DOE/ER/13370--4

DE89 011595

INTRODUCTION

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.

Previous accomplishments of this project have included: 1) the demonstration of a population shift from *Methanosarcina* to *Methanothrix* as the predominant aceticlastic methanogen in the bioreactor; 2) the demonstration that this shift was accompanied by significant changes of the digestor's response to temperature stress, and that these changes correlated well with the known temperature responses of the organisms involved; 3) the demonstration that hydrogenotrophic and acetotrophic methanogenic populations had markedly different responses to the methanogenic inhibitor 2-bromoethane sulfonate, showing that methanogenesis in a bioreactor should not be considered a single process; 4) the co-isolation of a two membered coculture which converts acetate to methane via oxidation and interspecies hydrogen tranfer, a novel mechanism; 5) 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; 6) the isolation of a thermophilic *Methanothrix* which grows much more rapidly than mesophilic strains and should be an excellent model organism for study of methanogenesis from acetate; 7) the isolation of the acetate-oxidizing member of coculture and demonstration that it is an acetogen; 8) the demonstration that this organism contains key acetogenic enzymes 9) the finding that entropy effects needed to be accounted for to explain the partial pressure of hydrogen found in the syntrophic acetate oxidizing coculture when growing at 60°C.

RECENT PROGRESS AND PROPOSED STUDIES

1. <u>Acetate-oxidizing coculture</u>. Three papers were published on this culture in 1988. Reprint #1 (Appl. Environ. Microbiol. 54:124-129, 1988) describes the isolation and characterization of the acetate oxidizing rod (AOR) and demonstration that it is an acetogen. Reprint # 2 (Arch. Microbiol. 150:513-518, 1988) demonstrates the presence of carbon monoxide dehydrogenase, a key enzyme of acetogenesis in both pure and mixed cultures of the AOR. Reprint #3, describes our finding that hydrogen levels were higher than predicted simply using the

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Nernst Equation, but were in line with predictions when entropy effects on chemical equilibrium at 60°C rather than the standard temperature of 25°C was taken into account.

Brian White, Univ. Illinois, has sequenced approximately 1000 bases of the 16S rRNA from the AOR, and David Stahl, also at Illinois has analyzed the data. The organism has the signature sequence of the Gram-positive bacteria, but shows no specific relationship with any of the Gram-positives tested, most likely meaning that it represents a separate branch of this group. We plan to submit a taxonomic note naming it perhaps *Versilibacter (Amphibacter?) coolhaasii* after V. C. Coolhaas, a Dutch researcher who gave an early description (1928) of a culture essentially identical with ours. The culture is not presently being worked on.

2. **Acetate Utilization by Thermophilic *Methanothrix* and *Methanosarcina*.** 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, a thermophilic methanosarcina isolated from our digestor. 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 *Methanosarcina* sp. strain CALS-1 did **not** follow Michaelis Menton kinetics in that rates were completely linear down to ca. 3 mM, followed by a rapid decrease in rate for lower concentrations. Acetate utilization showed a threshold value near 1 mM, which has not been reported before, although thresholds for hydrogen utilization by methanogens have been reported. We also have found that cultures of *Methanosarcina* growing on acetate do not completely consume it, and 0.3-0.8 mM acetate typically remained. The pattern for acetate utilization by *Methanothrix* strain CALS-1 was similar to that from *Methanosarcina* except that the point where 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 have examined utilization of ^{14}C-labeled acetate. There was threshold for acetate utilization near 15-20 μM acetate for *Methanothrix*. This pattern of acetate utilization is different from that predicted by Michaelis-Menton kinetics, but Ahring and Westermann (AEM 53:434, 1978) reported a threshold for acetate utilization by a thermophile they study, the TAM organism, and Clarens et al, from Narbonne, France, presented a poster at the Fifth Symposium of Anaerobic Digestion which showed a threshold for a thermophilic *Methanosarcina* which they've studied. This work

will be published in the February 1989 issue of AEM (Preprint # 1).

This difference in substrate affinities is most likely related to how *Methanosarcina* and *Methanothrix* take up and activate acetate. It is believed that acetate permeates through the membrane as the free acid, although carriers may be involved, and that the rate-determining factor in utilization is the activation of acetate, apparently to acetyl-coenzyme A. While the labs of Zeikus and Ferry have presented convincing evidence that acetate is activated via an acetate kinase-phosphotransacetylase system in either mesophilic or thermophilic strains of *Methanosarcina*, Kohler and Zehnder published a paper indicating that an acetate thiokinase system may be operating in the mesophile*Methanothrix soehngenii*. While thiokinases generally show greater affinities for acetate, they also theoretically expend an extra ATP equivalent if the pyrophosphate produced is not conserved. We are examining acetate activation in *Methanosthrix* sp. strain CALS-1 in order to compare the kinetics with those of acetate utilization in whole cells, and to examine the energetic consequences of utilization of a thiokinase pathway if present.

As described in Abstract #1, we have detected high acetate activating activity (0.4 U/mg protein) in crude extracts using the hydroxamate assay, which does not differentiate between acetyl phosphate and acetyl coenzyme A. The evidence so far favors this activity being due to the presence of a thiokinase type enzyme. The enzyme is oxygen stable and thermostable, showing nearly optimal activity at 80°C. Of particular interest is our finding that the enzyme has an apparent K_m near 5 mM acetate, considerably higher than that for acetate utilization by the organism. We are presently purifying the enzyme by FPLC so that activities of other enzymes will not interfere with more sensitive assays. Of particular importance is removing pyrophosphatase, so that we can determine whether pyrophophate is produced or not.

If pyrophoshphate is produced, as it is in other thiokinases, we can examine the fate of pyrophosphate. Our hypothesis is that the bond will either be conserved by either a pyrophosphate-acetate kinase system, as found by Peck's group for a *Desulfotomaculum* or a membrane-bound proton-translocating pyrophosphatase. We have found high levels of (ca. 1 U/mg protein) of pyrophosphatase in cells and will determine whether it is membrane bound.

We are also studying the CO dehydrogenase (CODH) and hydrogenase activities in strain CALS-1 (Abstract #2). We have found that extracts of strain CALS-1 have high CODH activity (5 μmol min^{-1} [mg protein]$^{-1}$). This enzyme has a temperature optimum near 85°C, considerably higher than the organisms optimal growth temperature of 60°C. It is of considerable interest to determine whether the *Methanothrix*, which only can use acetate, has more than one CODH or not.

We are examining this by FPLC. In a previous progress report we mentioned that *Methanothrix cultures* neither produce or consume hydrogen and that we cound not demonstrate hydrogenase in extracts. We have more recently detected very low levels of an uptake hydrogenase in extracts, but need to repeat and expand the results. The absence of hydrogenase or its presence at very low levels would make a hydrogen cycling mechanism, such as that proposed for *Desulfovibrio* by Odum and Peck (FEMS Lett. 12:47, 1981), unlikely to play a role in acetate catabolism in strain CALS-1. These studies will also increase our understanding of the interrelationship of hydrogen and acetate metabolism in methanogens.

3. **Other studies.** Min Hang has also isolated a thermophilic acetate-oxidizing spore-forming sulfate reducer (*Desulfotomaculum thermoacetoxidans).* 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_2-CO_2 and some of it is reduced to sulfide simultaneously. A first draft of a manuscript on the isolation and characterization of this sulfate reducer has been prepared. 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, a cigar-shaped sporeforming rod, the other two morphotypes being *Methanothrix* and an autofluorescent *Methanobacterium.* Other attempts to isolate the benzoate catabolizer have failed. Min Hang plans to study this enrichment culture further now that he has returned to China. A manuscript on inhibition of *Methanosarcina* by *Clostridium thermocellum* is also in first draft form.

4. **Other activities.** I was the first invited speaker at the Fifth International Symposium on Anaerobic Digestion, Bologna, Italy (Reprint #4), and spoke at the Second International Symposium on the Molecular Biology of Hydrogenase (Abstract #3).