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Progress Report

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Introduction and summary. The goal of this project is to gain a more complete understanding of the microorganisms converting a lignocellulose waste to methane in a thermophilic (58°C) anaerobic bioreactor. To accomplish this, we have directly examined microbial populations in the bioreactor and have examined the properties of microorganisms isolated from the bioreactor. The primary focus has been on anaerobic thermophiles involved in the formation and degradation of acetic acid, the precursor of two thirds of the methane produced in the bioreactor. Also, novel organisms of fundamental and practical significance have been isolated and characterized. As the project has progressed there has been greater emphasis on the physiology of pure cultures.

Among the accomplishments of the granting period (1985-90) are: 1) the demonstration that [U-^{14}C] glucose was metabolized directly to acetate by microbial populations in the bioreactor without the formation of intermediates such as propionate or butyrate. Addition of hydrogen caused significant lactate accumulation; 2) the isolation of a thermophilic *Methanothrix* (t_{opt} = 60°C) which grows much more rapidly (24 hr t_d) than do mesophilic strains; 3) the demonstration that both a thermophilic *Methanothrix* and a thermophilic *Methanosarcina* show thresholds for acetate utilization near 15 μM and 1 mM respectively. This is consistent with ecological data indicating that *Methanothrix* is favored by low acetate concentrations; 4) the demonstration of high acetyl-CoA synthetase activity in *Methanothrix* strain CALS-1 and the demonstration that the enzyme is apparently a homodimer with subunit molecular weight near 78,000 (see proposal); 5) the demonstration of high activities of carbon monoxide dehydrogenase in *Methanothrix* strain CALS-1 and that the activity is extremely thermostable; 6) the isolation of the acetate-oxidizing member of thermophilic syntrophic coculture and demonstration that it is an acetogen; 7) the demonstration that key acetogenic enzymes were present both in the axenic culture of the acetate oxidizer grown acetogenically as well as in the acetate-oxidizing coculture; 8) the finding that entropy effects needed to be taken into account to explain the partial pressure of hydrogen found in the syntrophic acetate oxidizing coculture when

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growing at 60°C; 9) the isolation of a thermophilic acetate oxidizing sulfate reducer, *Desulfotomaculum thermoacetoxidans*; 10) the demonstration of growth inhibition of thermophilic *Methanosarcina* cultures by culture supernatants of *Clostridium thermocellum*.

Thermophilic *Methanothrix*. We first described the presence of a thermophilic *Methanothrix* in our digestor in 1984 (Appl. Environ. Microbiol. 47:796-807). Reprint #1 describes the isolation and characterization of *Methanothrix* sp. strain CALS-1. The purity of the culture has been verified by H. Hippe and J. P. Touzel, making it apparently the first axenic culture of *Methanothrix*, since mesophilic cultures are contaminated (Touzel et al., Int. J. Syst. Bacteriol. 38:30-36, 1988). Among the salient features of the culture are the presence of gas vesicles, the ability to grow on acetate with a doubling time near 24 h (more than three times as rapid as mesophiles), the requirement of biotin as the only organic growth supplement, the optimum temperature near 60°C (5-10°C higher than that for *Methanosarcina* cultures), and the ability to use acetate rapidly at concentrations less than 1 mM.

At the time of publication, we had difficulty obtaining DNA suitable for determining G+C ratio. We have since used hydroxylapatite to purify the DNA and determined that it contains 50-52% G+C residues, similar to mesophilic *Methanothrix*. More recently, Touzel (pers. comm.) has determined that there is essentially no DNA-DNA hybridization between strain CALS-1 and mesophilic strains, and Rouviere (pers. comm.) has sequenced a good portion of the 16S rRNA from strain CALS-1 and found that the degree of relationship to mesophilic *Methanothrix* strains is similar to that between *Methanosarcina* and *Methanococcoides*. Nozhevnikova and Chudina (Microbiol. 53:618-624, 1985) named a thermophilic enrichment with strong morphological resemblance to strain CALS-1 *Methanothrix thermoacetophila*, and we may have to accommodate that name for ours. I am presently attempting to obtain a culture from them so that we can compare them, including by DNA-DNA hybridization studies in collaboration with J. P. Touzel. The isolation of this acetate utilizing methanogen was a major contribution to our understanding of the diversity of acetotrophic methanogens.

More recently Visiting Scientist Min Hang from Peoples Republic of China studied the kinetics of acetate utilization by *Methanothrix* sp. strain CALS-1 and by *Methanosarcina* sp. strain CALS-1, also isolated from our digestor (Reprint #2). It is

generally considered that while *Methanosarcina* can grow more rapidly on acetate, *Methanothrix* has an apparent K_m for acetate uptake an order of magnitude lower than *Methanosarcina*, making it more competitive at low acetate concentrations. For example, Schönheit et al.(Arch. Microbiol. 132:285, 1982) found an apparent K_m for acetate uptake by a mesophilic *Methanosarcina* of 3-5 mM, while Huser et al. (Arch. Microbiol. 132:1, 1982) reported a value of 0.7 mM for *Methanothrix soehngenii.*

Acetate utilization by washed concentrated *Methanosarcina* sp. strain CALS-1 did **not** follow Michaelis Menton kinetics in that rates were essentially constant at concentrations above ca. 3 mM, followed by a rapid decrease in rate for lower concentrations. Acetate utilization showed a minimum threshold value near 1 mM. We also have found that cultures of *Methanosarcina* growing on acetate for up to 40 days did not completely consume it, with 0.3-0.8 mM acetate typically remaining in the growth medium. The pattern for acetate utilization by *Methanothrix* strain CALS-1 was similar except that the point at which acetate was saturating was near 100 μM, considerably lower than previously reported for acetate-utilizing methanogens. To get more accurate measurements at such low concentrations, we examined utilization of ^{14}C-labeled acetate. There was a threshold for acetate utilization near 15-20 μM acetate for *Methanothrix*. Again, the pattern of acetate utilization was different from that predicted by Michaelis-Menton kinetics. Thresholds for hydrogen utilization by methanogens had been reported previously, and Ahring and Westermann (AEM 53:434, 1978) reported a threshold for acetate utilization by a thermophile they study, the TAM organism. In the same issue of Appl. Environ. Microbiol. as our paper appeared, Westermann, Ahring and Mah (AEM 55:514-515, 1989) published a paper describing thresholds for acetate utilization by mesophilic *Methanosarcina* and *Methanothrix* cultures. This represents a new paradigm for describing kinetics of acetate utilization by acetotrophic methanogens and understanding their competition.

We are presently doing biochemical studies on methanogenesis from acetate by *Methanothrix* sp. strain CALS-1. These studies are described in the research proposal.

Thermophilic acetate-oxidizing coculture. In 1984, we described a unique thermophilic two membered coculture which converted acetate to methane (Zinder and Koch, Arch. Microbiol. 138:263-272, 1984). One organism, now called the AOR (acetate-oxidizing rod), oxidized acetate to hydrogen and CO_2, while the other

organism, a hydrogenotrophic methanogen, produced methane from hydrogen and CO_2. The AOR was dependent on the methanogen to keep hydrogen partial pressures low enough so that acetate oxidation was endergonic. In a recent review, Rolf Thauer (Eur. J. Biochem. 176:497-508) said that we "surprised the scientific community with an organism that oxidizes acetate to 2 CO_2 using protons as electron acceptor....", perhaps an overstatement, but it illustrates the unique nature of the culture.

In the initial study, we were unable to grow the acetate oxidizing organism in axenic culture. After trying many potential substrates for axenic growth, we successfully isolated the organism on ethylene glycol. The isolation of the AOR and its properties are described in Reprint #3. Once isolated, the AOR was found to grow on H_2-CO_2 and produce acetate, precisely the opposite from the reaction it carries out in syntrophic coculture. This shows that the AOR is an acetogen, and which direction it carries out the reaction depends upon the concentrations of reactants and products.

Reprint #4 describes studies on the biochemistry of acetate oxidation by the two-membered coculture. On the basis of a very rapid exchange between the carboxyl group of acetate and CO_2, M. Koch and I predicted in 1984 that the pathway of acetate oxidation would resemble a reversal of the acetogenic pathway in acetogens. This would be the first new pathway for the oxidation of acetate since the tricarboxylic acid cycle and its variants, . We demonstrated that cultures of the AOR grown on H_2-CO_2 and two membered cultures grown on acetate showed high levels of CO dehydrogenase and formate dehydrogenase, key enzymes of that pathway, while levels of isocitric acid dehydrogenase were anabolic rather than catabolic. Activity stains of polyacrylamide gels suggested that a novel species of CO dehydrogenase was synthesized in cells growing on acetate. We recently examined extracts of the acetate oxidizing coculture using a Mono Q column, and only saw one major peak of CODH activity (and a minor peak which we believe is from the methanogen), indicating that either no new CODH was induced in these cells or if it was, it had the same binding to the Mono Q column. It is possible that the different bands we detected on gels represented different aggregation states of CODH or associations with other proteins.

We did not detect significant levels of formyltetrahydrofolate synthetase in cultures grown acetogenically or acetotrophically, which is in marked contrast with the Wood pathway in which it is a key enzyme. Bioassays for folates in the AOR done by D. Nagle and colleagues show that folate levels were much lower in the AOR than in typical acetogens. This finding, plus the finding of a compound with fluorescence similar to methenyl-tetrahydrofolate made us postulate that a pterin other than tetrahydrofolate was involved in acetogenesis and acetate oxidation in the AOR. Evidence supporting a reversal of the acetogenic pathway in acetate-oxidizing sulfate reducers was recently provided by Schauder, Thauer, Fuchs et al. (Arch. Microbiol. 145:162,1986; Arch Microbiol. 151:84-88, 1989; Spormann and Thauer, Arch. Microbiol. 150:374-380, 1988). Recently Länge et al. (Arch. Microbiol. 151:77-83, 1989) presented evidence that a pterin other than tetrahydrofolate, which had four glutamate residues on the side chain, serves as a one-carbon carrier in *Desulfobacterium autotrophicum*. If they purify enough of this cofactor, G. Fuchs has promised to send us some to assay with extracts of the AOR. Thus, our evidence supports a reversal of the acetogenic pathway, a pathway now known to exist in sulfate reducers.

We also examined the levels of hydrogen in the AOR-methanogen coculture during acetate oxidation, and the results of this study were published in Appl. Environ. Microbiol. 54:1457-1461 (1988). While many researchers have made predictions of what H_2 partial pressures should be in such cocultures, measurements typically have only been made on complex systems such as sewage sludge or lake sediments, where microenvironments and other reactions can complicate analysis. We found that H_2 partial pressures were about an order of magnitude higher (ca. 39 Pa) than predicted by simple thermodynamic calculations (ca. 3 Pa). We believe that we have found the basis for this discrepancy: entropy effects must be taken into account when doing calculations for the reaction at 60°C. What this predicts for nearly all anaerobic syntrophic ecosystems, is that H_2 partial pressures will nearly always become higher at higher temperature, and for this example, the difference between the predicted values at 0°C and 80°C is nearly 250 fold. This has important implications on the kinetics of hydrogen turnover at different temperatures. There is now evidence that inhibitory H_2 partial pressures are more than ten fold higher (~3000 Pa) in thermophilic (Ahring and Westermann, Appl. Environ. Microbiol. 54:2393-2397, 1988) than in mesophilic (100 Pa, Dwyer et al., Appl. Environ. Microbiol. 54:1354-1359, 1988) butyrate-oxidizing syntrophic cocultures, supporting our contention.

Presently left is to name the organism and offer an official taxonomic description. Bryan White and David Stahl have sequenced about 1000 base pairs of the 16S rRNA from the AOR, and have found that it falls in the Gram positive lineage, but shows no specific relationship to any particular group. Therefore, we will probably assign it a new genus name, perhaps *Amphibacter (Amphiacetum?, Versilibacter?) coolhaasii*, the species eptithet commemorating V. Coolhaas, who described a similar acetate utilizing enrichment in 1928.

Digestor Studies. At the beginning of the granting period, digestor studies were still underway. The last paper published on digestor work is Reprint #5. In it we described that ^{14}C-glucose, when added carrier-free to digestor sludge, was turned over within five seconds, and that the primary products were acetate and CO_2, with only miniscule amounts of lactate, ethanol, or fatty acids formed, even if they were "trapped" the addition of unlabeled substrate. The direct metabolism of glucose to acetate without any accumulation of intermediates indicates that hydrogen production by glucose consumers and hydrogen consumption by methanogens were tightly coupled processes. The acetate produced was eventually turned over to methane and CO_2.

When H_2 was added to the headspace and cultures were rapidly agitated, less labeled acetate accumulated, and significant amounts of lactate were formed instead, indicating that lactate was the primary electron sink for the glucose utilizing populations. Non labeled lactate accumulated as the primary reduced product in sludges incubated under H_2, suggesting that the response of the glucose utilizing populations to H_2 was representative of the overall response of the carbohydrate utilizing digestor populations. It would have been interesting to repeat these experiments with ^{14}C-cellobiose, which is more likely to be a product of cellulose hydrolysis, but it was not commercially available. We attempted to examine the breakdown of ^{14}C-cellulose, but the material sent to us by ICN Radiochemicals was not of sufficient radiochemical purity, while the preparation from New England Nuclear had too high a specific activity to be used in quantitative studies.

Other studies. We had done some preliminary studies on tricultures of *Clostridium thermocellum*, *Methanobacterium thermoautotrophicum*, and *Methanosarcina thermophila* strain CALS-1 in which the expected result was nearly stoichiometric

conversion of cellulose to methane. Instead we found that growth of the *Methanosarcina* was always inhibited by the *Clostridium.* This appeared to be due to production of an inhibitory substance by the *Clostridium* rather than nutrient depletion, since the effect was not reversed by adding back all of the nutrients in the medium. These studies were hampered by the apparent instability of the inhibitory substance. That this putative factor is unstable, perhaps to biological as well as chemical attack, is consistent with the coexistence of *Methanosarcina* and *C. thermocellum* in the digestor from which they were isolated. A brief paper describing the inhibition phenomenon will be sent to FEMS Microbiol. Ecol. after one more round of editing.

Visiting fellow Min Hang and I have also isolated a spore-forming thermophilic acetate-utilizing sulfate reducer from the digestor and have named it *Desulfotomaculm thermoacetoxidans* strain CAMZ. It can use a variety of simple organic compounds, and oxidizes substrates such as lactate completely to CO_2. Similar to the AOR, it also can grow on H_2-CO_2, with the primary product being acetate, although sulfate is required for growth with H_2CO_2 and acetate and sulfide were produced simultaneously. We had originally believed that this sulfate reducer used benzoate and could couple with methanogens via interspecies hydrogen transfer. Unfortunately, the isolated culture of this organism did not grow on benzoate. This was somewhat puzzling since it strongly resembled one of the three predominant morphotypes in the thermophilic benzoate enrichment from which it was isolated, the other two morphotypes being *Methanothrix* and an autofluorescent *Methanobacterium.* A manuscript describing this organism has been prepared, and as soon as some better photographs of strain CAMZ are taken, we will submit it to Int. J. Syt. Bacteriol.

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