and was highest at 60° C. Under the conditions shown in Table 5, the dye methylviologen was rapidly reduced in the absence of formate. Recently, this background reaction was reduced by elimination of dithiothreitol from the reaction mix. The enzyme was partially inhibited (about 30%) in extracts by sodium azide at 10 mM, and the activity remained in the soluble protein fraction after centrifugation for 1 hr at 100,000 x g.

Table 4. Metabolism of [^{14}C]Formate by Methanobacterium thermoautotrophicum.

Time (hr)	Strain	dpm x 10^6 in Fraction[a] Supernatant	Pellet	CO_2	CH_4
1	ΔH	45	0.0061		
	Marburg	38	0.0070		
	K^r	62	0.0074		
	RT-103	62	0.012		
46	ΔH	2.4	1.6	2.3	24
	Marburg	1.5	1.5	3.2	22
	K^r	2.1	2.4	6.5	47
	RT-103	2.2	2.2	3.3	42

[a][^{14}C]Formate was added to 10 ml cultures containing 3.7 mM formate to an approximate specific activity of 1.5 x 10^6 dpm/umol. Cultures were pressurized to 220 kPa overpressure with H_2:CO_2 (80:20) at 0, 16 and 40 hr.

Table 5. Formate Dehydrogenase Activity in M. thermoautotrophicum extracts.

Strain	Electron Acceptor	Specific Activity[a]
Marburg	FAD	-1.4
	Benzyl viologen	-0.5
	NAD	7.8
	NADP	8.4
RT-103	NAD	-0.6
	NADP	2.8

a. Anoxic extracts were incubated at 60°, in 20 mM MES buffer, pH 6.5, in 0.2 mM dithiothreitol. Reduction of electron acceptor (20 mM) was followed prior to addition of formate; formate was then added (20 mM) to initiate the reaction. The specific activity reported is corrected for the background (formate-independent) rate, and given in units of nmol reduced per min per mg protein. (Tanner et al., submitted for publication).

We will attempt to isolate a formate-utilizing strain of M. thermoautotrophicum by mutagenesis and selection. The hypothesis is that such a strain would overproduce the FDH activity, thereby able to use formate as sole energy source. A formate-utilizing derivative of strain Marburg would be the first methanogen engineered to use a new growth substrate. In addition to its novelty, it would help us to understand the relationship between formate and hydrogen/CO_2 metabolism in this species. Noteably, in M. vannielli, the pH optimum for growth on formate (about pH 8) is considerably higher than that for growth on hydrogen/carbon dioxide (about pH 7) [6]. A putatively anabolic enzyme converted to a catabolic role would be of interest.

IV. Prospects.

A basis for progress in the genetics and physiology of M. thermoautotrophicum has been established. The collection of resistant mutants will be used to establish a linkage map by use of the chromosomal DNA-dependent transformation system. The formate auxotroph, which has a phenotype not previously described in a bacterial species, opens a new view of one-carbon metabolism in the methanogens. The results of formate labeling and enzyme assays suggested a novel method of one carbon activation which will be pursued and described. New molecular techniques, such as additive transformation, can be applied to our genetic system.

Our physiological and genetic system, and the biochemical, structural, sequence, and regulatory studies from other laboratories will continue to add to basic knowledge of methanogens. The finding that some selectable genes can be introduced into recipient strains will be exploited in the future. New strains will be constructed with the ability to utilize new substrates or electron donors, or with increased resistance to toxic chemicals such as oxygen, solvents, and so on.

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V. Publications Acknowledging Office of Naval Research Support (07/1988-06/1989).

Tanner, R. S. 1989. Comparative evaluation of hard-surface disinfectants. J. **Indust. Microbiol. 4**: 145-154.

Nagle, D. P., Jr. 1989. Development of genetic systems in methanogenic archaebacteria. **Developments in Industrial Microbiology 30**: In the press.

Tanner, R. S. (1989). Monitoring sulfate-reducing bacteria: comparison of enumeration media. **J. Microbiol. Meth.**: In the press.

Previous Office of Naval Research Reports

Nagle, D. P., Jr., McInerney, M. J., and D. McCarthy. Development of a Gene Cloning System in Methanogens.
Annual Report, April 1986 to March 1987, DITC Number A179367, March, 1987; Final Report, April 1986 to June, 1988, DITC Number A197062, July, 1988.

Submitted or In Preparation

Tanner, R. S., M. J. McInerney, and D. P. Nagle, Jr. (in review). Formate auxotroph of Methanobacterium thermoautotrophicum Marburg. Submitted to **J. Bacteriol.**

Tanner, R. S., and D. C. Yang. (in preparation). Clostridium ljungdahlii PETC, a new acetogenic, gram-positive, anaerobic bacterium. For submission to **Int. J. Syst. Bacteriol.**

Trieu, V. N., and D. McCarthy. (in preparation). Improved plasmid shuttle vectors for Haemophilus influenzae and Escherichia coli. for submission to **Gene.**

Worrell, V. E., and D. P. Nagle, Jr. (in preparation). Genetics and physiology of the purine interconversion pathway of Methanobacterium thermoautotrophicum. For submission to **J. Bacteriol.**

VI. Abstracts and Presentations.

Nagle, D. P., Jr. 1988. Development of genetic systems for methanogenic archaebacteria. Abstr. Annu. Meeting Soc. Indust. Microbiol., S86. (invited presentation).

Nagle, D. P., Jr. R. S. Tanner, D. McCarthy, and V. E. Worrell. 1989. Genetic manipulation of methane-producing bacteria. Abstr. Ann. Mtg. Amer. Chem. Soc., symposium on Metabolic Pathway Engineering, No. 23. (invited presentation).

Worrell, V. E., and D. P. Nagle, Jr. 1989. Pathway for interconversion of purines and their derivatives in Methanobacterium thermoautotrophicum strain Marburg. Abstr. Ann. Mtg. Amer. Soc. Microbiol. I-22, p. 221.

Nagle, D. P., Jr. Genetics of methanogenic archaebacteria. Seminar presented at Oklahoma State University, Department of Microbiology, February, 1989.

Tanner, R. S. Formate auxotroph of Methanobacterium thermoautotrophicum. Seminar presented at University of Oklahoma, Department of Botany and Microbiology, March, 1989.

Tanner, R. S. Systematics in applied microbiology. Seminar presented University of Arkansas, Department of Chemical Engineering, April, 1989.

VII. Awards, Honors, Promotions.

August 1988

D. McCarthy promoted to Associate Professor, Department of Botany and Microbiology.

Effective August 1989

D. P. Nagle, Jr., promoted to Associate Professor, Department of Botany and Microbiology.

R. S. Tanner, Assistant Professor, Department of Botany and Microbiology (tenure track position).

VIII. Personnel supported.

Dr. Ralph S. Tanner was supported in part by this grant as a Visiting Assistant Professor in the Department of Botany and Microbiology.
Doris M. Kupfer was supported in part as a laboratory technician.
Ms. Veronica E. Worrell, a third year graduate student, was supported for the grant period (Hispanic female, USA).

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