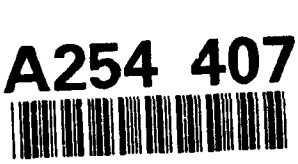

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This work focused on the elucidation of physiological aspects of the hyperthermophilic archaea *Pyrodictium brockii* and *Pyrococcus furiosus*. Continuous cultivation methods were developed for both organisms to facilitate metabolic studies. For *P. furiosus*, we demonstrated the bioenergetic importance of a novel glycolytic pathway centered around a previously reported tunsten-requiring oxido-reductase. We also showed that sulfur reduction is implicated in *P. furiosus* bioenergetics and examined this issue in maltose-limited cultures. Patterns of regulation were followed through the induction of several enzyme activities involved in the organism's energetics.

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FINAL REPORT

Hydrogen/Sulfur Autotrophy in the Hyperthermophilic Archaebacterium *Pyrodictium brockii*

Office of Naval Research - N00014-89-J-1591

January, 1989 to January, 1992

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Research Summary

Our objective in this project was to investigate the bioenergetics and physiology of high temperature bacteria. The initial focus was on a hyperthermophilic chemolithotroph, *Pyrodictium brockii* (Stetter et al., 1983; Parameswaran et al., 1988; Pihl et al., 1989). This bacterium was chosen for several reasons: it has the reported highest optimum growth temperature (105°C) for any organism and it clearly reduces sulfur to sulfide to fulfill some or all of its energetic requirements. Although small amounts of yeast extract stimulate growth, growth proceeds mainly through hydrogen/sulfur autotrophy apparently centered around a series of electron transfer reactions in the cell membrane (Pihl et al., 1989; Pihl et al., 1990). Initially, based on the work on less thermophilic archaebacteria (Danson, 1988), we thought that the absence of significant heterotrophy would simplify the analysis of energetics and allow us to follow growth stoichiometry through gas analysis (i.e., by following the patterns of utilization of CO_2, H_2 and polysulfides, and production of H_2S). An additional objective was to correlate membrane permeability with changes in energetic efficiency at different temperatures for the bacterium; the hypothesis is that the membranes of high temperature bacteria might be relatively permeable creating an energetic burden (McKay et al., 1982).

Continuous culture techniques, developed for *P. furiosus* (Brown and Kelly, 1989), were modified to grow *P. brockii* under sulfur- or hydrogen-limiting conditions. Methods for feeding suspensions of colloidal sulfur or polysulfides were developed and a technique to measure cell membrane permeability was adapted to high temperature. Considerable effort was directed at

determining the stoichiometry of hydrogen/sulfur autotrophy (Schicho et al., 1990). However, after much effort, it was found that difficulties in obtaining sufficiently quantitative data for determining maximal yields and maintenance requirements on hydrogen and sulfur made pursuing the planned study infeasible. Also, extremely low biomass yields (2E7 cells/ml corresponding to approximately 5 mg/l cell dry weight) available for proton motive force and membrane permeability measurements and determination of important enzyme activities caused us to re-assess our choice of bacterium for study.

As an alternative, we switched to *Pyrococcus furiosus*, an organism with which we had considerable experience. In many ways, this has proved to be a much better system for study, particularly in view of reproducibility of growth experiments and biomass yields. Also, some details of *P. furiosus*'s physiology have been worked out and a growing list of enzymes from this bacterium have been purified and studied (e.g., Adams, 1990; Aono et al., 1989; Blumentals et al., 1990; Brown et al., 1990; Brown and Kelly, in preparation; Bryant and Adams, 1989; Connaris et al., 1991; Costantino et al., 1990; Blumentals et al., in preparation; Eggen et al., 1990; Koch et al., 1990; Klingeberg et al., 1991). We have been able to use many of the techniques developed for our *P. brockii* experiments and have made significant progress in our study of *P. furiosus* bioenergetics and physiology. Some of the preliminary work is summarized in the attached manuscripts. More recent results will be outlined here.

Physiological Studies of *Pyrococcus furiosus*

Studies done in our laboratory and others have revealed some details of the growth physiology of *P. furiosus*. Growth can be achieved on artificial seawater-based media (Kester et al., 1967) containing a source of peptides with or without elemental sulfur. Significantly lower levels of peptide-containing extracts can be used if one of a variety of saccharides is included; however, glucose does not seem to be utilized directly for growth possibly because of its instability at high temperatures in hyperthermophilic media. Growth will not occur if some source of peptides is deleted from the media; mixtures of single amino acids will not suffice (Snowden et al., 1992). This obligate requirement for peptides has been described previously for other high temperature bacteria (Smith et al., 1975; Jannasch et al., 1988) and some tyope of peptide uptake system is likely in operation (Pine, 1980; Pittman et al., 1967; Payne, 1976; 1980). In fact, peptide-based extract is routinely added to growth media for extremely thermophilic and hyperthermophilic bacteria. Whether saccharides are present in the media or not, *P. furiosus*'s primary products include carbon dioxide, hydrogen, hydrogen sulfide (if grown on sulfur), acetate, ammonia and a variety of volatile fatty acids (Schicho, 1992), suggesting a fermentative metabolism (Schafer and

Schonheit, 1991). Blumentals et al. (1990) showed that soluble polysulfides are the form of sulfur reduced by the bacterium; these can be generated from elemental sulfur through nucleophilic attack of the crystalline sulfur-ring. Elemental sulfur reduction was found to be entirely dissimilatory. Although it is not clear yet as to the range of sulfur sources used by *P. furiosus* (both radioactive cysteine and methionine were assimilated into cell protein), sulfur compounds which can form polysulfides in aqueous solution can be reduced by *P. furiosus*.

The significance of sulfur in the bioenergetics of *P. furiosus* has been of great interest in our present studies. We have observed the appearance/disappearance of several protein bands on SDS-PAGE if elemental sulfur was added or deleted from growth media (Kelly and Deming, 1988; Brown and Kelly, 1989; Schicho et al., in preparation). We have recently shown, from dilution rate profiles using continuous culture under a maltose-limitation (energy-limiting chemostat), that the maximal growth yield on maltose is approximately 100% higher if a colloidal sulfur suspension is fed to the system. These same studies revealed that the maintenance coefficient is essentially the same for these two cases. (Maltose consumption can be separately attributed to growth and maintenance using simple chemostat theory (Bailey and Ollis, 1986)). There appears to be no change in fermentation pattern as the primary products are the same; here, hydrogen sulfide is counted as hydrogen for growth on sulfur. However, the relative amounts of fermentation products appears to differ for sulfur and non-sulfur cases (Schicho, 1992). This result is provocative and suggests that sulfur reduction, directly or indirectly, influences energetics. This may be the result of the de-bottlenecking of a fermentation pathway through the conversion of H_2 to H_2S or involve alternative energy-generating processes. Prospects now being pursued are the existence of a significant proton motive force coupled to sulfur reduction through a membrane-associated electron transport system and the existence of other ATP-producing glycolytic and amino acid-utilizing pathways.

Recently, Mukund and Adams (1990,1991) suggested that the tungsten-based aldehyde ferredoxin oxidoreductase (AOR) they purified and characterized from *P. furiosus* was central to a novel adaptation of the Entner-Doudoroff pathway; they referred to this pathway as *pyroglycolysis*. Recognizing the potential significance of such a pathway in our studies, we have collaborated with Adam's group at the University of Georgia. Our joint objective is to develop an understanding of the relationship between the operation of the pyroglycolytic pathway, sulfur reduction, and the regulation of several hydrolytic enzymes we have studied. This will be put in the perspective of the growth physiology of *P. furiosus*. Preliminary results suggest that there is such a relationship (Schicho et al., in preparation). Also, further work by Adams and co-workers has implicated tungsten as an essential trace element in the metabolism of other hyperthermophiles

(Adams et al., 1991). Complementary to Adams's efforts to elucidate the biochemical and enzymological significance of tungsten in hyperthermophilic proteins, we are investigating some of the metabolic implications.

The attached manuscript describes our preliminary results investigating the relationship of the pyroglycolytic pathway to the growth physiology of *P. furiosus* (Schicho et al., submitted). In brief, it was determined that low levels of tungsten included in the medium fed to an energy-limited chemostat (maltose-limitation) resulted in significant stimulation of growth. Tungsten addition to continuous culture in which a peptide source (i.e., tryptone) was the energy-limiting substrate showed no such stimulation. These experiments were done in sulfur-free media although this pattern was also seen if colloidal sulfur was added, albeit to a lesser extent. Assays performed on cell-free extracts from these experiments revealed significant stimulation of the AOR in the presence of tungsten.

There were other interesting patterns that were discerned in these preliminary experiments (see manuscript). In the presence of maltose, α-glucosidase (Costantino et al., 1990) activity was stimulated with the highest specific activities noted in the maltose + tungsten case. Total intracellular proteolytic activity decreased as maltose and maltose+tungsten was added to the medium. Hydrogenase (Bryant and Adams, 1989) activity was significantly higher for tryptone and maltose + tungsten than for maltose alone. All of these activities were measured specific to the total protein of the sample cell-free extracts which could create some artifacts. However, the patterns observed are very interesting and support the significance of this pathway in cellular bioenergetics. Clearly, complementary analyses (i.e., transcription events, Western blot analysis) of these phenomena would make interpretation of the results more definitive.

In addition to the influence of tungsten on the growth physiology of *P. furiosus*, we have investigated the action of several hydrolytic enzyme activities. Brown et al. (1990) showed that numerous amylolytic activities were stimulated by the presence of a range of saccharides (Brown, 1992) and that these activities were distributed intracellularly and extracellularly. The previously mentioned α-glucosidase (Costantino et al., 1990; Brown and Kelly, in preparation) which is intracellular and an extracellular enzyme exhibiting both amylase and pullulanase activity (Brown and Kelly, in preparation) have been purified and characterized. These enyzme activities are stimulated significantly by maltose and higher saccharides. If glucose is ultimately the energy substrate for *P. furiosus* growing on maltose or more complex saccharides, the action of these enzymes should be coupled in some way to a glycolytic pathway - especially under an energy-limitation. Intracellular proteolysis has also been examined in *P. furiosus* (Snowden et al., 1992) and other hyperthermophiles (Blumentals et al., in preparation). Among the preliminary findings

in our study was evidence for the existence of multicatalytic proteolytic complexes in these bacteria reminiscient of the non-lysomal structures found universally in eukaryotic cells (Tanaka and Ichihara, 1990; Orlowski, 1990) and more recently in extremely thermophilic archaebacteria (Dahlmann et al., 1989; Zwickl et al., 1991). The regulation of intracellular proteolytic activity in these bacteria may provide additional insight into the basis for life at high temperatures.

The work to this point has led to a conceptual framework within which *P. furiosus*'s energetics and physiology can be examined. As additional information becomes available, through our efforts or others, the framework can be expanded. Admittedly, this is only a beginning but this framework will be useful in broadening the scope of our study to other hyperthermophiles. Ultimately, detailed investigation of catabolic and anabolic pathways, replication apparatus, molecular genetics, membrane transport etc. can be pursued with *P. furiosus* or a more appropriate model organism the focus.

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BOOKS AND BOOK CHAPTERS

Pihl, T.D., R.N. Schicho, L. Black, R.J. Maier, and R.M. Kelly, "Hydrogen-Sulfur Autotrophy in the Growth of the Hyperthermophilic Archaebacterium, *Pyrodictium brockii*, *Biotechnology & Genetic Engineering Reviews - Vol. 8*, Intercept Ltd., Hampshire, UK (1990).

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PRESENTATIONS RELATED TO THIS PROGRAM SUPPORT

Kelly, R.M., J.A. Schuliger and S.H. Brown, "Characterization of Amylolytic Enzymes from High Temperature Bacteria," presented at the Annual Meeting of the AIChE, Los Angeles, CA, November, 1991.

Kelly, R.M., I.I. Blumentals and S.H. Brown, "Hydrolytic Enzymes from Hyperthermophilic Archaebacteria," presented at the International Conference on Marine Biotechnology, Baltimore, MD, October, 1991.

Kelly, R.M., I.I. Blumentals and S.H. Brown, "Hydrolytic Enzymes from Hyperthermophilic Archaebacteria," presented at the Spring meeting of the ACS, Atlanta, GA, April, 1991.

Kelly, R.M., "Hyperthermophilic Archaebacteria," presented at the NIADDK, National Institutes of Health, Bethesda, MD, December, 1990.

Schicho, R.N., D.S. Schmidt and R.M. Kelly, "Hydrogen/Sulfur Autotrophy: Growth and Metabolism of the Hyperthermophilic Archaebacterium *Pyrodictium brockii*," presented at the Annual Meeting of the AIChE, Chicago, Ill., November, 1990.

GRADUATE STUDENTS SUPPORTED IN SOME PART THROUGH THIS PROGRAM

Richard Schicho	Ph.D., April, 1992 (Warner-Lambert, Lowell, MA)
	Bioenergetics of Hyperthermophilic Bacteria
Stephen Brown	Ph.D., August, 1992 (Life Technologies, Inc., Gaithersburg, MD)
	Physiological and Biochemical Characterization of Amylolytic Enzymes from Hyperthermophilic Archaebacteria
Jeffrey Schuliger	M.S.E., August, 1991 (US Army, Aberdeen, MD)
	Growth Physiology and Biochemical Studies of a Novel, Deep Sea Hyperthermophilic Archaebacterium, ES4
Lesley Snowden	M.S.E., August, 1991 (Battelle Northwest, Richland, WA)
	Metabolic Patterns in the Hyperthermophilic Archaebacterium, *Pyrococcus furiosus*

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