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The objective of this project is to provide an understanding of thermophilic anaerobic microorganisms capable of breaking down acetic acid, the precursor of two-thirds of the methane produced by anaerobic bioreactors. Recent results include: 1) the isolation of *Methanothrix* strain CALS-1, which grows much more rapidly than mesophilic strains; 2) the demonstration that thermophilic cultures of *Methanosarcina* and *Methanothrix* show minimum thresholds for acetate utilization of 1-2.5 mM and 10-20 μM respectively, in agreement with ecological data indicating that *Methanothrix* is favored by low acetate concentration; 3) the demonstration of high levels of thermostable acetyl-coA synthetase and carbon monoxide dehydrogenase in cell-free extracts of *Methanothrix* strain CALS-1; 4) the demonstration of methanogenesis from acetate and ATP in cell free extracts of strain CALS-1. Methanogenesis occurred at a high rate (100-300 nmol min^{-1} [mg protein]$^{-1}$) 5) the demonstration that methanogenesis from acetate required 2 ATP/methane, and, in contrast to *Methanosarcina*, was independent of hydrogen and other electron donors; 6) the finding that entropy effects must be considered when predicting the level of hydrogen in thermophilic syntrophic cultures. This prediction has been verified by others and has a considerable impact on the modeling of electron transfer in thermophilic anaerobic bioreactors; 7) the isolation and characterization of the *Desulfotomaculum thermoacetoxidans*. Current research is centered on factors which allow thermophilic *Methanothrix* to compete with *Methanosarcina*.

phosphotransacetylase route which is used by *Methanosarcina*. While acetate activation by ACS may explain the higher affinity for acetate in *Methanothrix*, it is surprising that *Methanothrix* would use such an energetically costly process for acetate activation.

Graduate Student Yok Lan Teh therefore verified that the thermophilic strain CALS-1 also had high ACS activity (4 μmol min^{-1} [mg protein]$^{-1}$), and purified it to homogeneity and determined its physical and kinetic properties. The ACS is apparently a homodimer with subunit molecular weight near 78 kdal, and a total molecular weight near 165 kdal. The temperature optimum is near 70°C, and the K_m values for acetate and ATP were 2-4 mM and 5.5 mM respectively, while the K_i values for AMP and pyrophosphate were 5 mM and 1.5 mM respectively. The ACS could use ITP with 20% of the activity of ATP, and Mn^{2+} gave 113% of the activity of Mg^{2+}. Propionate was activated at 4% the rate of acetate, while all other organic acids, including glycolate and fluoroacetate, gave less than 1% of the activity of acetate. It is of interest that the K_m for acetate utilization by ACS is higher than that of the whole cells, suggesting that either this enzyme is not responsible for acetate utilization or acetate activation is not the rate-limiting step. We have also found high pyrophosphatatase activity in cell free extracts, and have found it not to be associated with the cell membrane, even in gently lysed extracts. A membrane bound pyrophosphatase could conceivably conserve some of the energy in the pyrophosphate by pumping ions, analogous to a F ATPase. We are sending a manuscript to FEMS Microbiology Letters describing the ACS.

2. Methanogenesis from acetate by cell-free extracts. A major goal of this project has been to obtain cell-free extracts of strain CALS-1 which convert acetate to methane. This has been studied by Graduate Student George Allen. Our initial attempts used extracts from cells grown in 1-liter bottles or 9-liter carboys. These extracts never showed significant activity, perhaps because of the wide pH swings caused by cycles of acetate consumption and acetic acid addition necessary to obtain sufficient cell density. However, we we obtained much better results with cells grown in a 10 liter fermentor in which the pH was maintained at 6.5 using acetic acid fed, on demand, by a pH controller (a pH auxostat). Such cultures could be grown to optical densities approaching 1.0, and provided us with very active cells. 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.

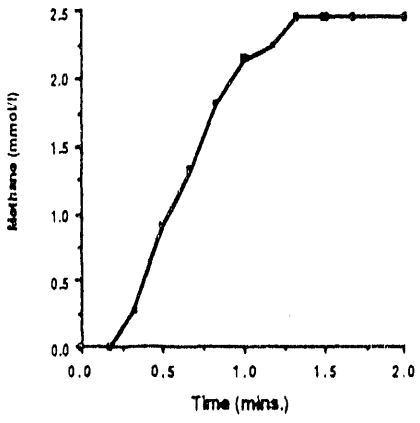

Figure 1. Representative plot of methanogenesis from acetate by a cell free extract of *Methanothrix* strain CALS-1. The assay volume was 1 ml and contained 25 mM HEPES buffer, pH 7.5, 10 mM sodium acetate, 5 mM ATP, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM titanium citrate, ca. 2.5 mg protein, and 10% glycerol.

As shown in Figure 1, there was usually a brief lag after addition of acetate and ATP, followed by rapid nearly linear methanogenesis. Cessation was due to ATP depletion, as repetitive

ATP additions led to proportionately more methanogenesis, with the proportion being near one CH_4 produced per two ATP added, which is consistent with acetate activation by ACS. Methanogenesis was dependent on ATP addition, and much lower rates of methanogenesis (ca. 10 nmol min^{-1} [mg protein]$^{-1}$) were obtained if acetyl CoA or acetyl phosphate replaced acetate and ATP. Centrifuging extracts at 100,000 x g for 2 h did not abolish activity, suggesting that the activity is "soluble", but we suspect there is still a significant amount of membrane material in the extract (see below). Methanogenesis from acetate was optimal at 65°C. Bromoethane sulfonate and cyanide inhibited methanogenesis from acetate, suggesting the involvement of coenzyme M and carbon monoxide dehdyrogenase, respectively.

Probably the most significant finding is that methanogenesis from acetate in the cell free extracts of *Methanothrix* strain CALS-1 was independent of addition of H_2 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 H_2. Indeed, H_2 may be an obligatory intermediate in methanogenesis from acetate in *Methanosarcina*. These results point to major differences in electron transport in these two organisms.

We are presently studying the involvement of ATP and CoA in methanogenesis from acetate in the cell free extracts. We are in the process of writing up a manuscript about these results as well along with those on H_2 and CO metabolism in whole cells presented below and will submit them to J. Bacteriol.

3. Cell membranes and ATPase. It is clear that the cell membrane in strain CALS-1 must play a crucial role in energy conservation and probably in electron transfer during methanogenesis from acetate by strain CALS-1. Graduate Student Yok Lan Teh has developed a two-step ultracentrifugation procedure to purify CALS-1 cell membranes. In these membranes, we have detected cytochromes b and c, typical of mesophilic *Methanothrix* and other methylotrophic methanogens. Two of the heaviest bands in an SDS protein gels from membranes co-purify on a Superose gel permeation column with a DCCD-sensitive ATPase activity. We believe these bands, with molecular weights near 66 kdal, 50 kdal, along with another band near 32 kdal, are the alpha, beta, and gamma subunits of the ATPase, making it more similar to the vacuolar-type ATPases found in other archaebacteria. The temperature optimum for this membrane bound activity was near 70°C, and we intend to purify the ATPase and study it further.

As stated previously, extracts prepared of strain CALS-1 with a French Press at 20,000 psi and centrifuged at 100,000 x g for 2 h still converted acetate to methane. However, we have recently found that these extracts still contain nearly half of their DCCD-sensitive ATPase activity, suggesting that such extracts contain a significant amount of membrane material. We are going to prepare extracts with more gentle lysis and stronger centrifugation to determine more accurately the role of membranes. It is possible that this methanogenesis system, and other systems which have been fractionated in a similar manner, are not really "soluble".

4. Carbon monoxide dehydrogenase. We have previously found that extracts of strain CALS-1 has high carbon monoxide dehydrogenase (CODH) activity, which is as expected, since CODH is considered to be an integral enzyme in the "disassembly" of acetyl CoA. We have found, by size exclusion chromatography, that the CODH in exists in two forms in CALS-1 extracts, one which has a molecular weight near 200,000, with subunits near 90 kdal and 20 kdal, and the other is a high molecular weight complex near 1000 kdal, with at least four subunit types. We see no sign of a fifth subunit described in *Methanosarcina*. One problem in purifying the CODH, is that we obtained poor recovery of activity from the Mono Q ion exchange column, which is our primary means of purification, and there was poor resolution of CODH from methylreductase. We have recently found that we get good resolution and excellent recovery of CODH activity using a hydroxylapatite column. If indeed the CODH complex is missing a subunit

found in to *Methanosarcina* one, it will be interesting to compare the acetyl-CoA assembly and disassembly activities present in the CALS-1 enzyme.

5. H_2 and CO metabolism. Using a reduction gas detector (obtained with DOE funds), capable of detecting sub-part-per-million partial pressures of CO and H_2, Technician Tim Anguish found that while *Methanosarcina* strain TM-1 produced negligible CO and large amounts of H_2 during methanogenesis from acetate, *Methanothrix* strain CALS-1 produced negligible amounts of H_2 and small, but significant, amounts of CO during methanogenesis followed by CO consumption after methanogenesis ceased. These studies were hampered by the large CO emissions from black Bellco butyl rubber stoppers and the CO present in purified gasses. Grey butyl stoppers were found to be more satisfactory, although we still could not obtain background CO levels below ca. 1 ppm. CO dehydrogenase activity was high, and surprisingly, so was formate dehydrogenase. The results corroborate those above on the lack of n H_2 involvement with methanogenesis from acetate by strain CALS-1 extracts and will be published along with those results. We have also found that strain CALS-1 has negligible hydrogenase activity.

Other Studies

1. High temperature methanogenesis from acetate. There are several known hydrogenotrophic methanogens capable of growth at temperatures greater than 70°C, as well as several fermentative bacteria which produce acetate. Therefore, it is reasonable to expect that there are acetate utilizing methanogens capable of growth at temperatures greater than 70°C, yet no one has cultured one, including Karl Stetter who has repeatedly tried (personal communication). Any scheme of anaerobic digestion at temperatures >70°C would need to include acetate-utilizing methanogens to efficiently convert a substrate to methane.

I have been soliciting samples from geothermal areas to enrich for such methanogens. Freshwater samples have included samples from geothermal areas in New Zealand and California, both of which were positive for high temperature anaerobic heterotrophs, yet no acetotrophic methanogens were found. More recently (10/91), John Baross, University of Washington, sent a sample from a flange from the Juan de Fuca ridge. From these samples, we obtained yeast extract utilizing coccoid bacteria at 75° C and 90°C, most likely *Thermococcus*, but no methanogens using either acetate or H_2-CO_2. We will try other samples as they become available.

2. Role of acetotrophic methanogens in a thermophilic pilot scale sludge blanket reactor treating vinasse. Brazil produces about 12 billion liters of ethanol from sugar cane per year using a yeast fermentation, mostly for use as an automotive fuel. This process generates ca. 150 billion liters per year of vinasse, a distillery waste with high organic content (organic acids, plant components, etc.). This waste is applied to the soil in sugar plantations because of its high mineral nutrient content, but the high organic content can cause environmental problems. Since this waste is already heated, it makes sense to convert the organic material to methane using thermophilic anaerobic digestion, thereby converting the organic material to methane, while maintaining the mineral content.

During spring 1990, Ms. Rosana Vazoller, from the University of São Paulo, São Carlos campus, visited my laboratory for three weeks to learn anaerobic techniques. In March 1991, I visited Brazil for a week to give a course on thermophilic methanogenesis, and to consult with Brazilian microbiologists and engineers. I visited the São Martinho alcohol distillation plant which produces ca 2.5 million liters of ethanol per day. At the plant , there was a pilot-scale 75,000 liter thermophilic (55-57°C) upflow anaerobic sludge blanket (UASB) reactor, operated by Dr. M. E. Souza, to treat the vinasse. The bioreactor was operated with a retention time of 10 h and reduced COD by 70%. The reactor was not being operated at the time I visited since it was during the three months between harvest times.

Microscopic examination of the sludge granules using brightfield and epifluorescence microscopy revealed that *Methanosarcina* was numerous, as was *Methanobacterium*, while *Methanothrix* was not detected. Ms. Vazoller, under the guidance of Dr. E. Foresti, is studying the microbiology and kinetics of acetate utilization by the sludge, as well as the effects of various environmental conditions on this process. She plans on visiting my laboratory this Spring for several months to isolate acetate utilizing methanogens from the reactor material (she has recently informed me that an acetate oxidizing rod (AOR) type organism is also numerous), and to do kinetic studies on the cultures she isolates in order to compare them with results from the reactor material. We will also examine the sulfide tolerance of methanogens since the sulfide concentration is high in the reactor, which may inhibit *Methanothrix*. This research is congruent with our earlier ecological studies on thermophilic bioreactors, and may yield some interesting practical results.

3. Methanogen PCE and acetogen PCE. In collaboration with J. Gossett in the Department of Environmental Engineering, we have been studying a mesophilic methanol-fed anaerobic enrichment culture which completely dechlorinates the solvent tetrachloroethylene (PCE) to ethylene (DiStefano, Gossett, and Zinder, Appl. Environ. Microbiol. 57:2287, 1991). During the period when the culture was fed low concentrations of PCE (ca. 3 μM every two days), the culture was methanogenic, and the predominant morphotype resembled small aggregates of *Methanosarcina*. We isolated this organism on methanol , and have characterized it. It did not significantly dechlorinate PCE in a preliminary test. Since our DOE project involves characterization of novel acetate metabolizing methanogens and their relatives, I thought that it was appropriate that Technician Tim Anguish characterize the culture further. It grew on methylated compounds, but did <u>not</u> grow on acetate or H_2-CO_2, making it more physiologically similar to the methylotrophic methanogens *Methanolobus* and *Methanococcoides*. However, these two genera are marine, while the isolate cannot grow at salt concentrations above one fifth those of seawater, and shows no proclivity to adapt to saltwater as does *Methanosarcina*. Furthermore, its G+C ratio is near 37%, lower than *Methanosarcina* (ca. 42%), but similar to *Methanolobus*. One-dimensional SDS gel electrophoresis studies of cellular proteins also show patterns distinct from *Methanosarcina*, and more similar to *Methanolobus*, although the results are not completely clearcut. We are ready to publish a description of the organism, and will probably assign it as a new freshwater species of *Methanolobus*.

When the PCE-degrading methanol enrichment culture was fed high concentrations of PCE (550 μM every two days), it ceased to make methane, and made acetate instead. We have recently isolated a methylotrophic acetogenic Gram positive coccus in chains, most likely a *Peptostreptococcus*. Again, description of this organism is congruent with our mission of better understanding anaerobic acetate metabolism. While it is not clear whether this organism is a dechlorinator, it does have the interesting property of converting methanol and H_2S to millimolar quantities of dimethyl sulfide, a novel reaction. Our progress studying this organism has been impeded because it requires a growth factor(s), present in low concentrations in anaerobic sewage sludge and rumen fluid. We have tried, without success, a large number of compounds that are known to support the growth of anaerobic bacteria. Until we find a better source of this growth factor, it is difficult to get densities of the culture greater than $OD_{600nm} = 0.1$. The studies on this growth factor do not involve DOE personnel, and only minimal effort effort by DOE personnel is being expended on these two novel organisms.

Recent Publications supported by DOE

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