OSMOREGULATION IN METHANOGENS

Progress Report

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Progress Report (5/91-1/93)

Publications Acknowledging DOE support (2 copies of each included):

- (1) M. V. Krishna Sastry, D. Robertson, J. A. Moynihan, and M. F. Roberts, "2,3-Diphosphoglycerate Is the Product of Cyclic-2,3-diphosphoglycerate Degradation in *Methanobacterium thermoautotrophicum*," *Biochemistry* **31**, 2926 (1992).
- (2) D.E. Robertson, D. Noll, and M.F. Roberts, "Free Amino Acid Dynamics in Marine Methanogens: β -Amino Acids as Compatible Solutes," *J. Biol. Chem.* **267**, 14893 (1992).
- (3) D. E. Robertson, M.-C. Lai, R. P. Gunsalus, & M. F. Roberts, "Composition, Variation, & Dynamics of Major Compatible Solutes in *Methanohalophilus* strain FDF1," *Appl. Environ. Microbiol.* **58**, 2438 (1992).
- (4) M. F. Roberts, M.-C. Lai, & R. P. Gunsalus, "Biosynthetic Pathways of the Osmolytes N^ε-acetyl- β -lysine, β -Glutamine, and Betaine in *Methanohalophilus* strain FDF1 Deduced from NMR Analyses", *J. Bacteriol.* **174**, 6688 (1992).

Submitted Manuscripts:

- (5) C. Clougherty, S. Krishnan, R. Ciulla, D. Byrd, and M. F. Roberts, "Halotolerance of *Methanobacterium thermoautotrophicum* strain ΔH ".
- (6) R. Ciulla, S. Krishnan, and Mary F. Roberts, "Internalization of Carbohydrates and Amino Acids by *Methanococcus thermolithotrophicus*".
- (7) A. Gorkovenko and M. F. Roberts, "Cyclic 2,3-diphosphoglycerate as a component of a new branch in gluconeogenesis in *Methanobacterium thermoautotrophicum* strain ΔH ".

The major goal of our work has been to develop and use NMR techniques to study how methanogenic archaeobacteria deal with osmotic stress with the hope of providing insights into increasing the salt tolerance of other cells. The project has three main sections: (i) *in vivo* studies of methanogens; (ii) use of ¹³C- and ¹⁵N- labeled potential precursors and *in vitro* analyses of specific label uptake for elucidation of osmolyte dynamics and biosynthetic pathways of osmolytes in these organisms, and isolation of key biosynthetic enzymes; and (iii) collaborative studies on identification of organic solutes in other methanogens. While we have had some sensitivity and viability problems with the first goal (discussed below), we have made significant progress on goals (ii) and (iii). This is detailed in the following section (numbers refer to the manuscripts listed above).

In two strains of marine methanogens, *Methanogenium cariaci* and *Methanococcus thermolithotrophicus*, four key organic solutes were observed: L- α -glutamate, β -glutamate, N^ε-acetyl- β -lysine, and betaine (2). The first three of these were synthesized *de novo*; betaine was transported into the *Mg. cariaci* cells from the medium. Mesophilic *Mg. cariaci* preferentially transported betaine from the extracellular medium if it was present to counterbalance the external NaCl. In its absence it synthesized N^ε-acetyl- β -lysine as the dominant osmolyte. In *Mc. thermolithotrophicus*, the zwitterionic β -amino acid only appeared in cells grown in medium with $\geq 6\%$ NaCl and became the major osmolyte at higher NaCl concentrations. Intracellular potassium ion concentrations, determined by ³⁹K NMR spectroscopy and atomic absorption, differed significantly in the two cells. In *Mc. thermolithotrophicus* intracellular K⁺ was balanced by the total

concentration of anionic amino acid species, glutamate and β -glutamate. Turnover of the organic solutes was monitored using ^{13}C -pulse/ ^{12}C -chase, and ^{15}N -pulse/ ^{14}N -chase experiments. Both β -amino acids exhibited slower turnover rates when compared to L- α -glutamate or aspartate, consistent with their roles as compatible solutes. Biosynthetic information for the β -amino acids was also provided by ^{13}C -labeling experiments. β -glutamate showed a lag in ^{13}C -uptake from $^{13}\text{CO}_2$, indicative of its biosynthesis from a precursor (probably a macromolecule) not in equilibrium with the soluble L- α -glutamate pool. Confirmation of a novel route for β -glutamate synthesis and the production of the β -lysine moiety from the diaminopimelate pathway was deduced from $^{13}\text{C}_2$ -acetate labeling patterns. Preliminary assays of crude protein mixtures from *Mc. thermolithotrophicus* grown in 8% NaCl detected a lysine-2,3-aminomutase activity. The same activity could not be detected in cells grown in 4% NaCl, consistent with the observation that N $^{\epsilon}$ -acetyl- β -lysine accumulates in 8% but not in 4% NaCl-challenged cells.

In the process of examining ^{13}C -labeling patterns of *Mc. thermolithotrophicus* it was noticed that a variety of solutes including carbohydrates (sucrose, glucose, trehalose, cellobiose, and inositol), amino acids and derivatives (glycine, betaine, and proline) when present in the external medium at high concentrations (e.g., 200 mM) could be internalized to a significant extent (6). ^{13}C NMR spectroscopy showed that the accumulation occurred via a facilitated passive pathway. In the case of a fixed concentration of sucrose, methanogenesis was affected as the NaCl in the external medium was increased. These results may apply to a wider selection of methanogens and may provide an easy route for getting exogenous solutes into methanogens.

A collaboration with the research group of Dr. Robert Gunsalus at U.C.L.A. studying *Methanohalophilus* strain FDF1 has elucidated organic osmolytes (3) and biosynthetic pathways (4) for these solutes in that organism as well. *Mh.* FDF1 can grow over a range of external NaCl concentrations from 1.2 M to 2.9 M and utilize methanol, trimethylamine, and dimethyl sulfide as substrates for methanogenesis. It produces the osmolytes glycine betaine, β -glutamine, and N $^{\epsilon}$ -acetyl- β -lysine with increasing external NaCl, but the relative ratio of these zwitterions depends primarily on the methanogenic substrate and less on the external osmolarity. When the cells are grown on methanol in defined medium, accumulation of glycine betaine predominates over the other zwitterionic solutes. The cells also synthesized a carbohydrate which was not detected in cells grown on trimethylamine. This negatively charged compound, identified as α -glucosylglycerate from the ^{13}C and ^1H chemical shifts, does not act as an osmoregulatory solute in the salt range 1.4-2.7 M in this methanogen as evidenced by its invariant intracellular concentration. $^{13}\text{CH}_3\text{OH}$ -pulse/ $^{12}\text{CH}_3\text{OH}$ -chase experiments were used to determine half-lives for these organic solute pools in the cells. L- α -glutamate showed a rapid loss of heavy isotope, indicating that L- α -glutamate functions as a biosynthetic intermediate in these cells. Measurable turnover rates for both β -glutamine, which acts as an osmolyte, and α -glucosylglycerate suggest they may function as metabolic intermediates as well. Molecules which function solely as osmolytes (glycine betaine and N $^{\epsilon}$ -acetyl- β -lysine) showed a slower turnover consistent with their roles as osmotic solutes in *Methanohalophilus* strain FDF1. $^{13}\text{CH}_3\text{OH}$ -label incorporation by the cells provided information on the biosynthetic pathways of these organic osmolytes. The labeling studies indicate that *Methanohalophilus* strain FDF1 produces glutamate and β -glutamine via a partial oxidative Krebs pathway. ^{13}C -labeling of betaine is consistent with methylation of glycine generated from serine (via serine hydroxymethyltransferase). The labeling pattern for N $^{\epsilon}$ -acetyl- β -lysine is consistent with the synthesis of its precursor α -lysine occurring by the diaminopimelate pathway in these cells.

Another collaboration involved the research group of Dr. Lacy Daniels, University of Iowa, and compared the osmotic response of *Methanobacterium thermoautotrophicum* strain Marburg (grown by Negash Belay in Iowa) to strain ΔH (grown by us) (5). Both of these strains could be adapted to grow in medium containing up to 0.6 M NaCl. In the ΔH strain, from 0.01 to

0.5M NaCl, there was a slight inhibition of cell growth, but methane production was only slightly affected. Total cell protein increased dramatically (reminiscent of 'heat-shock' proteins) and reached a maximum ~0.2 M NaCl. While there were large changes in cell protein, the major intracellular small organic solutes, cyclic-2,3-diphosphoglycerate (cDPG) and glutamate, increased only slightly between 0.01 and 0.4 M NaCl. [cDPG degradation and synthesis have been studied as an offshoot of this work and are summarized in manuscripts (1) and (7).] Total potassium detected by atomic absorption and ^{39}K NMR was roughly constant up to 0.4M, then decreased as the external NaCl increased further. The high intracellular K^+ was balanced by the negative charges of the organic osmolytes. Under these conditions, the cells were clearly hypertonic in low NaCl-containing media. Around 0.4-0.5 M external NaCl, the external osmotic strength and internal osmolarity were similar. The dramatically reduced growth at 0.6 M NaCl and the lack of growth at higher concentrations could reflect the inability of the cells to balance external osmotic pressure. *M. thermoautotrophicum* strain Marburg could be adapted for growth in mineral media with up to 0.52 M NaCl. Growth occurred in up to 0.65 M NaCl if yeast extract was added to the medium; this additive also lessens the long lags observed in mineral media. These cells synthesize a series of organic solutes including cDPG, glutamate, and a novel solute tentatively identified as 2-carboxyglutarate (continuing work will further refine this assignment). The tricarboxylate compound showed the largest changes with increasing external NaCl while cDPG and L- α -glutamate had only modest increases (for comparison, 2-carboxyglutarate was also observed in strain ΔH , but decreased with increasing external NaCl). Cells grown in the presence of exogenous choline internalized this compound and converted it to betaine which was observed as the major organic solute in the cells. Glycine was also internalized from the medium and partially converted to betaine, but the suppression of endogenous osmolyte synthesis occurred to a much lower extent. These results emphasize considerable differences in physiology in the two *M. thermoautotrophicum* strains.

The last collaborative effort involved Dr. Karl Stetter's group at Regensburg. We have tentatively identified a novel inositol phosphodiester, 1-L-myoinositol-phospho-1-D-myoinositol, as a major solute in the extreme thermophilic methanogen *Methanococcus igneus*. Present work involves elucidating its biosynthesis (presumably from glucose) and examining its turnover compared to the α and β -glutamate isomers which are the major osmolytes in these cells.

In vivo NMR work has proceeded at a much slower pace. The major problem has been to provide sufficient substrates for methanogenesis by the high density of cells necessary for observation in the spectrometer. We have preliminary studies of *Ms. barkeri* (using acetate and methanol as soluble substrates) and *Mc. thermolithotrophicus* (using formate) grown in their normal media. Because the methanococcus has a greater NaCl tolerance, and hence a higher internal solute production, we have decided to concentrate on that species (*Ms. barkeri* also had a large tendency to 'clump' at higher NaCl levels). *Mc. thermolithotrophicus* has been adapted to growth on formate (the cells synthesize formate dehydrogenase and use it to generate H_2 and CO_2 *in situ*) and we are presently investigating (*in vitro* NMR studies) the NaCl tolerance of this organism when grown on this substrate. As we have already shown for *Mh. FDF1*, a change in methanogenesis substrate can have a profound effect on osmolyte balance.

Proposed Work - Year 3:

(i) Further *in vivo* ^{13}C , ^{15}N , and ^{39}K NMR studies with *Mc. thermolithotrophicus* adapted to growth on formate. Initially we will finish the *in vitro* studies of NaCl-tolerance of formate grown cells and how organic osmolytes are affected. We have planned $\text{H}^{13}\text{COO}^-$ -pulse/ $\text{H}^{12}\text{COO}^-$ -chase, $^{15}\text{NH}_4^+$ -pulse/ $^{14}\text{NH}_4^+$ -chase experiments for cells at 3, 6, and 8% NaCl. Experiments are planned to increase external NaCl of cells metabolizing in the NMR tube to see how both methanogenesis and osmolyte production are transiently affected. ^{39}K NMR studies will also be pursued, but we have found that the lanthanide shift reagents used to differentiate external and

internal K^+ appear to be somewhat toxic to this methanogen. We are instead trying to develop a spin-echo sequence to exploit T_1 and T_2 differences between internal and external K^+ as a means of selectively following changes in K^+ as external ionic strength is altered.

(ii) N^ϵ -acetyl- β -lysine only accumulates in *Mc. thermolithotrophicus* in cells grown in $\geq 6\%$ NaCl. In working out the biosynthesis of osmolytes we have identified lysine-2,3-aminomutase as a key activity to be monitored. We are presently in the process of trying to purify and characterize this enzyme. We are hoping to get it adequately pure for preliminary N-terminal sequencing. If we can accomplish this we may be able to prepare a DNA probe for use in pulling out the gene from a *Mc. thermolithotrophicus* genomic library (which we have already constructed for another molecular biology study of ATCase in this organism).

(iii) Firm identification of the two novel compounds tentatively assigned as 2-carboxyglutarate and the inositol phosphodiester must be made. This will require purifying both solutes (ion exchange chromatography), confirmation of ^{13}C , 1H (^{31}P) parameters, and possibly MS analysis. Biosynthetic information will also be gathered from specific ^{13}C NMR labeling experiments.

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