AIIM

Association for Information and Image Management

1100 Wayne Avenue, Suite 1100
Silver Spring, Maryland 20910

301/587-8202

Centimeter

Inches

MANUFACTURED TO AIIM STANDARDS
BY APPLIED IMAGE, INC.

1

PROGRESS REPORT- FEBRUARY, 1994
STEPHEN H. ZINDER, CORNELL UNIVERSITY
GRANT: DE-FG02-85ER13370

INTRODUCTION

The primary goal of this research is to gain a better understanding of conversion of acetic acid to methane by thermophiles. Acetate is the precursor of approximately two-thirds of the methane produced by anaerobic bioreactors and many other methanogenic habitats. Besides their intrinsic interest, thermophilic acetotrophic methanogenic cultures usually grow at least twice as fast as their mesophilic counterparts, making them more amenable to study. Moreover, they play important roles in thermophilic (50-60°C) anaerobic bioreactors, which have considerable potential in treating industrial and other organic wastes.

In recent years, our attention has been mainly focused on the thermophilic acetate utilizing methanogen *Methanotherix* strain CALS-1. *Methanotherix*, also called *Methanosaeta*, is one of only two methanogenic genera known to convert acetate to methane, the other being *Methanosarcina*. The faster-growing more versatile *Methanosarcina* has been better studied. However, when one examines anaerobic digester contents, *Methanotherix* is often the dominant acetate-utilizing methanogen.

Ecological studies, including our own, have demonstrated that low acetate concentrations favor *Methanotherix* over *Methanosarcina*. While this competition had generally been considered in terms of Michaelis-Menten kinetics, our studies (11) demonstrated that a thermophilic *Methanosarcina* culture had a minimum threshold concentration for acetate utilization near 1 mM, while that for *Methanotherix* CALS-1 was 10-20 μ M. This threshold phenomenon has been confirmed in other acetotrophic methanogens (3, 8, 15). Besides competition for substrate, other factors can also favor *Methanotherix*, such as its ability to form more compact granules in sludge blanket reactors apparently due to its greater stickiness than *Methanosarcina*.

Thus, both *Methanosarcina* and *Methanotherix* carry out the same reaction, methanogenesis from acetate, yet *Methanotherix* carries it out at acetate concentrations ca. 100-fold lower than *Methanosarcina*, suggesting that there are profound biochemical differences between them. *Methanosarcina* biochemistry is being studied in several laboratories worldwide, while to my knowledge, no other group besides our own are actively studying *Methanotherix* biochemistry. Research on *Methanosarcina*, mainly done with cell-free extracts, has shown that acetate is first activated to acetyl-coenzyme A (CoA), which is then disassembled to a methyl group and a carbonyl group by an enzyme complex called carbon monoxide dehydrogenase, followed by electron transport from the carbonyl to the methyl group, thereby reducing the methyl group to methane. It is clear that methanogenesis from acetate follows a similar pattern in *Methanotherix*, but there are also some interesting differences.

As described in previous progress reports, we have achieved methanogenesis from acetate in cell-free extracts of *Methanotherix* strain CALS-1 grown in a pH auxostat. Using these cell extracts, specific activities for methanogenesis from acetate and ATP of 100-300 nmol min⁻¹ [mg protein]⁻¹ were routinely obtained, levels comparable to the rate in whole cells, which is not usually the case in methanogenic extracts. Probably the most significant finding is that methanogenesis from acetate in the cell free extracts of *Methanotherix* strain CALS-1 was independent of addition of H₂ or any other external reductant. This is in marked contrast with results in cell-free extracts from *Methanosarcina*, in which methanogenesis is directly dependent on an external reductant, usually H₂. Indeed, H₂ may be an obligatory intermediate in methanogenesis from acetate in *Methanosarcina*, while our results in whole cells (16) and extracts indicate that this is not the case for *Methanotherix*. These results point to major differences in electron transport in these two organisms.

RECENTLY OBTAINED RESULTS

1. **Methanogenesis in crude extracts.** One aspect we have been examining is the potential role of acetyl-CoA in methanogenesis by cell-free extracts. By analogy to *Methanosarcina*, it seems likely that acetyl-CoA is involved, but the evidence we have obtained so far has not supported this hypothesis. Treatment of extracts with activated charcoal, as done by Fischer and Thauer (2) abolished activity, which was not reversed by adding CoA, perhaps due to adsorption of another important cofactor by the charcoal. Adding CoA to the endogenous pool, which we estimated as 13 μ M, did not stimulate methanogenesis from acetate, and, in fact, concentrations over 200 μ M were inhibitory. A more recent experiment is shown below.

Figure 1. Methanogenesis from acetate and ATP, acetyl-phosphate, or acetyl-CoA by cell-free extracts of *Methanotherix* strain CALS-1.

Acetyl-CoA was actually the poorest substrate for methanogenesis, even when a small amount of ATP was added to potentially activate the system. Surprisingly, acetyl-phosphate was a fairly good substrate, despite our not finding phosphotransacetylase (PTA) in the extracts. We need to re-examine extracts for low levels of this enzyme. A likely explanation for the poor activity with acetyl-CoA is the buildup of free CoA causing inhibition. We are testing this hypothesis by adding acetyl-phosphate and exogenous PTA to the extract, since this system can regenerate acetyl-CoA without buildup of CoA. *B. stearothermophilus* PTA purchased from Sigma did not give enhanced methanogenesis from acetyl-phosphate, but we are not certain that the enzyme was active in our extracts. We have recently obtained, from J. G. Ferry's laboratory, PTA from *Methanosarcina thermophila* from cloned genes overexpressed in *E. coli*. This enzyme is highly active and should be ideal for our studies.

A related question is the involvement of acetyl-CoA synthetase (ACS) in the reaction. This enzyme cleaves ATP to AMP and pyrophosphate, and if the pyrophosphate is cleaved, the result is the net loss of two high-energy phosphate bonds for acetate activation. We have verified that *Methanotherix* strain CALS-1 extracts have high ACS activity, and that the purified enzyme

resembles that from the mesophile (6, 13). We have recently purified mg quantities of ACS in order to prepare antibodies against it. We will use these antibodies to immunoprecipitate ACS from extracts to determine its involvement in the pathway. If we lose activity from this procedure, we will add purified ACS back. A similar approach was used to verify the importance of carbon monoxide dehydrogenase in acetate catabolism in *M. barkeri* (9). This experiment should settle whether this expensive enzyme is actually used by *Methanotherix*.

2. Role of the cell membrane in methanogenesis from acetate. It is clear that the cell membrane plays an important role in methanogenesis and energy conversion in methanogens. Our initial studies on membranes in *Methanotherix* were centered on a membrane-bound DCCD-sensitive ATPase, which was a good entry into learn how to study and manipulate membrane-bound enzymes from this organism. Unfortunately, soon after we achieved purification and preliminary characterization of this ATPase, Inatomi et al (5) published a paper on an ATPase from another thermophilic *Methanotherix* strain. Their results and ours were essentially identical, and we did not pursue this line of studies further. A low pyrophosphatase activity we have detected in the membrane fraction has not proved to be stable after membrane disruption with a variety of detergents.

Despite indirect evidence that the cell membrane plays an important role in methanogenesis from acetate, previous biochemical studies, including our own, have indicated that this system is "soluble". However, these extracts are typically prepared by disruption with a French pressure cell at 20,000 psi. Using the membrane-bound DCCD-sensitive ATPase as a marker for membrane fragments, we have found that over half of the membrane material is still contained in the supernatant fraction of these extracts centrifuged at 200,000 x g for an hour. Since *Methanotherix* has a much more fragile outer envelope than non-marine strains of *Methanosarcina*, we can disrupt cells of this organism at the more moderate pressure of 6,000 psi. Under the microscope, one can see many more μm -sized vesicles than at 20,000 psi, yet only a few whole cells (which are removed by low-speed centrifugation). About 90% of the ATPase is now sedimented in the pellet of these extracts. We have fractionated these preparations into soluble and pellet fractions and have tested them for methanogenic activity.

Figure 2. Methanogenesis by supernatant and pellet fractions of an extract of *Methanothrix* sp. strain CALS-1 from cells broken in a French pressure cell at ca. 6,000 psi.

Not surprisingly, there was no activity in the pellet, presumably containing the membrane fraction. There was slight activity of the supernatant, but the two fractions combined gave significantly enhanced activity. This is the first biochemical evidence for direct involvement of the cell membrane in methanogenesis from acetate. We are going to examine the membrane fraction for various activities, and will be most interested in examining its role in carbon and electron flow.

3. Carbon monoxide dehydrogenase (CODH). We have been examining CODH in *Methanothrix* for several years and have found that it exists mainly as a high molecular weight complex, similar to one described in *M. thermophila* (14). This is in contrast to the results of Jetten et al. (7) who found only a two-subunit enzyme from a mesophilic strain of *Methanothrix*. We have been unable to purify enough of this enzyme complex to adequately characterize it. In the small amounts that we have purified, we have detected only four bands in SDS gels, in contrast to the *Methanosarcina* enzyme which has five subunit types.

We recently began collaborating with Dr. D. Grahame, Uniformed Services University, who demonstrated cleavage of acetyl-CoA by purified enzyme preparations of CODH (4). We sent Dr. Grahame a pellet harvested from a 10-liter fermentor run, and he and colleagues used his large-scale anaerobic enzyme purification facilities to partially purify the CODH complex by fractionation with a Sepharose 6B column. The resulting complex only contains a single band near 104 kDa as a major contaminant. In his preliminary analyses, some interesting results have been obtained. The CODH complex contained 65% of the corrinoid measured in the extracts, with the remaining 35% of the corrinoid eluting with membrane material in the void volume. This is in marked contrast to *M. barkeri*, where only 8.3% of the corrinoid was associated with CODH and nearly all of the rest is associated with a soluble fraction (which is presently being studied by Dr. J. Krzycki et al at Ohio State).

The CODH complex was capable of synthesizing acetyl-CoA from methyl-tetrahydrosarcinapterin and either CO, or CO₂ + Ti³⁺ + Fd (from *Methanosarcina*) with higher

activity than *Me. barkeri* CODH using the former substrate and lower activity using the latter. Dr. Grahame has studied the subunit structure of the purified enzyme and has found that the doublet we have detected near 52 kDa, in standard Laemmli gels using Tris-glycine buffer, representing the corrinoid protein, became a triplet structure in SDS gels using Tricine buffer. More importantly, Dr. Grahame has antibodies against all five subunits of the *M. barkeri* enzyme. Although the antibodies gave somewhat weaker reactions than they do with the *M. barkeri* enzyme, they clearly demonstrated that all five subunits were present in the *Methanotherix* enzyme. Thus the *Methanotherix* CODH complex has the following structure:

Table 1. Subunit structure of CODH complexes from acetotrophic methanogens. Weights are in kDa.

Subunit type	<i>Methanosarcina barkeri</i>	<i>Methanosarcina thermophila</i>	<i>Methanotherix</i> strain CALS-1
Alpha	88.5	82.2	80.7
Beta	61.1	57.2	52.4
Gamma	49.2	51.2	54.7
Delta	47.2	46.9	50.4
Epsilon	20.5	20.7	21.1
Sum	266.5	258.2	259.3

Note how the weight of the beta subunit is lower in *Methanotherix* but the gamma and delta subunits are higher so that they co-migrate, and that the total molecular weights of the five subunits is nearly equal for each organism

Thus, in a small period of time, the collaboration with Dr. Grahame has generated some interesting data, and we intend to continue it. We are particularly interested in the large amount of corrinoid protein that is associated with the membrane in this organism. A significant fraction of it is likely to be a tetrahydromethanopterin-CoM methyltransferase, a central enzyme in carbon flow and energy conversion in methanogens.

4. Novel thermophilic cultures converting acetate to methane. A continuing goal of this project is to describe novel acetate-utilizing cultures, especially those that can grow at temperatures greater than 70° C, since none have been described. Dr. Hugh Morgan, University of Waikato, New Zealand, paid a visit to Cornell before serving on the DOE review panel on lignocellulose. In late October, after returning to New Zealand, Dr. Morgan sent us a sample from a hot spring which, from his experience, contained numerous thermophilic anaerobes. A sample obtained from Dr. Morgan several years ago did not yield methanogens.

We used the sample to inoculate our standard growth medium containing either glucose (positive control) or acetate as a substrate, and incubated cultures at 57° C and at 75° C. The 57° C acetate cultures produced methane from acetate within a few days and were dominated by an organism clearly resembling *Methanotherix*. Since it appeared so similar to strain CALS-1 and to other described thermophilic *Methanotherix* cultures, we did not pursue its isolation. The 75° C sample produced methane at a somewhat slower rate, but eventually converted all of the acetate added to methane. There was no sign of a *Methanotherix*-like organism present, but the predominant organism was a large, sometimes bent, rod which showed autofluorescence characteristic of F₄₂₀-containing methanogens when viewed with an epifluorescence microscope. This culture was transferred to tubes with acetate growth medium which were incubated at either 70° C or 80° C. Growth and methanogenesis was achieved at either temperature, although they were more rapid at 70° C. Transfer of this culture to H₂/CO₂ medium yielded fairly rapid growth

with an organism with essentially the same morphotype as the acetate cultures predominating. We have very recently isolated this organism on H_2/CO_2 .

There are at least two possible explanations for these findings. One is that we have isolated a methanogen which can use either H_2/CO_2 or acetate. As such, it would resemble a partly characterized rod-shaped methanogen called the TAM organism (1) which uses H_2/CO_2 , formate, or acetate and grows at temperatures up to 70°C . If the isolate does not grow on acetate, it raises a second possibility, that this high temperature culture is an acetate-oxidizing two-membered culture similar to the one we originally described in 1984 (17), in which the methanogen is using the H_2/CO_2 (or formate) produced by the acetate oxidizer. In fact, Rintala et al (12) recently described a similar enrichment, although its maximum temperature was 70°C .

Thus, our next step is to test the H_2/CO_2 isolate for growth on acetate (and other potential methanogenic substrates such as formate and ethanol). If this culture grows on acetate, it is a novel methanogen, and can be readily characterized. This will include sequencing the DNA encoding its 16S rRNA, which our laboratory now has the capability of doing (see below). If it does not grow on acetate, we may characterize it further anyway, especially examining whether it can grow at 80°C as the original culture did since *Methanobacterium thermoautotrophicum* is considered to be incapable of growth at temperatures exceeding 75°C . Since it seems to grow optimally near 70°C , it is unlikely to be *Methanothermus* which grows optimally at 83°C . If the methanogenic culture is a syntrophic association, it will probably be much more challenging and time-consuming to isolate the acetate-oxidizing organism. Our first approach will be to determine whether the culture grows rapidly on more energetic substrates such as ethanol or ethylene glycol as the AOR does (10). This would facilitate isolation, since it would increase the numbers of the acetate-oxidizer relative to other members of the culture. Lawns of the methanogen can be used to culture the acetate oxidizer (17). The growth kinetics and temperature responses of this culture will be studied.

5. Methanol-utilizing methanogen. In previous progress reports, we described a mesophilic methanol-utilizing methanogen, strain PCE, from an enrichment culture being fed both methanol and tetrachloroethylene (PCE). This organism forms packets resembling *Methanosarcina*, but is unable to utilize acetate or substrates other than methylated compounds. It also seems unable to adapt to medium containing more than 100 mM salt. Finally, the %G+C of its DNA is 37, significantly lower than *Methanosarcina* cultures (ca. 42%), but closer to that of some strains of the methylotrophic methanogen *Methanolobus*, all presently known strains of which are marine. We have tried other taxonomic tests, such as one-dimensional SDS-PAGE of the proteins, but have not definitively resolved the taxonomic position of this isolate within the *Methanosarcinaceae*. We have recently used the polymerase chain reaction (PCR) using universal 16S rRNA primers to amplify the 16S rDNA from strain PCE, and intend to directly sequence the first 300 bp of the PCR product (direct sequencing of PCR products is less prone to errors than sequencing a cloned PCR product, which immortalizes mistakes made by Taq polymerase) using the universal primer. We have determined, examining rRNA sequences obtained from the Ribosomal Database Project, University of Illinois, that there should be enough divergence in that fragment to use its sequence as a diagnostic test to determine whether strain PCE shows any affinity to known methylotrophic methanogens. If no affinity is detected, we may have to sequence a larger portion of the PCR product. This should complete our characterization of this organism and the results would be submitted to the International Journal of Systematic Bacteriology.

REFERENCES

1. **Ahring, B. K., and P. Westermann.** 1984. Isolation and characterization of a thermophilic acetate-using methanogenic bacterium. *FEMS Microbiol. Lett.* **25**: 47-52.
2. **Fischer, R., and R. Thauer.** 1988. Methane formation from acetyl phosphate in cell extracts of *Methanosarcinabarkeri*. *FEBS Lett.* **228**: 249-253.
3. **Fukazaki, S., N. Nishio, and S. Nagai.** 1990. Kinetics of the methanogenic fermentation of acetate. *Appl. Environ. Microbiol.* **56**: 3158-3163.
4. **Grahame, D. A.** 1991. Catalysis of acetyl-CoA cleavage and tetrahydrosarcinapterin methylation by a carbon monoxide dehydrogenase-corrinoid enzyme complex. *J. Biol. Chem.* **266**: 22227-22233.
5. **Inatomi, K., Y. Kamagata, and K. Nakamura.** 1993. Membrane ATPase from the aceticlastic methanogen *Methanothrix-Thermophila*. *J. Bacteriol.* **175**: 80-84.
6. **Jetten, M. S. M., A. J. M. Stams, and A. J. B. Zehnder.** 1989. Isolation and characterization of acetyl-coenzyme A synthetase from *Methanothrix soehngenii*. *J. Bacteriol.* **171**: 5430-5435.
7. **Jetten, M. S. M., A. J. M. Stams, and A. J. B. Zehnder.** 1989. Purification and characterization of an oxygen stable carbon monoxide dehydrogenase of *Methanothrix soehngenii*. *Eur. J. Biochem.* **181**: 437-441.
8. **Jetten, M. S. M., A. J. M. Stams, and A. J. B. Zehnder.** 1990. Acetate threshold values and acetate activating enzymes in methanogenic bacteria. *FEMS Microbiol. Ecol.* **73**: 339-344.
9. **Krzycki, J. A., L. J. Lehman, and J. G. Zeikus.** 1985. Acetate catabolism by *Methanosarcina barkeri*: evidence for involvement of carbon monoxide dehydrogenase, methyl coenzyme M and methylreductase. *J. Bacteriol.* **163**: 1000-1006.
10. **Lee, M. J., and S. H. Zinder.** 1988. Isolation and characterization of a thermophilic bacterium which oxidizes acetate in syntrophic association with a methanogen and which grows acetogenically on H_2 - CO_2 . *Appl. Environ. Microbiol.* **54**: 124-129.
11. **Min, H., and S. H. Zinder.** 1989. Kinetics of acetate utilization by two thermophilic acetotrophic methanogens: *Methanosarcina* sp. strain CALS-1 and *Methanothrix* sp. strain CALS-1. *Appl. Environ. Microbiol.* **55**: 488-491.
12. **Rintala, J., S. Lepisto, and B. Ahring.** 1993. Acetate degradation at 70° C in upflow anaerobic sludge blanket reactors and temperature response of granules grown at 70° C. *Appl. Environ. Microbiol.* **59**: 1742-1746.
13. **Teh, Y. L., and S. H. Zinder.** 1992. Acetyl-coenzyme A synthetase in the thermophilic, acetate-utilizing methanogen *Methanothrix* Sp. strain CALS-1. *FEMS Microbiol. Lett.* **98**: 1-8.
14. **Terlesky, K. C., M. J. K. Nelson, and J. G. Ferry.** 1986. Isolation of an enzyme complex with carbon monoxide dehydrogenase activity containing corrinoid and nickel from acetate-grown *Methanosarcina thermophila*. *J. Bacteriol.* **168**: 1053-1058.
15. **Westermann, P., B. K. Ahring, and R. A. Mah.** 1989. Threshold acetate concentrations for acetate catabolism by acetoclastic methanogenic bacteria. *Appl. Environ. Microbiol.* **55**: 514-515.
16. **Zinder, S. H., and T. Anguish.** 1992. Carbon monoxide, hydrogen, and formate metabolism during methanogenesis from acetate by thermophilic cultures of *Methanosarcina* and *Methanothrix*. *Appl. Environ. Microbiol.* **58**: 3323-3329.
17. **Zinder, S. H., and M. Koch.** 1984. Non-aceticlastic methanogenesis from acetate: Acetate oxidation by a thermophilic syntrophic coculture. *Arch. Microbiol.* **138**: 263-272.

RECENT PUBLICATIONS SUPPORTED BY DOE

- Zinder, S.H. 1990. Conversion of acetic acid to methane by thermophiles. *FEMS Microbiol. Rev.* **75**:125-138.
- Min, H., and S.H. Zinder. 1990. Isolation and characterization of a thermophilic sulfate reducing bacterium *Desulfotomaculum thermoacetoxidans* sp. nov. *Arch. Microbiol.* **153**:399-404.
- Zinder, S. H. 1992. Methanogenesis. *In*: J. Lederberg, Ed. *Encyclopedia of Microbiology*, Vol. 3, 80-96. Academic Press, NY
- Zinder, S. H., and T. Anguish. 1992. Carbon monoxide, hydrogen, and formate metabolism during methanogenesis from acetate by thermophilic cultures of *Methanosarcina* and *Methanotherix*. *Appl. Environ. Microbiol.* **58**:3323-3329.
- Teh, Y. L., and S. H. Zinder. 1992. Acetyl-coenzyme A synthetase in the thermophilic, acetate-utilizing methanogen *Methanotherix* Sp. strain CALS-1. *FEMS Microbiol. Lett.* **98**:1-8.
- Zinder, S. H. 1993. Physiological ecology of methanogens, pp 128-206 *In*: J. G. Ferry, Ed. *Methanogenesis*. Chapman Hall Routledge, NY.
- Zinder, S. H. Syntrophic acetate oxidation and "reversible" acetogenesis. *In*: H. L. Drake, Ed. *Acetogenesis*. Chapman Hall Routledge. In press.

Abstracts:

- Teh, Y.-L. and S.H. Zinder. Acetyl-CoA synthetase from *Methanotherix* strain CALS-1. Proceedings, Ninety-first Annual Meeting, American Society for Microbiology, Paper No. I-6, 1991.
- Allen, G., C. Hand, T. Anguish, and S.H. Zinder. Methanogenesis from acetate in cell-free extracts and whole cells of *Methanotherix* sp. strain CALS-1. Proceedings, Ninety-first Annual Meeting, American Society for Microbiology, Paper No. I-6, 1991.
- Teh, Y. L., and S. H. Zinder. 1993. Characterization of membrane-bound ATPase activity in *Methanotherix* sp. strain CALS-1. Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993.
- Allen, G., C. Hand, B. Myers, And S. H. Zinder. 1993. Methanogenesis from acetate in cell-free extracts of the thermophilic methanogen *Methanotherix* sp. Strain CALS-1. Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993.

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DATE

FILMED

6 / 29 / 94

END