Narrative.

In the 14 calendar years (12 actual years of funding and two no-cost extensions) of support from 1985 until 1999 provided by DOE Grant DE-FG02-85ER13370 there were 30 articles, reviews, and book chapters that resulted from this support, with two more in press and a few more in preparation.

The initial project dealt with the ecology and physiology of thermophilic anaerobic methanogens converting acetic acid to methane. The PI was involved in the first isolation of all of the three thermophilic species known to carry out this reaction, which accounts for two thirds of the methane produced by anaerobic bioreactors. These three cultures are *Methanosarcina thermophila* (2), a thermophilic *Methanothrix* (now called *Methanosaeta)* strain CALS-1 (5), and an acetate-oxidizing coculture converting acetate to methane using interspecies hydrogen transfer (7-9). The latter organism was the most original contribution, since the reaction it carried out had never been described before. In the twelve years since the description of the two membered oxidizing culture, no novel thermophilic methanogenic acetate-utilizing cultures have been isolated despite numerous attempts by the PI as well as those of K. Stetter or B. Ahring. We also isolated a thermophilic acetate-utilizing sulfate reducer (13).

During this period of support, we demonstrated that the acetate-utilizing coculture could be resolved into an acetate-oxidizing rod (AOR) and a methanogen by culturing the former organism on pyruvate or ethylene glycol (7). Moreover, we demonstrated that the levels of hydrogen in the culture were higher than those predicted by the Nernst Equation and that entropic effects of temperature must be considered (8), a finding confirmed most notably by R. Conrad. We also demonstrated that the AOR oxidized acetate through the carbon monoxide pathway rather than the tricarboxylic acid cycle, the first organism to be demonstrated to do so (9).

Much of our work centered on the thermophilic *Methanosaeta* CALS-1. It had long been known that *Methanosaeta* was often present at low acetate concentrations, whereas, *Methanosarcina* was present when acetate was abundant. It was proposed that this could be explained by a higher K_s for acetate in *Methanosarcina*. Our kinetic studies (11) showed that this difference was due to a much lower threshold for acetate (ca. 5 μM) in *Methanosaeta* than in *Methanosarcina* (ca. 1 mM). This was the first demonstration that a threshold model could explain this important facet of competition between these two organisms, and this was confirmed by a concurrent paper by Ahring and Mah, as well as by later work by Jetten and Zehnder.

Thus, two organisms carrying out the same reaction had considerably different thermodynamics and kinetics of reaction. It was of interest to determine biochemical differences between *Methanosaeta* and *Methanosarcina* that could explain these physiological differences. One aspect is how electrons flow from acetate to methane. In *Methanosarcina*, several laboratories showed that H_2 was in thermodynamic equilibrium with acetate metabolism, suggesting a mechanism of hydrogen cycling, a finding we confirmed. We demonstrated that in strain *Methanosaeta* CALS-1, however, H_2 was not produced or consumed, and that CO acted as though it were in thermodynamic equilibrium.

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Another aspect is how acetate is activated. The work of the Ferry laboratory established that *Methanosarcina* used an acetate kinase-phosphotransacetylase system for acetate activation to acetyl-CoA, whereas the Zehnder group demonstrated the presence of an acetyl-CoA synthetase (ACS) in *Methanosaeta*. The former enzyme uses one ATP equivalent per acetate activated whereas the latter enzyme uses two ATP equivalents, an enormous amount of energy to sacrifice. We demonstrated that indeed the only acetate-activating enzyme in the strain CALS-1was ACS and provided expanded data on its kinetics (16), including the inhibitory nature of pyrophosphate.

To better understand the biochemistry of methanogenesis from acetate in strain CALS-1, we developed a cell-free methanogenesis system (22). We found that we obtained much better activity if the cells were grown in a pH auxostat rather than in batch culture. Indeed, this cell-free system showed a similar specific activity to whole cells, a first for a cell-free methanogenesis system. We demonstrated that methanogenesis from acetate was not dependent upon H_2 in strain CALS-1 as the *Methanosarcina* system was, in agreement with our previous findings that H_2 was not in equilibrium with methanogenesis in whole cells. We determined that about 2 mol ATP were needed per mol of methane produced, consistent with ACS activation of acetate. In subsequent studies (31), we demonstrated that coenzyme M, methanopterin, coenzyme B, and coenzyme A were required for methanogenesis from acetate. Moreover, when antibodies against ACS were used to immunoprecipitate it from extracts, there was a complete loss of activity which could be restored by adding back purified ACS, thus conclusively demonstrating the role of ACS in methanogenesis from acetate in strain CALS-1. Similarly, centrifuging out cell membranes led to loss of activity that could be restored by adding back a membrane preparation.

Despite the progress made in these studies, it was difficult to find graduate students interested in studying physiology and biochemistry of methanogenesis from acetate, and studies moved to physiology, biochemistry, and molecular biology of nitrogen fixation in *Methanosarcina barkeri*. Our previous studies showed that *M. barkeri* could fix nitrogen, that the nitrogenase enzymes themselves showed very low specific activity, and that the genes encoding the nitrogenase structural proteins resembled those of the gram-positive eubacterium *Clostridium pasteurianum*. We also showed that ammonia in the medium led to both repression and switch-off of the nitrogenase system. In partially DOE funded studies (21), we demonstrated that a nitrogenase transcript could not be found in *M. barkeri* growing with ammonia, and that this transcript was processed. We also found in gel-shift assays that a DNA-binding complex could be found associated with the promoter region the nitrogenase genes in N_2-grown cell and not in ammonia-grown cells (25). Moreover, there was a substance in ammonia-grown cells, most likely a protein, that inhibited DNA binding by proteins in extracts from N_2-grown cells.

In studies on the physiology of nitrogen fixation, we found that despite the high sensitivity of the nitrogenase enzymes in vitro to inhibition by salt, the intact *M. barkeri* cells could fix nitrogen in the presence of up to 0.8 M NaCl in the medium. This led to a study on the effect of osmolytes on nitrogen fixation (27). We found, as other had previously, that glutamate and N^{ε}-acetyl-β-lysine accumulated in cells in high NaCl, and that potassium glutamate was actually stimulatory to nitrogenase in this organism. There was no shift away from these nitrogen-rich and therefore energetically costly osmolytes when fixing nitrogen.

We also recently demonstrated that the so-called *nifD1* gene from *M. barkeri* resembles that of a V nitrogenase from the cyanobacterial eubacterium *Anabaena*. Indeed, the two predicted gene products are 80% identical at the amino acid level, an unprecedented level of identity between a eubacterial and archaeal protein, strongly implying gene transfer. (30). We also demonstrated the presence of a *vnfG* gene between *nifD1* and *nifK1*, further evidence for a vanadium nitrogenase, and demonstrated stimulation of nitrogen fixation by V added to the medium. Manuscripts are in preparation describing glutamate accumulation in *M. barkeri* subjected to switch-off, and further characterization of the nitrogenase proteins from *M. barkeri*.

DDOE support, including personnel and equipment, allowed the PI to collaborate with others at Cornell. Collaborations that led to the PI's name on a published paper include one demonstrating that methane gas bubbles produced from methanol by *Methanosarcina barkeri* could impede liquid flow in soils (18). The PI also helped in the physiological and phylogenetic characterization of tannin resistant anaerobes from animal rumen populations (20, 26). A collaboration with a laboratory studying methane oxidation (28) led to the development of a method using *Methanosaeta* strain CALS-1 to synthesize ^{14}C-labeled methane from methyl-labeled ^{14}C-acetate. This methane is considerably purer and of higher specific activity than either chemically or biologically synthesized methane using previously developed methods, and it has been adapted by the Reeburgh laboratory (personal communication) to produce tritiated methane for methane oxidation studies.

The PI has been invited to write many reviews and book chapters related to DOE supported work. These include reviews on thermophilic waste treatment (3) and on thermophilic acetate utilizers (10, 12, 19). The PI is particularly proud of a review on physiological ecology of methanogens (17), which is one of the articles R. Thauer (personal communication) provides new members of his laboratory to understand the field. The PI has also written general reviews about methods to study methanogens (24), bacterial diversity (23), and has co-authored a prefatory chapter on microbial ecology with Abigail Salyers in the 2000 edition of Bergey's Manual. Finally, the PI was chair of the 1999 Gordon Research Conference on Archaea, ironically after he had lost DOE support to study Archaea. This conference was very successful, and the PI received many compliments for it, as well as a "grade" of 1.4 on a scale of 1-5 in evaluations of the conference, in which 1.0 is the top score.

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