# ABSTRACT

HAN, CHAE JOON. Physiological Studies of Extremely Thermoacidophilic Microorganisms under Normal and Stressed Conditions. (Under the direction of Professor Robert M. Kelly.)

The purpose of this research was to study thermoacidophilic physiology for the purpose of developing a new strategy for metal and sulfur biotransformation processes. *Metallosphaera sedula*, an extremely thermoacidophilic archaeon growing mixotrophically on organic and inorganic substrates at pH 2.0 and 74$^{o}$C, was used as a model organism to address this issue. The manipulation of *M. sedula*'s energetics under thermally and chemically challenged states led to increase in specific leaching rates. A dual-limited continuous culture, at a dilution rate of 0.04 $hr^{-1}$, was used to provide a steady-state operation at which the cellular and sub-cellular responses were monitored and the biocatalytic efficiency of cells was evaluated. Under the thermal stress, *M. sedula* showed acquired thermotolerance and "stressed-phase" growth at 81$^{o}$C, which is 2$^{o}$C is higher than its normal maximum growth temperature, and over-synthesized (~6-fold increase) a 66 kDa heat shock protein (MseHSP60), which is immunologically related to a molecular chaperone (Thermophilic Factor 55) from *Sulfolobus shibatae.* More importantly, however, there was a significant increase in specific iron turnover rate presumably through disruption of cellular proton network. In addition, although less dramatic when compared to thermal stress effects, similar results could be obtained when *M. sedula* was subjected to the chemical stress. By temporarily short-circuiting *M. sedula*'s proton network with uncouplers, the respiration capacity of cells was disrupted, leading to the increased $Fe^{2+}$ to $Fe^{3+}$ iron turnover rate. The work described here should propose a new approach for analyzing physiological responses under extreme growth conditions and thereby points to

novel strategies for improving whole-cell catalytic efficiency of thermoacidophilic microorganisms.

# PHYSIOLOGICAL STUDIES OF EXTREMELY THERMOACIDOPHILIC MICROORGANISMS UNDER NORMAL AND STRESSED CONDITIONS

**by**

**CHAE JOON HAN**

A Dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

**CHEMICAL ENGINEERING**

Raleigh

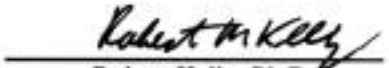
1998

APPROVED BY:

David Ollis, Ph.D.

Steven Peretti, Ph.D.

Walter Dobrogosz, Ph.D.



Robert Kelly, Ph.D.
Chair of Advisory Committee

# BIOGRAPHY

Chae Joon Han was born August 26, 1968 to Hwan Bok and Chun Taik Han in Seoul, Korea. He was educated in the primary and secondary schools in Seoul until 1986. After that, he was transferred to J. M. Hanks High School at El Paso, Texas for his senior year. Chae attended the University of Texas at Austin and received the degree of Bachelor of Science in Chemical Engineering with Honors (December, 1992). In August, 1993, he started graduate studies in the Department of Chemical Engineering at the North Carolina State University,

# Acknowledgments

First, I thank God who accompanied and lifted me in the times of triumphs and trials. I know He was the wing beneath my wing throughout all these curious years. I am always grateful to my parents and my sisters who supported me with constant love and prayers. They are the ones who believe in my abilities and encourage my dreams every day.

I must thank my graduate advisor Dr. Robert Kelly for his guidance and support over these years. His patience and understanding during my times of exhilaration and disappointment are greatly appreciated. I will always remember my co-workers and friends who not only provided technical support and scientific insight but also made me enjoy working at our lab. Thanks go to Tina Rinker (whose charming personality cheered me up on many rainy days) and Bob Shepherd, Sheryl and Mike Bauer, Paula Hicks, Kimberly Parker, Lance Driskill, Jun Gao, Mike Hess, and Guy Duffaud (who revitalized my interest in classical music).

I am very grateful to Dr. JoonSoo Lee at Harvard for all his advice and friendship. His insight and enthusiasm for science has enlightened me ever since my undergraduate years. Many thanks go to SeaHoon (Sam) Park at Georgia Tech who worked for me as like my own ‘clone’.

Lastly, I thank my Bible study group members. Their love and prayers will be always remembered. Special thanks go to OkJin and KyungJin Min whose faith led me to plant my own.

# TABLE OF CONTENTS

Page

LIST OF TABLES .....vii

LIST OF FIGURES .....viii

CHAPTER 1. THERMOACIDOPHILE PHYSIOLOGY

Introduction .....1
Background
Thermophiles .....4
Archaea .....5
Thermoacidophiles .....6
1. Genus *Sulfolobus* .....8
2. Genus *Acidianus* .....9
3. Genus *Desulfurolobus* .....10
4. Genus *Metallosphaera* .....11
5. Genus *Thermoplasma* .....13
6. Genus *Picrophilus* .....14
Microbial Desulfurization / Bioleaching .....14
Bioenergetics .....21
Stress Response .....25
Research Issues .....31
Literature Cited .....34

CHAPTER 2. ACQUIRED THERMOTOLERANCE AND STRESSED-PHASE GROWTH OF THE EXTREMELY THERMOACIDOPHILIC ARCHAEON *METALLOSPHAERA SEDULA* IN CONTINUOUS CULTURE

Abstract .....62
Introduction .....63
Materials and Methods
Cell cultivation.....66
Preparation of cell extracts .....67
One-dimensional SDS-PAGE .....67
Two-dimensional gel electrophoresis .....68
Immunoblotting .....68
Thermal acclimation experiments .....69
Results
Response of *M. sedula* to thermal stress
in continuous culture .....69
Characterization of thermal stress response of
*M. sedula* in continuous culture .....71

Discussion ..... 72
Literature Cited ..... 75

CHAPTER 3. A PROTEASE FROM THE EXTREMELY THERMOACIDOPHILIC ARCHAEON *METALLOSPHAERA SEDULA*: EVIDENCE OF PROTEASOME-LIKE INTRACELLULAR PROTEINASE IN THE KINGDOM *CRENARCHAEOTA*

Introduction ..... 86
Materials and Methods
Microorganism and Preparation of Cell Extracts ..... 89
Purification ..... 90
Proteolytic Activity Assay ..... 90
Electrophoresis ..... 91
Results and Discussion ..... 91
Literature Cited ..... 95

CHAPTER 4. BIOOXIDATION CAPACITY OF THE EXTREMELY THERMOACIDOPHILIC ARCHAEON *METALLOSPHAERA SEDULA* UNDER BIOENERGETIC CHALLENGE

Abstract ..... 108
Introduction ..... 109
Materials and Methods
Cell cultivation ..... 111
Pyrite ..... 112
Batch leaching experiments ..... 112
Continuous culture ..... 112
Temperature effect on bioleaching of *M. sedula* ..... 113
Temperature shifts and thermal stress ..... 113
Chemical stress ..... 113
Iron assays ..... 114
Results
Temperature effect on bioleaching rates within normal growth temperature ranges ..... 114
Effect of thermal stress on bioleaching ..... 115
Effect of respiratory uncouplers on bioleaching ..... 115
Continuous and pulsed addition of nigericin to continuous culture of *M. sedula* ..... 116
Discussion ..... 116
Literature Cited ..... 120

CHAPTER 5. BIOTECHNOLOGICAL APPLICATIONS AND FUTURE RESEARCH DIRECTIONS OF THERMOACIDOPHILES AND HYPERTHERMOPHILES

Introduction ..... 135
Thermoacidophiles as biocatalysts under energetically challenged states ..... 136
Future research directions ..... 139
Literature Cited ..... 142

APPENDICES

APPENDIX 1. A KINETIC ASSESSMENT OF PYRITE OXIDATION BY METALLOSPHAERA SEDULA USING COMPUTER SIMULATION .....147

APPENDIX 2. CULTIVATION OF HYPERTHERMOPHILIC AND EXTREMELY THERMOACIDOPHILIC MICROORGANISMS

1.0 General considerations .....153
2.0 Cultivation strategies and challenges
2.1 Availability and handling of extremely thermophilic microorganisms .....154
2.2 Media development .....156
2.3 Cultivation equipment and strategies .....161
3.0 Growth of hyperthermophilic, heterotrophic anaerobes: *Thermococcus litoralis*
3.1 Media formulation .....165
3.2 Culture preservation .....167
3.3 Batch cultivation in serum bottles .....168
3.4 Continuous cultivation .....170
3.5 Large scale cultivation .....172
3.6 Product formation .....173
3.7 Exopolysaccharide production and biofilm formation .....173
4.0 Growth of extreme thermoacidophilic archaea: *Metallosphaera sedula*
4.1 Media formulation .....177
4.2 Culture preservation .....178
4.3 Batch cultivation in reaction flasks .....178
4.4 Continuous cultivation .....179
5.0 New development in the isolation and characterization of high temperature microorganisms .....181
6.0 Literature Cited .....183

# LIST OF TABLES


Page

Table 1.1. Differentiating characteristics of the three domains of life .....52

Table 1.2. Metabolic characteristics of extreme thermoacidophiles .....53

Table 2.1. Polypeptide numbering and isoform ratios for the 66 kDa protein of interest in sample under thermally stressed versus sample under normal growth conditions .....79

Table 4.1. Protonophore (2,4-DNP) effects on specific leaching rates of *M. sedula* .....125

Table 4.2. Ionophore (nigericin) effects on specific leaching rates of *M. sedula.* .....125

Table A.2.1. Biodiversity of Extreme Environments .....201

Table A.2.2. The Extremely Thermophilic Genera (Topt ≥ 75°C) .....202

Table A.2.3. Hyperthermophile Media .....204

Table A.2.4. Trace Mineral Solutions .....205

Table A.2.5. Vitamin Solution .....206

# LIST OF FIGURES


Page

Figure 1.1. Phylogenetic tree based on 16S rRNA .....58

Figure 1.2. Conformational changes in the GroEL oligomer during the functional cycle .....59

Figure 1.3. Proposed conformational cycle of archaeosome .....60

Figure 1.4. Schematic of *Metallosphaera sedula* network .....61

Figure 2.1. Heat shock of *M. sedula* in 10-liter continuous culture .....80

Figure 2.2. Thermal acclimation and heat shock of *M. sedula* in 10-liter continuous culture .....81

Figure 2.3. Gradual and abrupt exposure to thermal stress on *M. sedula* in 10-liter continuous culture .....82

Figure 2.4. The levels of total protein per cell and average cell densities for conditions shown in Figure 2.3 .....83

Figure 2.5. Effect of temperature and thermal acclimation on protein patterns in *M. sedula* cell extracts .....84

Figure 2.6. Two-dimensional BDH(2%)/SDS(10%) polyacrylamide gel analysis of proteins from *M. sedula* at (a) 74°C and (b) 81°C in continuous culture .....85

Figure 3.1. Three-dimensional structure of the proteasome from the archaea *Thermoplasma acidophilum* .....100

Figure 3.2. Model of HslVU (ClpYQ) complex system .....101

Figure 3.3. Ammonium sulfate ($(NH_4)_2SO_4$) precipitation of *M. sedula* crude extract .....102

Figure 3.4. Total protein assay on ammonium sulfate cut supernatants and (re-suspended) precipitated proteins from *M. sedula* .....103

Figure 3.5. Proteolytic activity of fractions from Mono Q ion-exchange column .....104

Figure 3.6. Proteolytic activities in a cell extract supernatant from cells of *M. sedula* fractionated by DEAE ion exchange column .....105

Figure 3.7. Effect of pH on the proteolytic activity of the partially purified proteinase from *M. sedula* .....106

Figure 3.8. Effect of temperature on the proteolytic activity of the partially purified proteinase from *M. sedula* .....107

Figure 4.1. Generalized chemiosmotic network for extreme thermoacidophiles under normal growth conditions .....126

Figure 4.2. Temperature effect on bioleaching kinetics by *M. sedula* .....128

Figure 4.3. Nigericin effect on overall ferric iron released by *M. sedula* in batch cultures .....129

Figure 4.4. *M. sedula* biooxidation kinetics in continuous culture in the presence of 1 µM nigericin .....131

Figure 4.5. Pulse injection of nigericin and its impact on *M. sedula* biooxidation kinetics in continuous culture .....133

Figure 4.6. Proposed mechanism for *M. sedula*'s chemiosmotic network under nigericin stress .....134

Figure A.1.1. Time courses of the cell density and the ferric iron production during the dissolution of pyrite by *Metallosphaera sedula.* .....151

Figure A.2.1. Chemostat for continuous cultivation of anaerobic extreme thermophiles .....207

Figure A.2.2. *T. litoralis* biofilm formation .....208

Figure A.2.3. Chemostat for continuous cultivation of extreme thermoacidophiles .....209

# Chapter 1

## Thermoacidophile Physiology

### INTRODUCTION

Increased cost of extraction and processing of strategic metals (e.g. copper and uranium), precious metals (e.g. gold and silver), and conventional minerals (e.g. coal), as well as the depletion of high grade reserves and stricter environmental regulations, have stimulated the consideration of alternative technologies by the mining industry. Microbial desulfurization/bioleaching processes have been recognized as an attractive alternative to the traditional physical and chemical treatments, due to potential reductions in process energy requirements and fewer adverse environmental consequences (Moffat, 1994; Goldstein *et al*., 1993). Commercial mining companies have already begun using acidophilic bacteria to leach valuable metals from low-grade ores. Gold particles, for instance, are often trapped in iron pyrite ($FeS_2$) and the best way to extract them is to expose the ore to an acid solution containing bacteria that break down the pyrite matrix. The gold is then easily recovered by precipitating it out of solution (Pool, 1990). Several companies in North and South America, Australia and Africa have already developed and implemented bioprocessing of gold and copper extraction using mesophiles and moderately thermophilic bacteria (Moffat, 1994; Brierley, 1994).

Despite the promising future that bioprocessing offers, there are number of obstacles to overcome before significant utilization of such approaches can be realized. These obstacles include the heterogeneity of sulfur forms embedded in metal ores (e.g. iron pyrite, chalcopyrite, arsenopyrite, etc.), the inherent lability of biological systems, and the comparatively slow rates of sulfur removal and/or metal oxidation by microorganisms. At this point, the metabolic complexity and lack of genetic information

for these microorganisms necessitates bioprocessing optimization through manipulation of the metabolism of native organisms by nutritional and/or environmental means. Therefore, detailed understanding and investigation of the physiology and bioenergetics of the particular microorganism are critical for the development of a successful bioprocess.

Microbial leaching technology has primarily been developed with mesoacidophilic *Thiobacillus* species. The major contribution of *Thiobacillus ferrooxidans* to metal extraction is its ability to attack sulfide-containing minerals and to convert the insoluble sulfides of metals, such as copper, lead, zinc, or nickel, to their soluble metal sulfates. However, recent investigations have revealed the presence of extreme thermoacidophiles (optimal growth temperature above 70°C) in leaching operations. As the oxidation of pyrite by oxygen is a highly exothermic reaction ($\Delta H_r^o = -1481$ kJ/mol $FeS_2$), microbial oxidation of high-pyritic materials would result in an increase of the temperature in the reactor such that the growth of, and pyrite oxidation by, mesophilic microorganisms would be partially or completely inhibited unless the reactor was cooled. In fact, the cooling of such a large reactor would be uneconomical. In this case, thermophilic, pyrite-oxidizing microorganisms would be more appropriate to oxidize the metal sulfur species in ores. The potential benefits of high temperature bioprocessing with extreme thermophiles are higher kinetic rates and extents of both chemical and biological reactions, in addition to elimination of the above mentioned cooling requirements expected for mesophile-based bioreactors and operations. Extreme thermoacidophilic species, such as those in the archaeal genera *Sulfolobus* (Brierley and Murr, 1973), *Acidianus* (Segerer *et al*., 1986), and *Metallosphaera* (Huber *et al*., 1989), have been found to achieve higher oxidation rates than mesophiles (Peeples and Kelly, 1993). Studies aimed at improving upon thermophile-based bioleaching have focused on the isolation of more effective cultures and on the optimization of processing conditions. However, the scale-up and operation of

thermophile-based bioleaching processes will be best done if the physiology and bioenergetics of the particular microorganism are sufficiently understood. As yet, designed variations in operating temperature, pH or medium composition that may effectively increase the reaction rates have received little or no attention.

Thus, if simple manipulations of process conditions could be used to improve bioleaching rates, or strategies developed to make organisms more tolerant to thermal, nutritional, and other environmental changes, bioleaching technology could be expanded dramatically. While genetic systems for thermoacidophiles, especially chemolithotrophic organisms, are not yet available, several advances in this area suggest that metabolic engineering approaches can ultimately be considered to improve bioleaching efficiency in specific organisms (Rawlings and Kusano, 1994). In the work proposed here, we will focus on the understanding of the microbial physiology and bioenergetics of extremely thermoacidophilic microorganisms and on the exploitation of stress response to accelerate biooxidation process. Recent efforts in our laboratory have focused on a particular thermoacidophile, *Metallosphaera sedula* (Peeples and Kelly, 1993), and its response to environmental stress (Peeples and Kelly, 1995). Based on preliminary studies, systematic exposure of *M. sedula* to certain types of stress will be performed to demonstrate the potential improvements in pyrite leaching resulting from *M. sedula*'s response to stress. Subsequently, we will investigate this issue further to examine approaches and strategies that can exploit the stress responses of microorganisms, in either pure or mixed cultures, to improve overall bioprocessing kinetics.

## BACKGROUND

### Thermophiles

Organisms capable of growth in high temperature geothermal environments are thermophilic or "heat loving". Over the past decade, thermophiles have generated great interest both from fundamental and applied science perspectives. Fundamental research on thermophiles has focused on their molecular biology, genetics, biochemistry, evolution, taxonomy, and ecology. From an applied or biotechnological point of view, thermophiles are of interest as sources of unique enzymes with unusual properties, as the active agents in high-temperature fermentation, in waste-treatment processes, and in mineral leaching (Brock, 1986; Kelly and Brown, 1993; Kelly *et al*., 1994). Researchers have tried to understand what enables these organisms, and their constituent biomolecules, to not just survive but thrive in high temperature environments.

In general, an organism is considered thermophilic when it is capable of living at temperatures at or near the maximum for the taxonomic group of which it is a part. For bacteria, the thermophile boundary is set at 55 to 60$^{o}$C. This boundary was selected because of the rarity of natural environments above 50$^{o}$C, and the fact that 60$^{o}$C is the present upper limit for eukaryotic life (Brock, 1986). Among the thermophiles, there are physiologically distinct groups. For example, methanogens are found in organic materials, combining carbon with hydrogen to form methane. Other thermophiles generate energy by reducing sulfur with hydrogen to form hydrogen sulfide (Pool, 1990). Thermophilic organisms are further classified as moderate thermophiles, extreme thermophiles, and hyperthermophiles as growth range and optimal temperature increase. A hyperthermophilic species is one capable of living at 90$^{o}$C, with an optimal growth temperature of 80$^{o}$C or above, and nearly all are archaea. Extreme thermophiles live

between 70°C and 90°C. Moderately thermophilic organisms live in the range of 50 to 70°C (Kelly *et al*., 1994).

## Archaea

The evaluation of thermophiles and other organisms from extreme environments has identified a new branch of the phylogenetic tree and the designation of a new kingdom of life, the *archaea* (see Figure 1.1). The archaea are unique and distinct from bacteria and eucarya but possess specific relationships to both of the two other domains. A variety of physiological characteristics has been used to delineate the three domains (see Table 1.1), including 16S ribosomal RNA sequencing (Woese, 1993; Woese *et al*., 1990). Even though archaea are prokaryotes, they differ from eubacteria in many ways and show some molecular and genetic characteristics that are more typical of eukaryotes (Woese, 1987). These organisms occupy a variety of ecological niches, and are interesting targets for research in that they possess attributes that are potentially useful to industry. For example, many species of archaea thrive under environmental conditions (such as high temperature, high salinity, high or low pH, and anoxia) that preclude the survival of most other life forms. The archaea fall into two distinct groups. The *Euryarchaeota* include methanogens, extreme halophiles, sulfate-reducing species and two thermophilic genera. The *Crenarchaeota* include thermoacidophiles, sulfur-dependent archaebacteria, and extreme thermophiles. Several moderately thermophilic bacteria have been characterized as well as a few extremely thermophilic bacteria (Brock and Freeze, 1969; Bonch-Osmolovskaya *et al*., 1990; Burggraf *et al*., 1990; Huber *et al*., 1986; Huber *et al.*, 1992; McKay *et al*., 1982; Miroshnichenko *et al*., 1989; Svetlichny *et al*., 1991). However, the majority of extreme thermophiles and hyperthermophiles are found among the archaea.

Organisms isolated from sulfur-rich acid springs and certain soils have been shown to be acidophilic. These include the *Sulfolobales* (Brock, 1986; Brock et al., 1972) and several of the *Thermoproteales* (Zillig *et al*., 1990; Zillig *et al*., 1982; Zillig *et al*., 1981). From neutral pH boiling springs, *Pyrodictales* and thermophilic archaea have been isolated (Stetter *et al*., 1983). From deep sea hydrothermal vents, the *Thermococcales*, *Methanogens*, and *Archaeglobales* dominate (Jannasch *et al*., 1988; Jones *et al*., 1983; Neuner *et al*., 1990; Ruby *et al*., 1981; Zillig *et al*., 1987; Zellner *et al*., 1989). There is a great deal of metabolic diversity among the archaea (Adams, 1990). Organisms may grow organotrophically (with fermentative or respiratory pathways) or lithotrophically (fixing $CO_2$ and turning over inorganic compounds for energy generation). There are aerobes and anaerobes. Most species can grow facultatively under a variety of modes. This adaptive physiology can be seen in cellular energetics as organotrophic/lithotrophic growth and aerobic/anaerobic growth within the same strain.

Research on archaea is of particular interest for an understanding of early stages of the evolution of organisms, the evolution of survival strategies, and structure-function relations of specific proteins, especially of membrane components involved in energy transduction. For example, the remarkable stability of the membranes of many thermophilic and thermoacidophilic archaea, allowing them to withstand high temperatures, is partially based on the structure of cell surface layer with unique tetraether- and diether-linked lipids (Baumeister and Lembcke, 1992; Sprott, 1992).

## Thermoacidophiles

All thermoacidophiles of the archaeal *Crenarchaeota* branch are members of the *Sulfolobaceae* (Stetter, 1989; Woese *et al*., 1990). Table 1.2 is a list of thermoacidophiles with their metabolic characteristics and the environments from which they have been

isolated. Up to now the genera *Sulfolobus*, *Acidianus*, *Desulfurolobus*, and *Metallosphaera* have been described (Brock *et al*., 1972; Segerer *et al*., 1986; Zillig *et al*., 1986; Huber *et al*., 1989). They share a regular to irregular coccoid shape, a thermoacidophilic mode of life, and the ability to oxidize elemental sulfur (Stetter, 1989). The different genera within the *Sulfolobaceae* can be distinguished from each other by their metabolic and biochemical properties. Members of *Sulfolobus* exhibit a G+C DNA content of around 37 mol% and are able to utilize sugars, amino acids, and complex organic substances as energy and carbon sources (Brock *et al*., 1972; Zillig *et al*., 1980). *Acidianus* and *Desulfurolobus* are closely related to each other (Huber et al., 1987). They have a G+C content of about 31 mol% and a facultatively aerobic metabolism with elemental sulfur as electron donor or acceptor (Segerer *et al*., 1986; Zillig *et al*., 1986). *Metallosphaera* is characterized by a G+C content of 45 mol% and a strong ore-leaching capacity (Huber *et al*., 1989). The subdivision into the genera on the basis of the metabolic and biochemical properties is further supported by the lack of significant DNA/DNA hybridization and the incomplete serological cross-reaction of their DNA-dependent RNA polymerases between member of them (Stetter, 1989).

Other thermoacidophiles belong to the order *Thermoplasmales* of the archaeal *Euryarchaeota* branch. The genera *Thermoplasma* and *Picrophilus* have been described to date (Darland *et al*., 1970; Segerer *et al*., 1988; Schleper *et al*., 1995). The organisms in these two genera share several physiological properties; i.e., they grow heterotrophically, preferably on yeast extract as a carbon source, in the same temperature range (45 to 65°C) under strongly acidic conditions (pH ~0 to 4).

## 1. Genus *Sulfolobus*

Within the genus *Sulfolobus*, the four species *S. acidocaldarius, S. solfataricus, S. shibatae,* and *S. metallicus* can be distinguished from each other by DNA/DNA hybridization (Segerer *et al*., 1986; Grogan *et al*., 1990; Huber and Stetter, 1991) and by 16S rRNA sequencing (Woese *et al*., 1984; Grogan *et al*., 1990; Huber and Stetter, 1991).

*Sulfolobus acidocaldarius,* 'lobed sulfur-oxidizing organism which lives in acid thermal habitats', was first isolated from hot spring in Yellowstone National Park (Brock *et al*., 1972). It is a facultative autotroph, growing on sulfur or on a variety of simple organic compounds, and its membrane lacks peptidoglycan. With a pH optimum of 2-3, it grows optimally at 83$^{o}$C and over the range of 50-90$^{o}$C. The DNA base composition (G+C content) of this organism is around 37 mol%.

*Sulfolobus solfataricus* was isolated from hot acidic volcanic springs at Pisciarelli Solfatara in Italy (Zillig *et al*., 1980). It grows between 50 and 88$^{o}$C with an optimum temperature at 87$^{o}$C and over the pH range of 3.5-5. The G+C content of this organism is around 35 mol%. Elemental sulfur and/or yeast extract is used as the energy source. *S. solfataricus* has a similar G+C DNA content and the same general properties as *S. acidocaldarius,* but differs significantly from the latter species in the molecular weights of the 11 components of its RNA polymerase and in the salt requirements of this enzyme. Whereas the enzyme from *S. acidocaldarius,* similar to the *Escherichia coli* polymerase, works best at a moderate ionic strength, the enzyme from *S. solfataricus* prefers very low ionic strength.

*Sulfolobus shibatae,* formerly known as *Sulfolobus* isolate B12, was obtained from acidic geothermal spring in Japan (Grogan *et al*., 1990). This organism is aerobic, weakly motile, and facultatively chemolithoautotrophic by sulfur oxidation. DNA

composition is around 35 mol% G+C. Optimal growth temperature is approximately 81°C and pH around 3. This isolate yields a pattern of chromosomal restriction fragments quite different from those of *S. solfataricа* or *S. acidocaldarius*, yet it resembles these species with respect to a wide array of physiological characteristics (Grogan, 1989). Based on DNA-DNA cross-hybridization, $S_{AB}$ value, and RNA polymerase pattern, *S. shibatae* is more closely related to *S. solfataricus* than to *S. acidocaldarius*. This microorganism has proven particularly interesting for its UV-inducible amplification of a 15.5-kb plasmid (Yeats *et al*., 1982), the subsequent release of virus-like particles (SSV1) containing this circular DNA (Martin *et al.*, 1984), and recent identification of a prominent heat shock protein TF55 (Thermophilic Factor 55) (Trent *et al*., 1991).

*Sulfolobus metallicus* was obtained from Icelandic solfataric fields (Huber and Stetter, 1991). It is strict chemolithotroph gaining energy by oxidation of $S^o$ and sulfidic ores. This isolate grows between 50 and 75°C and pH 1-4.5 and tolerate NaCl concentrations of up to 3%. The G+C content of its DNA is 38 mol%. It resembles members of *Sulfolobus* in its morphology, its ability to oxidize reduced sulfur compounds and its G+C content. However, this organism is different in its strictly chemolithoautotrophic mode of life, DNA/DNA hybridization and incomplete serological cross-reaction of RNA polymerase.

### 2. Genus *Acidianus*

Within the genus *Acidianus*, two species - *A. brierleyi* and *A. infernus* - have been described so far (Brierley and Brierley, 1973; Segerer *et al*., 1986). The cells have an irregular coccoid morphology and are non-motile, facultative aerobes. Lithotrophic growth occurs aerobically by means of $S^o$ oxidation or anaerobically by means of $S^o$ reduction with $H_2$. The purified DNA has a G+C content of about 31 mol%.

*Acidianus brierleyi* was first isolated from an acidic solfataric spring (Brierley and Brierley, 1973). Growth is chemolithotrophic by means of oxidation or reduction of $S^o$ or oxidation of ferrous iron. Organotrophic growth occurs in the presence of $O_2$ and yeast extract, peptone, tryptone, beef extract, or Casamino Acids. The temperature range for growth is 45 to 75°C with the optimum of 70°C, while the pH range is 1 to 6 with the optimum around 2. The DNA dependent RNA polymerase consists of nine subunits with different molecular weights (Segerer *et al*., 1986; Zillig *et al*., 1980).

*Acidianus infernus* was obtained from hot water, mud, and marine sediments at geothermal springs in Italy, the Axores, Iceland, and the United States (Segerer *et al*., 1986). Cells grow at temperatures ranging from 65 to 96°C, with optimum growth at around 90°C. The pH suitable for growth ranges from 1.0 to 5.5, with the optimum pH around 1.5 to 2. *A. infernus* shows a distinct similarity to the type species of *Sulfolobus*, *S. acidocaldarius*, in its morphology, its extreme thermoacidophily, its lithotrophy based on $S^o$ oxidation, and its similar strongly acidic biotope. However, *A. infernus* diverges from the *Sulfolobus* by its lower G+C content and its ability to grow anaerobically by means of $S^o$ reduction. *A. brierleyi* has the same G+C content as *A. infernus* and is also able to grow anaerobically by means of $S^o$ reduction. However, it differs from *A. infernus* by its ability to grow organotrophically without $S^o$ in the presence of $O_2$, by its much lower growth temperature, and by the lack of significant DNA homology.

### 3. Genus *Desulfurolobus*

Within the genus *Desulfurolobus*, 'the sulfur reducing lobe', one species, *D. ambivalens*, is recognized to date (Zillig *et al*., 1986). Cells are facultatively aerobic or anaerobic chemolithoautotrophs capable of utilizing $CO_2$ as sole carbon source and either $S^o + O_2$ (yielding sulfuric acid) or $S^o + H_2$ (yielding $H_2S$) as energy sources. DNA G+C

content is around 32 mol%. According to its phenotype, including the shape of the cells and their ability to grow aerobically via sulfur oxidation, the *Desulfurolobus* undoubtedly belongs to the *Sulfolobaceae* family. However, in view of the apparently incomplete immunochemical cross-reaction, by its quite different G+C content, and by its ability to grow facultatively via sulfur reduction, the *Desulfurolobus* is different genus from the *Sulfolobus*. There are many similarities between the *Desulfurolobus* and the *Acidianus*, including the identity of the modes of growth and the G+C content. However, the maximum temperature tolerated by the *Desulfurolobus* (87°C), the absence of a significant DNA/DNA cross hybridization, and the differences of the mobilities of homologous RNA polymerase components contradicts a presumed close relationship of the *Desulfurolobus* and the *Acidianus*.

*Desulfurolobus ambivalens* was isolated from the Pisciarelli hot springs in Italy (Zillig *et al.*, 1986). The isolate is obligate chemolithoautotroph and grows between pH 1 and 3.5 with the optimum pH of 2.5. The growth temperature optimum is about 80°C and a maximal growth temperature is 87°C. *D. ambivalens* contains several plasmids, one of which, pSL10, is amplified during anaerobic growth mode. This amplification is accompanied by the appearance of virus-like particles resembling SSV1 described for *Sulfolobus shibatae*. However, in contrast to the situation in the *S. shibatae* harboring SSV1, pSL10 occurs exclusively as a plasmid, not in the integrated state. Also, pSL10 has less than half the size of the SSV1 genome.

### 4. Genus *Metallosphaera*

Within the genus *Metallosphaera*, two species - *M. sedula* and *M. prunae* - have been characterized to date (Huber et al., 1989; Fuchs et al., 1995). These organisms are gram-negative irregular cocci. Aerobic, facultative chemolithoautotrophic growth occurs

on sulfidic ores like pyrite, chalcopyrite and sphalerite and on elemental sulfur. The cells also grow organotrophically on yeast extract, peptone and beef extract, but do not utilize sugars.

*Metallosphaera sedula*, 'the efficient metal-mobilizing sphere', is the type species of the genus *Metallosphaera* and was isolated from a solfataric field in Italy (Huber *et al*., 1989). Cells grow between 50°C and 79°C (optimum around 74°C) and pH 1.0 to 4.5 (optimum around 2). It has DNA G+C content of 45 mol%.

By this organism's acidophilic mode of life and the presence of benzothiophenquionone and glycerol-dialkyl-nonitol tetraethers they resemble members of the genera *Sulfolobus* and *Acidianus* (De Rosa and Gambacorta, 1988). Due to its ore-leaching capacity and its ability to grow by oxidation of elemental sulfur and organic material, it is reminiscent of *Acidianus brierleyi* (Brierley and Brierley, 1973; Segerer *et al*., 1986). In contrast to *A. brierleyi* though, this organism exhibits a nearly round shape, grows up to 79°C and tolerates pH values up to 5. A further difference with all members of *Acidianus* and *Desulfurolobus* is the inability of this organism to grow anaerobically (Segerer *et al*., 1986). Also, its G+C content is about 14 mol% higher than those of *Acidianus* and *Desulfurolobus*. By its strictly aerobic mode of life, this isolate resembles the *Sulfolobus* species. However, this organism can be distinguished easily from the *Sulfolobus* species by its inability to grow on sugars and lack of DNA/DNA hybridization with *Sulfolobus*. This isolate mobilizes metal ions from sulfidic ores, like chalcopyrite, about 10 times faster than *A. brierleyi* and are, therefore, highly efficient ore leachers suitable for reactor leaching and for *in situ* leaching of geothermally heated ore deposits (Huber *et al*., 1989).

*Metallosphaera prunae* was isolated from smoldering slag heap of a uranium mine in Thüring, Germany. The cells grow between 55°C and 80°C (optimum around 75°C)

and pH 1.0 and 4.5. The G+C content of its DNA is 46 mol%. The physiological and biochemical characteristics of this organism is very similar to those of *M. sedula*. However, in contrast to *M. sedula*, *M. prunae* requires $O_2$ concentrations between 8 and 12% (v/v) for optimal growth on molecular hydrogen (*M. sedula*: 0.5% v/v). In addition, *M. prunae* possess flagella and a fibrillar surface coat most likely of carbohydrates, lacking in *M. sedula* cultures.

### 5. Genus *Thermoplasma*

The genus *Thermoplasma* belongs to the order *Thermoplasmales* of the kingdom *Euryarchaeota*. *Thermoplasma* cells are highly irregular in shape due to the absence of a cell wall and envelope. Within this genus, the two species - *T. acidophilum* and *T. volcanium* - have been described to date (Segerer *et al*., 1988).

*T. acidophilum* was isolated from self-heated coal refuse piles (Darland *et al*., 1970; Brock, 1978). G+C content of its DNA is about 46 mol%, and it grows between 45 and 63°C (optimum 59°C) and pH 0.5-4.0 (optimum pH 1-2). *T. volcanium* was isolated from submarine and continental solfataras at Vulcano island, Italy, continental solfataras and a tropical swamp in Java and continental solfataras in Iceland and the Yellowstone National Park (Segerer et al., 1988). It has a G+C content of 38 mol% DNA and grows between 33 and 67°C (optimum 60°C) with optimum pH 2.0. Both of these species show general archaeal features like resistance against eubacterial cell wall-, ribosome- and transcription antibiotics and the presence of an ADP-ribosylable elongation factor G-like protein. The cells grow on extracts of yeast, meat, and eubacteria and archaea. Anaerobic growth is strongly enhanced by elemental sulfur, which is reduced to $H_2S$ by sulfur respiration.

### 6. Genus *Picrophilus*

The genus *Picrophilus* is a member of the order *Thermoplasmales*. Within this genus, *Picrophilus oshimae* and *Picrophilus torridus* have been described so far (Schleper *et al*., 1995). They resemble *Thermoplasma* spp. by their moderate temperature (50 to 55°C) and their strong acidity (pH <0.5 to 2.2). Unlike *Thermoplasma* spp., however, these organisms have a S-layers and clearly surpass in acidophily. They grow well even around pH 0 and optimally at pH 0.7. Only a few eukaryotic fungi and an alga have also been reported to be capable of growth at pH values around 0 (Brock, 1978).

## Microbial Desulfurization / Bioleaching

The microbially accelerated oxidation of pyrite ($FeS_2$) has important economic and environmental consequences. For example, acidic ferric sulfate solutions resulting from pyrite oxidation assist in the commercial recovery of copper and uranium from ores and wastes, and microbial leaching has begun to be applied as a pretreatment step in the commercial recovery of gold from sulfidic deposits (Moffat, 1994). In addition, pyrite is the dominant form of inorganic sulfur in coal, and many studies have shown the potential for microbial coal cleaning through pyrite oxidation. Conversely, uncontrolled microbial pyrite oxidation in active and abandoned coal and metal mines leads to acid mine drainage (AMD), a serious water pollution problem (Evangelou and Zhang, 1995). Therefore, microorganisms play a key role in both natural and commercial processes. The rate-determining step in pyrite oxidation in water is the oxidation of ferrous iron, which microorganisms accelerate in acid mine water by a factor of $10^6$ or more above the abiotic rate (Singer and Stumm, 1970).

Pyrite is oxidized by oxygen to ferrous iron and sulfate. The reaction is slow, even at elevated temperatures:

$$FeS_2 + 3.5O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+$$

The microbial pyrite oxidation is considered to be either direct or indirect. During direct oxidation, pyrite is solubilized to ferric ion and sulfate:

$$4FeS_2 + 15O_2 + 2H_2O \xrightarrow{\text{Microorganisms}} 2Fe_2(SO_4)_3 + 2H_2SO_4$$

During the indirect process, the ferrous ion produced in the pyrite oxidation is oxidized to ferric ion by the microorganisms. Ferric iron is a strong oxidant and readily oxidizes pyrite:

$$FeS_2 + 2Fe^{3+} \rightarrow 3Fe^{2+} + 2S^0$$

Any elemental sulfur formed can be oxidized either by the microorganisms or by $Fe^{3+}$:

$$2S^0 + 3O_2 + 2H_2O \xrightarrow{\text{Microorganisms}} 2H_2SO_4$$

$$S^0 + 6Fe^{3+}\ 4H_2O \rightarrow 6Fe^{2+} + SO_4^{2-} + 8H^+$$

The principal difference between the two mechanisms is whether the microorganisms are closely associated with the pyrite surface, which is needed for the direct mechanism, or if the microorganisms simply promote the chemical reactions through ferric ion. The

conversion of $Fe^{2+}$ to $Fe^{3+}$ is slow in the absence of microbial activity and is the rate-limiting step for pyrite oxidation without microorganisms:

$$2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$$

It is possible that both mechanisms take place at the same time. The overall reaction can then be summarized as follows:

$$4FeS_2 + 15O_2 + 2H_2O \rightarrow 2Fe_2(SO_4)_3 + 2H_2SO_4$$

Note that one mole of $H_2SO_4$ is produced for every two moles of $Fe^{2+}$ is oxidized to $Fe^{3+}$.

The most commonly investigated microorganism for microbial ore leaching is the mesoacidophile *Thiobacillus ferrooxidans*, which grows autotrophically on pyrite as an energy source, oxidizing iron and sulfur to produce acidic ferric sulfate solutions. At low pH, the acidophilic, chemoautotrophic mesophile catalyzes and accelerates the oxidation of $Fe^{2+}$ by a factor larger than $10^6$.

Rates of pyrite oxidation by *T. ferrooxidans* are usually determined by measuring the rate of production of soluble iron. However, the rates of pyrite oxidation by *T. ferrooxidans* reported in the literature are difficult to compare, complicating the evaluation of different bacterial strains and leaching processes (Suzuki *et al*., 1990). Recently, an inter-laboratory comparison of NIST reference pyrite leaching by *T. ferrooxidans* showed that the mean rate of bioleaching was 12.4 mg of Fe per liter per hour (Olson, 1991). A standardized procedure and a single strain of *T. ferrooxidans* were used in that study. Some researchers investigated whether *T. ferrooxidans* can be used for bioleaching of metal sulfides other than iron pyrite, such as molybdenum sulfide (Blaustein *et al*., 1993;

Pistorio *et al*., 1994; Attia and El Zeky, 1990; Sugio *et al*., 1990). Others tried to improve the rate of pyrite oxidation by *T. ferrooxidans* utilizing some particulate solids to modify the oxidizing activity (Loi *et al*., 1993).

Although relatively simple processes involving *T. ferrooxidans* can be used for bioleaching, the rates of sulfur removal are still slow compared to conventional chemical/physical methods. Also, *T. ferrooxidans* is ineffective in removing organic sulfur. Duarte *et al*. (1993) did a comparative study of thermophiles and mesophiles, and reported the problem of incomplete leaching typically associated with the use of mesophiles. Thus, efforts at improving biological removal of sulfur have been directed at identifying more effective organisms for bioleaching and trying new approaches to optimize removal rates. Several extreme thermoacidophiles of the genera *Sulfolobus*, *Acidianus*, and *Metallosphaera* have been shown to oxidize pyrite faster than *T. ferrooxidans* (Larrson *et al*., 1990; Peeples and Kelly, 1993; Norris and Owen, 1993). The potential advantages of using thermoacidophiles in bioleaching over the use of mesophilic *Thiobacillus*-like species are the following: 1) operation of the system at high temperatures (70-90°C) improves the rate of chemical oxidation of pyritic sulfur by ferric iron produced by microbial oxidation of pyrite present in coal, and 2) the process can be operated at high cell and coal concentrations without expensive cooling systems.

Kargi and Robinson (1982, 1985) found that more than 90% of initial pyritic sulfur was removed by *S. acidocaldarius* from bituminous coal samples. In their study, the optimal temperature and pH values resulting in maximum rate of sulfur leaching were 70°C and 1.5, respectively. They suggested the higher sulfur removal rate obtained at pH 1.5 may be due to higher microbial activity at low pH (pH 1.5) and/or due to increased contribution of acid leaching at pH 1.5.

Boogerd *et al*. (1990) studied the effects of increasing temperature on oxygen and carbon dioxide mass transfer and abiotic leaching rates. They concluded that the growth of thermophiles in continuous cultures at high temperature would more likely to be limited by carbon dioxide than oxygen. Their study underlined the importance of the effect of temperature on the chemical oxidation of $FeS_2$ by ferric iron. An empirical rate equation was proposed for the chemical oxidation as a function of temperature and was used to determine that chemical oxidation rates increase significantly from 30°C to 70°C. By assuming that biotic rates remain constant, they estimated the relative contributions of the biotic and abiotic reactions for pyrite oxidation vary from mostly biotic at 30°C to about 45% abiotic at 70°C.

However, their assumption of constant biotic reaction rate at different temperatures is not necessarily true, since recent results in our laboratory showed that significantly higher biotic leaching rates could be achieved at higher temperatures. The effects of the growth kinetics of bacteria on solid substrate and in solution on the total rate of pyrite oxidation were also not taken into consideration. In addition, some of the ferric ions formed precipitate (jarosite), reducing the potential to oxidize pyrite. Therefore, the actual contribution of the biotic reaction rate at higher temperature may be much higher than predicted.

A number of studies of bacterial leaching have indicated that the attachment or adsorption of organism to the mineral surface may play an important role in the leaching process (Silverman, 1967; Corpe, 1980; Daniels, 1980; Marshall, 1985; Stotzky, 1985; Shrihari *et al*., 1993). It was proposed that reversible adsorption was due to long-range attraction such as van der Waals forces and electrical repulsion energy in the overlapping double layers surrounding the bacterial and substratum surfaces. Irreversible adsorption was attributed to short-range forces such as electrostatic, hydrogen and hydrophobic

bonding. Also, irreversible adhesion is thought to involve production of extracellular polymers necessary to bridge the electrostatic barrier holding the cell at a finite distance from the substratum surface. These polymers are typically glycoproteins or lipopolysaccharides and assume the form of capsular holdfasts, thin pili, or extracellular secretions (Nicolaus *et al*., 1993). However, the actual adsorption mechanism that takes place in this process is not understood yet. Silverman (1967) proposed two mechanisms of bacterial pyrite oxidation operating concurrently: a direct contact mechanism requiring physical contact between bacteria and pyrite particles for biological pyrite oxidation, and an indirect mechanism whereby bacteria regenerate the ferric ions required for chemical oxidation of pyrite by oxidizing ferrous ions to ferric ions. Several researchers tried to elucidate and model the adsorption/desorption processes, since it is crucial to understand the details of mechanism in order to design an efficient bioleaching process.

Bagdigian and Myerson (1986) studied these adsorption phenomena using *T. ferrooxidans* on coal surfaces. They reported that the adsorption process was at least 90% complete within the first two minutes. Also, scanning electron micrographs of inoculated coal samples showed that *T. ferrooxidans* selectively adsorbed to exposed pyrite phases dispersed throughout the organic coal matrix. They suggested this selective (preferential) adsorption by *T. ferrooxidans* may be attributable to the lower surface free energy effects of pyrite inclusions in coal matrix. As such, they proposed that the relatively rapid (< 2 min) irreversible adsorption may be due to compatibility of the substratum and the molecular structure of the microbial surface, rather than due to the production of biopolymers appendages and/or secretions because such processes require the input of metabolic energy and therefore generally time-dependent. Based on their experimental results, this group hypothesized that there may be a two-stage adsorption process involving both reversible and irreversible events.

Vitaya and Toda (1991a and b) confirmed that the adsorption of *S. acidocaldarius* on surfaces of coal actually occurred in two stages. In the first stage, the adsorption occurred rapidly in 1 min after the contact (initial irreversible adsorption). In the second stage, desorption followed the adsorption and reached equilibrium after about 30 min (a combination of both reversible and irreversible adsorptions). From the scanning electron micrographs of pyrite surfaces, they could see some extracellular slime appeared to be excreted from the cell membrane. This indicated that the irreversible adsorption after 30 min was physiological and time-dependent. Based on these observations, they concluded that the rapid adsorption in the first stage was related to the hydrophobicity between coal and cell membrane and the adsorption in second stage was related with the oxidation of pyrite. However, they did not suggest the exact mechanism which was involved in the second stage of adsorption. It was speculated that the organism has to utilize endogenous carbon and energy sources derived from an electrical potential difference in order to synthesize the slime, which may include key proteins involved in this stage of adsorption.

Attempts to model the bioleaching process in a reactor have included microbial growth kinetics on the solid substrates (Lindström, 1993; Okereke and Stevens, Jr., 1991). However, due to poor understanding of adsorption/desorption phenomena, most of those proposed kinetics are relatively simple and not mechanistic. Gormely *et al.* (1975) presented a kinetic model for the continuous bioleaching of ZnS in an iron-free liquid medium. Sanmugasunderam *et al.* (1985) applied and extended the Gormely model for one continuous reactor to the bioleaching of ZnS in two continuous reactors. Chang and Myerson (1982) developed a continuous bioleaching model of $FeS_2$ which included bacterial growth on a soluble substrate (ferrous iron), as well as the growth of bacteria adsorbed on the $FeS_2$ surface. In these models proposed by Gormely *et al.* (1975) and Chang and Myerson (1982), it was assumed that the growth rate of bacteria adsorbed on

the mineral is proportional only to the concentration of adsorbed cells. Konishi *et al.* (1990 and 1992) claimed that the growth of bacteria adsorbed on a pyrite surface is proportional to the product of the number of adsorbed cells and the fraction of solid surface unoccupied by cells. However, they assumed that the net formation rate of $Fe^{2+}$ was zero and pyrite was not dissolved chemically with $Fe^{3+}$. These assumptions may not be applicable to a leaching system where the temperature is high and the chemical reaction cannot be neglected. In recent work, Vitaya *et al.* (1994) proposed a model which included both direct and indirect mechanisms. From their work, it is suggested that the contributions of direct (biological) and indirect (chemical) reactions to the total rate of pyrite oxidation were in a ratio of 2:1.

**Bioenergetics**

Because biological leaching occurs as a result of the energetic requirements of particular microorganism, it is important to understand the factors that affect membrane integrity and energetic driving forces. According to Mitchell's chemiosmotic hypothesis, the transmembrane electrochemical gradient of protons plays a central role in energy transduction and transport processes in mitochondria, chloroplasts, and bacteria (Mitchell, 1966). It is now generally accepted that organelles and organisms contain membrane-bound proton pumps that generate a transmembrane proton motive force composed of a transmembrane electrical potential ( $\psi$) and a transmembrane pH gradient ( pH) (measured in millivolts):

$$\Delta p = \Delta\Psi - 2.303\ R\ T\ \Delta pH\ /\ F$$

where p is the proton motive force, R is the universal gas constant, T is the absolute temperature and F the Faraday constant. The most striking bioenergetic characteristic of acidophiles is their unique ability to maintain a large pH gradient, even under highly stressful conditions of pH and nutrition. This large pH gradient is generated by the proton pumps of the cytoplasmic membrane (Cobley and Cox, 1983; Michels and Bakker, 1985; Matin *et al*., 1982). There is disagreement, however, on whether the pH is maintained passively or through active chemiosmotic mechanisms. The idea that a passive mechanism is involved was first proposed by Hsung and Haug (1977), who reported that the pH in *Thermoplasma acidophila* was not affected by treatment with 2,4-dinitrophenol (DNP) or azide or even when the cells were subjected to boiling; the particular passive mechanism which they postulated was a Donnan potential of positively charged membrane-impermeable molecules that presumably kept H+ out of the cell. This view is supported by the studies of Oshima *et al*. (1977) and those of Cox *et al*. (1984), which showed that a large pH was maintained in the presence of protonophores or respiratory inhibitors. In contrast, Krulwich *et al*. (1978) and Michels and Bakker (1985) reported that DNP completely abolished pH in *Bacillus acidocaldarius*, and the protonophores as well as respiratory chain inhibitors stopped proton motive force ( p) linked transport functions; these authors concluded that pH in this organism was actively maintained. Recently, Matin (1990a and b) reported that in completely inactive cells a sizable pH was retained and proposed that it is a generation of H+ diffusion potentials which inhibits the complete collapse of pH.

A metabolic proton circuit enables species, such as the mesoacidophile *T*. *ferrooxidans*, to thrive in ecological niches, rich in metal sulfides. Their extracellular pH may be less than 2.0, but intracellular pH must be close to neutrality for normal cellular functions (Matin, 1990a and b). Based on above chemiosmotic theory, protons enter the

cell through an ATPase, which results in the storage of cellular energy, and are shuttled out of the cell through an electron transport chain. The net flux is coupled with the transfer of electrons from substrates to molecular oxygen as the terminal electron acceptor. Cox *et al*. (1979) showed that *T. ferrooxidans* growth on ferrous ions at pH 2.0 resulted in a p of -256 mV, composed of pH of 4.5 (-265 mV) and a membrane potential of +10 mV. Respiratory chain inhibitors and uncouplers were found to significantly reduce p by inducing a large, negative membrane potential, while not affecting the pH gradient to a significant extent.

Very few studies have focused on the relation between proton motive force and energy substrate turnover as they relate to chemiosmotic coupling in extreme thermoacidophiles. The generation and maintenance of the p for extreme thermophiles are complicated by the need for temperature stabilization of the cell membrane. Proton-translocating activity coupled to respiration has been found in *Sulfolobus* species (Moll and Schäfer, 1988). Also, the existence of a membrane-associated ATPase has been reported (Konishi *et al*., 1990; Lübben and Schäfer, 1987). In fact, previous whole cell experiments with *S. acidocaldarius* have proved that both of these ATP-synthesis and proton extrusion are coupled to cellular respiration (Lübben et al., 1987). In this experiment, researchers showed prolonged anaerobiosis caused cell death and lysis, followed by decrease in ATP content. Furthermore, the effect of DCCD (*N*, *N'*-dicyclohexyl-carbodiimide) revealed this operation of a proton coupled ATP-synthase (Lübben and Schäfer, 1989).

Lately, it has been shown that electron transport-coupled phosphorylation in *S. acidocaldarius* involves a membrane-associated F-type ATPase rather than V-type ATPase (Lübben and Schäfer, 1989; Schäfer *et al*., 1990). Energy stored in the electrochemical gradient of protons across the plasma membrane of *S. acidocaldarius* is utilized by these

$F_0F_1$-type ATPases of bacteria and eukarya. This is an integral membrane complex with a peripheral protein moiety representing the large catalytic head piece, connected to an ion channel by a stalk operating as a conformational energy transducer. It is generally thought that archaeal ATP synthases also have a corresponding spatial structure. Other molecular modules of the energy transduction pathway on the level of phosphotransferases are the adenylatekinase, buffering the cellular energy charge, and the cytosolic pyrophosphatase as a thermodynamic sink in coupled activation processes.

Although most of the essential components for the electron transport and respiration system of *Sulfolobus* were identified and have been characterized in great detail (Iwasaki *et al.*, 1995a, b and c), no homogeneous data on direct respiratory measurements exist. In particular, there is only limited information on the turnover of membrane-bound electrons in terms of oxygen consumption. In addition, available data from various laboratories have been determined under widely different conditions with respect to temperature and pH. Information on cellular proton extrusion is limited at pH below 2-3; thus, most reported experiments were conducted at higher medium pH (3.5-6.0), and the extrapolations to optimum growth conditions for *Sulfolobus* have to be made. However, it has been clearly shown that these thermoacidophilic archaea have unique bioenergetic features related to the level of substrate metabolism, formation and utilization of high energy phosphate bonds, and primary energy conservation in respiratory electron transport. In particular, through chemiosmotic coupling, the extracytoplasmic oxidation of mineral substrates, such as pyrite and sulfur, is linked to the shuttling of protons across an impermeable barrier, i.e., the cell membrane of these microorganisms. Therefore, it will be interesting to see whether the factors affecting membrane permeability and/or proton pumps affect the turnover rate of mineral substrates of thermoacidophiles. These issues were addressed in Chapter 4 in more detail.

## Stress Response

Acidophilic organisms of all types may be subjected to thermal and/or nutritional stress in their natural environment or in bioleaching processes. It has been demonstrated that when any cell is subjected to various forms of external stress, there is a shift in cellular processes, which can be beneficial in a particular application (Matin, 1992). Most notably, the production of stress-response proteins which stabilize cellular constituents in acidophiles have been identified (Jerez *et al*., 1988; Matin, 1992; Trent *et al*., 1990). For example, the acidophilic bacteria, *Leptospirrilum ferrooxidans* and *T. ferrooxidans*, were shown to produce several heat shock proteins upon a 11°C shift in temperature; a shift in pH from (3.5 to 1.5) also elicited a stress response (Jerez, 1988). These stress-response or heat-shock proteins, which are members of so called 'molecular chaperones', are now known to participate in a large variety of cellular functions.

Until recently, it had been assumed that protein folding *in vivo* generally occurs spontaneously in an energy-independent fashion. This view has changed profoundly over the past ten years, due to discovery of molecular chaperones, which occur ubiquitously in eubacteria, archaea and in eukaryotic cells (Ellis, 1987; Martin and Hartl, 1994). The need for molecular chaperones arises because the high concentrations of transiently interacting protein surfaces inside the cells allow the formation of incorrect non-functional structures. These molecular chaperones assist in *de novo* protein folding, stabilize proteins under stress conditions and maintain polypeptide chains in a loosely folded state competent for translocation across organellar membranes (Martin and Hartl, 1994; Ellis, 1994). These proteins are thought to recognize the hydrophobic sequences or surfaces of proteins that are typically exposed to solvent in the unfolded, but not in the native, state. Apparently, they do not interact with a specific consensus sequence motif and, thus, have the ability to

prevent the incorrect intra- and inter-molecular folding and association of many different proteins (Martin and Hartl, 1994).

The most thoroughly studied family of molecular chaperones are called the chaperonins, the archetypal examples being the GroEL and GroES proteins of *E. coli* (Martin and Hartl, 1994) (see Figure 1.2). These GroE chaperonins, which can be found in chloroplasts, mitochondria, and eubacteria, are distantly related to another group, called the t-complex polypeptide 1 (TCP-1) chaperonins, found in archaea and the eukaryotic cytosol (Willison *et al*., 1986; Silver *et al*., 1987). The chaperonins all have in common a large oligomeric structure consisting of two stacked rings of subunits, each about 60 kDa in size and possessing ATPase activity. Each ring surrounds a central cavity that plays a key role in the functioning of this molecule. The GroE chaperonins have seven subunits per ring, and the TCP-1 chaperonins have eight or nine. The GroE also include another smaller oligomer consisting of a single ring of seven subunits, each about 10 kDa in size. The larger oligomer from *E. coli* is called GroEL, whereas the smaller oligomer is called GroES. They combine together in 1:1 ratio to form an asymmetric binary complex in the presence of adenine nucleotides. Several different types of models are proposed to describe how GroEL and GroES function to correctly refold proteins, and each of them is currently under intense debate (Ellis and Hartl, 1996).

The term *stress* or *heat-shock response* refers to the reaction of a living cell to potentially harmful physical or chemical agents. An increase in the temperature of a cell's environment elicits a heat-shock response by that cell to counteract the stressful effects on it. In bacteria and eukaryotes, heat shock causes the protein products of many genes to diminish or become undetectable, while stimulating the production of other proteins - the heat shock proteins (HSPs). According to Schlesinger (1986), a heat shock protein is defined by two criteria: (a) its synthesis is strongly stimulated by an environmental stress,

in particular, that resulting from a change in temperature a few degrees centigrade above the normal physiological case; and, (b) its gene contains a conserved sequence of 14 base pairs in the 5' non-coding region, the Pelham box (Pelham, 1982). This sequence serves as the promoter for HSP mRNA transcription. These proteins are the hallmark of the heat shock response, and have been grouped, according to their molecular mass, into the 100, 90, 70, 60, 40 and 30 kDa families. The functions of the family members are heterogeneous and there is even some variation in molecular mass within the families. The initial studies on cloned heat shock genes and purified proteins led to two important results. First, the heat shock proteins proved to be very highly conserved among widely divergent organisms. Second, several of the major heat shock proteins are members of gene (proteins) families that include proteins normally present and, in most cases, essential for cell function. It is commonly accepted that the function of the heat shock proteins is either to protect cells against subsequent heat stress or to enhance the ability of cells to recover from the toxic effects of heat or other stresses.

In *E. coli*, twenty-four proteins are induced by a shift-up in temperature; of these, twenty are under the control of a single gene, *htpR* (or *rpoH*), which encodes a small protein called a sigma factor 32 ($\sigma^{32}$), which confers specificity on RNA polymerase (Neidhardt *et al*., 1990). Among those studied in detail, some proteins, including GroEL, GroES, DnaK, DnaJ and GrpE, are essential for growth at all temperatures, whereas others (Lon, LysU and HtpG) are dispensable under normal growth conditions (Yura *et al*., 1990). Reports show that changes in the cellular level of $\sigma^{32}$ are directly responsible for increased transcription from heat shock promoters, suggesting $\sigma^{32}$ plays a central role in heat shock response in *E. coli*. Lately, the level of general stress response protein $\sigma^{S}$, which is encoded by the *rpoS* gene, is shown to induced by heat shock (Muffler *et al*.,

1997). Despite its induction, however, $\sigma^S$ does not seem to contribute to heat adaptation but may induce cross-protection against different stresses.

Archaea have the ability to mount a heat-shock (stress) response that differs in some aspects from those of eubacteria and eukaryotes. For example, heat shocks of 60 min or longer, which would exceed the ability of other organisms to respond, still induce an intense response. Archaeal species are so diverse, even within each main branch (i.e. *Euryarchaeota* or *Crenarchaeota*), that diversity in the heat shock response is to be expected. Mesophilic archaea, such as *Methanococcus voltae* and *Methanosarcina mazei*, respond to heat shock by a decrease in the activity of many genes, and an increase in others - the heat shock genes (Hebert *et al*., 1991; Clarens *et al*., 1995). Eubacterial cells respond in a similar way; the response to most stressors involves a common set of heat shock proteins. However, in extreme thermophiles or hyperthermophiles, a further elevation of temperatures induces the synthesis of very few proteins, frequently only one (de Macario and Macario, 1994).

The nature of heat shock response by extreme thermophiles and hyperthermophiles is intriguing, considering the fact that they thrive at temperatures far above the upper limits for most cells. Holden and Baross (1993) showed the hyperthermophilic archaeon ES4, a heterotrophic sulfur reducer, is capable of acquiring thermotolerance from thermal stress at temperatures above its optimum for growth. Two proteins with molecular masses of approximately 90 and 150 kDa significantly decreased in abundance with increasing growth temperature, while a 98 kDa protein, present at very low levels at normal growth temperatures (76 to 99$^o$C), was more abundant when the growth temperature was shifted from 95 to 102$^o$C. *Pyrodictium occultum*, one of the most hyperthermophilic organisms known to date (growing up to 110$^o$C), synthesized increased levels of an novel ATPase complex (later referred to as thermosome) when culture temperature was shifted from 102

to 108°C (Phipps *et al*., 1991). This complex was composed of equal quantities of two polypeptides of 56- and 59 kDa. It exhibited an extremely thermostable ATPase activity with a temperature optimum at 100°C. Similar types of thermosomes were also identified from hyperthermophiles *Methanopyrus kandleri* and *Thermoplasma acidophilum* (Andrä *et al*., 1996; Waldmann *et al*., 1995). All of these thermosomes are found to be closely related to eukaryotic chaperonin TRiC of Group II chaperonins.

Most studies to date on heat shock response in extremely thermophilic microorganisms have focused on members of the order *Sulfolobales* in the kingdom *Crenarchaeota*. The response of *S. acidocaldarius* to heat was examined by increasing the temperature from 70°C to 85°C (Jerez, 1988). Heat shock caused a decrease in the synthesis of many proteins, but induced the synthesis of 86, 66, 64, 38, and 22 kDa proteins. The synthesis of these proteins appears to be up- and down-regulated by a temperature level centered at 70°C. The heat shock response of *S. shibatae* strain B12, which grows optimally at 70°C, was investigated by one- and two-dimensional gel electrophoresis using $^{35}$S-methionine to radiolabel newly synthesized proteins (Trent *et al*., 1990). The protein patterns of cells subjected to heat shock at 88 and 92°C for 60 min were analyzed. A 55 kDa band on 10% SDS-PAGE, induced by heat shock at 88°C, was also seen at 92°C. Two proteins of 35 and 25 kDa also increased in cultures at 88°C. This group showed that *S. shibatae* possessed an acquired thermotolerance, and purified a prominent heat hock protein TF55 (Thermophilic Factor 55) in this organism. TF55 was shown to be related to a chaperone-component in the eukaryotic cytosol and proposed to be representative of a new class of molecular chaperones (Trent *et al*., 1991). Later, Kagawa *et al*. (1995) found that *S. shibatae* has a thermosome (or rosettasome), which is related to TCP1, and TF55 is one of its two subunits. This thermosome exists in two distinct conformational states - closed and open complex states - that are part of its

functional cycle (Quaite-Randall *et al*., 1995) (see Figure 1.3). Knapp *et al*. (1994) purified the TF55 from *S. solfataricus* and showed it to be composed of two similar subunits formed into a 9-fold symmetrical complex. Further investigations showed that chaperonin from *S. solfataricus* promotes correct refolding and thermal aggregation of proteins *in vivo* (Guagliardi *et al*., 1994; Guagliardi *et al*., 1995).

Since the archaea constitute a repository of heat shock and molecular chaperone genes that function in diverse, hostile environmental conditions, it is evident that the proteins which constitute the archaeal stress response have great biological potential as they have evolved to deal with such harsh conditions. In particular, the reports shown above indicate that thermotolerance can be increased by a preparatory heat shock, even in an organism that normally thrives at high temperatures, such as extreme thermophiles and hyperthermophiles. Therefore, the temperatures at which these unique organisms live may be used for bioconversions, bioremediation, or for the production of industrially useful molecules. But more importantly, the temperatures at which these organisms live may be increased even higher with a preparatory heat shock, to some extent, to enable biotechnological applications that require very high temperatures to be carried out. However, the physiological roles of these heat-shock related genes and proteins, specially TF55 and other archaeal chaperonins (i.e. thermosomes), in thermoacidophiles have yet to be elucidated. Nor have their metabolic responses to variations in temperature, pH, and nutritional conditions that could be encountered in bioprocessing steps been examined. In order to address these issues, the main focus of this work was devoted to elucidate stress response from *M. sedula* and to investigate whether this physiological response can be exploited in bioleaching processes as described in Chapter 2 and Chapter 4, respectively.

## RESEARCH ISSUES

This project will address several issues related to improving biooxidation rates for recovery of gold from ores and/or removal of sulfur from coals by extremely thermoacidophilic microorganisms. To date, only temperatures to about 70°C and pH's to 1.7 have been considered, even though higher temperatures and lower pH's may produce better overall results. At temperatures and pH's above/below the normal growth optima, the bioenergetics and nutritional patterns of thermoacidophilic organisms, such as *M. sedula*, have not been investigated, nor has their capacity to catalyze pyrite dissolution.

To address these issues, the following questions can be considered:

### 1. Will higher temperature lead to better leaching rate?

According to the Arrhenius equation, it is expected that the rate of chemical oxidation of pyrite by ferric iron will accelerate with increasing temperature. Boogerd *et al*. (1990) showed that only 2% of pyrite was oxidized chemically by ferric iron at 30°C, but 43% at 70°C. Although the Arrhenius relationship holds for only a limited range for biological activity, the metabolic activity of microorganisms may be stimulated temporarily at higher temperatures. It has been shown that only few degrees of temperature shift-up was enough to significantly enhance pyrite leaching rates by *M. sedula*. Then, it will be interesting to see whether the thermoacidophile with higher growth temperature (e.g. *S. solfataricus*, 87°C) than *M. sedula* can be a better metal leacher at elevated temperatures.

**2. What happens when the microorganisms are exposed to thermal, pH, and/or chemical stress?**

A number of changes can occur in the heat-shocked cells. Among these are the cessation of DNA synthesis and condensation of chromatin, the dephosphorylation of ribosomal protein S6, collapse of the intermediate filament network, increase in intracellular calcium, and shifts to glycolytic metabolism (Schlesinger, 1986). Similar phenomena, including spore formation and protease production, may occur in pH-shocked and/or nutritionally starved cells (Matin, 1990a and b). Also, for the metal leaching organisms, the attachment patterns on the solid surface may be influenced under stress. It is possible that more cells may be found in the attached state under stressful conditions, especially in nutritionally-limited situations, because it seems that the unattached (free) cells can only survive through feeding on the substrates released by the attached cells (Shrihari *et al*., 1993).

Many studies have shown that a pre-shock treatment can render a biological system more resistant to a subsequent stress and that this protection effect is transient. Evidence is accumulating that acquired thermotolerance can be developed in prokaryotes upon heat shock (Holden and Baross, 1993; Trent *et al*., 1990 and 1994; Zellner and Kneifel, 1993; Carper *et al*., 1987; Burdon, 1986 and 1988; Schlesinger, 1990). Positive correlations exist between the amount of heat shock proteins present and the degree of tolerance. Although it is not understood how the heat shock proteins induce enhanced thermotolerance in the cells under stress, it is clear that this defense mechanism can lead to increased tolerance to other types of stress which the cells may encounter in the actual bioreactor operation.

### 3. Can periodic exposure to stress enhance overall leaching rates?

Currently, the metabolic characteristics of *M. sedula* are not well defined. Figure 1.4 shows the metabolic network based on existing knowledge of *M. sedula's* nutritional requirements as well as a theoretical chemiosmotic framework. Under normal growth conditions, the cells will generate the proton motive force ( p) by translocating protons across the cytoplasmic membrane utilizing the electron transport chain (ETC) as a proton pump. Then, the p can be used to drive a variety of energy-linked processes, including (1) entrance of certain substrates into the cell against a concentration gradient; (2) maintaining the cell's interior at a proper pH; and (3) generating ATP from ADP (Neidhardt *et al*., 1990).

However, when the cells are subjected to the sub-lethal or lethal temperatures and/or pHs, the membrane will likely become permeable to $H^+$ ions, and more protons will transfer to inside of the cell's cytoplasmic membrane. To survive, the organism must compensate the required energy. To generate more needed energy (i.e. to expel protons), the cells have to turnover more iron (i.e. to release electrons) for the energy source. Therefore, under the stress conditions, the iron turnover rate may be enhanced to compensate the cell's increased maintenance requirement.

## LITERATURE CITED


1. **Adams, M. W. W.** 1990. The metabolism of hydrogen by extremely thermophilic, sulfur-dependent bacteria. FEMS Microbiol. Rev. **75:** 219-238.
2. **Andrä, S., G. Frey, M. Nitsch, W. Baumeister, and K. O. Stetter.** 1996. Purification and structural characterization of the thermosome from the hyperthermophilic archaeum *Methanopyrus kandleri*. FEBS Lett. **379:** 127-131.
3. **Attia, Y. A.., and M. A. E. Zeky.** 1990. Bioleaching of non-ferrous sulfides with adapted thiophilic bacteria. Chem. Eng. J. **44:** B31-B40.
4. **Bagdigian, R. M., and A. S. Myerson.** 1986. The adsorption of *Thiobacillus ferrooxidans* on coal surfaces. Biotechnol. Bioeng. **28:** 467-479.
5. **Baumeister, W., and G. Lembcke.** 1992. Structural features of archaebacterial cell envelopes. J. Bioenerg. Biomem. **24:** 567-575.
6. **Blake, R. C., E. A. Shute, M. M. Greenwood, G. H. Spencer, and W. J. Ingledew.** 1993. Enzymes of aerobic respiration on iron. FEMS Microbiol. Rev. **11:** 9-18.
7. **Blaustein, B. D., J. T. Hauck, G. J. Olson, and J. P. Baltrus.** 1993. Bioleaching of molybdenum from coal liquefaction catalyst residues. Fuel. **72:** 1613-1618.
8. **Bonch-Osmolovskaya, E. A., M. L. Miroshnichenko, N. A. Kostrikina, N. A. Chernych, and G. A. Zavarzin.** 1990. *Thermoproteus uzoniensis* sp. nov., a new extremely thermophilic archaebacterium from Kamchatka continental hot springs. Arch. Microbiol. **154:** 556-559.
9. **Boogerd, F. C., P. Bos, J. G. Keunen, J. J. Heijnen, and R. G. J. M. v. d. Lans.** 1990. Oxygen and carbon dioxide mass transfer and the aerobic,

autotrophic cultivation of moderate and extreme thermophiles: a case study related to the microbial desulfurization of coal. Biotechnol. Bioeng. **35:** 1111-1119.

10. **Boogerd, F. C., C. V. D. Beemd, T. Stoelwinder, P. Bos, and J. G. Kuenen.** 1991. Relative contributions of biological and chemical reactions to the overall rate of pyrite oxidation at temperatures between 30 and 70°C. Biotechnol. Bioeng. **38:** 109-115.
11. **Brierley, C. L., and J. A. Brierley.** 1973. A chemoautotrophic and thermophilic microorganism isolated from an acid hot spring. Can. J. Microbiol. **19:** 183-188.
12. **Brierley, C. L., and L. E. Murr.** 1973. Leaching: use of a thermophilic and chemoautotrophic microbe. Science. **179:** 488-490.
13. **Brierley, J. A.** 1994. Biooxidation-heap technology for pretreatment of refractory sulfidic gold ore. BIOMINE'94.
14. **Brock, T. D., and H. Freeze.** 1969. *Thermus aquaticus* gen. n. and sp. n., a non-sprouting extreme thermophile. J. Bacteriol. **98:** 298-297.
15. **Brock, T. D., K. M. Brock, R. T. Belly, and R. L. Weiss.** 1972. *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. Arch. Microbiol. **84:** 54-68.
16. **Brock, T. D.** 1978. Thermophilic microorganisms and life at high temperatures. Springer-Verlag, New York.
17. **Brock, T. D.** 1986. in *The Thermophiles: General, molecular, and applied microbiology* (John Wiley & Sons, New York).
18. **Burdon, R. H.** 1986. Heat shock and the heat shock proteins. Biochem. J. **240:** 313-324.

19. **Burdon, R. H.** 1988. The heat shock proteins. Endeavor. **12:** 133-138.

20. **Burggraf, S., H. W. Jannasch, B. Nicolaus, and K. O. Stetter.** 1990. *Archaeglobus profundus* sp. nov., represents a new species within the sulfate-reducing archaebacteria. System. Appl. Microbiol. **13:** 24-28.

21. **Carper, S. W., J. J. Duffy, and E. W. Gerner.** 1987. Heat shock proteins in thermotolerance and other cellular processes. Cancer Res. **47:** 5249-5255.

22. **Chang, Y. C., and A. S. Myerson**. 1982. Growth models of the continuous bacterial leaching of iron pyrite by *Thiobacillus ferrooxidans*. Biotechnol. Bioeng. **24:** 889-902.

23. **Clarens, M., A. J. L. Macario, and E. Conway de Macario.** 1995. The archaeal *dnaK-dnaJ* gene cluster: Organization and expression in the methanogen *Methanosarcina mazei*. J. Mol. Biol. **250:** 191-201.

24. **Cobley, J. G., and J. C. Cox.** 1983. Energy conservation in acidophilic bacteria. Microbiol. Rev. **47:** 579-595.

25. **Corpe, W. A..** 1980. In *Adsorption microorganism to surfaces*, Microbial surface components involved in adsorption of microorganisms onto surfaces. 105-139

26. **Cox, J. C., D. G. Nicholls, and W. J. Ingledew.** 1979. Transmembrane electrochemical potential and transmembrane pH gradient in the acidophile *Thiobacillus ferrooxidans*. Biochem. J. **178:** 195-200.

27. **Cox, J. C., and M. D. Brand.** 1984. in *Microbial chemoautotrophy*. Iron oxidation and energy conservation in chemoautotrophs: the thermodynamics of ATP synthesis in the acidophile *Thiobacillus ferrooxidans* (Ohio State University Press, Columbus).

28. **Daniels, S. L.** 1980. In *Adsorption microorganisms to surfaces*, Mechanisms involved in sorption of microorganisms to solid surfaces. 7-50

29. **Danson, M. J.** 1989. Central metabolism of the archaebacteria: an overview. Can. J. Microbiol. **35:** 58-64.

30. **Darland, G., T. D. Brock, W. Samsonoff, and S. F. Conti.** 1970. A thermophilic acidophilic *Mycoplasma* isolated from a coal refuse pile. Science **170:** 1416-1418.

31. **de Macario, E. C., and A. J. L. Macario.** 1994. Heat-shock response in archaea. Trends Biotechnol. **12:** 512-518.

32. **De-Rosa, M., and A. Gambacorta.** 1988. The lipids of archaebacteria. Prog. Lipid Res. **27:** 153-175.

33. **Duarte, J. C., P. C. Estrada, P. C. Pereira, and H. P. Beaumont.** 1993. Thermophilic vs. mesophilic bioleaching process performance. FEMS Microbiol. Rev. **11:** 97-102.

34. **Ellis, R. J.** 1987. Proteins as molecular chaperones. Nature. **328:** 378-379.

35. **Ellis, R. J.** 1994. Roles of molecular chaperones in protein folding. Curr. Op. Struct. Biol. **4:** 117-122.

36. **Ellis, R. J., and F.-U. Hartl.** 1996. Protein folding in the cell: competing models of chaperonin function. FASEB Journal **10:** 20-26.

37. **Evangelou, V. P., and Y. L. Zhang.** 1995. A review: pyrite oxidation mechanisms and acid mine drainage prevention. Critical Reviews in Environmental Science and Technology. **25:** 141-199.

38. **Fuchs, T., H. Huber, K. Teiner, S. Burggraf, and K. Stetter.** 1995. *Metallosphaera prunae*, sp. nov., a novel metal-mobilizing, thermoacidophilic archaeum, isolated from a uranium mine. System. App. Microbiol. **18:** 560-566.

39. **Goldstein, A. H., R. D. Rogers, and G. Mead. 1993.** Mining by microbe. Bio/Tech. **11:** 1250-1254.

40. **Gormely, L. S., D. W. Duncan, R. M. R. Branion, and K. L. Pinder.** 1975. Continuous culture of *Thiobacillus ferrooxidans* on a zinc sulfide concentrate. Biotechnol. Bioeng. **17:** 31-49.

41. **Grogan, D. W. 1989.** Phenotypic characterization of the archaebacterial genus *Sulfolobus*: comparison of five wild-type strains. J. Bacteriol. **171:** 6710-6719.

42. **Grogan, D., P. Palm, and W. Zillig.** 1990. Isolate B12, which harbours a virus-like element, represents a new species of the archaebacterial genus *Sulfolobus*, *Sulfolobus shibatae*, sp. nov. Arch. Microbiol. **154:** 594-599.

43. **Guagliardi, A., L. Cerchia, S. Bartolucci, and M. Rossi.** 1994. The chaperonin from the archaeon *Sulfolobus solfataricus* promotes correct refolding and prevents thermal denaturation in vitro. Protein Science **3:** 1436-1443.

44. **Guagliardi, A., L. Cerchia, and M. Rossi.** 1995. Prevention of *in vitro* protein thermal aggregation by the *Sulfolobus solfataricus* chaperonin. J. Biol. Chem. **270:** 28126-28132.

45. **Hebert, A. M., A. M. Kropinski, and K. F. Jarrell.** 1991. Heat shock response of the archaebacterium *Methanococcus voltae*. J. Bacteriol. **173:** 3224-3227.

46. **Holden, J. F., and J. A. Baross.** 1993. Enhanced thermotolerance and temperature-induced changes in protein composition in the hyperthermophilic archaeon ES4. J. Bacteriol. **175:** 2839-2843.

47. **Hsung, J. C., and A. Haug.** 1977. Membrane potential of *Thermoplasma acidophila*. FEBS Lett. **73:** 47-50.

48. **Huber, R., T. A. Langworthy, H. König, M. Thomm, C. R. Woese, U. B. Sleytr, and K. O. Stetter.** 1986. *Thermotoga maritima* sp. nov.

represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch. Microbiol. **144:** 324-333.

49. **Huber, R., J. K. Kristjansson, and K. O. Stetter.** 1987. *Pyrobacculum* gen. nov., a new genus of neutrophilic, rod-shaped archaebacteria from continental solfataras growing optimally at 100 C. Arch. Microbiol. **149:** 95-101.

50. **Huber, G., C. Spinnler, A. Gambacorta, and K. O. Stetter.** 1989. *Metallosphaera sedula* gen. and sp. nov. represents a new genus of aerobic, metal-mobilizing, thermophilic archaebacteria. System. Appl. Microbiol. **12:** 38-47.

51. **Huber, H., and K. O. Stetter.** 1990. *Thiobacillus cuprinus* sp. nov., a novel facultatively organotrophic metal-mobilizing bacterium. Appl. Environ. Microbiol. **56:** 315-322.

52. **Huber, R., T. Wilharm, D. Huber, A. Trincone, S. Burggraf, H. König, R. Rachel, I. Rockinger, H. Fricke, and K. O. Stetter. 1992.** *Aquifex pyrophilus* gen. nov. sp. nov, represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. System. Appl. Microbiol **15:** 340-351.

53. **Iwasaki, T., K. Matsuura, and T. Oshima.** 1995a. Resolution of the aerobic respiratory system of the thermoacidophilic archaeon, Sulfolobus sp. strain 7. I. The archaeal terminal oxidase supercomplex is a functional fusion of respiratory complexes III and IV with no c-type cytochromes. J. Biol. Chem. **270:** 30881-30892.

54. **Iwasaki, T., K. Matsuura and T. Oshima.** 1995b. Resolution of the aerobic respiratory system of the thermoacidophilic archaeon, Sulfolobus sp. strain 7. II. Characterization of the archaeal terminal oxidase subcomplexes and implication for the intramolecular electron transfer. J. Biol. Chem. **270:** 30893-30901.

55. **Iwasaki, T., K. Matsuura and T. Oshima.** 1995c. Resolution of the aerobic respiratory system of the thermoacidophilic archaeon, Sulfolobus sp. strain 7. III. The archaeal novel respiratory complex II (succinate:caldariellaquinone oxidoreductase complex) inherently lacks heme group. J. Biol. Chem. **270:** 30902-30908.

56. **Jannasch, H. W., C. O. Wirsen, S. J. Molyneaux, and T. A. Langworthy.** 1988. Extremely thermophilic fermentative archaebacteria of the genus *Desulfurococcus* from deep-sea hydrothermal vents. Appl. Environ. Microbiol. **54:** 1203-1209.

57. **Janssen, K.** 1995. in *Current Protocols in Molecular Biology,* Vol. 2 (John Wiley & Sons, Inc.)

58. **Jerez, C. A.** 1988. The heat shock response in mesophilic and thermoacidophilic chemolithotrophic bacteria. FEMS Microbiol. Lett. **56:** 289-294.

59. **Jones, W. J., J. A. Leigh, F. Mayer, C. R. Woese, and R. S. Wolfe.** 1983. *Methanococcus jannaschii* sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. Arch. Microbiol. 254-261.

60. **Kagawa, H. K., J. Osipiuk, N. Maltsev, R. Overbeek, E. Quaite-Randall, A. Joachimiak, and J. D. Trent.** 1995. The 60 kDa heat shock proteins in the hyperthermophilic archaeon *Sulfolobus shibatae*. Journal of Molecular Biology **253:** 712-725.

61. **Kargi, F., and J. M. Robinson.** 1982. Microbial desulfurization of coal by thermophilic microorganism *Sulfolobus acidocaldarius.* Biotechnol. Bioeng. **24:** 2115-2121.

62. **Kargi, F., and J. M. Robinson.** 1985. Biological removal of pyritic sulfur from coal by the thermophilic organism *Sulfolobus acidocaldarius.* Biotechnol. Bioeng. **26:** 41-49.

63. **Kelly, D. P. 1971.** Autotrophy: concepts of lithotrophic bacteria and their organic metabolism. Ann. Rev. Microbiol. **25:** 177-210.

64. **Kelly, R. M., and S. H. Brown.** 1993. Enzymes from high temperature microorganisms. Curr. Opin. Biotech. **4:** 188-193.

65. **Kelly, R. M., T. L. Peeples, S. B. Halio, K. R. Rinker, and G. D. Duffaud.** 1994. Extremely thermophilic microorganisms: metabolic strategies, genetic characteristics and biotechnological potential. An. New York Ac. Sci. **745:** 409-425.

66. **Knapp, S., I. Schmidt-Krey, H. Hebert, T. Bergman, H. Jornvall, and R. Ladenstein.** 1994. The molecular chaperonin TF55 from the thermophilic archaeon *Sulfolobus solfataricus.* J. Mol. Biol. **242:** 397-407.

67. **Konishi, J., K. Denda, T. Oshima, T. Wakagi, E. Uchida, Y. Oshumi, Y. Anraku, T. Matsumoto, T. Wakabayashi, Y. Mukohata, K. Ihara, K.-I. Inatomi, K. Kato, T. Ohta, W. S. Allison, and M. Yoshida.** 1990. Archaebacterial ATPases: relationship to other ion-translocating ATPase families examined in terms of immunological cross-reactivity. J. Biochem. **108:** 554-559.

68. **Konishi, Y., S. Asai, and H. Katoh.** 1990. Bacterial dissolution of pyrite by *Thiobacillus ferrooxidans.* Bioproc. Eng. **5:** 231-237.

69. **Konishi, Y., H. Kudo, and S. Asai.** 1992. Bioleaching of zinc sulfide concentrate by *Thiobacillus ferrooxidans.* Biotechnol. Bioeng. **39:** 66-74.

70. **Krulwich, T. A., L. F. Davidson, S. J. F. Jr., R. S. Zuckerman, and A. A. Guffanti.** 1978. The proton motive force and ß-galactoside transport in *Bacillus acidocaldarius*. J. Biol. Chem. **253:** 4599-4603.

71. **Larsson, L., G. Olsson, O. Holst, and H. T. Karlsson.** 1990. Pyrite oxidation by thermophilic archaebacteria. Appl. Environ. Microbiol. **56:** 697-701.

72. **Liang, P., L. Averboukh, and A. B. Pardee.** 1993. Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. Nucleic Acids Res. **21:** 3269-3275.

73. **Lindstrom, E. B., S. Wold, N. Kettaneh-Wold, and S. Saaf.** 1993. Optimization of pyrite bioleaching using *Sulfolobus acidocaldarius*. Appl. Microbiol. Biotechnol. **38:** 702-707.

74. **Loi, G., A. Mura, N. Passarini, P. Trois, and G. Rossi.** 1993. Modification of oxidizing activity of *Thiobacillus ferrooxidans* by some particulate solids. Fuel. **72:** 1607-1611.

75. **Lübben, M., S. Anemüller, and G. Schäfer.** 1987. Interaction of DCCD with the ATP synthase of the archaebacterium *Sulfolobus acidocaldarius*. Bio. Chem. Hoppe-Seyler **368:** 555.

76. **Lübben, M., and G. Schäfer.** 1987. A plasma-membrane associated ATPase from the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. Eur. J. Biochem. **164:** 533-540.

77. **Lübben, M., and G. Schäfer.** 1989. Chemiosmotic energy conversion of the archaebacterial thermoacidophile *Sulfolobus acidocaldarius*: oxidative phosphorylation and the presence of an $F_0$-related N, N'-dicyclohxylcarboiimide-binding proteolipid. J. Bacteriol. **171:** 6106-6116.

78. **Marshall, K. C.** 1985. in *Bacterial Adhesion*, Mechanisms of bacterial adhesion at solid-water interfaces. 136-156.

79. **Martin, A., S. Yeats, D. Janekovic, W.-D. Reiter, W. Aicher, and W. Zillig.** 1984. SAV1, a temperate UV-inducible DNA virus-like particle from the archaebacterium *Sulfolobus acidocaldarius* isolate B12. EMBO Journal. **3:** 2165-2168.

80. **Martin, J., and F.-U. Hartl.** 1994. Molecular chaperones in cellular protein folding. BioEssays. **16:** 689-692.

81. **Matin, A.** 1978. Organic nutrition of chemolithotrophic bacteria. Ann. Rev. Microbiol. **32:** 433-468.

82. **Matin, A., B. Wilson, E. Zychlinsky, and M. Matin.** 1982. Proton motive force and the physiological basis of delta pH maintenance in *Thiobacillus acidophilus*. J. Bacteriol. **150:** 582-591.

83. **Matin, A.** 1990a. Bioenergetics parameters and transport in obligate acidophiles. Biochim. Biophys. Acta. **1018:** 267-270.

84. **Matin, A.** 1990b. Keeping a neutral cytoplasm; the bioenergetics of obligate acidophiles. FEMS Microbiol. Rev. **75:** 307-318.

85. **Matin, A.** 1992. in *Biochemical Engineering VII*, Genetics of bacterial stress response and its applications (New York Academy of Sciences). 1-15.

86. **McKay, A., J. Quilter, and C. W. Jones.** 1982. Energy conservation in the extreme thermophile *Thermus thermophilus* HB8. Arch. Microbiol. **131:** 43-50.

87. **Michels, M., and E. P. Bakker.** 1985. Generation of a large protonophore-sensitive proton motive force and pH difference in the acidophilic bacteria *Thermoplasma acidophilum* and *Bacillus acidocaldarius*. J. Bacteriol. **161:** 231-237.

88. **Miroshnichenko, M. L., E. A. Bonch-Osmolovskaya, A. Neuner, N. A. Kostrikina, M. A. Chernych, and V. A. Lekseev.** 1989. *Thermococcus stetteri* sp. nov., a new extremely thermophilic marine sulfur-metabolizing archaebacterium. System. Appl. Microbiol. **12:** 257-262.

89. **Mitchell, P.** 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev. **41:** 445-504.

90. **Moffat, A. S.** 1994. Microbial mining boosts the environment, bottom line. Science. **264:** 778-779.

91. **Moll, R., and G. Schäfer.** 1988. Chemiosmotic proton cycling across the plasma membrane of the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. FEBS Lett. **232:** 359-363.

92. **Muffler, A., M. Barth, C. Marschall, and R. Hengge-Aronis.** 1997. Heat shock regulation of $\sigma^s$ turnover: a role for DnaK and relationship between stress responses mediated by $\sigma^s$ and $\sigma^{32}$ in *Escherichia coli*. J. Bacteriol. **179:** 445-452.

93. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter.** 1990. in *Physiology of the Bacterial Cell: A Molecular Approach* (Sinauer Associates, Inc., Sunderland, Massachusetts).

94. **Neuner, A., H. W. Jannasch, S. Belkin, and K. O. Stetter.** 1990. *Thermococcus litoralis* sp nov.: a new species of extremely thermophilic marine archaebacteria. Arch. Microbiol. **153:** 205-207.

95. **Nicolaus, B., M. C. Manca, I. Romano, and L. Lama.** 1993. Production of an exopolysaccharide from two thermophilic archaea belonging to the genus *Sulfolobus*. FEMS Microbiol. Lett. **109:** 203-206.

96. **Norris, P. R., and J. P. Owen.** 1993. Mineral sulphide oxidation by enrichment cultures of novel thermoacidophilic bacteria. FEMS Microbiol. Rev. **11:** 51-56.

97. **O'Farrell, P. H.** 1975. High-resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:** 4007-4021.

98. **Okereke, A., and S. E. Steves Jr.** 1991. Kinetics of iron oxidation by *Thiobacillus ferrooxidans.* Appl Environ Microbiol. **57:** 1052-1056.

99. **Olson, G. J.** 1991. Rate of pyrite bioleaching by *Thiobacillus ferrooxidans* results of an interlaboratory comparison. Appl. Environ. Microbiol. **57:** 642-644.

100. **Oshima, T., H. Arakawa, and M. Baba.** 1977. Biochemical studies on an acidophilic thermophilic bacterium *Bacillus acidocaldarius*: isolation of bacteria, intracellular pH and stabilities of biopolymers. J. Biochem. **81:** 1107-1113.

101. **Peeples, T. L., S. Hirosue, V. Muralidharan, and R. M. Kelly.** 1991. Coal sulfur transformations monitored by hyperthermophilic archaebacteria. Fuel. **70:** 599-604.

102. **Peeples, T. L., and R. M. Kelly.** 1993. Bioenergetics of the metal/sulfur oxidizing extreme thermoacidophile, *Metallosphaera sedula*. Fuel. **72:** 1619-1624.

103. **Peeples, T. L., and R. M. Kelly.** 1995. Bioenergetic response of the extreme thermoacidophile *Metallosphaera sedula* to thermal and nutritional stress. Appl. Environ. Microbiol. **61:** 2314-2321.

104. **Pelham, H. R. B.** 1982. A regulatory upstream promoter element in the Drosophilla HSP70 heat-shock gene. Cell. **30:** 517-528.

105. **Phipps, B. M., A. Hoffmann, K. O. Stetter, and W. Baumeister.** 1991. A novel ATPase complex selectively accumulated upon heat shock is a major cellular component of thermophilic archaebacteria. EMBO J. **10:** 1711-1722.

106. **Pistorio, M., G. Curutchet, E. Donati, and P. Tedesco.** 1994. Direct zinc sulphide bioleaching by *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*. Biotech. Lett. **16:** 419-424.

107. **Pool, R.** 1990. Pushing the envelope of life. Science. **247:** 158-160.

108. **Quaite-Randall, E., J. D. Trent, R. Josephs, and A. Joachimiak.** 1995. Conformational cycle of the archaeosome, a TCP1-like chaperonin from *Sulfolobus shibatae*. J. Biol. Chem. **270:** 28818-28823.

109. **Rawlings, D. E., and T. Kusano.** 1994. Molecular genetics of *Thiobacillus ferrooxidans*. Microbiol. Rev. **58:** 39-55.

110. **Rawlings, D. E., and S. Silver.** 1995. Mining with microbes. Bio/Tech. **13:** 773-778.

111. **Rottenberg, H.** 1979. The measurement of membrane potential and pH in cells, organelles and vesicles. Methods Enzymol. **LV:** 547-569.

112. **Ruby, E. G., C. O. Wirsen, and H. W. Jannasch.** 1981. Chemolithotrophic sulfur-oxidizing bacteria from the Galapagos Rift hydrothermal vents. Appl. Environ. Microbiol. **42:** 317-324.

113. **Sanmugasunderam, V., R. M. R. Branion, and D. W. Duncan.** 1985. A growth model for the continuous culture microbiological leaching of zinc sulfide concentrate by *Thiobacillus ferrooxidans*. Biotechnol. Bioeng. **27:** 1173-1180.

114. **Schäfer, G., M. Lübben, and S. Anemüller.** 1990. Electron transport-phosphorylation and its catalysts in the archaebacterium *Sulfolobus acidocaldarius*. Biochim. Biophys. Acta. **1018:** 271-274.

115. **Schleper, C., G. Puehler, I. Holz, A. Gambacorta, D. Janekovic, U. Santarius, H. Klenk, and W. Zillig.** 1995. *Picrophilus* gen. nov., fam. nov.:

a novel aerobic, heterotrophic, thermoacidophilic genus and family comprising archaea capable of growth around pH 0. J. Bacteriol. **177:** 7050-7059.

116. **Schlesinger, M. J.** 1986. Heat shock proteins: the search for functions. J. Cell Biol. **103:** 321-325.
117. **Schlesinger, M. J.** 1990. Heat shock proteins. J. Biol. Chem. **265:** 12111-12114.
118. **Segerer, A., A. Neuner, J. K. Kristjansson, and K. O. Stetter.** 1986. *Acidianus infernus* gen. nov., sp. nov. and *Acidianus brierleyi* comb. nov.: facultatively aerobic, extremely acidophilic thermophilic sulfur-metabolizing archaebacteria. Int. J. Syst. Bacteriol. **36:** 559-564.
119. **Segerer, A., T. A. Langworthy, and K. O. Stetter.** 1988. *Thermoplasma acidophilum* and *Thermoplasma volcanium* sp. nov. from solfatara fields. System. Appl. Microbiol. **10:** 161-171.
120. **Shrihari, S. R. Bhavaraju, J. M. Modak, R. Kumar, and K. S. Gandhi.** 1993. Dissolution of sulphur particles by *Thiobacillus ferrooxidans*: substrate for unattached cells. Biotechnol. Bioeng. **41:** 612-616.
121. **Silver, L. M., K. C. Kleene, R. J. Distel, and N. B. Hecht.** 1987. Synthesis of mouse t complex proteins during haploid stages of spermatogenesis. Devl. Biol. **119:** 605-608.
122. **Silverman, M. P.** 1967. Mechanism of bacterial pyrite oxidation. J. Bacteriol. **94:** 1046-1051.
123. **Singer, P., and W. Stumm.** 1970. Acidic mine drainage: the rate-determining step. Science. **167:** 1121-1123.
124. **Sprott, G.** 1992. Structures of archaebacterial membrane lipids. J. Bioenerg. Biomem. **24:** 555-566.

125. **Stetter, K. O., Helmut, König, and E. Stackerbrandt.** 1983. *Pyrodictium* gen. nov., a new genus of submarine disc-shaped sulphur reducing archaebacteria growing optimally at 105ºC. System. Appl. Microbiol. **4:** 535-551.

126. **Stetter, K. O.** 1989. Order III. *Sulfolobales* ord. nov. In *Bergey's Manual of Systematic Bacteriology*. **3:** 2250-2253.

127. **Stotzky, G.** 1985. In *Bacterial adhesion*, Mechanisms of adhesion to clays, with reference to soil system. 195-245

128. **Sugio, T., Y. Tsujita, K. Inagaki, and T. Tano.** 1990. Reduction of cupric ions with elemental sulfur by *Thiobacillus ferrooxidans*. Appl. Environ. Microbiol. **56:** 693-696.

129. **Suzuki, and Isamu**. 1990. Ferrous iron and sulfur oxidation and ferric iron reduction activities of *Thiobacillus ferrooxidans* are affected by growth on ferrous iron, sulfur or a sulfide ore. Appl. Environ. Microbiol. **56:** 1620-1626.

130. **Svetlichny, V. A., T. G. Sokolova, M. Gerhardt, M. Ringpfeil, N. A. Dostrinkina, and G. A. Zavarzin.** 1991. *Carboxydothermus hydrogenoformans* gen nov., sp. nov., a CO-utilizing thermophilic anaerobic bacterium from hydrothermal environments of Kunashir Island. System. Appl. Microbiol. **14:** 254-260.

131. **Trent, J. D., J. Osipiuk, and T. Pinkau.** 1990. Acquired thermotolerance and heat shock in the extremely thermophilic archaebacterium *Sulfolobus* sp. Strain B12. J. Bacteriol. **172:** 1478-1484.

132. **Trent, J. D., E. Nimmesgern, J. S. Wall, F.-U. Hartl, and A. Horwich.** 1991. A molecular chaperone from a thermophilic archaebacterium is related to the eukaryotic protein t-complex polypeptide-1. Nature. **354:** 490-493Q.

133. **Trent, J. D., M. Gabrielsen, B. Jensen, J. Neuhard, and J. Olsen.** 1994. Acquired thermotolerance and heat shock proteins in thermophiles from the three phylogenetic domains. J. Bacteriol. **176:** 6148-6152.

134. **Vitaya, V. B., and K. Toda.** 1991a. Kinetics and mechanism of the adsorption of *Sulfolobus acidocaldarius* on coal surfaces. Biotechnol. Prog. **7:** 427-433.

135. **Vitaya, V. B., and K. Toda.** 1991b. Physiological adsorption of Sulfolobus *acidocaldarius* on coal surfaces. Appl. Microbiol. Biotechnol. **35:** 690-695.

136. **Vitaya, V. B., J. Koizumi, and K. Toda.** 1994. A kinetic assessment of substantial oxidation by *Sulfolobus acidocaldarius* in pyrite dissolution. J. Ferment. Bioeng. **77:** 528-534.

137. **Waldmann, T., E. Nimmesgern, M. Nitsch, J. Peters, G. Pfeifer, S. Müller, J. Kellermann, A. Engel, F.-U. Hartl, and W. Baumeister.** 1995. The thermosome of *Thermoplasma acidophilum* and its relationship to the eukaryotic chaperonin TRiC. Eur. J. Biochem. **227:** 848-856.

138. **Welsh, J., K. Chada, S. S. Dalal, R. Cheng, D. Ralph, and M. McClelland.** 1992. Arbitrarily primed PCR fingerprinting of RNA. Nucleic Acids Res. **20:** 4965-4970.

139. **Willison, K. R., D. Dudley, and J. Potter.** 1986. Molecular cloning and sequence analysis of a haploid expressed gene encoding t complex polypeptide 1. Cell. **44:** 727-738.

140. **Woese, C. R., R. Gupta, C. M. Hahn, W. Zillig, and J. Tu.** 1984. The phylogenetic relationships of three sulfur dependent archaebacteria. System. Appl. Microbiol. **5:** 97-105.

141. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:** 221-271.

142. **Woese, C. R., O. Kandler, and M. L. Wheelis.** 1990. Towards a natural system of organisms: proposal for the domains archaea, bacteria and eucarya. Proc. Natl. Acad. Sci. USA. **87:** 4576-4579.

143. **Woese, C. R.** 1993. The archaea: their history and significance. In *The Biochemistry of Archaea (Archaebacteria)*; 1st ed.; M. Kates et al., Ed.; Elsevier, Amsterdam. **26:** vii-xxix.

144. **Yeats, S., P. McWilliam, and W. Zillig.** 1982. A plasmid in the archaebacterium *Sulfolobus acidocaldarius*. EMBO J. **1:** 1035-1038.

145. **Yeh, T. Y., J. R. Godshalk, G. J. Olson, and R. M. Kelly.** 1987. Use of epifluorescence microscopy for characterizing the activity of *Thiobacillus ferrooxidans* on iron pyrite. Biotechnol. Bioeng. **30:** 138-146.

146. **Yura, T., Y. Kawasaki, N. Kusukawa, H. Nagai, C. Wada, and R. Yano.** 1990. Roles and regulation of the heat shock σ factor $\sigma^{32}$ in *Escherichia coli*. Ant. v. Leewen. **58:** 187-190.

147. **Zaritsky, A., M. Kihara, and R. Macnab.** 1981. Measurement of membrane potential in *Bacillus subtilis*: a comparison of lipophilic cations, rubidium ion and a cyanine dye as probes. J. Mem. Biology. **63:** 215-231.

148. **Zellner, G., E. Stackebrandt, H. Kneifel, P. Messner, U. B. Sleytr, E. C. D. Marcario, H.-P. Zabel, K. O. Stetter, and J. Winter.** 1989. Isolation and characterization of a thermophilic, sulfate reducing archaebacterium, *Archaeglobus fulgidus* Strain Z. System. Appl. Microbiol. **11:** 151-160.

149. **Zellner, G., and H. Kneifel.** 1993. Caldopentamine and caldohexamine in cells of *Thermotoga* species, a possible adaptation to the growth at high temperatures. Arch. Microbiol. **159:** 472-476.

150. **Zillig, W., K. O. Stetter, W. Wunderl, W. Schulz, H. Priess, and I. Scholz.** 1980. The *Sulfolobus*- *"Caldariella"* group: taxonomy on the basis of the structure of DNA-dependent and RNA-polymerases. Arch. Microbiol. **125:** 259-269.

151. **Zillig, W., K. O. Stetter, W. Schäfer, D. Janekovic, S. Wunderl, I. Holz, and P. Palm.** 1981. Thermoproteales: a novel type of extremely thermoacidophilic anaerobic archaebacterial isolated from Icelandic Solfataras. ZBl. Bakt. Hyg. **2:** 205-277.

152. **Zillig, W., K. O. Stetter, D. Prangishvilli, W. Schäfer, S. Wunderl, D. Janekovic, I. Holz, and P. Palm.** 1982. *Desulfurococcaceae,* the second family of the extremely thermophilic, anaerobic sulfur-respiring *Thermoproteales*. ZBl. Bakt. Hyg. **3:** 304-317.

153. **Zillig, W., S. Yeats, I. Holz, A. Böck, M. Rettenberger, F. Gropp, and G. Simon.** 1986. *Desulfurolobus ambivalens*, gen. nov., sp. nov., an autotrophic archaebacterium facultatively oxidizing or reducing sulfur. System. Appl. Microbiol. **8:** 197-203.

154. **Zillig, W., I. Holz, H.-P. Klenk, J. Trent, S. Wunderl, D. Janekovic, E. Imsel, and B. Haas.** 1987. *Pyrococcus woesei*, sp. nov., an ultra-thermophilic marine archaebacterium, representing a novel order, *Thermococcales*. System. Appl. Microbiol. **9:** 62-70.

155. **Zillig, W., I. Holz, D. Janekovic, H.-P. Klenk, E. Imsel, J. Trent, S. Wunderl, V. H. Forjaz, R. Coutinho, and T. Ferreira.** 1990. *Hyperthermus butylicus*, a hyperthermophilic sulfur-reducing archaebacterium that ferments peptides. J. Bacteriol. **172:** 3959-3965.

**Table 1.1. Differentiating Characteristics of the Three Domains of Life (adapted from Kelly *et al.* 1994)**

| Characteristic | Bacteria | Archaea | Eucarya |
|---|---|---|---|
| Hyperthermophilic species | + | + | - |
| Murein cell wall | + | - | - |
| Ester-linked membrane lipids | + | - | + |
| Ether-linked membrane lipids | - | + | - |
| Polyunsaturated fatty acids | -(+) | - | + |
| 70S Ribosomes | + | + | -(+) |
| DNA positively super-coiled in hyperthermophiles | + | + | - |
| DNA introns | - | + | + |
| Tungsten enzymes | -(+) | + | - |
| $CO_2$ fixation via Calvin-Benson pathway | + | -(+) | + |
| Photosynthesis | + | -(+) | + |
| Salt tolerance >3.5-4.5 NaCl | - | + | - |

**Table 1.2. Metabolic Characteristics of Extreme Thermoacidophiles**

**Order *Sulfolobales***

| organism | ref | pH {pHopt} | T (°C) {Topt} | growth mode | Doubling time | Habitat | energetics |
|---|---|---|---|---|---|---|---|
| *Acidianus brierleyi* DSM 1651 | [1] [2] [3] | 1.0-6.0 {1.5-2.0} | 65-90 {70} | $O_2$ -, A $O_2$+, M | 7.8hr | acidic solfatara hydrothermal sea water thermal spring drainage Yellowstone Piscarelli, Italy | anaerobic respiration: $S^\circ$, $H_2$ -> $H_2S$ aerobic respiration: metal sulfides, $S^\circ$, $Fe^{2+}$, $O_2$, organics -> $H_2SO_4$, $CO_2$ |
| *Acidianus infernus* DSM 3191 | [1] [3] | 1.0-5.0 {2} | 65-90 {90} | f $O_2$ + A | 2hr | solfatara crater | anaerobic respiration: $S^\circ$, $H_2$ -> $H_2S$ aerobic respiration: metal sulfides, $S^\circ$, $Fe^{2+}$, $O_2$, organics -> $H_2SO_4$, $CO_2$ |
| *Desulfurolobus ambivalens* DSM 3772 | [4] | 2.5- 3.5 | 70-87 {80} | f $O_2$ - f A | 4hr | solfatara hot springs | fermentation; $S^\circ$, $O_2$--> $H_2SO_4$ sulfur reduction: $S^\circ$, $H_2$--> $H_2S$ |

**Table 1.2 continued**

| organism | ref | pH {pHopt} | T (°C) {Topt} | growth mode | Doubling time | Habitat | energetics |
|---|---|---|---|---|---|---|---|
| *Desulfurolobus ambivalens* DSM 3772 | [4] | 2.5- 3.5 | 70-87 {80} | f $O_2$ - f A | 4hr | solfatara hot springs | fermentation; S°, $O_2$--> $H_2SO_4$ sulfur reduction: S°, $H_2$--> $H_2S$ |
| *Metallosphaera sedula* DSM 5348 | [5] [2] [3] | 1.0-4.5 {1.7} | 50-79 {75} | $O_2$ + A (M) | 5.6hr | solfatara | aerobic respiration: metal sulfides, S°, $Fe^{2+}$ ,$O_2$, organics -> $H_2SO_4$ , $CO_2$ |
| *Metallosphaera prunae* DSM 10039 | [9] | 1.0-4.5 {1.7} | 55-80 {75} | $O_2$ + A (M) | 3.5hr | uranium mine | aerobic respiration: metal sulfides, S°, $Fe^{2+}$ ,$O_2$, organics -> $H_2SO_4$ , $CO_2$ |

**Table 1.2 continued**

| organism | ref | pH {pHopt} | T (°C) {Topt} | growth mode | Doubling time | Habitat | energetics |
|---|---|---|---|---|---|---|---|
| *Sulfolobus acidocaldarius* DSM 639 | [5] [2] [3] | 0.9-5.8 {2.0} | 55-90 {83} | $O_2$ + f A (M) | 6.5 hr | solfatara acid springs | aerobic respiration: metal sulfides, $S^\circ$, $Fe^{2+}$, $O_2$, organics -> $H_2SO_4$, $CO_2$ |
| *Sulfolobus solfataricus* DSM 1616 | [6] | 3.5-5 {4.5} | 50-88 {87} | $O_2$ + f A (M) | 11 hr | hot acidic volcanic springs | aerobic respiration: $S^\circ$, yeast extract --> $H_2SO_4$ |
| *Sulfolobus shibatae* DSM 5389 | [7] | 3-4 {3} | 75-86 {81} | $O_2$ + f A (M) | ? 15 hr | acidic geothermal springs | aerobic respiration: $S^\circ$, yeast extract --> $H_2SO_4$ |
| *Sulfolobus metallicus* DSM 6482 | [8] | 1-4.5 | 50-75 {65} | $O_2$ + A | 8 hr | solfataric fields | aerobic respiration: metal sulfides, $S^\circ$, $Fe^{2+}$, $O_2$, --> $H_2SO_4$ |

**Table 1.2 continued**

**Order *Thermoplasmales***

| organism | ref | pH {pHopt} | T (°C) {Topt} | growth mode | Doubling time | Habitat | energetics |
|---|---|---|---|---|---|---|---|
| *Thermoplasma acidophilum* DSM 1728 | [10] | 0.5-4 {1-2} | 45-63 {59} | f $O_2$ - H | 5.5hr | solfataric fields, coal refuse | aerobic respiration: anaerobic respiration: $S^{\circ}$ , $H_2$ -> $H_2S$ |
| *Thermoplasma volcanium* DSM 4300 | 10] | 1-4 {2} | 33-67 {60} | f $O_2$ - H | 5hr | solfataric fields | aerobic respiration: anaerobic respiration: $S^{\circ}$ , $H_2$ -> $H_2S$ |
| *Picrophilus oshimae* DSM 9789 | [11] | 0-2.2 {0.7} | 45-65 {60} | $O_2$ + H | 6hr | hot springs | aerobic respiration: organics -> $H_2SO_4$ , $CO_2$ |
| *Picrophilus torridus* DSM 9790 | [11] | 0-2.2 {0.7} | 45-65 {60} | $O_2$ + H | 6hr | hot soil | aerobic respiration: organics -> $H_2SO_4$ , $CO_2$ |

**Table 1.2 continued**

TABLE LEGEND

| Growth Mode | | | |
|---|---|---|---|
| $O_2$ + | aerobe | H | heterotroph |
| f $O_2$ + | facultative aerobe | (M) | mixotroph |
| o $O_2$ + | obligate aerobe | A | autotroph |
| $O_2$ - | anaerobe | f A | facultative autotroph |
| o $O_2$ - | obligate anaerobe | | |

| References | |
|---|---|
| 1). A. Segerer, et al., 1986 | 7) D. Grogan, et al., 1990 |
| 2). T. L. Peeples and R. M. Kelly, 1993 | 8) G. Huber and K. O. Stetter, 1991 |
| 3). G. Huber, et al., 1992 | 9) T. Fuchs, et al., 1995 |
| 4). W. Zillig, et al., 1986 | 10) A. Segerer, et al., 1988 |
| 5). G. Huber, et al., 1989 | 11) C. Schleper, et al., 1995 |
| 6) W. Zillig, et al., 1980 | |

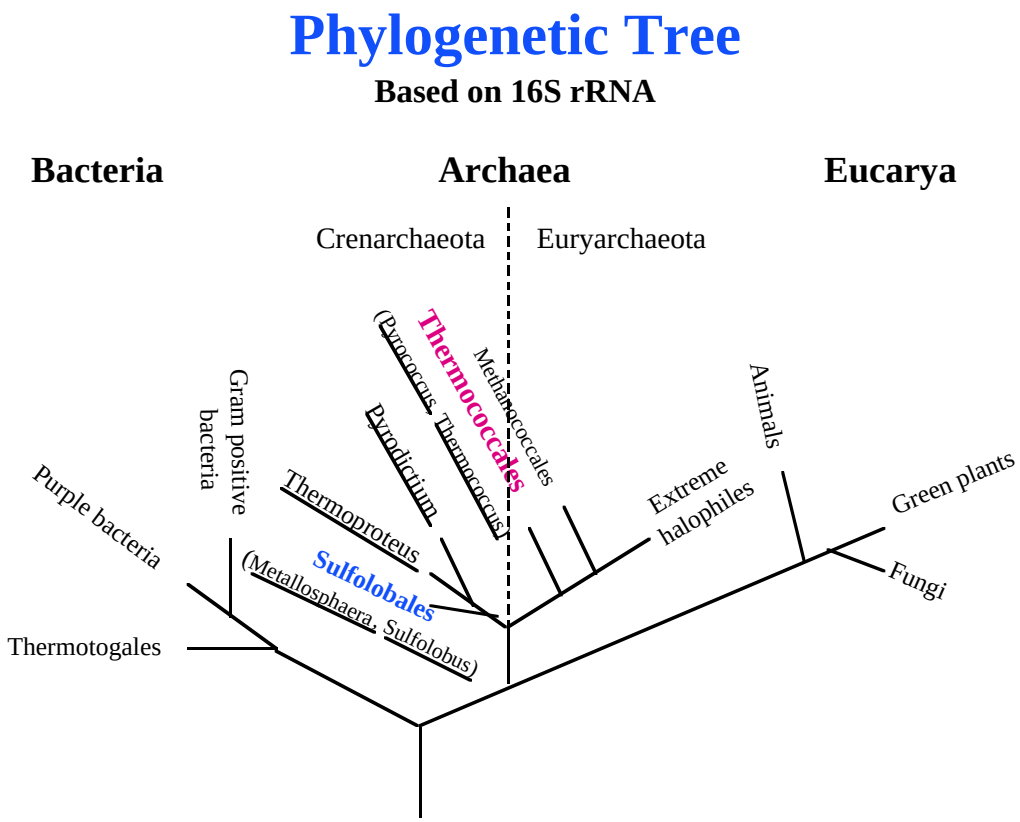



**Figure 1.1. Phylogenetic tree based on 16S rRNA (Woese, 1993)**

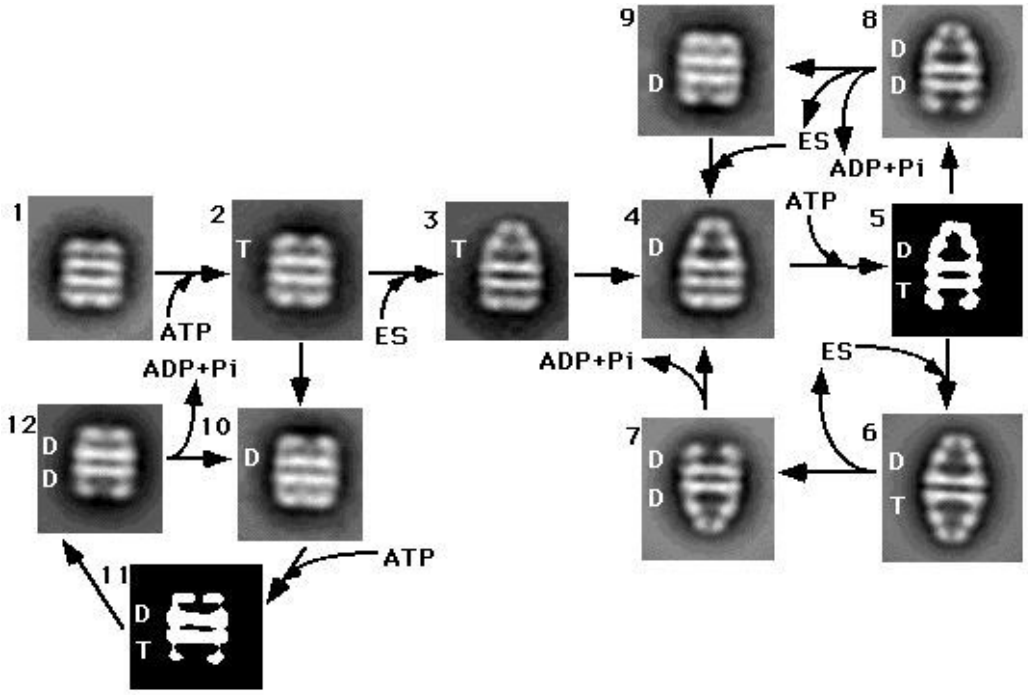



**Figure 1.2. Conformational changes in the GroEL oligomer during the functional cycle.** A hypothetical model for the GroEL-GroES cycle in the absence and presence of GroES (Lorca *et al*., 1997).

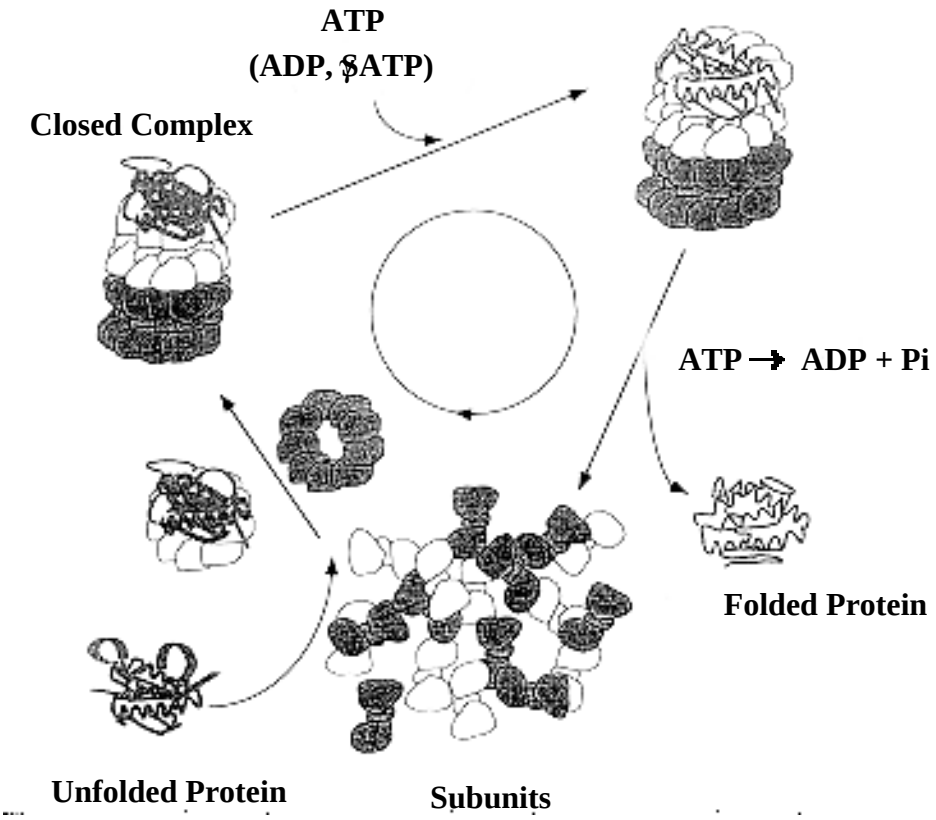



**Figure 1.3. Proposed conformational cycle of archaeosome.** This model depicts the hypothetical conformational cycle and protein binding of the *Sulfolobus shibatae* chaperonin (archaeosome) during thermotolerance and protein folding (Quaite-Randall *et al*., 1995).

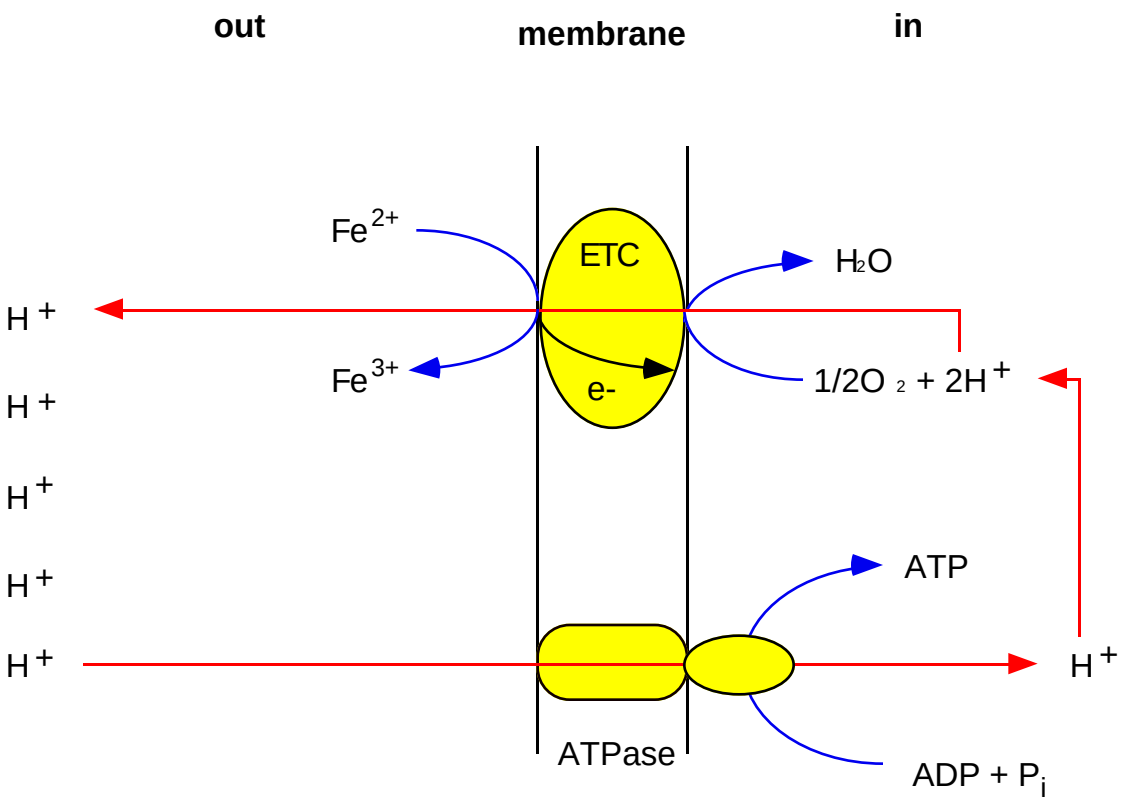



**Figure 1.4. Schematic of *Metallosphaera sedula* network:** This metabolic network is based on existing knowledge of *M. sedula*'s nutritional requirements as well as a theoretical chemiosmotic framework.

# Chapter 2

## Acquired Thermotolerance and Stressed-Phase Growth of the Extremely Thermoacidophilic Archaeon *Metallosphaera sedula* in Continuous Culture

Chae J. Han, Sea H. Park*, and Robert M. Kelly

(* undergraduate who assisted the maintenance of cont. culture)

Portions of this chapter were published

in *Applied and Environmental Microbiology* **63:** 2391-2396, 1997

### Abstract

The response of an extremely thermoacidophilic archaeon *Metallosphaera sedula* (growth temperature range 50-79°C, $T_{opt}$ 74°C and $pH_{opt}$ 2.0) to thermal stress was investigated using a 10-liter continuous cultivation system. *M. sedula*, growing at 74°C, pH 2.0, and dilution rate of 0.04 $hr^{-1}$, was subjected to both abrupt and gradual temperature shifts in continuous culture to determine the response of cell density levels and protein synthesis patterns. An abrupt temperature shift from 74°C to 79°C resulted in little, if any, changes in cell density and a small increase in total protein/cell. When the culture temperature was shifted further to 80.5°C, cell density dropped to below $5x10^6$ cells/ml from $2x10^8$ cells/ml, leading to washout of the culture. Operation at this temperature and slightly higher temperatures, however, could be achieved by exposing the culture to thermal stress more gradually (0.5°C increments). As a result, stable operation could be maintained at temperatures up to 81°C, and the washout temperature could be increased to 82.5°C. Continuous culture operation at 81°C for 100 hours

("stressed phase") led to an approximately 7-fold lower steady state cell density than observed for operation at or below 79°C. However, SDS-PAGE analysis (both one- and two-dimensional) revealed significantly higher levels (6-fold increase) of a 66 kDa stress response protein (MseHSP60), immunologically related to Thermophilic Factor 55 from *Sulfolobus shibatae* (Trent, J. D., J. Osipiuk, and T. Pinkau. 1990. *J. Bacteriol.* **172**:1478-1484). If the acclimated culture was returned to lower temperatures (i.e., 74°C), the amount of MseHSP60 returned to levels observed prior to thermal acclimation. Furthermore, when the previously acclimated culture (at 81°C) was shifted back from 74°C to 81°C, without going through gradual acclimation steps, the result was the immediate onset of washout, suggesting no residual thermotolerance. This study shows that gradual thermal acclimation of *M. sedula* could only extend the temperature range of stable growth for this organism by 2°C above its maximal growth temperature, albeit at reduced cell densities. Also, this investigation illustrates the utility of continuous culture for characterizing heat shock response and assessing maximum growth temperatures for extremely thermophilic microorganisms.

## Introduction

As the thermal environment for a particular microorganism moves to higher temperatures, cellular function is eventually compromised to such an extent that growth ceases, despite the stabilizing influences that intrinsic and extrinsic factors might provide for individual biomolecules. The thermal history of each microorganism can affect its upper temperature limit for growth, which may be elevated by thermal acclimation to supraoptimal temperatures (3). Cellular response to supraoptimal temperatures seems to have certain universal features independent of optimal growth temperature; namely,

increased synthesis of one or more heat shock proteins thought to be involved in protein turnover and prevention of protein aggregation and denaturation *in vivo* (4,24,25).

The nature of heat shock response by extremely thermophilic microorganisms is intriguing, considering the fact that they thrive at temperatures far above the limits for most cells (5,25). In some organisms, stress induces elevated levels of certain intracellular compatible solutes (2,7,15,28), some of which have been shown to provide thermal stabilization to proteins *in vitro* (21). Evidence has also been provided that thermal stress induces synthesis of specific proteins. The hyperthermophilic archaeon ES4, growing optimally at 98-100°C (20), was found to produce increased levels of a 98 kDa protein when the growth temperature was shifted from 95°C to 102°C (8). Furthermore, some degree of thermotolerance could be conferred for survival of ES4 at 105°C by first exposing 95°C grown cells to 102°C for periods of 90 minutes. Another hyperthermophile, *Pyrodictium occultum*, produced increased levels of an ATPase complex (i.e., thermosome) when culture temperatures were shifted from 102°C to 108°C (18,19). This complex, comprised of 56 and 59 kDa polypeptides, was a significant fraction of total cellular protein at 108°C (73%) but a much smaller fraction at 100°C (11%). A similar ATPase complex has been identified in *Thermoplasma acidophilum* (26).

Most work to date on heat shock and thermal (acclimation) in thermophilic microorganisms has focused on members of the order Sulfolobales in the kingdom *Crenarchaeota*. Many of these chemolithotrophic organisms grow aerobically and typically have optimal ($T_{opt}$) and maximal ($T_{max}$) growth temperatures below 100°C. It appears that, unlike many other mesophilic organisms and cells, there is only one prominent heat shock protein noted in the Sulfolobales examined to date, which has a subunit molecular weight of approximately 60 kDa (25). Several Sulfolobales have been studied. Evidence of a heat shock response in *Sulfolobus acidocaldarius* was first reported by Jerez (10), who

observed significantly increased levels of a protein with molecular weight of 64-66 kDa for cells shifted from 70°C to 85°C. Trent et al. (22) showed that *Sulfolobus shibatae* strain B12 exhibited a heat shock response when the cells were shifted to 88°C, accompanied by the increased synthesis of predominant 55 kDa protein (Thermophilic Factor 55 or TF55). *S. shibatae* was found to acquire some thermotolerance at 92°C by first incubating cells at 88°C for 60 minutes or longer. This major heat shock protein in *S. shibatae* was shown to be unrelated to DnaK or GroEL, but similar to a family of eukaryotic protein, known as t-complex polypeptide-1(TCP1) (23), whose role as a molecular chaperone has not been established. Peeples and Kelly (17) showed the *Metallosphaera sedula*, which grows optimally at 74°C and pH 2.0 (9), produced increased amounts of a protein immunologically related to TF55 during temperature shift from 73 to 79°C. Shifts from 70 to 80°C were accompanied by significant reductions in cell density (from $10^7$ to $10^6$ cells/ml) and intracellular pH (from 5.5 to 3.3). Full recovery of the culture could be accomplished if the cells were shifted back to 70 from 80°C within 13 hours after heat shock in batch cultures.

Kagawa et al. (11) found that TF55 was actually composed of two subunits forming two homo-oligomeric rings joined in a structure called a rosettasome, and referred to this complex as the archaeal HSP60 (designated here as SshHSP60). The α- and β-subunits (MW of 59.72 kDa and 59.68 kDa, respectively) were found to be approximately 55% identical at the amino acid level. Western blot analysis using antibodies against these subunits revealed that the SshHSP60 heat shock protein produced by *S. shibatae* was similar to those found in other members of the *Crenarchaeota*, although not evident in the *Euryarchaeota*. A similar version of SshHSP60 was found in *Sulfolobus solfataricus* (12) and was shown to function as a molecular chaperonin *in vitro* (6).

Given that members of the Sulfolobales appear to contain a single, prominent heat shock protein, they represent an interesting model with which the regulation of thermal stress response can be studied. These organisms are mostly thermoacidophilic, typically growing at high temperatures and low pH. Because contamination is alleviated to a great extent under these extreme growth conditions, Peeples and Kelly (17) showed that a particular Sulfolobale, *Metallosphaera sedula*, can be maintained in continuous culture for extended periods of time. The study of heat shock response and thermotolerance in continuous culture could be advantageous since complications arising from transients in growth phase and rates can be eliminated. The work reported here examines *M. sedula* response to thermal stress and addresses the growth physiology of this organism at supraoptimal temperatures with and without prior thermal acclimation. At the same time it illustrates the utility of continuous culture in studying the stress response of thermophilic organisms.

## Materials and Methods

**Cell cultivation.** *Metallosphaera sedula* (DSMZ 5348) was obtained from the Deutsch Sammlung von Microorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) and cultivated using a slightly modified method from that previously described (17). A 10-liter glass chemostat (Ace Glass, Vineland, NJ) was used for thermal stress response studies. The continuous culture was agitated at 150 rpm with an impeller and aerated by sterile (0.2 μm filter) house air at a rate of 100 cc/min. Residence time distribution studies showed that reactor approximated a continuous stirred tank reactor which behaved as a first order system from a mixing standpoint. A Digi-Sense temperature controller (Cole-Parmer, Chicago, IL), connected to a Teflon-coated probe, was used to monitor and adjust temperature of the culture. Temperature control was typically ± 0.2°C,

as confirmed periodically with a mercury glass thermometer inserted into the culture. A dilution rate of 0.04 $hr^{-1}$ (corresponding to a 17 hour doubling time) was used to minimize chances of premature washout when thermal stress was applied to the culture. Samples were taken regularly and fixed with glutaraldehyde to monitor cell density using epifluorescence microscopy (27).

*Sulfolobus shibatae* (DSMZ 5389) was obtained from the DSMZ (Braunschweig, Germany) and cultivated using the culture media compositions provided from DSMZ. *S. shibatae* was grown in batch cultures at 75°C with gentle agitation to late log phase to generate biomass for gel analysis and immunoblotting.

**Preparation of cell extracts.** Cells were centrifuged at 7500 rpm (9500 g) in a Sorvall RC5C centrifuge (DuPont, Newtown, CT) for 40 min. Supernatant was discarded and pellets were washed at least twice with 10 mM $H_2SO_4$ at pH 2. To lyse the cells, the pellets were re-suspended in 100 mM sodium phosphate buffer (pH 7.5) and then disrupted using a sonicator (Model XL2020, Heat systems Inc., Farmingdale, NY) and/or a French Pressure Cell Press (SLM Instrument Inc., Urbana, IL) at 16,000 psig. Lysates were centrifuged again at 4000 rpm for 30 min. Soluble cell extracts were stored at -20°C for gel electrophoresis analysis (SDS-PAGE) and immunoblotting. Total protein concentration was determined with a dye-staining method (1) with BSA as a standard (Bio-Rad Laboratories, Richmond, CA).

**One-dimensional SDS-PAGE.** A modified Laemmli method (13) was used to separate *M. sedula* cell extracts on SDS-PAGE. Cell extracts were incubated at 100°C in 5X dissociation buffer, containing 400 mM Tris pH 6.8, 10% SDS and 400 mM dithiothreitol, for 10 min prior to loading on 10% or 12.5% discontinuous SDS-PAGE.

For one-dimensional SDS-PAGE, equal amounts of total protein (typically 40 µg) were loaded in each lane. Gels were stained with Coomassie blue. The following pre-stained molecular weight markers (Gibco-BRL, Gaithersburg, MD) were used for protein size references: lysozyme (14,300), ß-lactoglobulin (18,200), carbonic anhydrase (27,830), ovalbumin (43,760), bovine serum albumin (71,460), phosphorylase B (110,760), and myosin (216,760). Densitometry was performed using an Eagle Eye II system (Stratagene, La Jolla, CA).

**Two-dimensional gel electrophoresis.** Two-dimensional gel was performed according to the method of O'Farrell (16) by Kendrick Labs, Inc. (Madison, WI) as follows: Isoelectric focusing was carried out in glass tubes of inner diameter 2.0 mm using 2.0% pH 4-8 ampholines (BDH from Hoefer Scientific Instruments, San Francisco, CA) for 9,600 volt-hrs. 40 ng of an IEF internal standard, tropomyosin protein, with lower reference point at MW 33,000 and pI 5.2, were added to the samples. The following proteins (Sigma Chemical Co., St. Louis, MO) were added as molecular weight standards: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000) and lysozyme (14,000). Gel quantification was performed by Kendrick's Phoretix 2D software.

**Immunoblotting.** Gels were transferred to nitrocellulose for Western blot analysis. Proteins were probed with antibodies directed against SshHSP60 (provided by J. D. Trent, Argonne National Laboratories) in 1% gelatin. The secondary antibody was a rabbit Ig, horseradish peroxidase conjugate (Amersham, Little Chalfont, Buckinghamshire, England). Detection was performed by utilizing the ECL Western blotting kit (Amersham, Little Chalfont, Buckinghamshire, England).

**Thermal acclimation experiments.** To study thermotolerance and heat shock response in *M. sedula*, the continuous culture was subjected to various degrees of long-term thermal stress. The continuous culture was shifted abruptly (2°C increment) or gradually (0.5°C increments), after the culture stabilized at a particular temperature. Dilution rate of culture was 0.04 $hr^{-1}$. Cells were harvested from continuous culture after the new steady state was achieved, typically after 3 to 4 reactor volume changes which corresponds to 95% and 98% approaches to steady state, respectively, based on mixing characteristics.

## Results

**Response of *M. sedula* to thermal stress in continuous culture.** Most studies focusing on the response of cells and organisms to thermal stress are done in batch culture in which abrupt temperature shifts are applied. Even experiments in which some degree of thermal acclimation is sought, sudden temperature shifts to batch cultures are typically used. Here, continuous culture was used to eliminate problems in interpreting results that may arise from transient growth conditions and to permit flexibility in adjusting the time course of temperature shifts.

*M. sedula* has a reported growth temperature optimum ($T_{opt}$) of 74°C-75°C, with a maximum temperature for growth ($T_{max}$) of 79°C. In 10 liter (working volume) continuous culture at pH 2.0 and dilution rate 0.04 $hr^{-1}$, *M. sedula* was subjected to series of temperature shifts within the normal growth range as well as above it. In the first series of experiments (see Figure 2.1), the culture temperature was shifted from 73°C -> 76°C -> 79°C over a period of 220 hours, with no significant change in cell density. After holding the culture at 79°C for over 100 hours (4 reactor volume changes), the temperature was shifted to 80.5°C, resulting in a dramatic reduction in culture cell density from $10^8$ cells/ml

to less than 5 x $10^6$ cells/ml, indicating the onset of washout. At this point, the temperature was shifted back to 74°C, resulting in the complete recovery of the culture cell density to over $10^8$ cells/ml. The recovery rate after the shift of the cells at 80.5°C to 74°C, computed from the increase in cell density from 280 to 350 hours, corresponded to a doubling time of approximately 17 hours; this is equivalent to the dilution rate. It was apparent from this experiment, that an abrupt temperature shift upwards of only 1.5°C at 79°C was sufficient to cause the culture to washout, even at this relatively low dilution rate.

Having determined the sensitivity of the culture to temperature changes around the reported $T_{max}$, operation of the culture at and above this temperature was examined, as shown in Figure 2.2. Here, the culture was brought to 79°C from 74°C and then to 80°C and then held at 80°C for 125 hours. Unlike the first experiment (in which the shift was from 79°C to 80.5°C, as shown in Figure 2.1), the shift from 79°C to 80°C resulted in little, if any, change in culture cell density. The culture was then shifted in increments of 0.5°C. Upon shifting to 80.5°C from 80°C, no immediate drop in cell density was noted, although a further shift to 81°C caused the cell density to drop to approximately 2 x $10^7$ cells/ml. Subsequent 0.5°C shifts upward in temperature (81°C -> 81.5°C -> 82°C) resulted in further decreases in cell density until evidence for the onset of culture washout at 82.5°C was noted. At this point, the culture could be stabilized by decreasing the temperature to 81°C, where a steady state operation could be maintained at a cell density of 2 x $10^7$ cells/ml. By dropping the temperature back to near $T_{opt}$ (74°C), the culture cell density gradually recovered to the same levels (~$10^8$ cells/ml) observed prior to imposition of thermal stress.

The significance of thermal acclimation for *M. sedula* can be seen from the results of the experiment shown in Figure 2.3. If the cells were first acclimated at 79-80°C, the continuous culture could be operated at 81°C in steady state, albeit at a reduced cell

density. If the culture operating at 81°C was shifted back to 74°C, the cell density returned to the levels observed previously at 74°C prior to thermal acclimation. A similar trend was also observed in amounts of total protein per cell. Figure 2.4 shows when the culture temperature was shifted back to 74°C, the protein/cell level returned to the one observed prior to 81°C exposure. It also shows a slight increase in the protein/cell level between 74 and 80°C as temperature increases and 5 to 6-fold increase in protein/cell level during thermal stress at 81°C. However, if the culture at 74°C was then shifted abruptly (without first gradual exposure to 79-80°C), cell density plummeted immediately to less than $8x10^6$ cells/ml and the onset of culture washout was observed. These results suggest that the culture that had been acclimated to a supraoptimal growth temperature passed along no lasting capacity to withstand an abrupt temperature shift, when it returned to normal growth temperatures.

**Characterization of thermal stress response of *M. sedula* in continuous culture.** Studies of other cells and microorganisms exposed to thermal stress have revealed changes in metabolic patterns in addition to increases and decreases in the synthesis or expression of specific proteins (4, 24,25). It was shown previously that *M. sedula* produced increased amounts of an approximately 60 kDa heat shock protein (designated MseHSP60), which cross reacted with antibodies generated against a molecular chaperone/heat shock protein (SshHSP60) identified in *S. shibatae* (17). Changes in the level of MseHSP60 in *M. sedula* was indicative of the organism's response to thermal stress.

Samples taken from the continuous culture experiment shown in Figure 2.3 were examined by one-dimensional and two-dimensional gel electrophoresis in addition to Western blot analysis. Figure 2.5 shows one-dimensional SDS-PAGE gel at 74°C (lane

2), 79°C (lane 3), 80°C (lane 4), 81°C (lane 5), and 74°C (lane 6). It is clear that the band corresponding to an approximately 66 kDa protein (MseHSP60) intensifies as the temperature increases from 74°C to 81°C. As the culture is returned to 74°C, the intensity of this band returns to the levels comparable to that observed initially at 74°C. Using densitometry on the SDS-PAGE in Figure 2.5a, a 3-fold increase in the integrated density value of these 66 kDa bands were measured for the 81°C compared to the 74°C, both before and after thermal stress (data not shown) . Figure 2.5b confirms this trend in heat shock protein levels by Western blot analysis of the same samples using SshHSP60 antibodies.

To quantify changes in MseHSP60 more accurately under different thermal conditions, two-dimensional electrophoresis was used to compare samples taken from 74°C (before heat shock) and 81°C (stressed phase) continuous cultures (Figure 2.6). Note that smaller amounts of the thermally stressed sample (81°C) was loaded (approximately one-third the amount of the 74°C sample) to resolve highly expressed 66 kDa MseHSP60. Table 2.1 shows the intensities of spots associated with prominent 66 kDa polypeptides in the stressed (81°C) and unstressed (74°C) cases. An approximately 6-fold overall increase in MseHSP60 isoforms could be observed in samples from 81°C compared to 74°C. In particular, the volume intensity of one isoform (pI 5.8, MW 668,989) was 73-fold higher in 81°C compared to 74°C. It was also noted that no other prominent polypeptide spot was observed in the thermally stressed sample relative to the unstressed case.

## Discussion

The work described here illustrates the sensitivity of the extremely thermoacidophilic archaeon *M. sedula* to the shifts in growth temperature at or near the upper growth temperature limit. Thermotolerance is usually defined as a transient resistance to lethal

thermal treatment which can be influenced by prior exposure to sub-lethal heat treatment (14). The temperature of the sub-lethal level and length of exposure to this temperature are apparently important in imparting thermotolerance (23). Continuous culture provides a mechanism to adjust these two parameters in a systematic way, as demonstrated here. The use of continuous culture also provides some clear advantages over batch culture approaches for studying thermotolerance and thermal stress as eliminates the complications arising transients in growth phase. It also can be used to select for a culture that manages to grow, and not just to survive, under stressed conditions. By comparing the characteristics of continuous cultures under thermal stress prior to and during washout, insights into the physiological basis for growth in relation to survival may be gained.

Gradual thermal acclimation of the continuous culture of *M. sedula* led to the establishment of a "stressed phase" at 81°C, which could be maintained in steady state for extended periods of time. The culture is particularly vulnerable at this temperature and dilution rate, as even an increase of 0.5°C led to the onset of washout. This stressed phase was characterized by a significant reduction in cell density (~7-fold), in addition to the increased synthesis of a heat shock protein (MseHSP60). This single 66 kDa molecular species appears to be the only prominent heat shock protein in *M. sedula*. This is consistent with other reports focusing on heat shock response in the thermophilic archaea which show that only single major heat shock protein is typically produced (5, 25). Table 2.1 shows the ratio of MseHSP60 isoforms appearing on two-dimensional electrophoresis at 74$^{o}$C and 81$^{o}$C, confirming significant increase in the MseHSP60 level during the thermal stress. It also appears here that the increased level of MseHSP60 is positively related to acquired thermotolerance.

The reasons for the reduced cell density in the "stressed phase" at 81°C is not clear. Peeples and Kelly (17) showed that *M. sedula,* when shifted to temperatures above 80°C

for any period of time, appears to lose its capability to maintain internal pH at the levels measured during growth at optimal temperatures. (Presumably, this is the result of damaging membrane integrity and/or functional problems for particular proteins at non-optimal temperatures and pH. In this sense, thermoacidophiles may be more limited in developing thermotolerance because of the potential problem of proton leakage from an external environment at pH 2.0. Efforts are underway to determine if this is the case. There also appears to be no hysteresis involving the development of thermotolerance, at least for the conditions examined in this study. Figure 2.3 shows that cells returned to 74°C after having grown at 81°C for extended periods are equally sensitive to an abrupt temperature shift to 81°C as cultures that had not been previously acclimated. From this perspective, it appears that thermotolerance is a transient characteristic which is inducible but not permanent

Despite slow, gradual exposure to elevated temperatures, the maximum temperature for stable growth of *M. sedula* could only be extended by approximately 2°C, from 79°C to 81°C. Reported attempts to impart thermotolerance to *S. shibatae* (22) and to *Pyrococcus* strain ES4 (8) were evaluated by the length of survival time at temperatures far in excess of the normal growth temperature. In essence, these experiments focus on the transition from stationary to death phase. In neither case did the cultures survive at supramaximal temperatures for significant periods of time and no net increase or even stabilization in levels of cell density or cell protein was observed. It remains to be seen how approaches such as the one used here will influence the level of thermotolerance of other high temperature organisms and whether long-term acclimation makes any difference compared to short-term exposure to excessive temperatures in this regard. It also raises the question whether the maximal temperature for growth of other extremely thermophilic organisms can be extended by more than a just a few degrees by thermal acclimation.

## Literature Cited


1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. **72:** 248-254.
2. **Ciulla, R., C. Clougherty, N. Belay, S. Krishnan, C. Zhou, D. Byrd, and M. F. Roberts.** 1994. Halotolerance of *Methanobacterium thermoautotrophicum* H and Marburg. J. Bacteriol. **176:** 3177-3187.
3. **Coleman, J. S., S. A. Heckathorn, and R. L. Hallberg.** 1995. Heat shock proteins and thermotolerance: linking molecular and ecological perspectives. Trends Ecol. Evol. **10:** 305-306.
4. **Craig, E. A., B. D. Gambill, and R. J. Nelson.** 1993. Heat shock proteins: molecular chaperones of protein biogenesis. Microbiol. Rev. **57:** 402-414.
5. **de Macario, E. C., and A. J. L. Macario.** 1994. Heat-shock response in archaea. Trends Biotechnol. **12:** 512-518.
6. **Guagliardi, A., L. Cerchia, and M. Rossi.** 1995. Prevention of *in vitro* protein thermal aggregation by the *Sulfolobus solfataricus* chaperonin. J. Biol. Chem. **270:** 28126-28132.
7. **Hensel, R., and H. Konig.** 1988. Thermoadaptation of methanogenic bacteria by intracellular ion concentration. FEMS Microbiol. Lett. **49:** 75-79.
8. **Holden, J. F., and J. A. Baross.** 1993. Enhanced thermotolerance and temperature-induced changes in protein composition in the hyperthermophilic archaeon ES4. J. Bacteriol. **175:** 2839-2843.

9. **Huber, G., C. Spinnler, A. Gambacorta, and K. O. Stetter.** 1989. *Metallosphaera sedula* gen. and sp. nov. represents a new genus of aerobic, metal-mobilizing, thermophilic archaebacteria. System. Appl. Microbiol. **12:** 38-47.

10. **Jerez, C. A.** 1988. The heat shock response in mesophilic and thermoacidophilic chemolithotrophic bacteria. FEMS Microbiol. Lett. **56:** 289-294.

11. **Kagawa, H. K., J. Osipiuk, N. Maltsev, R. Overbeek, E. Quaite-Randall, A. Joachimiak, and J. D. Trent. 1995.** The 60 kDa heat shock proteins in the hyperthermophilic archaeon *Sulfolobus shibatae*. J. Mol. Biol. **253:** 712-725.

12. **Knapp, S., I. Schmidt-Krey, H. Hebert, T. Bergman, H. Jornvall, and R. Ladenstein.** 1994. The molecular chaperonin TF55 from the thermophilic archaeon *Sulfolobus solfataricus*. J. Mol. Biol. **242:** 397-407.

13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680-685.

14. **Laszlo, A.** 1988. The relationship of heat-shock proteins, thermotolerance, and protein synthesis. Exp. Cell Res. **178:** 401-414.

15. **Martins, L. O., and H. Santos.** 1995. Accumulation of mannosyl glycerate and di-myo-inositol phosphate by *Pyrococcus furiosus* in response to salinity and temperature. Appl. Environ. Microbiol. **61:** 3299-3303.

16. **O'Farrell, P. H.** 1975. High-resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:** 4007-4021.

17. **Peeples, T. L., and R. M. Kelly.** 1995. Bioenergetic response of the extreme thermoacidophile *Metallosphaera sedula* to thermal and nutritional stress. Appl. Environ. Microbiol. **61:** 2314-2321.

18. **Phipps, B. M., A. Hoffmann, K. O. Stetter, and W. Baumeister.** 1991. A novel ATPase complex selectively accumulated upon heat shock is a major cellular component of thermophilic archaebacteria. EMBO J. **10:** 1711-1722.

19. **Phipps, B. M., D. Typke, R. Hegerl, S. Volker, A. Hoffmann, K. O. Stetter, and W. Baumeister.** 1993. Structure of a molecular chaperone from a thermophilic archaebacterium. Nature **361:** 475-477.

20. **Pledger, R. J., and J. A. Baross.** 1991. Preliminary description and nutritional characterization of a chemoorganotrophic archaeobacterium growing at temperatures of up to 100°C isolated from a submarine hydrothermal vent environment. J. Gen. Microbiol. **137:** 203-211.

21. **Scholz, S., J. Sonnenbichler, W. Schäfer, and R. Hensel.** 1992. Di-myo-inositol-1,1'-phosphate: a new inositol phosphate isolated from *Pyrococcus woesei*. FEBS Lett. **306:** 239-242.

22. **Trent, J. D., J. Osipiuk, and T. Pinkau.** 1990. Acquired thermotolerance and heat shock in the extremely thermophilic archaebacterium *Sulfolobus* sp. Strain B12. J. Bacteriol. **172:** 1478-1484.

23. **Trent, J. D., E. Nimmesgern, J. S. Wall, F.-U. Hartl, and A. Horwich.** 1991. A molecular chaperone from a thermophilic archaebacterium is related to the eukaryotic protein t-complex polypeptide-1. Nature. **354:** 490-493Q.

24. **Trent, J. D., M. Gabrielsen, B. Jensen, J. Neuhard, and J. Olsen.** 1994. Acquired thermotolerance and heat shock proteins in thermophiles from the three phylogenetic domains. J. Bacteriol. **176:** 6148-6152.

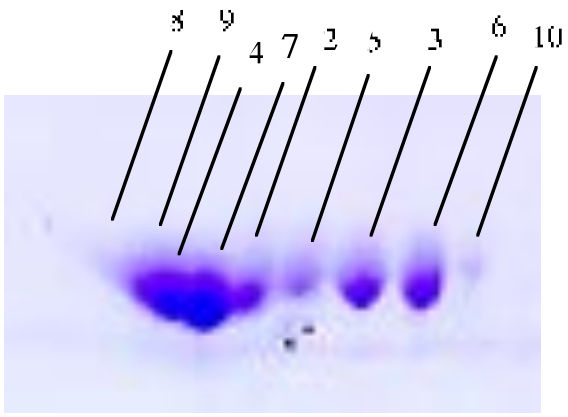
25. **Trent, J. D.** 1996. A review of acquired thermotolerance, heat-shock proteins, and molecular chaperones in archaea. FEMS Microbiol. Rev. **18:** 249-258.

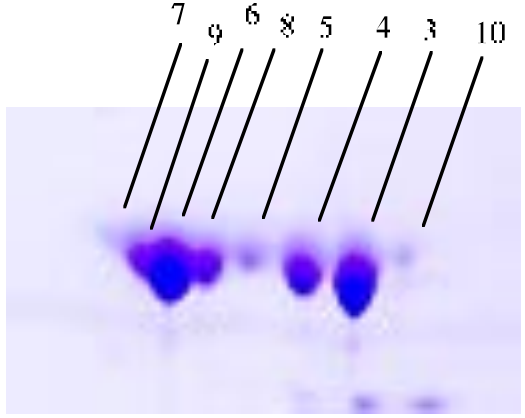
26. **Waldmann, T., E. Nimmesgrern, M. Nitsch, J. Peters, G. Pfeifer, S. Müller, J. Kellermann, A. Engel, F.-U. Hartl, and W. Baumeister.** 1995. The thermosome *of Thermoplasma acidophilum* and its relationship to the eukaryotic chaperonin TRiC. Eur. J. Biochem. **227:** 848-856.

27. **Yeh, T. Y., J. R. Godshalk, G. J. Olson, and R. M. Kelly.** 1987. Use of epifluorescence microscopy for characterizing the activity of *Thiobacillus ferrooxidans* on iron pyrite. Biotechnol. Bioeng. **30:** 138-146.

28. **Zellner, G., and H. Kneifel.** 1993. Caldopentamine and caldohexamine in cells of *Thermotoga* species, a possible adaptation to the growth at high temperatures. Arch. Microbiol. **159:**472-476.

**Table 2.1.** Polypeptide numbering and isoform ratios for the 66 kDa protein of interest in sample under thermally stressed (left) versus sample under normal growth conditions (right). Protein loading for thermally stressed cell was 34% of that for normal cells to facilitate quantification of intensity spots.




sample at 81°C (left above)

sample at 74°C (right above)

| Spot | pI | MW | Corrected Volume | Spot | pI | MW | Volume | **Ratio** |
|---|---|---|---|---|---|---|---|---|
| 8 | - | - | 2364 | - | - | - | - | - |
| 9 | 5.8 | 66899 | 10694 | 7 | 5.8 | 66899 | 396 | **7 3** |
| 4 | 5.9 | 65494 | 22337 | 9 | 5.9 | 65494 | 3286 | **1 8** |
| 7 | 5.9 | 64440 | 29330 | 6 | 5.9 | 64440 | 21833 | **4** |
| 2 | 6.0 | 65494 | 19695 | 8 | 5.9 | 65494 | 4582 | **1 1** |
| 5 | 6.0 | 68849 | 9708 | 5 | 6.0 | 68849 | 1688 | **1 5** |
| 3 | 6.1 | 67477 | 19963 | 4 | 6.1 | 67477 | 8529 | **6** |
| 6 | 6.2 | 67477 | 19226 | 3 | 6.2 | 67477 | 15588 | **3** |
| 10 | 6.3 | 68506 | 1308 | 10 | 6.3 | 68506 | 766 | **4** |

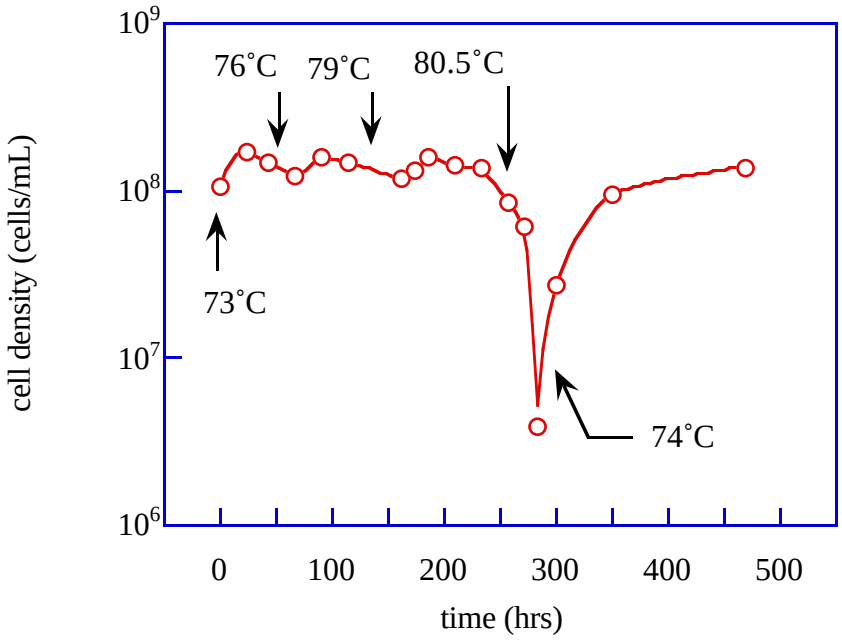



**Figure 2.1. Heat shock of *M. sedula* in 10-liter continuous culture.** The cell density levels (O) prior to (0 - 250 hours), during (250 - 300 hours), and after (300 - 500 hours) heat shock are shown. Arrows indicate the temperature shifts made at the specified time points. Temperature shifts were made from 73° to 79°C (*M. sedula* normal growth range) to 80.5°C (heat shock) back to 74°C (optimal temperature). The onset of washout occurred at 80.5°C.

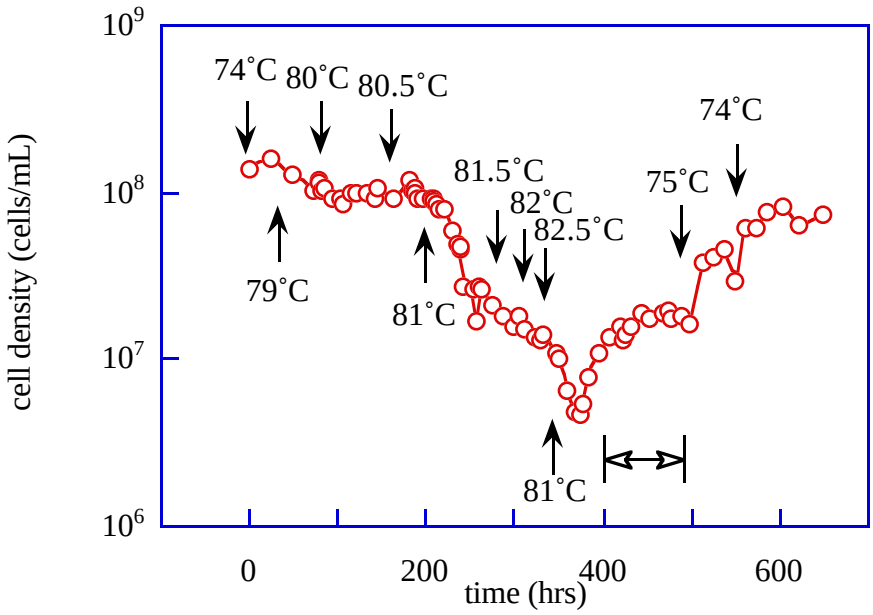



**Figure 2.2. Thermal acclimation and heat shock of *M. sedula* in 10-liter continuous culture.** The culture was subjected to gradual increases in temperature over the course of the experiment. Temperature shifts were made from 74°C→79°C (*M. sedula* normal growth range) to 80°C→80.5°C→81°C→81.5°C→82°C→82.5°C→81°C (thermal stress) to 75°C→74°C (optimal growth range). The cell density levels (O) prior to (0 - 100 hours), during (100 - 500 hours), and after (500 - 650 hours) exposure to thermal stress are shown. Arrows indicate the temperature shifts made at the specific time points. Double-headed arrow indicate the 81°C "stressed phase." Note that the cell density is about 7-fold lower at 81°C compared to 74°C. The onset of washout occurred at 82.5°C.

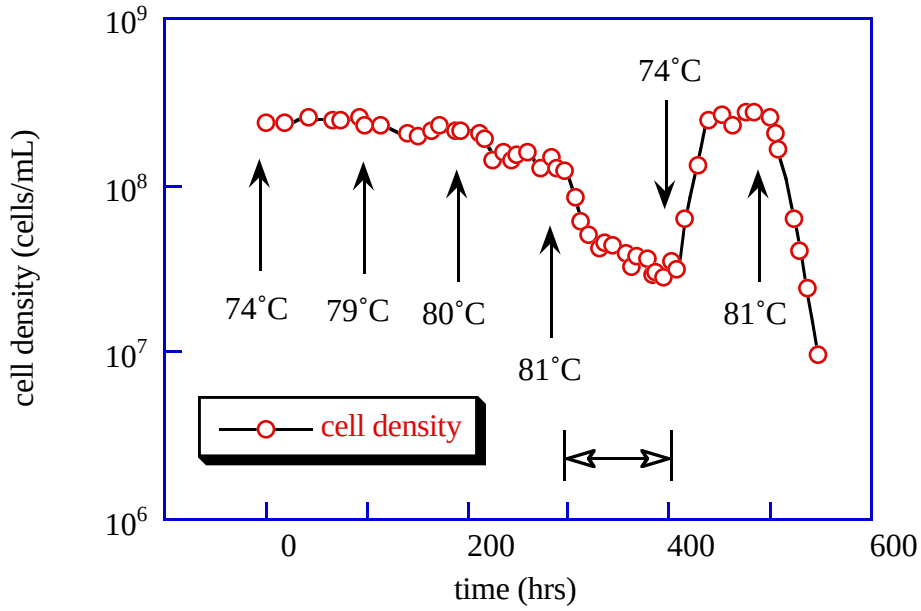



**Figure 2.3. Gradual and abrupt exposure to thermal stress on *M. sedula* in 10-liter continuous culture.** This experiment examined whether thermotolerance acquired by *M. sedula* through gradual exposure to thermal stress created any residual capacity to withstand subsequent abrupt thermal stress. Arrows indicate the temperature shifts made at the specific time points. Double-headed arrow indicates the "81°C stressed phase" during 300 and 400 hours. Temperature shifts were done in five phases: 0 to 100 hrs, normal to maximal growth range; 100 to 300 hrs, gradual thermal stress; 300 to 400 hrs, stressed phase; 400 to 500 hrs, recovery period; 500 to 600 hrs, abrupt thermal stress and subsequent washout.

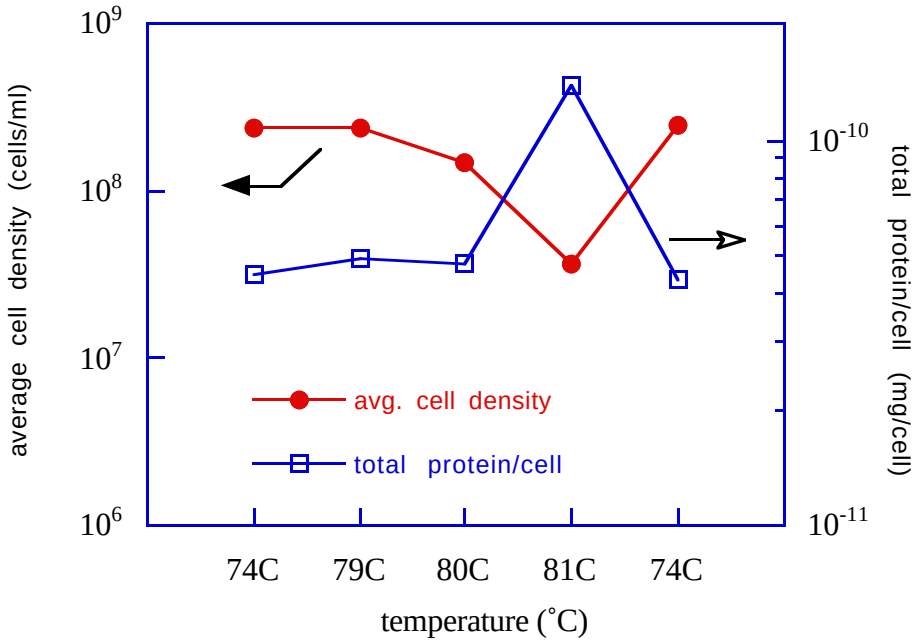



**Figure 2.4. The levels of total protein per cell and average cell densities for conditions shown in Figure 2.3.** The plot shows the increased amount of total protein per cell (about 3-fold) and the reduction of cell density (about 7-fold) at 81°C ("stressed phase") relative to other phases. This increase in total protein per cell at 81°C appears to be mostly the result of over-production of heat shock protein (MseHSP60) (see Figures 5 and 6).

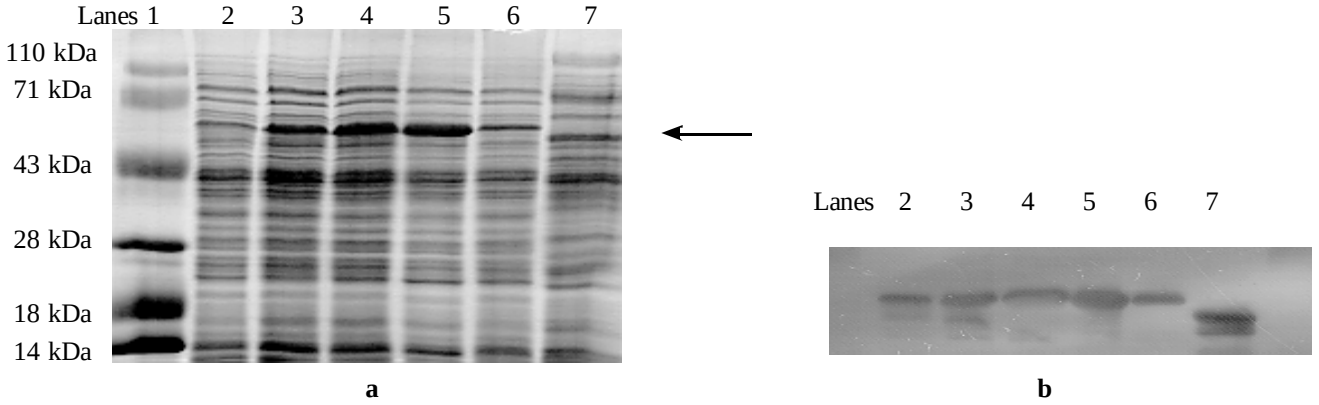
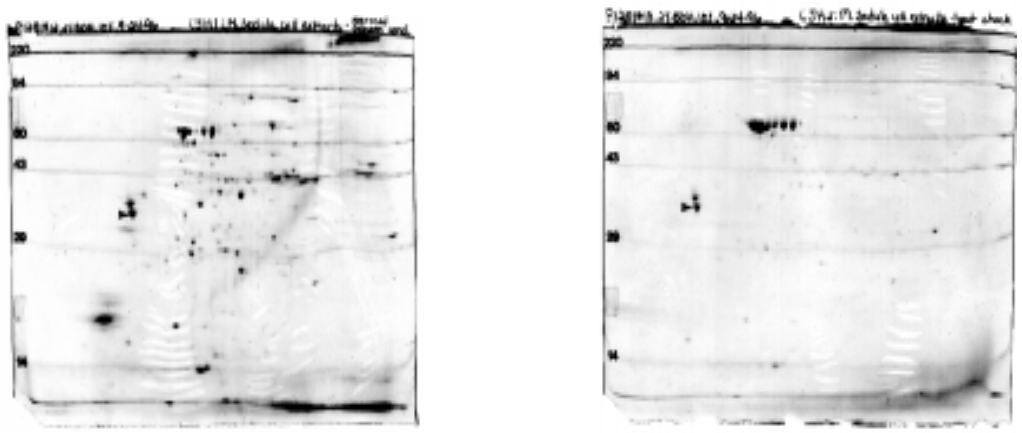



**Figure 2.5. Effect of temperature and thermal acclimation on protein patterns in *M. sedula* cell extracts. (a)** Samples run on 12.5% SDS-PAGE and stained with Coomassie blue. The arrow to the right indicates the locations of heat shock protein (MseHSP60) in lanes 2 to 6. Lanes: 1, prestained protein molecular weight standards (as described in Materials and Methods section); 2, 74°C; 3, 79°C; 4, 80°C; 5, 81°C; 6, 74°C; 7, cell extract from *S. shibatae* grown at 75°C. Note that the *S. shibatae* heat shock protein subunits (SshHSP60) appears to be slightly lower in molecular mass than MseHSP60. **(b)** Western blot (against Thermophilic Factor 55 antibodies) analysis of *M. sedula* cell extracts from **(a).** Only bands from *M. sedula* and *S. shibatae* in the range of 60 to 70 kDa cross reacted with TF55 antibodies. Lanes: (see above). Cell extracts from *S. shibatae* show that two bands cross-reacted with the antibodies (lane 7), presumably a- and b- subunits of SshHSP60. Note that there are increased levels of cross-reactivity of MseHSP60 bands as the culture temperatures is increased.



**a** **b**

**Figure 2.6. Two-dimensional BDH(2%)/SDS(10%) polyacrylamide gel analysis of proteins from *M. sedula* at (a) 74°C and (b) 81°C in continuous culture.** One-third less protein (16.9 mg) was loaded at 81°C compared to 74°C (50 mg) to resolve the isoforms of MseHSP60. For comparison and quantification of spots, the spot volume minus background volume was multiplied by 2.9 to correct for loading. In general, the spot intensities were similar in both cases except those proteins corresponding to MseHSP60 (circled). Quantification showed that the MseHSP60 isoforms increased approximately 6-fold at 81°C as compared to 74°C, and in particular, one isoform (MW 66,899 Da and pI 5.8) increased 73-fold at the higher temperature. A total of 8 to 9 isoforms could be identified. An IEF internal standard (MW 33,000 Da and pI 5.2) was added as indicated by the arrow.

# Chapter 3

## A Protease from the Extremely Thermoacidophilic Archaeon *Metallosphaera sedula*: Evidence of Proteasome-like Intracellular Proteinase in the Kingdom *Crenarchaeota*

### Introduction

In recent years much research effort has been focused on the isolation and characterization of enzymes or proteins from thermophilic microorganisms. This growing interest is justified by their biotechnological potential and by the insights that they may provide to elucidate the general mechanism of thermostability. Thermophilic proteases seem especially promising, and several proteolytic enzymes have recently been isolated from both archaeal and eubacterial sources (Burlini et al, 1992; Fusi et al., 1991; Saravani et al., 1989; Kwon et al., 1988; Cowan et al, 1985). Furthermore, proteasomes from thermophilic archaea have received particular attention (Löwe et al., 1995; Grziwa et al., 1994; Dahlmann et al., 1992).

Proteasomes are multisubunit non-lysosomal proteases and are the central enzymes of protein degradation in both the cytosol and the nucleus (Goldberg and Rock, 1992). They are involved in many important biological processes, including the removal of abnormal, misfolded or improperly assembled proteins, stress response, cell cycle control, cell differentiation and metabolic adaptation to environmental changes (Goldberg, 1995; Rechsteiner et al., 1993; Hilt et al., 1993). These cellular functions are linked to ubiquitin- and ATP-dependent protein degradation involving the 26S proteasome, whose core and proteolytic chamber is formed by the 20S proteasome and by two polar 19S

complexes containing ATPase activity which associate the termini of its core. The 26S proteasome differs in its enzymatic properties from the 20S particle. The ATP-requiring degradation of ubiquitinated and of certain non-ubiquitinated proteins is linked to the 26S complex, whereas the 20S proteasome only degrades unfolded proteins in an energy-independent manner.

Proteasomes are ubiquitous in all three domains of life. The discovery of 20S proteasomes in the archaeon, *Thermoplasma acidophilum*, which has several molecular features reminiscent of eukaryotes, has greatly facilitated the analysis of the structure of this high molecular mass proteinase, since this archaeal enzyme is composed of only two types of subunit (Dahlmann et al., 1989). The primary structure of both subunits, designated α and ß, has been determined (Zwickl et al., 1992a and 1992b) and has been analyzed by X-ray crystallography at 3.4Å resolution (Löwe et al., 1995) (see Figure 3.1). This barrel-shaped particle is made of two inner rings composed of seven ß subunits each, and two outer disks, each composed of seven α subunits (Hegerl et al., 1991; Grziwa et al., 1991; Pühler et al., 1992). To date, 20S proteasomes were found in four archaeal species, all of them belonging to the kingdom *Euryarchaeota*: *T. acidophilum* (Dahlmann et al., 1992; Zwickl et al., 1992; Zwickl et al., 1994), *Methanosarcina thermophila* (Maupin-Furlow and Ferry, 1995), *Methanococcus jannaschii* (Bult et al., 1996), and *Pyrococcus furiosus* (Bauer et al., 1997). However, no proteasome has ever been identified among the members of the kingdom *Crenarchaeota.*

Although the proteasome of the archaea *T. acidophilum* contains only two different subunits, α and ß (25.8 kDa and 22.3 kDa, respectively), proteasomes from eukaryotes have a more complex subunit pattern, consisting of multiple α and ß type subunits (12 to 14 subunits) related to either α or ß subunits of the *Thermoplasma* proteasome. The yeast *Saccharomyces cerevisiae* 20S proteasome is similar in size and shape to the archaeal

proteasome but is much more complex, having seven different α-type and seven different ß-type subunits, all of which have been cloned and sequenced and could be categorized by sequence homology (Hilt et al., 1993). It has trypsin-like (basic), chymotrypsin-like (hydrophobic), and peptidylglutamyl peptide hydrolytic (PGPH) (acidic) activities. It has been reported that five of seven ß-type subunits are synthesized as proproteins, from which propeptides are cleaved during proteasome maturation. This prosequence, the role of which is speculated to be for masking the active sites before maturation, is not required in the archaea for its proteasome assembly (Chen and Hochstrasser, 1995; Seemüller et al., 1996).

Eubacteria contain at least two soluble ATP-dependent proteases: Lon protease and Clp protease. The Clp protease, a major multimeric protease of *Escherichia coli*, consists of a proteolytic component, ClpP, in association with an ATP-hydrolyzing, chaperonin-like component, ClpA (Kessel et al., 1995). ClpP is composed of two rings of seven subunits superimposed in bipolar fashion along the axis of rotational symmetry. This structure is similar to that formed by ß subunits of the eukaryotic and archaeal proteasomes. In the presence of MgATP, ClpA forms an oligomer with 6-fold symmetry. There is virtually no sequence homology between ClpP and the proteolytic subunits of proteasome and only limited similarity between the ATPase binding site of ClpA and the ATPase components of the 26S proteasome (Dubiel et al., 1992). Despite the lack of sequence similarity between ClpAP from eubacteria and proteasomes from archaea and eukaryotes, it seem that these multimeric proteases nevertheless have a profound similarity in the underlying biochemical mechanisms by which these enzymes bind, unfold, and deliver protein substrates to their proteolytic active sites. Recently, ClpQ (HslV) from *E. coli*, a homolog of the ß-subunits of the 20S proteasome from *E. coli* was purified along with ClpY (HslU), an ATPase homologous protease chaperone, ClpX (Kessel et al.,

1996). In the active ClpYQ complex, cleavage of small peptides and proteins requires ATP. EM analysis showed that ClpQ and ClpY are both ring-shaped particles and that the active ClpYQ complex is a cylindrical four-ring structure, composed of ClpQ, a two-ring dodecamer, sandwiched between ClpY rings (Goldberg et al., 1997) (see Figure 3.2).

In this report, the first evidence of proteasome-like proteinase from *Metallosphaera sedula* and its partial purification is described. *M. sedula* is an obligatory aerobic, extremely thermoacidophilic archaeon which grows optimally at 75$^{o}$C by a mixotrophic growth mode, growing lithotrophically on sulfidic ores like pyrite, sphalerite and chalcopyrite, and on elemental sulfur, and growing organotrophically on beef extract, Casamino Acids, peptone, tryptone and yeast extract, but not on any sugars. This is the first report of a proteasome in a member of the *Crenarchaeota*.

## Materials and Methods

**Microorganism and Preparation of Cell Extracts.***Metallosphaera sedula* (DSMZ 5348) was obtained from the Deutsch Sammlung von Microorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) and cultivated using a slightly modified method from that previously described (Han et al., 1997). Cells were centrifuged at 7500 rpm (9500 g) in a Sorvall RC5C centrifuge (DuPont, Newtown, CT) for 40 min. Supernatant was discarded and pellets were washed at least twice with 10 mM $H_2SO_4$ at pH 2. To lyse the cells, the pellets were re-suspended in a buffer A (100 mM sodium phosphate buffer (pH 7.5)) and then disrupted using a sonicator (Model XL2020, Heat systems Inc., Farmingdale, NY) and/or a French Pressure Cell Press (SLM Instrument Inc., Urbana, IL) at 16,000 psig. Lysates were centrifuged again at 4000 rpm for 30 min to remove cell debris.

**Purification.** Proteins were precipitated with $(NH_4)_2SO_4$ and separated from the lysate by centrifugation at 11,000 rpm for 10 min in an Eppendorf Centrifuge 5415C (Fisher Scientific, Raleigh, NC). The protease activity was strongest in the pellet resulting from addition of 40% (0.242 g of ammonium sulfate per mL of lysate). The pellet was dissolved in buffer B (50 mM Tris (pH 8)) and dialyzed overnight against the same buffer. Dialyzate was concentrated and applied to various columns as follows. The lysate was applied to a DEAE Sepharose column (Pharmacia LKB). The column was washed with 1 liter of buffer B, followed by a salt gradient in buffer B (0 to 1 M NaCl). In separate experiments, the lysate was applied to a Mono Q column (Pharmacia HR5/5) equilibrated in buffer B and eluted with a linear gradient of 1 M NaCl. Pooled fractions with proteolytic activity were concentrated in an Amicon stirred cell and subsequently applied to Superose 6 column (10/30) (Pharmacia LKB) which had been previously equilibrated to buffer B (with 150 mM NaCl) to estimate molecular mass of proteinase. The column was calibrated with the following proteins (Sigma Chemical): thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and albumin (66 kDa). Protein concentrations were determined with a Bio-Rad protein assay based on dye-binding (Bradford, 1976).

**Proteolytic Activity Assay.** Proteinase activity was measured by cleavage of synthetic MCA-linked peptides (Sigma Chemical Co., St. Louis, MO) on a CytoFluor 2350 microtiter plate reader with excitation at 360 nm and emission at 460 nm (Millipore, Bedford, MA). For each assay, the substrate (e.g. 0.1 mM *N*-succinyl-valine-lysine-methionine (VKM) -7-amido-4-methyl coumarin (MCA)) and sample (10 µl of the lysate or the partially purified proteinase) or blank (10 µl of 50 mM sodium phosphate, pH 6.0) were separately equilibrated to the assay temperature (typically at 78°C). The reaction was

initiated by mixing 100 µl of substrate in 50 mM sodium phosphate buffer (pH 6.0) with a sample or a blank. After 15 min of incubation at desired temperatures, the reaction was terminated by quenching on ice. Enzyme (proteolytic) activity was corrected for thermal autocatalytic degradation of the substrate. All assays were done in triplicate.

**Electrophoresis.** A modified Laemmli method (1970) was used to separate *M. sedula* cell extracts on SDS-PAGE. Cell extracts were incubated at 100°C in 5X dissociation buffer, containing 400 mM Tris pH 6.8, 10% SDS and 400 mM dithiothreitol, for 10 min prior to loading on 10% or 12.5% discontinuous SDS-PAGE. For one-dimensional SDS-PAGE, equal amounts of total protein (typically 40 µg) were loaded in each lane. Gels were stained with Coomassie blue. The following pre-stained molecular weight markers (Gibco-BRL, Gaithersburg, MD) were used for protein size references: lysozyme (14,300), ß-lactoglobulin (18,200), carbonic anhydrase (27,830), ovalbumin (43,760), bovine serum albumin (71,460), phosphorylase B (110,760), and myosin (216,760).

## Results and Discussion

The purification procedure reported here yielded only a partially purified proteasome-like proteinase from the crude extracts of *M. sedula*. The proteinase was partially purified using $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography, and gel filtration as described in Materials and Methods. The proteinase was found quantitatively in the protein pellet of the $(NH_4)_2SO_4$ precipitate (at 0.242 g $(NH_4)_2SO_4$/mL lysate) as shown in Figure 3.3. Lower concentrations of $(NH_4)_2SO_4$ did not increase the proteolytic activity on VKM-MCA substrate, suggesting all of the proteinase had precipitated at 0.242 g $(NH_4)_2SO_4$/mL lysate. However, this suggested most other intracellular proteins also precipitated at this particular concentration of $(NH_4)_2SO_4$ as shown in Figure 3.4. This

protein pellet with proteolytic activity was dialyzed against 50 mM Tris (pH 8.0) and applied to a Mono Q ion-exchange column. The proteinase was eluted at a salt concentration of 400 mM NaCl from the Mono Q (fractions 11 and 12). Figure 3.5 shows proteolytic activity of all the fractions from the Mono Q run, including fractions 11 and 12 which had highest proteolytic activity against VKM-MCA substrate. Proteolytic activities (as reported in fluorescence unit) were more than 4,000 fold higher than any other fractions that eluted from the Mono Q run. However, comparing this proteolytic activity from *M. sedula* to the one from *P. furiosus*, we found that this enzymatic activity from *M. sedula* was at least 100 fold lower than that from *P. furiosus*. This clearly indicated that the proteinase activity and/or the constitutive level of this proteinase from *M. sedula* is far less than other organisms, such as *P. furiosus*. The fractions 11 and 12 with highest proteolytic activity were pooled together and concentrated. This concentrate was applied to Superose 6 (10/30) to purify the proteinase further and to estimate its molecular mass. However, the eluted fraction from this gel filtration was too dilute to measure any significant proteolytic activity or to run a gel electrophoresis (data not shown).

The activity of this partially purified proteinase was tested on various MCA-linked substrates. This enzyme cleaved peptidyl linkages on the carboxy side of hydrophobic residues of M and F in VKM-MCA and AAF-MCA, respectively. Only slight activity was shown with Y residue of the substrate LLVY-MCA. Figure 3.6 shows proteolytic activity (in fluorescence unit) from DEAE Sepharose column fractions. The enzyme showed no activity toward basic substrates (AFK-MCA, FVR-MCA, RR-MCA, GGR-MCA) and some other hydrophobic substrates (IIW-MCA, AAPV-MCA, GPLGP-MCA). This clearly indicated that this particular proteinase from *M. sedula* has very limited substrate specificity unlike other eukaryotic proteasomes. However, this characteristics of narrow

substrate specificity is similar to proteasomes from *T. acidophilum* and *P. furious* (Dahlmann et al., 1989; Bauer et al., 1997).

Assays were performed to determine the effect of pH on proteolytic activity. The enzyme fractions were dialyzed against desired pH buffer and substrates were pre-equilibrated to the desired temperature and pH prior to reaction. The following pH buffers were prepared to cover desired pH ranges: 50 mM sodium acetate-acetic acid at pH 4.4 to 5.6, 50 mM sodium phosphate at pH 5.6 to 8.0, and 50 mM glycine-NaOH at pH 7.9 to 9.0. As shown in Figure 3.7, the optimum pH of the proteinase from *M. sedula* was acidic at 5.8. This is consistent with *M. sedula*'s intracellular pH which is about 5.5 (Peeples, 1994).

Assays were also conducted to determine the optimum temperature for this enzyme on VKM-MCA hydrolysis. Figure 3.8 shows the temperature optimum is about 85$^{o}$C, which is at least 10$^{o}$C above the cell's optimum growth temperature. Most of reported optimum temperature for proteasome is close to the growth temperature of the organism. However, in case of *T. acidophilum*, the reported temperature optimum of its proteasome was 91$^{o}$C which is far greater than its optimum growth temperature (59$^{o}$C) (Searcy, 1995).

From these purification procedures, structural features of this *M. sedula* proteinase could not be identified along with its biochemical properties, since only partially purified enzyme with in small quantities could be obtained. Therefore, it is difficult to say whether this enzyme is a proteasome or not. However, the data suggest this enzyme is similar to other archaeal proteasomes. First, this enzyme has a very large molecular mass. The fractions, which had a proteolytic activity, during the gel filtration step eluted along with the thyroglobulin (669 kDa) molecular marker. If this is accurate, then this multicomplex proteinase from *M. sedula* is about same size as proteasome from *P. furiosus* (640 kDa)

and *T. acidophilum* (650 kDa). Only the proteasomes with multi-catalytic proteinase activity have this high molecular mass range from archaea. The intracellular proteinases, which found to be non-proteasomes, from other members of the *Crenarchaeota*, such as *Sulfolobus solfataricus*, have far smaller molecular masses (Fusi et al., 1991; Burlini et al., 1992). Secondly, all the archaeal proteasomes characterized so far have very narrow substrate specificity. In contrast, the intracellular metallo-, serine-, and cystein-proteases from *S. solfataricus* have quite different substrate specificity with much broader specificity than proteasomes. Based on these considerations, the proteinase from *M. sedula* is more likely a proteasome rather than an other high molecular weight endo- or exo-protease.

To date, no proteasome has been found in the members of *Crenarchaeota*. This study also could not conclude that this proteinase from *M. sedula* is actually a proteasome. However, this study showed that this enzyme is similar to other archaeal proteasomes regarding its molecular size and substrate specificity. It will be interesting to see how the *Crenarchaeotal* proteasomes have evolved and/or are different from the *Euryarchaeotal* ones and what their roles are in the physiology of the prokaryotic archaea.

## Literature Cited


1. **Bauer, M. W., S. H. Bauer and R. M. Kelly.** 1997. Purification and characterization of a proteasome from the hyperthermophilic archaeon *Pyrococcus furiosus*. Appl. Environ. Microbiol. **63:** 1160-1164.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. **72:** 248-254.
3. **Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, G. G. Sutton, J. A. Blake, L. M. Fitzgerald, R. A. Clayton, J. D. Gocayne, A. R. Kerlavage, B. A. Dougherty, J. F. Tomb, M. D. Adams, C. I. Reich, R. Overbeek, E. F. Kirkness, K. G. Weinstock, J. M. Merrick, A. Glodek, J. L. Scott, N. S. M. Geoghagen, J. F. Weidman, J. L. Fuhrmann, D. Nguyen, T. R. Utterback, J.M. Kelley, H. P. Peterson-Klenk, C. M. Fraser, H. O. Smith, C. R. Woese, and J. C. Venter.** 1996. Complete sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science **273:** 1958-1073.
4. **Burlini, N., P. Magnani, A. Villa, F. Macchi, P. Tortora and A. Guerritore.** 1992. A heat-stable serine proteinase from the extreme thermophilic archaebacterium *Sulfolobus solfataricus*. Biochim. Biophys. Acta **1122:** 283-292.
5. **Chen, P., and M. Hochstrasser.** 1995. Biogenesis, structure and function of the yeast 20S proteasome. EMBO J. **14:** 2620-2630.
6. **Cowan, D., R. Daniel, and H. Morgan.** 1985. Thermophilic proteases: Properties and potential applications. Trends Biotechnol. **3:** 68-72.

7. **Dahlmann, B., F. Kopp, L. Kuehn, B. Niedel, G. Pfeifer, R. Hegerl, and W. Baumeister.** 1989. The multicatalytic proteinase (prosome) is ubiquitous from eukaryotes to archaebacteria. FEBS Lett. **251:** 125-131.

8. **Dahlmann, B., L. Kuehn, A. Grziwa, P. Zwickl and W. Baumeister.** 1992. Biochemical properties of the proteasome from *Thermoplasma acidophilum*. Eur. J. Biochem. **208:** 789-797.

9. **Dubiel, W., K. Ferrell, G. Pratt, M. Rechsteiner.** 1992. Subunit 4 of the 26S protease is a member of a novel eukaryotic ATPase family. J. Biol. Chem. **267:** 22699-22702.

10. **Fusi, P., M. Villa, N. Burlini, P. Tortora and A. Guerritore.** 1991. Intracellular proteases from the extremely thermophilic archaebacterium *Sulfolobus solfataricus*. Experientia **47:** 1057-1060.

11. **Goldberg, A., and K. Rock.** 1992. Proteolysis, proteasomes, and antigen presentation. Nature **357:** 375-379.

12. **Goldberg, A. L.** 1995. Functions of the proteasome. The lysis at the end of the tunnel. Science **268:** 522-523.

13. **Goldberg, A., T. N. Akopian, A. F. Kisselev, D. H. Lee and M. Rohrwild.** 1997. New insights into the mechanisms and importance of the proteasome in intracellular protein degradation. Biol. Chem. **378:** 131-140.

14. **Groll, M., L. Ditzel, J. Löwe, D. Stock, M. Bochtler, H. D. Bartunik, and R. Huber.** 1997. Structure of 20S proteasome from yeast at 2.4Å resolution. Nature **386:** 463-471.

15. **Grziwa, A., W. Baumeister, B. Dahlmann, and F. Kopp. 1991.** Localization of subunits in proteasomes from *Thermoplasma acidophilum* by immunoelectron microscopy. FEBS **290:** 186-190.

16. **Grziwa, A., S. Maack, G. Pühler, G. Wiegand, W. Baumeister, and R. Jaenicke.** 1994. Dissociation and reconstitution of the Thermoplasma proteasome. Eur. J. Biochem. **223:** 1061-1067.

17. **Han, C. J., S. H. Park and R. M. Kelly.** 1997. Acquired thermotolerance and stressed-phase growth of the extremely thermoacidophilic archaeon *Metallosphaera sedula* in continuous culture. Appl. Environ. Microbiol. **63:** 2391-2396.

18. **Hegerl, R., G. Pfeifer, G. Pühler, B. Dahlmann, and W. Baumeister. 1991.** The three-dimensional structure of proteasomes from *Thermoplasma acdiophilum* as determined by electron microscopy from random conical tilting. FEBS **283:** 117-121.

19. **Hilt, W., W. Heinemeyer and E. H. Wolf.** 1993. Studies on the yeast proteasome uncover its basic structural features and multiple in vivo functions. Enzyme Protein **47:** 189-201.

20. **Kessel, M., M. R. Maurizi, B. Kim, E. Kocsis, B. L. Trus, S. K. Singh and A. C. Steven.** 1995. Homology in structural organization between *E. coli* ClpAP protease and the eukaryotic 26S proteasome. J. Mol. Biol. **250:** 587-594.

21. **Kessel, M., W. Wu, S. Gottesman, E. Kocsis, A. C. Steven and M. R. Maurizi.** 1996. Six-fold rotational symmetry of ClpQ, the *E. coli* homolog of the 20S proteasome, and its ATP-dependent activator, ClpY. FEBS Lett. **398:** 274-278.

22. **Kwon, S. T., I. Terada, H. Matsuzawa, and T. Ohta.** 1988. Nucleotide sequence of the gene for aqualysin I (thermophilic alkaline serine protease) of

*Thermus aquaticus* YT-1 and characteristics of the deduced primary structure of the enzyme. Eur. J. Biochem. **173:** 491-497.

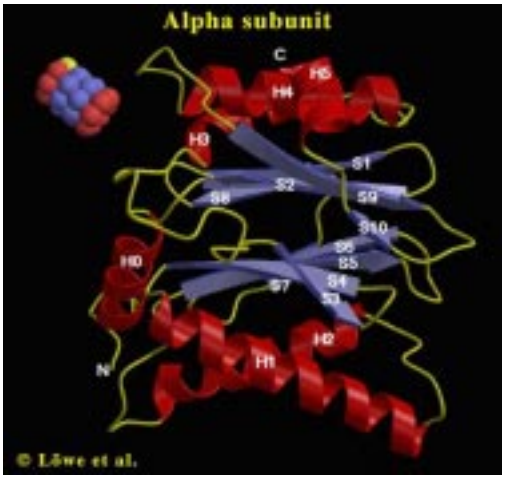
23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680-685.
24. **Löwe, J., D. Stock, B. Jap, P. Zwickl, W. Baumeister and R. Huber.** 1995. Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4Å resolution. Science **268:** 469-608.
25. **Maupin-Furlow, J. A., and J. G. Ferry.** 1995. A proteasome from the methanogenic archaeon *Methanosarcina thermophila*. J. Biol. Chem. **270:** 28617-28622.
26. **Peeples, T. L.** 1994. PhD Thesis. The Johns Hopkins University.
27. **Pühler, G., S. Weinkauf, L. Bachmann, S. Müller, A. Engel, R. Hegerl, and W. Baumeister.** 1992. Subunit stoichiometry and three-dimensional arrangement in proteasomes from *Thermoplasma acidophilum*. EMBO J. **11:** 1607-1611.
28. **Rechsteiner, M., L. Hoffman, and W. Dubiel.** 1993. The multicatalytic and 26S proteases. J. Biol. Chem. **268:** 6065-6068.
29. **Saravani, G. A., D. A. Cowan, R. M. Daniel, and H. W. Morgan.** 1989. Caldolase, a chelator-insensitive extracellular serine proteinase from a *Thermus* spp. Biochem. J. **262:** 409-416.
30. **Searcy, D.** 1995. Culture of Thermoplasma acidophilum, p. 3.51-3.61. *In Archaea*: a laboratory manual. Cold Springs Harbor Laboratory Press, Cold Springs Harbor, N.Y.
31. **Seemüller, E., A. Lupas, and W. Baumeister.** 1996. Autocatalytic processing of the 20S proteasome. Nature **382:** 468-370.

32. **Zwickl, P., F. Lottspeich, and W. Baumeister.** 1992a. Expression of functional *Thermoplasma acdiophilum* proteasomes in *Escherichia coli*. FEBS Lett. **312:** 157-160.

33. **Zwickl, P., A. Grziwa, G. Pühler, B. Dahlmann, F. Lottspeich, and W. Baumeister.** 1992b. Primary structure of the *Thermoplasma* proteasome and its implications to the structure, function, and evolution of the multicatalytic proteinase. Biochemistry **31:** 964-972.

34. **Zwickl, P., J. kleinz, and W. Baumeister.** 1994. Critical elements of proteasome assembly. Nat. Struct. Biol. **1:** 765-769.

A




B

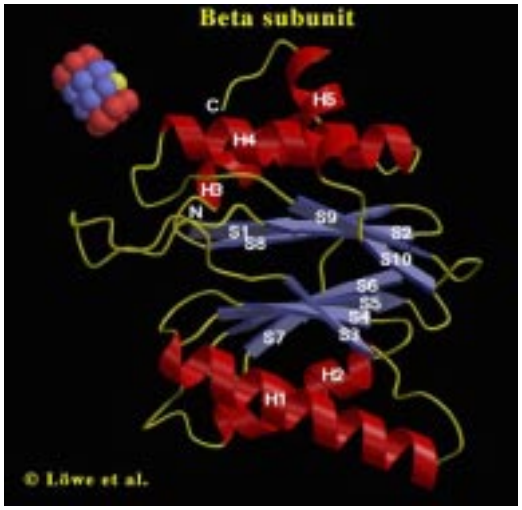



**Figure 3.1. Three-dimensional sturcture of the proteasome from the archaea *Thermoplasma acidophilum*.** Ribon drawings of (A) the α subunit and (B) the β subunit (Löwe et al., 1995).

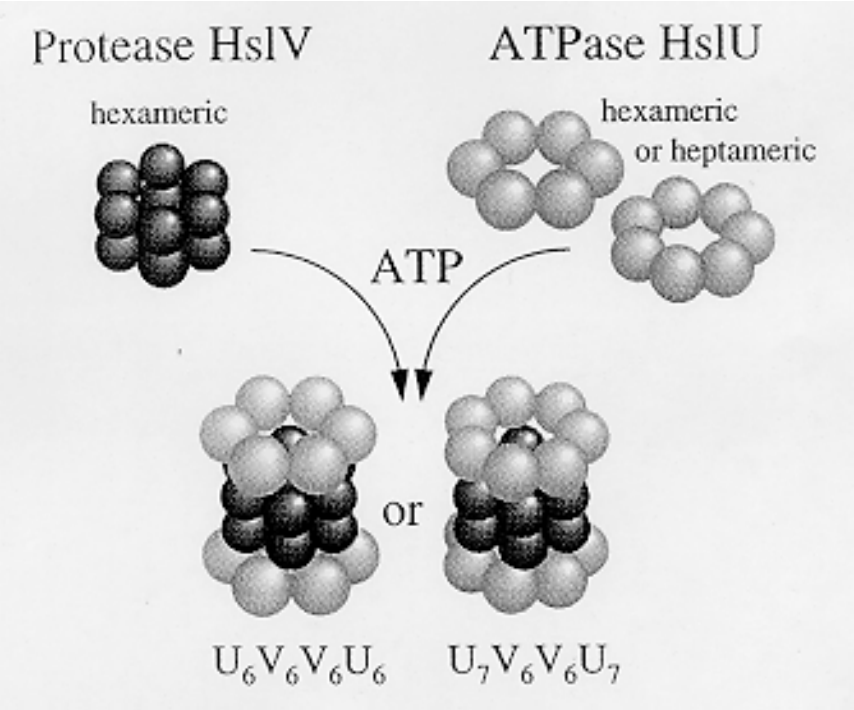



**Figure 3.2. Model of HslVU (ClpYQ) complex system.** HslV (ClpQ) forms a dodecamer spontaneously, but HslU (ClpY) needs the binding of ATP/ADP/AMP-PNP for oligomerization. Model proposes that an active system is formed from one dodecamer of HslV (ClpQ) and two rings of HslU (ClpY) in the presence of ATP or AMP-PNP (Goldberg et al., 1997).

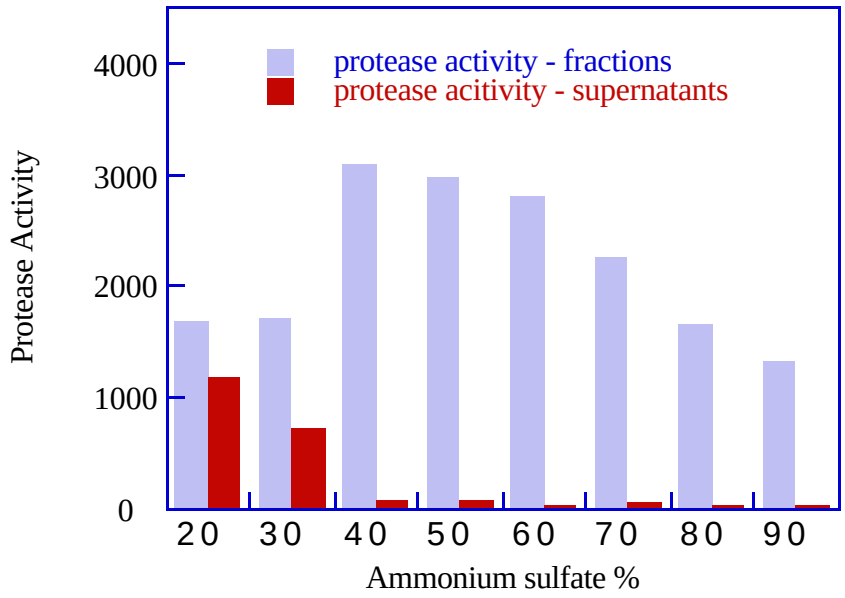



**Figure 3.3. Ammonium sulfate ($(NH_4)_2SO_4$) precipitation of *M. sedula* crude extract.** Various amounts of $(NH_4)_2SO_4$ were added to fractionate proteins by precipitation. Supernatants and precipitated proteins (which were resuspended in the buffer B) were dialyzed as described in Materials and Methods section. Assays were performed to measure proleolytic activity using VKM-MCA substrate at pH 5.8 and 85°C for 15 min.

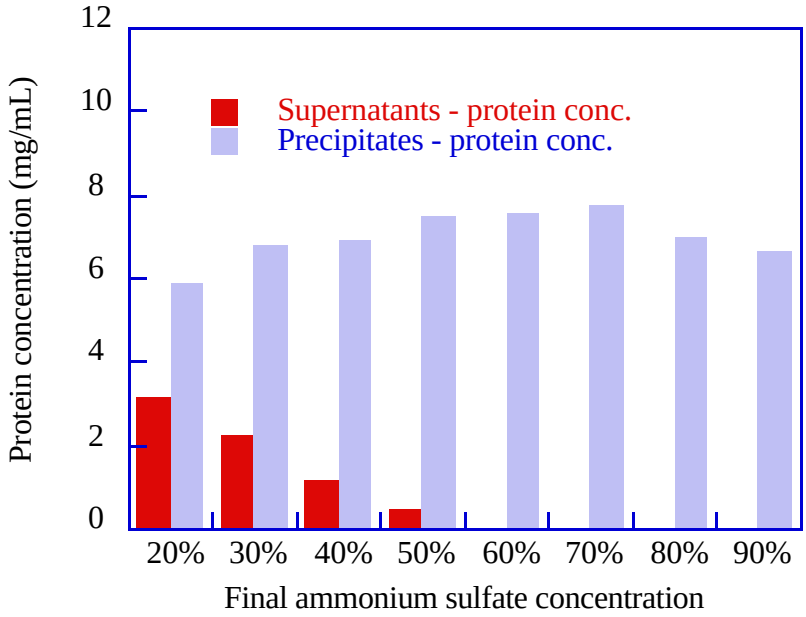



**Figure 3.4. Total protein assay on ammonium sulfate cut supernatants and (re-suspended) precipitated proteins from *M. sedula*.** Supernatants and precipitated proteins were dialyzed against buffer B and assayed for total protein using the Bio-Rad assay. Most of proteins were precipitated at 40 % of $(NH_4)_2SO_4$ or 0.242 g of $(NH_4)_2SO_4$.

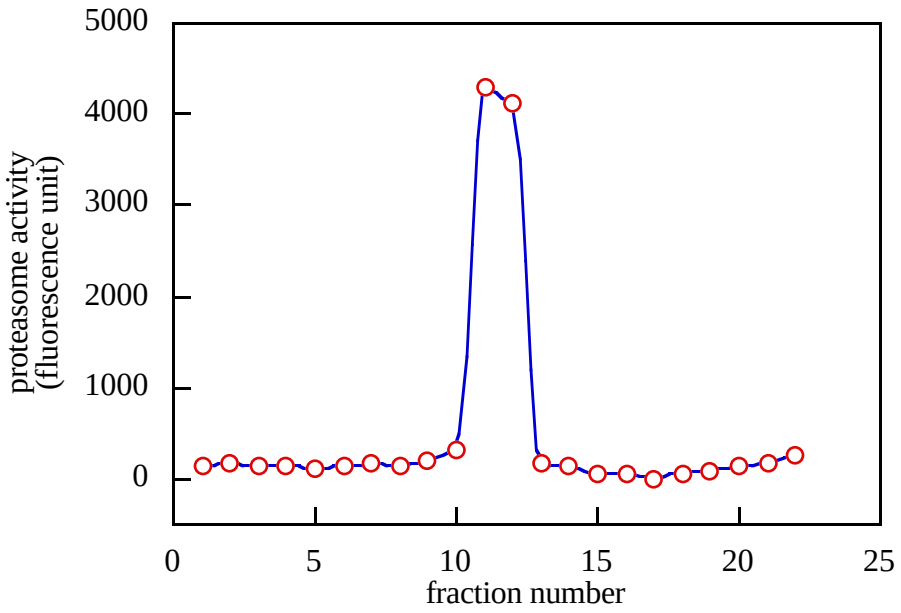



**Figure 3.5. Proteolytic activity of fractions from Mono Q ion-exchange column.** Proteolytic activity with substrate VKM-MCA was tested for all the fractions from Mono Q run with 0 - 1 M NaCl linear gradient. Fractions 11 and 12 shows the highest proteolytic activity toward substrate VKM-MCA.

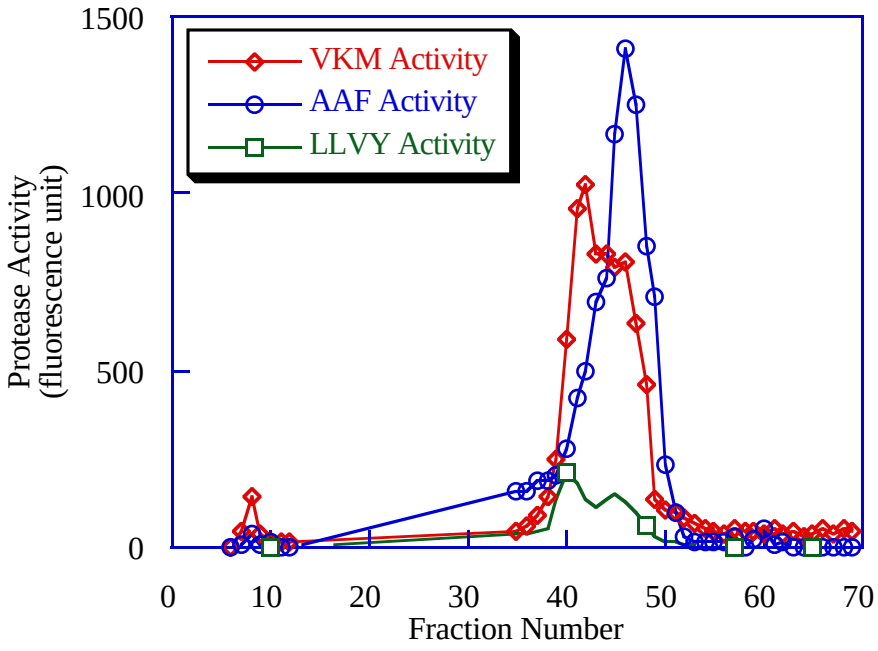



**Figure 3.6. Proteolytic activities in a cell extract supernatant from *M. sedula* fractionated by DEAE ion exchange column.** Protease activities were tested using the following substrates at the indicated concentrations: ( ) 0.1 mM VKM-MCA; (O) 0.1 mM AAF-MCA; (□) 0.1 mM LLVY-MCA. The tests were performed as described in Materials and Methods.

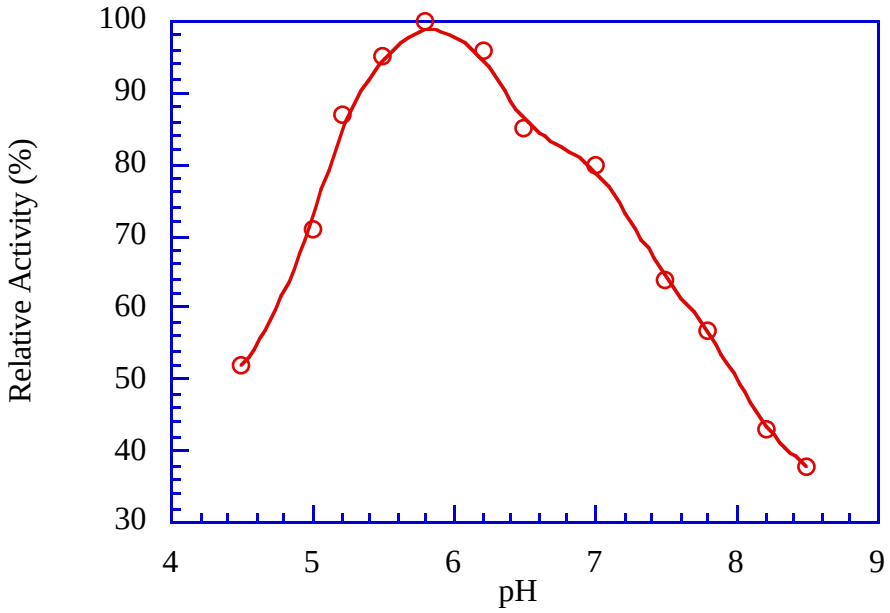



**Figure 3.7. Effect of pH on the proteolytic activity of the partially purified proteinase from *M. sedula*.** The amount of VKM-MCA released was measured after 15 min at 85°C. The specific activity was normalized to pH 5.8. All assays were performed in triplicate.

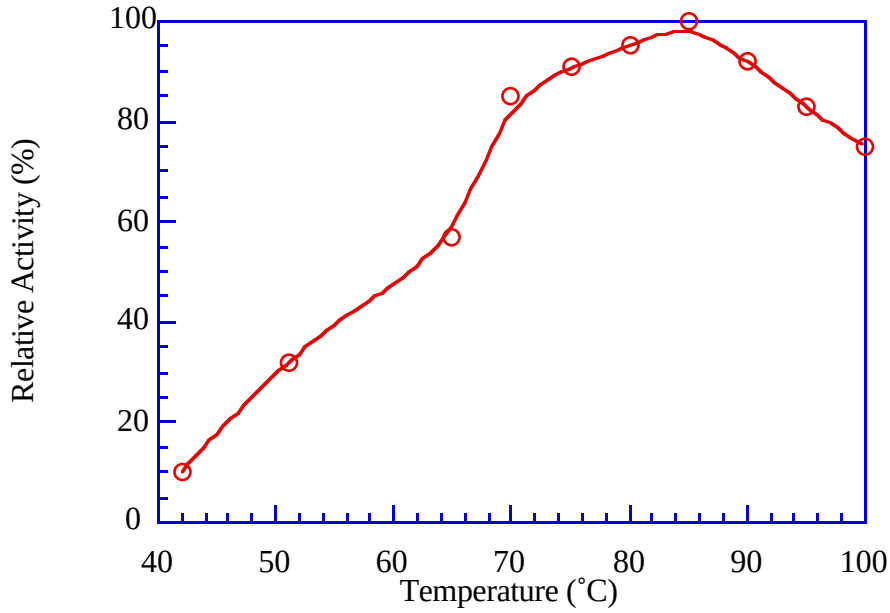



**Figure 3.8. Effect of temperature on the proleolytic activity of the partially purified proteinase from *M. sedula*.** The amount of VKM-MCA released was measured after 15 min at various temperatures and at pH 5.8. All assays were performed in triplicate.

# Chapter 4

## Biooxidation capacity of the extremely thermoacidophilic archaeon *Metallosphaera sedula* under bioenergetic challenge

Chae J. Han and Robert M. Kelly

Portions of this chapter were published

in *Biotechnology and Bioengineering*, 1998

**Keywords:** thermoacidophile, chemolithotroph, heat shock, chemical stress, continuous culture

Running title: Biooxidation of *Metallosphaera sedula* under bioenergetic challenge

### Abstract

The biooxidation capacity of an extremely thermoacidophilic archaeon *Metallosphaera sedula* (DSMZ 5348) was examined under bioenergetic challenges imparted by thermal or chemical stress in regard to its potential use in microbial bioleaching processes. Within the normal growth temperature range of *M. sedula* (70-79°C) at pH 2.0, upward temperature shifts resulted in bioleaching rates that followed an Arrhenius-like dependence. When the cells were subjected to supraoptimal temperatures through gradual thermal acclimation at 81°C (Han, C. J., Park, S. H., Kelly, R. M. 1997. Appl. Environ. Microbiol. **63:** 2391-2396), cell densities were reduced but 3 to 5 times faster specific leaching rates ($Fe^{3+}$ released from iron pyrite/cell/hr) could be achieved by

the stressed cells compared to cells at 79°C and 73°C, respectively. The respiration capacity of *M. sedula* growing at 74°C was challenged by poisoning the cells with uncouplers to generate chemical stress. When the protonophore 2,4-dinitrophenol (5-10 mM) was added to a growing culture of *M. sedula* on iron pyrite, there was little effect on specific leaching rates compared to a culture with no protonophore at 74°C; 25 µM levels proved to be toxic to *M. sedula*. However, a significant stimulation in specific rate was observed when the cells were subjected to 1 µM nigericin (+135%) and 2 µM (+63%); 5 µM levels of the ionophore completely arrested cell growth. The ionophore effect was further investigated in continuous culture growing on ferrous sulfate at 74°C. When 1 µM nigericin was added as a pulse to a continuous culture, a 30% increase in specific iron oxidation rate was observed for short intervals, indicating a potential positive impact on leaching when periodic chemical stress is applied. This study suggests that biooxidation rates can be increased by strategic exposure of extreme thermoacidophiles to chemical or thermal stress and this approach should be considered for improving process performance.

## Introduction

Extreme thermoacidophiles, mostly members of the archaeal order Sulfolobales, represent a unique type of extremophile in that they must deal with high temperature and low pH simultaneously. For example, *Metallosphaera sedula*, an extremely thermoacidophilic archaeon, grows optimally at 74°C and at pH 2.0 (Huber et al., 1989). This organism and other members of the Sulfolobales grow chemolithotrophically by oxidizing reduced iron and sulfur species through a membrane-bound transport system which coordinates electron and proton fluxes to produce ATP (Cobley and Cox, 1983; Lübben and Schäfer, 1989; Schäfer et al., 1990) (see Figure 4.1). Since these organisms are effective in leaching of metals and sulfur from ores and coal (Clark et al., 1993;

Peeples and Kelly, 1993), methods to induce higher rates of metal oxidation or desulfurization are desirable. Unfortunately, the metabolic complexity of iron/sulfur oxidation and the early stage of development for genetic systems in extreme thermoacidophiles make directed approaches to this end very difficult. Therefore, manipulation of process parameters currently represents the most available route for improvement.

Most studies done so far to improve bioleaching rates by extreme thermoacidophiles have dealt with manipulating process parameters only within normal growth ranges of temperature and pH (Boogerd et al., 1990; Kargi and Robinson, 1984; Lindström et al., 1993). Although these microorganisms already contend with growth conditions that are normally debilitating to other living cells and organisms, they can also be subjected to adverse conditions, such as heat shock, starvation, or chemical stress. Such exposures provoke an atypical or stress-induced metabolic response (De Macario and Macario, 1994; Han et al., 1997; Jerez, 1988; Lowe et al., 1993; Peeples and Kelly, 1995; Trent et al., 1994; Trent, 1996). If specific exposure to stress can be utilized and controlled, as has often been tried in medical (Burdon, 1988; Hall and Foster, 1996) and food (Archer, 1996; Jørgensen et al., 1996; Lou and Yousef, 1997; McDonald et al., 1990) applications, a new strategy for improving cellular bioleaching capacity may arise.

Studies of thermal stress in extreme thermoacidophiles have shown that either a small set of proteins or a single protein induced by heat shock has been associated with their prolonged survival at supraoptimal temperatures (Guagliardi et al., 1994; Han et al., 1997; Kagawa et al., 1995; Knapp et al., 1994; Peeples and Kelly, 1995; Trent et al., 1990; Waldmann et al., 1995). The exploitation of stress response in these organisms under bioenergetic challenges to accelerate leaching rates, however, has received little or no attention. Normally the intracellular pH of extreme thermoacidophiles is close to

neutral. A significant transmembrane pH gradient must be maintained across the cell membrane which, in fact, is required for chemiosmosis and subsequent electron translocation via iron turn-over (Cobley and Cox, 1983). If this energy network were to be disrupted partially or entirely by chemical or thermal stress, one possible result is excess accumulation of protons intracellularly and a drop in internal pH (Peeples and Kelly, 1995). To survive, thermoacidophiles would need to re-establish the proton gradient, possibly through higher iron or sulfur oxidation rates, to generate ATP to compensate for the additional bioenergetic burden. The objective of this study was to investigate whether adaptation or metabolic response of extreme thermoacidophiles to certain bioenergetic challenges (i.e., heat shock or respiratory uncouplers) could be exploited to increase biooxidation rates. If successful, strategic exposure to stress would provide a mechanism to improve bioleaching process performance.

## Materials and Methods

**Cell cultivation.** *Metallosphaera sedula* (DSMZ 5348) was obtained from the Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). Cells were cultivated in a sterilized basal medium consisting of the following components: 0.4 g/L $K_2HPO_4$, 0.4 g/L $NH_4SO_4$, 0.4 g/L $MgSO_4 \cdot 7H_2O$ (Fisher Scientific, Raleigh, NC). Casamino acids (A-2427; Sigma, St. Louis) (0.2 g/L) were added as an organic carbon source. $H_2SO_4$ (10 N) was added to the medium to adjust the pH to 2.0. Cultures were inoculated to a starting density of $10^6$ cell/mL. Cell samples (500 µL to 1 mL) were fixed with glutaraldehyde and stained with acridine orange for enumeration by epifluorescence microscopy (Yeh et al., 1987).

**Pyrite.** 100 grams of -165/+250 mesh (58 to 91 μm) Reference Material 8455 pyrite was obtained from National Institute of Standards and Technology (NIST, Gaithersburg, MD). Characterization of this reference material has been reported previously (Olson, 1991).

**Batch leaching experiments.** Small cultures (50 mL) were prepared by adding 1 to 2 grams of standard reference pyrite (NIST 8455) to sterile basal media with Casamino acids in shake flasks. The inoculum was subcultured from a continuous culture and all media were autoclaved prior to the beginning of a particular experiment. Typically, laboratory batch culture leaching experiments were performed at 74°C and pH 2.0 with agitation at 100 rpm in an oil bath (New Brunswick Scientific, Piscataway, NJ). Uninoculated controls were run to account for any abiotic leaching at a particular temperature and/or growth conditions. Typically, the abiotic release of ferric iron from pyrite in the controls was only about 1% of the biotic case.

**Continuous culture.** A 10-liter glass chemostat (Ace Glass, Vineland, NJ) was used to generate biomass for thermal and chemical stress response studies. The continuous culture was agitated at 150 rpm with an impeller and aerated by sterile (0.2 μm filter) house air at a rate of 100 cc/min. All chemostat oxidation experiments were performed by feeding filtered (0.2 μm pore size) and sterilized inorganic substrate (5 grams per liter of $FeSO_4 \cdot 7H_2O$) into the reactor vessel containing sterile basal medium with organic substrate. A dilution rate of 0.04 $hr^{-1}$ (corresponding to a 17 hour cell doubling time) was used to minimize chances of premature washout when thermal or chemical stress was applied to the culture; washout would normally be expected to occur at more than twice this dilution rate (Han and Kelly, data not shown).

**Temperature effect on bioleaching of *M. sedula***. Iron pyrite oxidation rates for *M. sedula* in batch culture were determined at 73°C (optimal growth temperature) and 79°C (maximal growth temperature). Equal number of starting cells (i.e. 1.0 x $10^8$ cells/mL) were directly transferred from continuous culture to the pre-heated shake flasks with basal medium at pH 2.0 without iron pyrite. After several hours of incubation at either 73 or 79°C, cell densities reached nearly high $10^7$ cells/mL at which time iron pyrite (2.0% w/v) was added to the culture. Periodically, samples for cell density enumeration were withdrawn and counted under epifluorescence microscopy with acridine orange stain (Yeh et al., 1987 ). Iron assay was performed to estimate the amount of total iron, ferrous iron, and ferric iron released in the solution.

**Temperature shifts and thermal stress.** For the characterization of stress response from temperature elevation or heat shock and corresponding changes in iron leaching capacity of *M. sedula* in batch culture, cells were transferred from the continuous culture and then subjected to varying extents of temperature shifts. Thermal stress to the continuous culture was performed as described previously (Han et al., 1997). Briefly, the continuous culture temperature was shifted gradually (0.5°C increments), after the culture stabilized at a particular temperature. A steady-state thermally-stressed phase (Han et al., 1997) in the continuous culture could be achieved at supraoptimal temperature (81°C) at dilution rate of 0.04 $hr^{-1}$.

**Chemical stress.** For the characterization of leaching capacity of chemically-challenged cells in batch culture, various concentrations of 2,4-dinitrophenol (Sigma Chemical, St. Louis, MO) and nigericin (Sigma Chemical, St. Louis, MO) were added after the culture had reached mid-log phase (~$10^7$ cells/mL). To assess the changes in biooxidation rates

for chemically-stressed cells in continuous culture, nigericin (1 μM) was either held constant or injected as a pulse to the continuous culture operating at steady-state.

**Iron assays.** Culture samples were analyzed for total and ferrous iron concentrations by 1,10-phenanthroline-based assay (Peeples and Kelly, 1993). The amount of ferric iron released in the solution was determined by difference.

## Results

**Temperature effect on bioleaching rates within normal growth temperature ranges.** Iron pyrite leaching rates by *M. sedula* were followed at 73°C (near optimal growth temperature) and 79°C (normal maximal growth temperature) over a 4 to 5 day period. Solution cell densities typically climbed from below $10^7$ to in excess of $10^8$ cell/mL during this period. Pyrite leaching was initiated when the cell density reached mid to high $10^7$ cells/mL. Although some cell attachment to iron pyrite was noted when samples were viewed under epifluorescence microscopy, this did not appear to be significant. Based on total protein assay only about 5% of the total protein in a given sample was attributed to attached cells compared to cells in solution (data not shown). Ferric iron levels rose rapidly after about 40 hours of incubation. Bioleaching rates were higher at 79°C (6.7 x $10^{-7}$ mg-$Fe^{3+}$/cell/hr) compared to 73°C (4.3 x $10^{-7}$ mg-$Fe^{3+}$/cell/hr), corresponding to an Arrhenius-like 60% increase for a 6°C temperature change. Uninoculated controls, however, showed no significant increase in ferric iron levels when temperature shit was made from 73 to 79°C.

**Effect of thermal stress on bioleaching.** To determine the effect of thermal stress on bioleaching capacity of *M. sedula*, samples from "stressed phase" continuous culture growing on ferrous sulfate at 81°C (Han et al., 1997) were transferred to batch culture at the same temperature without iron pyrite. Parallel cultures were grown at 73°C and 79°C. After two hours of incubation at the respective temperatures, iron pyrite (2.0% w/v) was added and cell density and iron concentration (total, ferrous and ferric) were followed as a function of time (Figure 4.2). After approximately 80 hours, total $Fe^{3+}$ released was highest for the 81°C case (see Figure 4.2b), despite the fact that this culture had a lower solution cell density at this point (see Figure 4.2a). As a result, the specific rate was 5-fold and 3-fold higher at 81°C than at 73°C and 79°C, respectively (see Figure 4.2c). Bioleaching rates accounted for abiotic leaching contributions determined from uninoculated controls. Abiotic leaching contributions, typically less than 1% of biotic case, were similar at all three temperatures.

**Effect of respiratory uncouplers on bioleaching.** The respiration capacity of *M. sedula* was challenged in batch culture by disrupting the proton and ion pumping capacity with various amounts of a protonophore (2,4-dinitrophenol at 0 to 25 µM) and ionophore (nigericin at 0 to 5 µM). Tables I and II show the specific leaching rate observed in chemically stressed and unstressed (control) cells. The differences between the controls for the two sets of uncoupler experiments arise from the different inocula used in the experiments. *M. sedula*'s bioleaching capacity was completely compromised at 2,4-DNP levels of 25 µM or nigericin levels of 5 µM. At 5 and 10 µM 2,4-DNP, the effect of specific bioleaching rate was slight. However, nigericin at either 1 or 2 µM was found to stimulate bioleaching rates considerably, resulting in 2.4-fold improvement in specific

rates at 1 μM concentrations. Figure 4.3 shows that overall $Fe^{3+}$ release rates also improved in the presence of 1 μM nigericin.

**Continuous and pulsed addition of nigericin to continuous culture of *M. sedula*.** Continuous culture (10 L working volume) was employed to determine the response of ferrous sulfate oxidation by *M. sedula* under constant and pulsed addition of nigericin to create respiratory stress. The culture was brought to steady state at 74°C and a cell density of approximately $1.5 \times 10^8$ cell/mL (Phase I) before nigericin was added and held constant at 1 μM (Phase II) (see Figure 4.4a). After nigericin was added, a slight decrease in cell density with time was noted and total $Fe^{3+}$ concentrations rose initially then declined with time to levels or below those observed in Phase I (Figure 4.4b). Specific iron oxidation rates increased slightly after the addition of nigericin (Figure 4.4c) and then returned to levels noted before the ionophore was added.

When nigericin was added as a pulse to bring the system initially to 1 μM, a drop in cell density and total $Fe^{3+}$ release was noted soon after ionophore injection (see Figures 4.5a and b). Cell densities returned to pre-pulse levels within 25 hours of nigericin injection, although total $Fe^{3+}$ concentrations increased throughout the rest of the experiment. Although not as dramatic as in the batch experiment, specific iron oxidation rates were higher after addition of nigericin (see Figure 4.5c).

## Discussion

The work described here suggests a potential strategy to accelerate iron biooxidation rates by extreme thermoacidophiles. By compromising cellular bioenergetics in various ways, iron or sulfur oxidation may be enhanced as a means to compensate for a nonideal situation. In fact, energy coupling by thermoacidophiles at high temperature and

low pH may be relatively inefficient even under normal growth conditions in view of the high maintenance coefficients and the high membrane permeability to protons found for these organisms when compared to mesoacidophiles (De Vrij et al, 1988; Farrand et al., 1983; Konings et al, 1992; Kuhn et al., 1980; McKay et al., 1982). On the other hand, Elferink et al. (1994) showed *Sulfolobus acidocaldarius* has a relatively low proton permeability at 80°C compared to *Escherichia coli* or *Bacillus stearothermophilus*. In any case, Extreme thermoacidophiles presumably need to invest additional metabolic energy for maintenance of a suitable intracellular pH under stress.

Within the normal growth temperature range for *M. sedula*, specific iron oxidation rates increase with temperature; at 73 and 79°C, cell densities were comparable although iron pyrite oxidation rates were 1.6-fold higher at the higher temperature. Through long-term thermal acclimation in continuous culture, *M. sedula* can be stabilized in a heat-shocked state ("stressed phase") at supraoptimal temperatures (i.e., 81°C), although cell densities here are reduced by 5-fold or more (Han et al., 1997). In this stressed state, maintenance requirements should be more significant to overcome additional burdens placed on the cell, such as leakage of protons into the cytoplasm. To compensate, *M. sedula* would need to enhance its proton pumping capacity, possibly fueled by ATP generated through additional dissimilatory iron and sulfur oxidation. This would explain the higher specific bioleaching rates at 81°C.

Collapse of proton pumping circuits under thermal stress can arise from increased membrane permeability to proton leakage. Thermal damage to membrane structural components or partial or total inactivation of proteins involved in maintaining the transmembrane pH potential would lead to an acidification in internal pH for thermoacidophiles. Uncouplers (e.g., protonophores and ionophores) to some extent mimic this effect by directly or indirectly compromising the pH gradient across the cell

membrane. Sub-lethal levels of these compounds have been found to induce a compensatory bioenergetic response in mesophilic microorganisms, which has been referred to as "low energy shock" (Lewis et al., 1994). De Melo and co-workers (1996) demonstrated protonophores, such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and nisin, caused a decline in proton motive force in *Staphylococcus aureus* and *E. coli*. This could be reestablished by the increased rate of proton extrusion through the respiratory chain and subsequent increase in $O_2$ consumption. Increased respiration rates were also observed for *Paracoccus denitrificans* under the influence of 2,4-DNP (Stouthamer and Bettenhausen, 1981). Gage and Neidhardt (1993) showed that wild-type *E. coli* could adapt to sub-lethal levels of 2,4-DNP, after an acclimation period, to grow at rates achieved before uncoupler addition. Concomitantly, increased synthesis of 53 proteins was observed in the chemically stressed cells, including a number of heat-shock proteins. Recovery from 2,4-DNP-induced chemical stress was accelerated if pyrroloquinoline quinone (PQQ), a cofactor for glucose dehydrogenase (Matsushita et al., 1987) was added, leading to the oxidation of glucose to gluconate. They suggested that the electrons generated from glucose oxidation passed through the electron transport chain, thus an alternative energetic pathway was utilized to maintain or reestablish proton motive force.

In this study, sub-lethal levels of 2,4-DNP had little, if any, effect on specific biooxidation rates by *M. sedula*. Other bioenergetic alternatives that did not impact bioleaching rates could have been operable as a means to reestablish proton motive force under "low energy shock" (Lewis et al., 1994). Sub-lethal levels of nigericin, an ionophore, however, were found to stimulate biooxidation rates by *M. sedula*. Why the ionophore effect on iron turnover was more significant than the protonophore is not clear. Perhaps, the different mechanisms by which these uncouplers work to challenge

respiration explains the variations in iron oxidation rates. Nigericin catalyzes the carrier-mediated antiport of $K^+$ for $H^+$ (Harold, 1986), extruding protons from outside to inside the cell membrane as a means of dissipating the proton gradient. *M. sedula* may have compensated for this by pumping these protons back out through the electron transport chain. The respiration rate would then increase, coupled to the increased rate of ferrous to ferric iron oxidation (see Figure 4.6). Additional work focusing on other uncouplers and their effect on proton motive force in this system needs to be done to investigate the above hypothesis.

There is still much to be learned about the relationship between chemical and thermal stresses and their impact on biooxidation capacity of extremely thermoacidophilic microorganisms. However, these results indicate this strategy might be exploited to improve biooxidation rates. It remains to be seen if such a strategy can be implemented in an optimal and economic way in a process setting. Alternatively, as techniques for genetic manipulation of these organisms develop, it may be possible to modify key bioenergetic pathways to render them less efficient and thereby improve overall biooxidation performance.

## Literature Cited


1. **Archer, D. L.** 1996. Preservation microbiology and safety: evidence that stress enhances virulence and triggers adaptive mutations. Trends Food Sci. Technol. **7:** 91-95.
2. **Boogerd, F. C., Bos, P., Keunen, J. G., Heijnen, J. J., Van der Lans, R. G. J. M.** 1990. Oxygen and carbon dioxide mass transfer and the aerobic, autotrophic cultivation of moderate and extreme thermophiles: a case study related to the microbial desulfurization of coal. Biotechnol. Bioeng. **35:** 1111-1119.
3. **Burdon, R. H.** 1988. The heat shock proteins. Endeavour **12:** 133-138.
4. **Clark, T. R., Baldi, F., Olson, G. J.** 1993. Coal depyritization by the thermophilic archaeon *Metallosphaera sedula*. Appl. Environ. Microbiol. **59:** 2375-2379.
5. **Cobley, J. G., Cox, J. C.** 1983. Energy conservation in acidophilic bacteria. Microbiol. Rev. **47:** 579-595.
6. **De Macario, E. C., Macario, A. J.** 1994. Heat-shock response in Archaea. Trends Biotechnol. **12:** 512-518.
7. **De Vrij, W., Bulthuis, R. A., Konings, W. N.** 1988. Comparitive study of energy-transducing properties of cytoplasmic membranes from mesophilic and thermophilic Bacillus species. J. Bacteriol. **170:** 2359-2366.
8. **Elferink, M. G. L., de Wit, J. G., Driessen, A. J. M., Konings, W. N.** 1994. Stability and proton-permeability of liposomes composed of archaeal tetraether lipids. Biochim. Biophys. Acta **1193:** 247-254.
9. **Farrand, S. G., Jones, C. W., Linton, J. D., Stephenson, R. J.** 1983. The effect of temperature and pH on the growth efficiency of the thermoacidophilic

bacterium *Bacillus acidocaldarius* in continuous culture. Arch. Microbiol. **135:** 276-283.

10. **Guagliardi, A., Cerchia, L., Bartolucci, S., Rossi, M.** 1994. The chaperonin from the archaeon *Sulfolobus sofataricus* promotes correct refolding and prevents thermal denaturation in vitro. Protein Science **3:** 1436-1443.
11. **Hall, H. K., Foster, J. W.** 1996. The role of fur in the acid tolerance response of *Salmonella typhimurium* is physiologically and genetically separable from its role in iron acquisition. J. Bacteriol. **178:** 5683-5691.
12. **Han, C. J., Park, S. H., Kelly, R. M.** 1997. Acquired thermotolerance and stressed-phase growth of the extremely thermoacidophilic archaeon *Metallosphaera sedula* in continuous culture. Appl. Environ. Microbiol. **63:** 2391-2396.
13. **Harold, F. M.** 1986. The vital force: a study of bioenergetics. Freeman, New York.
14. **Huber, G., Spinnler, C., Gambacorta, A., Stetter, K. O.** 1989. *Metallosphaera sedula* gen. and sp. nov. represents a new genus of aerobic, metal-mobilizing,thermophilic archaebacteria. System. Appl. Microbiol. **12:** 38-47.
15. **Jerez, C. A.** 1988. The heat shock response in mesophilic and thermoacidophilic chemolithotrophic bacteria. FEMS Mirobiol. Lett. **56:** 289-294.
16. **Jørgensen, F., Panaretou, B., Stephens, P. J., Knochel, S.** 1996. Effect of pre- and post-heat shock temperature on the persistence of thermotolerance and heat shock-induced proteins in *Listeria monocytogenes*. J. Appl. Bacteriol. **80:** 216-224.
17. **Kagawa, H. K., Osipiuk, J., Maltsev, N., Overbeek, R., Quaite-Randall, E., Joachimiak, A., Trent, J. D.** 1995. The 60 kDa heat shock

proteins in the hyperthermophilic archaeon *Sulfolobus shibatae*. J. Mol. Biol. **253:** 712-725.

18. **Kargi, F., Robinson, J. M.** 1985. Biological removal of pyritic sulfur from coal by the thermophilic organism *Sulfolobus acidocaldarius*. Biotechnol. Bioeng. **26:** 41-49.
19. **Knapp, S., Schmidt-Krey, I., Hebert, H., Bergman, T., Jornvall, H., Ladenstein, R.** 1994. The molecular chaperonin TF55 from the thermophilic archaeon *Sulfolobus solfataricus*. J. Mol. Biol. **242:** 397-407.
20. **Konings, W. N., Tolner, B., Speelmans, G., Elferink, M. G. L., De Wit, J. G., Driessen, A. J. M.** 1992. Energy transduction and transport processes in thermophilic bacteria. J. Bioenerg. Biomem. **24:** 601-609.
21. **Kuhn, H. J., Cometta, S., Fiechter, A.** 1980. Effects of growth temperature on maximal specific growth rate, yield, maintenance, and death rate in glucose-limited continuous culture of the thermophilic *Bacillus caldotenax*. European J. Appl. Microbiol. Biotechnol. **10:** 303-315.
22. **Lewis, K., Naroditskaya, V., Ferrante, A., Fokina, I.** 1994. Bacterial resistance to uncouplers. J. Bioenerg. Biomem. **20:** 639-646.
23. **Lindström, E. B., Wold, S., Kettaneh-Wold, N., Saaf, S.** 1993. Optimization of pyrite bioleaching using *Sulfolobus acidocaldarius*. Appl. Microbiol. Biotechnol. **38:** 702-707.
24. **Lou, Y., Yousef, A. E.** 1997. Adaptation to subleathal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. Appl. Environ. Microbiol. **63:** 1252-1255.

25. **Lowe, S. E., Jain, M. K., Zeikus, J. G.** 1993. Biology, ecology, and biotechnological applications of anaerobic bacteria adapted to environmental stresses in temperature, pH, salinity, or substrates. Microbiol. Rev. **57:** 451-509.

26. **Lübben, M., Schäfer, G.** 1989. Chemiosmotic energy conversion of the archaebacteral thermoacidophile *Sulfolobus acidocaldarius*: oxidative phosphorylation and the presence of an $F_0$-related N, N'-dicyclohxylcarboiimide-binding proteolipid. J. Bacteriol. **171:** 6106-6116.

27. **Matsushita, K., Nonobe, M., Shinagawa, E., Adachi, O., Ameyama, M.** 1987. Reconstitution of pyroroloquinoline quinone-dependent D-glucose oxidase respiratory chain of *Escherichia coli* with cytochrome *O* oxidase. J. Bacteriol. **169:** 205-209.

28. **McDonald, L. C., Fleming, H. P., Hassan, H. M.** 1990. Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. Appl. Environ. Microbiol. **56**: 2120-2124.

29. **McKay, A., Quilter, J., Jones, C. W.** 1982. Energy conservation in the extreme thermophile *Thermus thermophilus* HB8. Arch. Microbiol. **131:** 43-50.

30. **Olson, G. J.** 1991. Rate of pyrite bioleaching by *Thiobacillus ferrooxidans*: results of an interlaboratory comparison. Appl. Enviorn. Microbiol. **57:** 642-644.

31. **Peeples, T. L., Kelly, R. M.** 1993. Bioenergetics of the metal/sulfur oxidizing extreme thermoacidophile, *Metallosphaera sedula*. Fuel **72:** 1619-1624.

32. **Peeples, T. L., Kelly, R. M.** 1995. Bioenergetic response of the extreme thermoacidophile *Metallosphaera sedula* to thermal and nutritional stress. Appl. Environ. Microbiol. **61:** 2314-2321.

33. **Schäfer, G., Lübben, M., Anemüller, S.** 1990. Electron transport-phosphorylation and its catalysts in the archaebacterium *Sulfolobus acidocaldarius*. Biochim. Biophys. Acta **1018:** 271-274.

34. **Stouthamer, A. H., Bettenhaussen, C. W.** 1981. Influence of 2,4-dinitrophenol on the maxium specific growth rate and the respiration rate of chemostat cultures of *Paracoccus denitrificans*. FEMS Mirobiol. Lett. **10:** 33-35.

35. **Trent, J. D., Osipiuk, J., Pinkau, T.** 1990. Acquired thermotolerance and heat shock in the extremely thermophilic archaebacterium *Sulfolobus* sp. strain B12. J. Bacteriol. **172:** 1478-1484.

36. **Trent, J. D., Gabrielsen, M., Jensen, B., Neuhard, J., Olsen, J.** 1994. Acquried thermotolerance and heat shock proteins in thermophiles from the three phylogenetic domains. J. Bacteriol. **176:** 6148-6152.

37. **Trent, J. D.** 1996. A review of acquired thermotolerance, heat-shock proteins, and molecular chaperones in archaea. FEMS Microbiol. Rev. **18:** 249-258.

38. **Waldmann, T., Nimmesgern, E., Nitsch, M., Peters, J., Pfeifer, G., Müller, S., Kellermann, J., Engel, A., Hartl, F.-U., Baumerister, W.** 1995. The thermosome of *Thermoplasma acidophilum* and its relationship to the eukaryotic chaperonin TRiC. Eur. J. Biochem. **227:** 848-856.

39. **Yeh, T. Y., Godshalk, J. R., Olson, G. J., Kelly, R. M.** 1987. Use of epifluorescence microscopy for characterizing the activity of *Thiobacillus ferrooxidans* on iron pyrite. Biotechnol. Bioeng. **30:** 138-146.

**Table 4.1.** Protonophore (2,4-DNP) effects on specific leaching rates of *M. sedula.*

| | pyrite leaching rate (mg-$Fe^{3+}$/cell/hr) x $10^{10}$ | % increase |
|---|---|---|
| control | 7.0 | 0 |
| 5 μM DNP | 5.9 | -16 |
| 10 μM DNP | 7.8 | +11 |
| 25 μM DNP | - | - |

**Table 4.2.** Ionophore (nigericin) effects on specific leaching rates of *M. sedula.*

| | pyrite leaching rate (mg-$Fe^{3+}$/cell/hr) x $10^{10}$ | % increase |
|---|---|---|
| control | 1.9 | 0 |
| 1 μM nigericin | 4.5 | +135 |
| 2 μM nigericin | 3.1 | +63 |
| 5 μM nigericin | - | - |

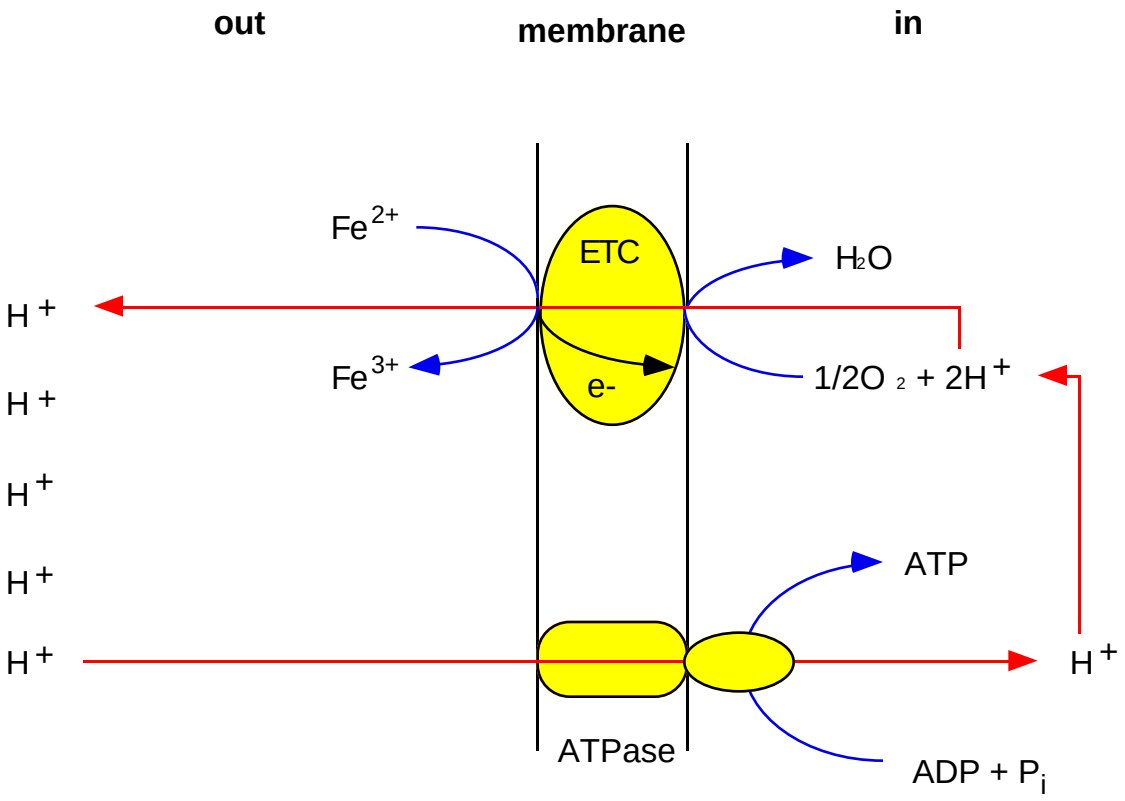
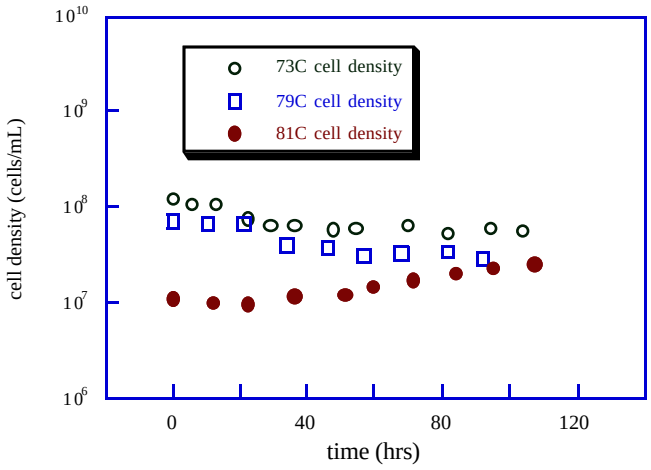
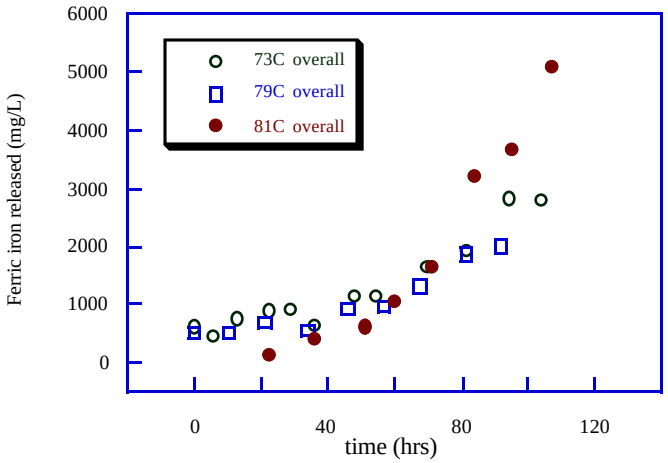


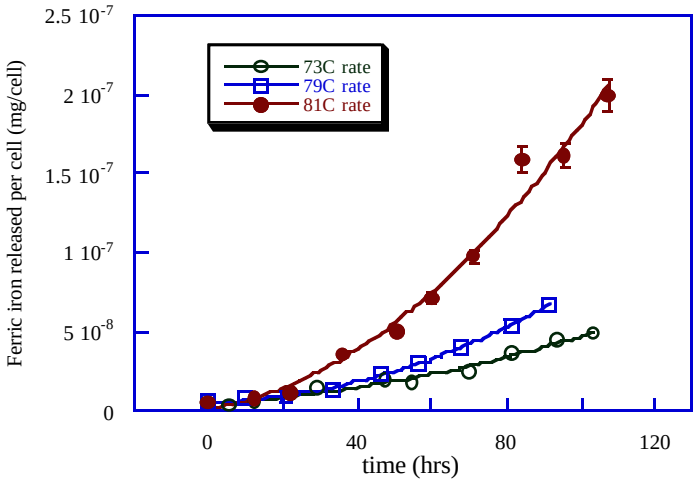

**Figure 4.1. Generalized chemiosmotic network for extreme thermoacidophiles under normal growth conditions.** This model (modified from Cobley and Cox, 1983) couples the generation of proton motive force to the phosphorylation of ATP and $Fe^{2+}$ oxidation. Outside protons are translocated to inside the cell via an ATPase channel and pumped out through the electron tranport chain (ETC). The electrons released from $Fe^{2+}$ oxidation are consumed by oxygen which acts as a terminal electron acceptor.


73C cell density
79C cell density
81C cell density
cell density (cells/mL)
time (hrs)

**a**


73C overall
79C overall
81C overall
Ferric iron released (mg/L)
time (hrs)

**b**




**c**

**Figure 4.2. Temperature effect on bioleaching kinetics by *M. sedula*.**

(a) Cell densities at 73°C (optimal growth temperature), 79°C (maximal growth temperature), and 81°C (heat shock) are shown, (b) Ferric iron released by *M. sedula* at 73°C, 79°C, and 81°C corrected for abiotic contributions at these temperatures, (c) Ferric iron released per cell per hour at 73°C, 79°C, and 81°C. Specific leaching rates (mg-$Fe^{3+}$/cell/hr x $10^{-10}$) were 4.1 (for 73°C), 6.6 (for 79°C), and 20.0 (for 81°C).

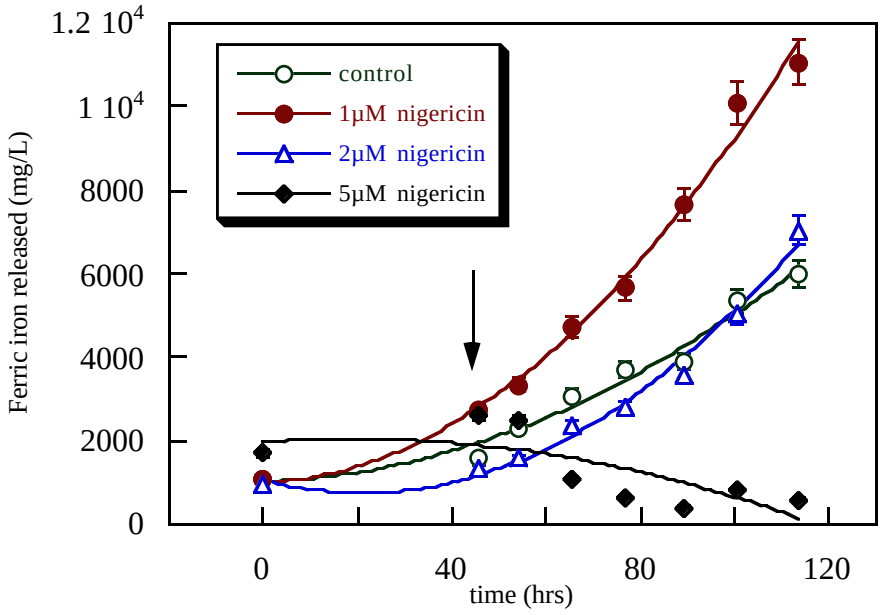
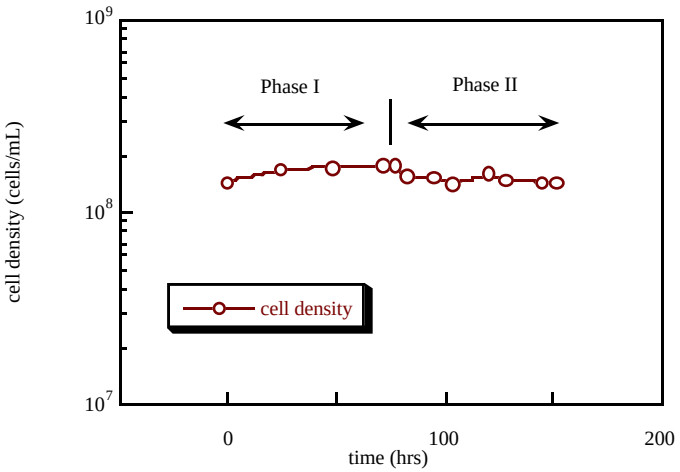
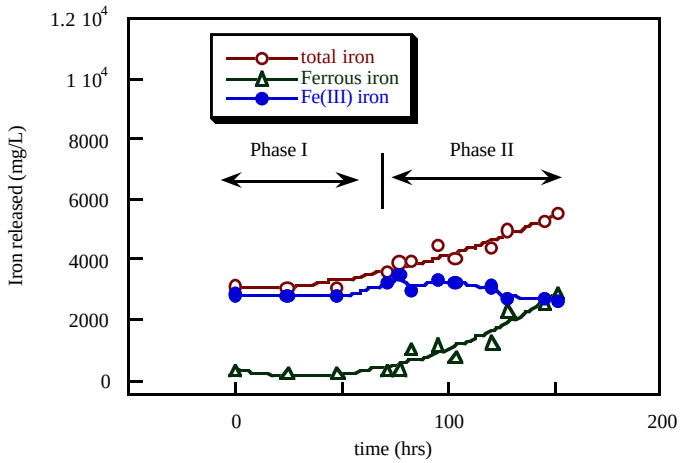



**Figure 4.3. Nigericin effect on overall ferric iron released by *M. sedula* in batch cultures.** The amount of ferric iron released in the solution (mg/L) for unstressed cells (control) and stressed (1 μM, 2 μM, and 5 μM nigericin) cells is shown. Arrow indicates the time at which nigericin was added.


$10^9$
$10^8$
$10^7$
cell density (cells/mL)
Phase I
Phase II
cell density
0
100
200
time (hrs)

**a**


1.2 $10^4$
1 $10^4$
8000
6000
4000
2000
0
Iron released (mg/L)
total iron
Ferrous iron
Fe(III) iron
Phase I
Phase II
0
100
200
time (hrs)

**b**

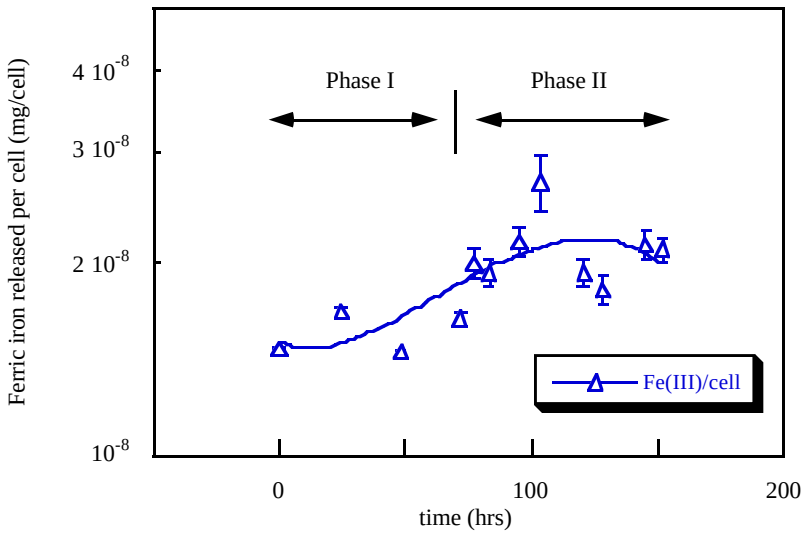
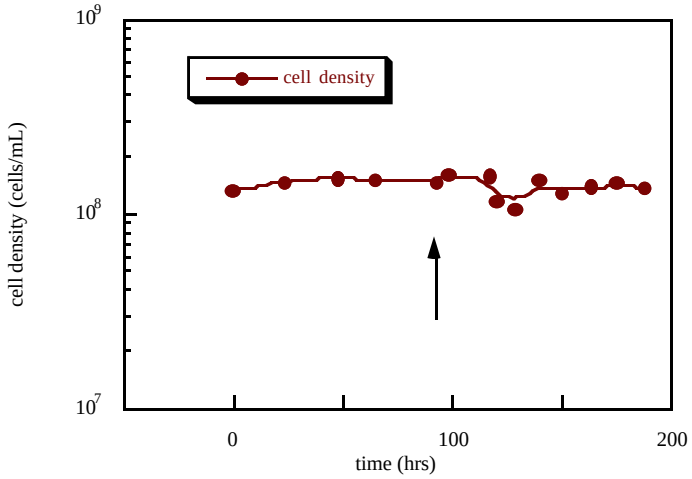
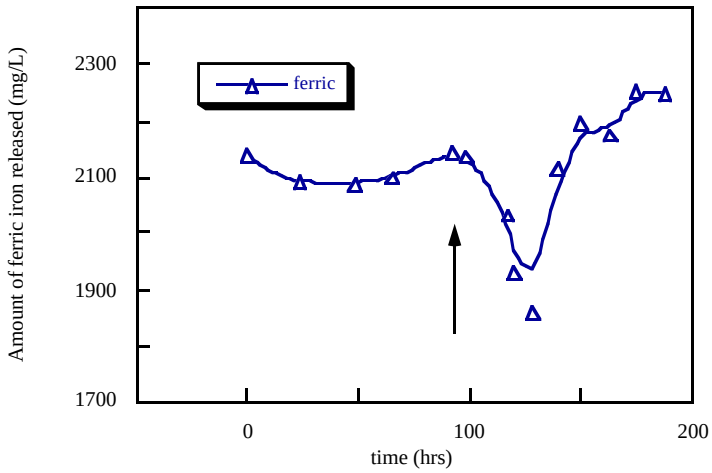



**Figure 4.4. *M. sedula* biooxidation kinetics in continuous culture in the presence of 1 µM nigericin.** (a) The cell density level (cells/mL) is shown before (phase I) and after (phase II) 1 µM nigericin addition, (b) Total, ferrous, and ferric iron released before (phase I) and after (phase II) nigericin addition, (c) Specific leaching rates for before (phase I) and after (phase II) nigericin addition.


cell density
cell density (cells/mL)
$10^9$
$10^8$
$10^7$
0
100
200
time (hrs)

**a**


ferric
Amount of ferric iron released (mg/L)
2300
2100
1900
1700
0
100
200
time (hrs)

**b**

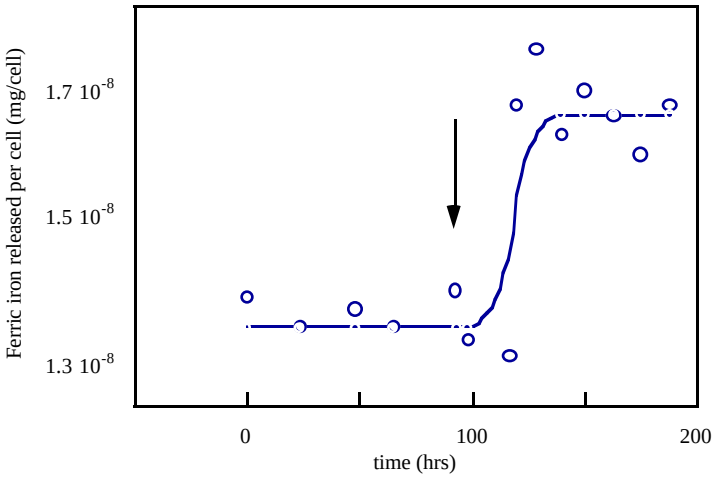



**Figure 4.5. Pulse injection of nigericin and its impact on *M. sedula* biooxidation kinetics in continuous culture.** (a) Cell density before and after 1 µM pulse of nigericin. Arrow indicates the time point when the pulse of nigericin was added, (b) Overall ferric iron released before and after 1 µM nigericin pulse, (c) Specific leaching rates before and after 1 µM nigericin pulse.

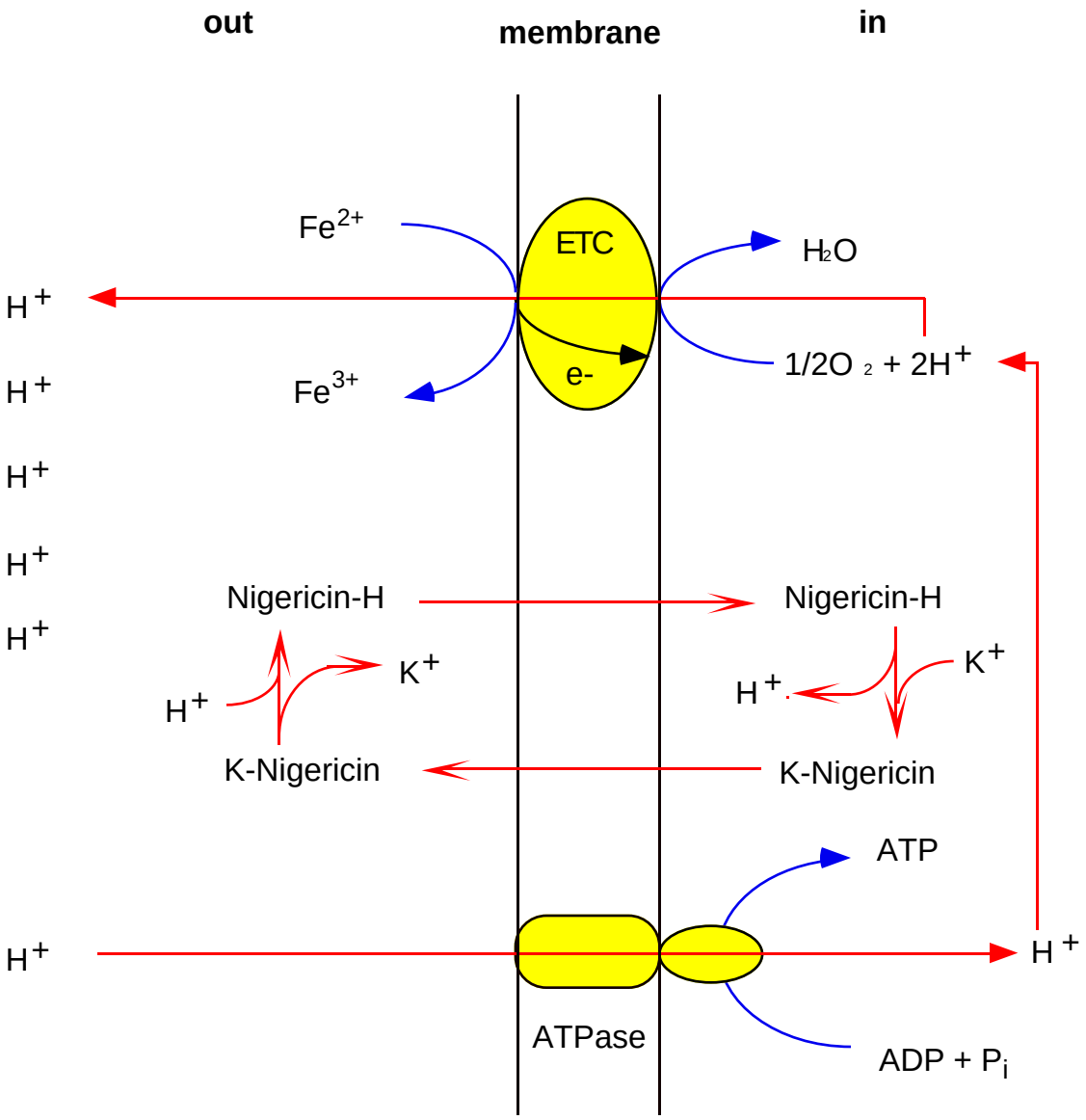



**Figure 4.6. Proposed mechanism for *M. sedula*'s chemiosmotic network under nigericin stress.** Nigericin catalyzes the antiport of $K^+$ for $H^+$, leading to a dissipation of the proton gradient. To re-establish the gradient, proton and electron translocations would need to increase, fueled by increased $Fe^{2+}$ oxidation rates.

# Chapter 5

## Biotechnological Applications and Future Research Directions of Thermoacidophiles and Hyperthermophiles

### Introduction

One of the principal goals of biochemical engineering research is to contribute to the discovery and/or identification of useful biocatalysts, in the forms of molecular compounds and/or whole-cell biotransformation, ultimately providing a foundation for commercial production. Microbial metabolic diversity offers a large genetic pool from which biochemical engineers can search for new as well as improved biocatalytic options. The archaeal domain of life contains promising microorganisms for whole-cell biotransformations and/or thermostable enzymes for potential industrial application. In the last fifteen years, considerable information has accumulated on the physiology and biochemistry of the extremophiles in archaea (Kates et al., 1993). Some aspects, such as bioenergetics, molecular biology and genetics, membrane lipids, etc., have been dealt with in various research groups, but the subject as a whole has not yet been addressed in comprehensive manner to be utilized by or applicable to the industrial settings. Knowledge of microbial physiology and cellular energetics is critical to understand the whole-cell biocatalysis and to make improvements on it.

The objective of this thesis project was to understand, pursue and apply characteristics of thermoacidophile physiology and energetics to seek a new metabolic strategy for bioleaching processes. Experiments were designed in such a way to simultaneously evaluate physiology and technical feasibility. A continuous culture was

utilized as a tool to provide conditions, such as various environmental or artificial factors (e.g. temperature and antibiotics), that affect biological system and biocatalytic efficiency of the cells.

**Thermoacidophiles as biocatalysts under energetically challenged states**

This project addressed several issues related to improving bioleaching rates by extremely thermoacidophilic microorganisms. In particular, the concept of improving biocatalysis through exploiting cellular stress response was investigated and evaluated. Since *Metallosphaera sedula* can utilize organic substrates for biomass generation and inorganic substrates (e.g. iron, iron pyrite) for energy sources, our goal was to minimize the cell population but maximize the leaching capacity of each cell. In other word, we sought a system that was the most biocatalytically efficient but the least biologically (or metabolically) efficient.

To date, mesoacidophiles, such as *Thiobacillus ferrooxidans*, are still primary choice of microorganisms in biooxdiation/bioleaching industry (Rawlings and Silver, 1995), even though thermoacidophiles like *M. sedula* appear to leach metals at higher rates than mesoacdiophiles (Huber *et al*., 1989). Limited understanding of thermoacidophile physiology and bioenergetics may be affecting the drive to scale-up and operate thermophile-based bioleaching processes. This is why this thesis project emphasized characterizing physiology, kinetics, and molecular behavior of cells under thermal and chemical stress conditions in addition to extreme growth environment that these cells face in their natural growth habitat. It is hoped that this framework will shed light on the relationship between the organism's metabolic energy sources and intracellular energy-requiring processes and eventually be useful for developing industrial bioleaching strategies.

Biological and biocatalytic characteristics of *M. sedula* in batch and continuous culture (Schicho et al., 1993), especially under nutrient limitation (Peeples and Kelly, 1993 and 1995), was studied previously. These studies showed that *M. sedula* favors mixotrophic growth, and higher ore leaching rate may be achieved through subjecting *M. sedula* to short periods of environmental stress. In addition, proton motive force was measured to understand the chemiosmotic network of these cells under different growth conditions in batch culture. The results showed that manipulating cellular stress response may be exploited to improve iron turn-over rates by the cells. These findings give us the first glimpse of a new strategy of improving iron leaching rate by the stressed cells.

In this project, changes in cellular energetics derived from stress response were monitored directly utilizing continuous culture. Operation of dual-limited continuous culture at a dilution rate of 0.04 $hr^{-1}$ facilitated the study of steady-state cellular responses under energetically-challenged phases, which could be analyzed for changes in growth patterns, behavior of molecular components (in particular, stress response proteins), and leaching capacity. First, the response of *M. sedula* to thermal stress in continuous culture was investigated. The cells were subjected under both abrupt and gradual temperature shifts to determine the responses of cell density levels and protein synthesis patterns. An abrupt temperature shift (short-term thermal stress) to 80.5$^{o}$C caused complete washout of the culture. On the contrary, when the culture was exposed to gradual (0.5$^{o}$C step increase) thermal stress, stable operation of culture could be maintained at temperatures of up to 81$^{o}$C, which is 2$^{o}$C higher than *M. sedula*'s maximum growth temperature. Operation at this temperature for 100 hours ('stressed phase') led to sevenfold lower steady-state cell densities than that observed at or below 79$^{o}$C. However, this also led to significant increase in the levels of a 66 kDa stress response protein (MseHSP60). This 66 kDa heat shock protein was found to be immunologically related to Thermophilic

Factor 55 from *Sulfolobus shibatae* (Trent et al., 1990). The study also revealed that acquired thermotolerance is only a transient phenomenon.

To examine the bioleaching capacity of *M. sedula* imparted by thermal stress, the cells' leaching potential at stressed phase (81°C) was compared to that at normal growth temperature ranges. Within the normal growth temperature range (70-79°C) at pH 2.0, upward temperature shifts resulted in bioleaching rates that followed an Arrhenius-like dependence. However, when the cells were subjected to thermal stress at 81°C, three to five times faster specific leaching rates ($Fe^{3+}$ released from iron pyrite/cell/hr) could be achieved by the thermally-challenged cells compared to cells at 79°C and 73°C, respectively.

The cellular response from the stress caused by poisoning the cells with uncouplers was monitored using both batch and continuous culture. When the protonophore 2,4-dinitrophenol (5-10 μM) was added to a growing culture of *M. sedula* on iron pyrite, there was little effect on the cell density level, but 25 μM levels proved to be toxic to the cells. Similar phenomena could be observed when various levels of ionophore Nigerian was added to the cell culture: the cell density was not affected under 1-2 μM of nigericin stress, but 5 μM levels completely arrested cell growth. No significant changes in cell density level could be noticed when 1 μM of nigericin was either held at constant level or injected as a pulse in the continuous culture. Protein distribution on two-dimensional gel revealed only minor changes in protein synthesis patterns (data not shown). Compared to cellular response under thermal stress, response to chemical stress was not as dramatic.

However, there were significant changes and increases in specific leaching rates by the uncoupler-poisoned cells. A significant stimulation in specific rate was observed when the cells were subjected to 1 μM nigericin (135% increase) and 2 μM nigericin (63%

increase). In addition, when 1 μM nigericin was added as a pulse to continuous culture, a 30% increase in specific iron oxidation rate could be observed for short intervals, suggesting a potential positive impact on leaching if periodic chemical stress is applied.

**Future research directions**

The challenge is then what kinds of tools and strategies one should use to interpret and analyze the physiological responses from cells under various growth conditions, such as those observed from *M. sedula* in continuous culture. The resulting information not only should help us to understand the fundamentals of cell physiology but also could be utilized in industrial applications more effectively. Microorganisms, which have limited abilities to change their environment or to escape, must alter their phenotype in order to meet changes in their growth conditions. Although only subsets of their genes are expressed in any given situation, these organisms make use of their entire genome. It is this ability to display differentiated gene expression that underlies the versatility of many microorganisms. Upon stressful or different growth conditions, such as nutritional limitation, heat shock or toxic poisoning, these organisms will respond in various pre-determined genetically-designed patterns, including *de novo* gene expression. Obviously, the investigation of differentially expressed genes, either individually or in groups, should be considered as a primary approach to understand these complex biological processes such as ‘stress response’ or ‘stress adaptation’. For this, the identification of those respective or functionally related genes is a key focus.

Efforts for completing total genome sequences of many cells and microorganisms are now underway. In the last two years, the genomes of three prokaryotes, *Haemophilus influenza* (1.8 Mb, 1,743 open reading frames (ORFs) [Fleischmann et al., 1995]), *Mycoplasma genitalium* (0.7 Mb, 470 ORFs [Fraser et al., 1995]), and *Methanococcus*

*jannaschii* (1.7 Mb, 1, 738 ORFs [Bult et al., 1996]), as well as the first eukaryotic genome *Saccharomyces cerevisiae* (14 Mb, 6,340 ORFs [Goffeau et al., 1996]), have been completed. Others in progress include the genome of the nematode *Caenorhabditis elegans* (100 Mb, ca. 20,000 ORFs) and the human genome project (3,000 Mb, ca. 50,000 ORFs). In addition, the genomes of *Staphylococcus aureus* (2.80 Mb, ca. 2,300 ORFs) and *Pyrococcus furiosus* (2.10 Mb, ca. 2,200 ORFs) are being sequenced to help in the development of new antibiotics and in order to find thermostable enzymes, respectively. However, completed genome sequence alone will not answer all questions, because the roles for more than 40 to 60% of these ORFs cannot be assigned a function based on homology sequence. Not only that, due to the complex nature of many adaptive responses, as we have seen in the case of *M. sedula* in chapters 2 and 4, the study of individual genes may not provide the necessary information to allow a holistic understanding of how the cell adapts to new conditions.

Another way to study gene is to look at differentially expressed genes at the level of translation instead of transcription. This new strategy, so called functional genomics or proteomics (Humphery-Smith et al., 1997), has gained support in the last few years both from academia and industry. The goal of this approach is to map and quantify all proteins expressed under different conditions within a cell, and then correlate them with their respective genes. This will allow us to decipher what unknown genes do, since proteins are gene-products, and how genes in overall interact together within an organism. To do this, variety of new methodologies are being developed, but in principle, the idea of systematically mapping and identifying the proteome arose over 20 years ago when the two dimensional (2D) gel electrophoresis was developed. The study of proteomics is, in general, based on the following scheme: First, proteins from total crude extracts will be separated by 2D gel electrophoresis as the basis for constructing proteome maps, and

second, mass spectrometry will allow automated identification of those proteins, and third, they will be matched with respective genes through established genome data banks (for reviews, see James, 1997; Fraser and Fleischmann, 1997; Figeys et al., 1996a and b; Garrels et al., 1997; Sazuka et al., 1997; VanBogelen et al., 1997; Fey et al., 1997; Humphery-Smith et al., 1997; Shevchenko et al., 1996; Yates et al., 1995; Mann and Wilm, 1994). Therefore, scanned images of 2D gels of cells in different physiological states can be analyzed using computer programs, and then this information will lead us to understand what goes on inside the cell at the metabolic level on a functional basis.

The study of physiological response of *P. furiosus*, grown with different glycosidic substrates, is currently in progress in our lab. Tools including continuous culture, 2D gel electrophoresis and mass spectrometry are being used in conjunction with the *P. furiosus* genome sequence data bank being generated at the University of Utah. So far, more than 90% of its genome sequence is completed. The initial plan is to detect differentially expressed levels of glycosyl hydrolases from this organism, such as α-glucosidase, ß-glucosidase, and many other sugar degrading proteins, under various types of sugar-limited conditions in continuous culture. These differentially expressed proteins on 2D gel electrophoresis will be identified by comparing them with genome data bank of its own as well as others. This can eventually lead to confirmation of known biosynthetic pathways and the discovery of new ones which can provide physiological insights to how this cell reorganize its genetic components to adapt to constantly changing growth conditions. Thus, by combining biochemical engineering, genomics, and analytical biochemistry, unprecedented understanding of the physiology of individual extremophiles can be obtained.

## Literature Cited


1. **Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, G. G. Sutton, J. A. Blake, L. M. Fitzgerald, R. A. Clayton, J. D. Gocayne, A. R. Kerlavage, B. A. Dougherty, J. F. Tomb, M. D. Adams, C. I. Reich, R. Overbeek, E. F. Kirkness, K. G. Weinstock, J. M. Merrick, A. Glodek, J. L. Scott, N. S. M. Geoghagen, J. F. Weidman, J. L. Fuhrmann, D. Nguyen, T. R. Utterback, J. M. Kelley, H. P. Peterson-Klenk, C. M. Fraser, H. O. Smith, C. R. Woese, and J. C. Venter.** 1996. Complete sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science **273:** 1958-1073.
2. **Fey, S. J., A. Nawrocki, M. R. Larsen, A. Görg, P. Roepstorff, G. N. Skews, R. Williams, and P. M. Larsen.** 1997. Proteome analysis of *Saccharomyces cerevisiae*: a methodological outline. Electrophoresis **18:** 1361-1372.
3. **Figeys, D., A. Ducret, I. J. R. Yates, and R. Aebersold.** 1996a. Protein identification by solid phase microextraction-capillary zone electrophoresis-microelectrospray-tandem mass spectrometry. Nat. Biotechnol. **14:** 1579-1583.
4. **Figeys, D., I. v. Oostveen, A. Ducret, and R. Aebersold.** 1996b. Protein identification by capillary zone electrophoresis/microelectrospray ionization-tandem mass spectrometry at the subfemtomole level. Anal. Chem. **68:** 1822-1828.
5. **Fraser, C. M., J. D. Gocayne, O. White, M. D. Adams, R. A. Clayton, R. D. Fleischmann, C. J. Bult, A. R. kerlavage, G. Sutton, J. M. Kelley, J. L. Fritchman, J. F. Weidman, K. V. Small, M. Sandusky, J. Fuhrmann, D. Nguyen, T. R. Utterback, D. M. Saudek,**

**C. A. Phillips, J. M. Merrick, J.-F. Tomb, B. A. Dougherty, K. F. Bott, P.-C. Hu, T. S. Lucier, S. N. Peterson, H. O. Smith, C. A. Hutchison III, and J. C. Venter.** 1995. The minimal gene complement of *Mycoplasma genitalium*. Science **270:** 397-403.

6. **Fraser, C. M., and R. D. Fleischmann.** 1997. Strategies for whole microbial genome sequencing and analysis. Electrophoresis **18:** 1207-1216.
7. **Garrels, J. I., C. S. McLaughlin, J. R. Warner, B. Futcher, G. I. Latter, R. Kobayashi, B. Schwender, T. Volpe, D. S. Anderson, R. Mesquita-Fuentes, and W. E. Payne.** 1997. Proteome studies of *Saccharomyces cerevisiae*: Identification and characterization of abundant proteins. Electrophoresis **18:** 1347-1360.
8. **Goffeau, A., B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Galibert, J. D. Hoheisel, C. Jacq, M. Johnston, E. J. Louis, H. W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin, and S. G. Oliver.** 1996. Life with 6000 genes. Science **274:** 546-567.
9. **Huber, G., C. Spinnler, A. Gambacorta, and K. O. Stetter.** 1989. *Metallosphaera sedula* gen. and sp. nov. represents a new genus of aerobic, metal-mobilizing, thermophilic archaebacteria. System. Appl. Microbiol. **12:** 38-47.
10. **Humphery-Smith, I., S. J. Cordwell, and W. P. Blackstock.** 1997. Proteom research: complementarity and limitations with respect to the RNA nad DNA worlds. Electrophoresis **18:** 1217-1242.
11. **James, P.** 1997. Breakthroughs and Views: Of Genomes and Proteomes. Biochem. Biophys. Res. Com. **231:** 1-6.
12. **Kates, M., D. J. Kushner, and A. T. Matheson.** 1993. The Biochemistry of Archaea (Archaebacteria). Elsevier. Amsterdam, The Netherlands.

13. **Mann, M., and M. Wilm.** 1994. Error-tolerant identification of peptides in sequence databases by peptide sequence tags. Anal. Chem. **66:** 4390-4399.

14. **Peeples, T. L., and R. M. Kelly.** 1993. Bioenergetics of the metal/sulfur oxidizing extreme thermoacidophile, *Metallosphaera sedula*. Fuel **72:** 1619-1624.

15. **Peeples, T. L., and R. M. Kelly.** 1995. Bioenergetic response of the extreme thermoacidophile *Metallosphaera sedula* to thermal and nutritional stress. Appl. Environ. Microbiol. **61:** 2314-2321.

16. **Rawlings, D. E., and S. Silver.** 1995. Mining with microbes. Bio/Tech. **13:** 773-778.

17. **Sazuka, T., and O. Ohara.** 1997. Towards a proteome project of cyanobacterium *Synechocystis* sp. strain PCC6803: Linking 130 protein spots with their respective genes. Electrophoresis **18:** 1252-1258.

18. **Schicho, R. N., K. Ma, M. W. W. Adams, and R. M. Kelly.** 1993. Bioenergetics of sulfur reduction in the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Bacteriol. **175:** 1823-1830.

19. **Shevchenko, A., O. N. Jensen, A. V. Podtelejnikov, F. Sagliocco, M. Wilm, O. Vorm, P. Mortensen, A. Shevchenko, H. Boucherie, and M. Mann.** 1996. Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels. Proc. Natl. Acad. Sci. USA **93:** 14440-14445.

20. **VanBogelen, R. A., K. Z. Abshire, B. Moldover, E. R. Olson, and F. C. Neidhardt.** 1997. *Escherichia coli* proteome analysis using the gene-protein database. Electrophoresis **18:** 1243-1251.

21. **Yates, J. R., J. K. Eng, A. L. McCormack, and D. Schieltz.** 1995. Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein data base. Anal. Chem. **67:** 1426-1436.

# APPENDICES

## Appendix 1

## A Kinetic Assessment of Pyrite Oxidation by *Metallosphaera sedula* Using Computer Simulation

The relative contributions of biological and chemical reactions to the total rate of pyrite oxidation in the presence of *Metallosphaera sedula* were studied on the basis of experimental work coupled with mathematical modeling developed by Vitaya et al. (1994). Following kinetic model was used:

**Rate of change of weight and specific area of pyrite**

$$\frac{dw_p}{dt} = -\frac{1}{Y_s}\mu_s X_s w_p A\left(1-\frac{X_s}{S_m}\right) - \frac{1}{14}\frac{k_f}{f_{Fe}} w_p A\left(1-\frac{X_s}{S_m}\right)[Fe^{3+}]$$

$$\frac{dA}{dt} = -\frac{8.9\times10^{-2}}{w_{p0}} A\frac{dw_p}{dt}$$

**Rate of change of cell density in solution**

$$\frac{dX_l}{dt} = \mu_{l\max}\frac{[Fe^{2+}]}{(K_s+[Fe^{2+}])}X_l - \frac{Aw_p k_a X_l(S_m - X_s)}{V} + \frac{Aw_p k_d X_s}{V}$$

**Rate of change of cell number per unit surface area of pyrite**

$$\frac{dX_s}{dt} = \mu_s X_s\left(1-\frac{X_s}{S_m}\right) + k_a X_l(S_m - X_s) - k_d X_s$$

**Rate of change of ferrous ions**

$$\frac{d[\mathrm{Fe}^{2+}]}{dt} = \frac{15k_f}{14V} w_p A\left(1-\frac{X_s}{S_m}\right)[\mathrm{Fe}^{3+}] + \frac{f_{\mathrm{Fe}}}{Y_s}\frac{\mu_s}{V} X_s w_p A\left(1-\frac{X_s}{S_m}\right) - \frac{1}{Y_{\mathrm{Fe}}}\mu_{l\max}\frac{[\mathrm{Fe}^{2+}]}{K_s+[\mathrm{Fe}^{2+}]} X_l$$

**Rate of change of ferric ions**

$$\frac{d[\mathrm{Fe}^{3+}]}{dt} = -\frac{1}{V} k_f w_p A\left(1-\frac{X_s}{S_m}\right)[\mathrm{Fe}^{3+}] + \frac{\mu_{l\max}}{Y_{\mathrm{Fe}}}\frac{[\mathrm{Fe}^{2+}]}{K_s+[\mathrm{Fe}^{2+}]} X_l$$

The numerical values of parameters were,

$w_{p0}$ = 2 (g) initial pyrite weight

$A_0$ = 0.0163 ($m^2 \cdot g^{-1}$) initial specific surface area of pyrite

$X_{l0} = 5.5 \times 10^{10}$ (cells$\cdot L^{-1}$) initial cell density in solution

$X_{s0} = 3.69 \times 10^7$ (cells$\cdot m^{-2}$) initial number of adsorbed cells per unit area of pyrtie surface

$[Fe^{2+}]_0$ = 2.5 ($g \cdot L^{-1}$) initial ferrous ion concentration

$[Fe^{3+}]_0$ = 1.0 ($g \cdot L^{-1}$) initial ferric ion concentration

$S_m = 5.87 \times 10^9$ (cells$\cdot m^{-2}$) maximum number of adsorbed cells per unit area of solid surface

$K_s$ = 0.04 ($g \cdot L^{-1}$) saturation constant of substrate

$k_a = 1.8 \times 10^{-13}$ ($L \cdot cell^{-1} \cdot hr^{-1}$) apparent adsorption coefficient

$k_d$ = 1.56 ($hr^{-1}$) apparent desorption coefficient

$k_f$ = 1.07 ($L \cdot m^{-2} \cdot hr^{-1}$) rate constant of chemical reaction between ferric ions and pyrite

V = 0.1 (L) volume of solution

$\mu_s$ = 0.005 ($hr^{-1}$) specific growth rate on pyrite surface

$\mu_{l\,max}$ = 0.01 ($hr^{-1}$) maximum specific growth rate in liquid phase

$Y_s = 1.6 \times 10^8$ (cells$\cdot$(g-$FeS_2$)$^{-1}$) growth yield of cells per gram of pyrite

$Y_{fe} = 2.0 \times 10^8$ (cells$\cdot$(g-$Fe^{2+}$)$^{-1}$) growth yield of cells per gram of ferrous ion

$f_{Fe}$ = 0.5 fraction of Fe in $FeS_2$

The computer algorithm (programmed with MATLAB software) is following:

```
function xdot=hanfn(t,x)
global wp0 A0 Xl0 Xs0 Fe20 Fe30 Sm Ks ka kd kf V MUs MUlmax
global Ys Yfe fFe
xdot(1)=-1/Ys*MUs*x(4)*x(1)*x(2)*(1-x(4)/Sm)-1/14*kf/fFe*x(1)*x(2)...
        *(1-x(4)/Sm)*x(6);
xdot(2)=-x(2)*8.9e-2/wp0*xdot(1);
xdot(3)=MUlmax*x(5)/(Ks+x(5))*x(3)-x(2)*x(1)*ka*x(3)*(Sm-x(4))/V...
        +x(2)*x(1)*kd*x(4)/V;
xdot(4)=MUs*x(4)*(1-x(4)/Sm)+ka*x(3)*(Sm-x(4))-kd*x(4);
xdot(5)=15*kf/V*x(1)*x(2)*(1-x(4)/Sm)*x(6)/14+fFe/Ys*MUs/V...
        *x(4)*x(1)*x(2)*(1-x(4)/Sm)-1/Yfe*MUlmax*x(5)/(Ks+x(5))*x(3);
xdot(6)=-1/V*kf*x(1)*x(2)*(1-x(4)/Sm)*x(6)+MUlmax/Yfe*x(5)*x(3)/(Ks+x(5));
```

```
tx=[0 45.5 54 65.5 77 89 100.5 113.5];
dx3=[5.02e7 8.11e7 1.04e8 1.10e8 1.18e8 1.35e8 1.41e8 1.39e8];
dx6=[1102.3 1587.8 2312.9 3391.4 3725.3 3884.0 5332.4 6002.9];
subplot(211);plot(tx,dx3.*1000,'o');
subplot(212);plot(tx,dx6./1000,'o');

global wp0 A0 Xl0 Xs0 Fe20 Fe30 Sm Ks ka kd kf V MUs MUlmax
global Ys Yfe fFe
wp0=2; A0=0.0163; Xl0=5.5e10; Xs0=3.69e7; Fe20=2.5; Fe30=1.0;
Sm=5.87e9; Ks=0.04; ka=1.8e-13; kd=1.56; kf=1.07; V=0.1;
MUs=0.005; MUlmax=0.01; Ys=5.0e8; Yfe=1.5e9; fFe=0.5;
t0=0; tf=120;
x0=[wp0 A0 Xl0 Xs0 Fe20 Fe30]';
[t1,x1]=ode23('hanfn',t0,tf,x0);
t=1:0.5:120;t=t';
xn=interp1(t1,x1,t);

subplot(211);plot(t,xn(:,3),tx,dx3*1000,'bo');ylabel('cell
density(cells/mL)');xlabel('time(hr)')
subplot(212);plot(t,xn(:,6),tx,dx6/1000,'bo');ylabel('Fe(III)
released(g/L)');xlabel('time(hr)')
```

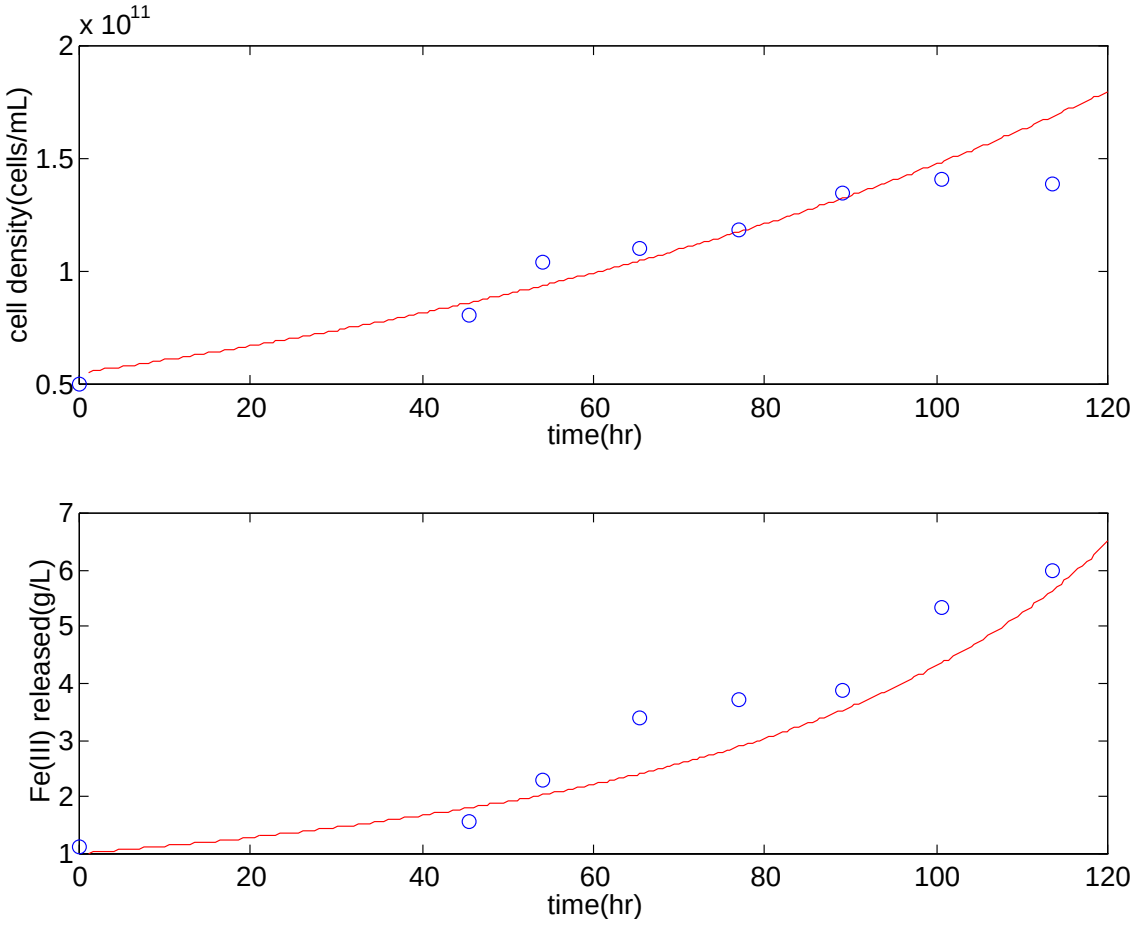



**Figure A.1.1. Time courses of the cell density and the ferric iron production during the dissolution of pyrite by *Metallosphaera sedula*.** Figure shows (A) cell density (cells/mL) vs. time (hr) and (B) amount of ferric iron released (g/L) vs. time. Solid line represents simulations from the kinetic modelings and circles (O) represent actual experimental data.

# Appendix 2

# Cultivation of Hyperthermophilic and Extremely Thermoacidophilic Microorganisms

Kristina D. Rinker, Chae J. Han*, Michael W. W. Adams, and Rober M. Kelly

(* principal author of the section 4 of this manuscript)

Running Title: Cultivation of thermophilic and hyperthermophilic microorganisms

Portions of this section is *in press* for:

ASM Manual of Industrial Microbiology and Biotechnology

Chapter 9

## 1.0 General Considerations

Over the past decade, there has been considerable interest in microorganisms growing at extremes of temperature, pressure, pH, and ionic strength (Table A.2.1). The unusual conditions under which extremophiles grow have necessitated the development of cultivation methodologies that, while based on conventional approaches to growing microorganisms, require modifications related to the organism's growth environment. As a result, many interesting schemes for cultivating extremophiles have been recently reported. These have been the basis for both the discovery of new biocatalysts and for investigations into physiological and metabolic issues associated with these microorganisms (1,2,3).

To date, most efforts on extremophilic microorganisms have centered on growth at high temperatures, for reasons stemming from the interest in thermostable biocatalysts (2,3), as well as the proposed placement of thermophilic organisms in evolutionary biology (139). While there has been some confusion concerning nomenclature for this group of organisms, for the purposes of this discussion, extreme thermophiles are considered to be those growing optimally at 75°C and above, and hyperthermophiles, a subset of extreme thermophiles with maximal growth at or above 90°C (Table A.2.2). Extreme thermophiles and, for that matter, many extremophilic microorganisms, are predominantly in the domain of Archaea (139); two exceptions among the extreme thermophiles are the bacterial genera *Aquifex* (29, 59) and *Thermotoga* (14,55), both of which are also hyperthermophilic (growth 90°C). Although it is clear that the constituent biomolecules of extreme thermophiles are designed for function at high temperatures, it remains to be seen how the physiological and metabolic characteristics of these organisms are related to the growth environment. This, of course, becomes important in developing strategies for isolation and subsequent cultivation of specific microorganisms.

## 2.0 Cultivation Strategies and Challenges

Planning and executing the cultivation of extremely thermophilic microorganisms under laboratory conditions brings into account a number of issues. These include the location from which the organism was isolated, media development, methodology, and equipment necessary to culture particular organisms on a specified scale. While many approaches for less thermophilic organisms apply, there are complications that arise because of the extremely high growth temperatures, e.g., the stability of media components, evaporation of fluid from high temperature baths, logistics for culture maintenance and storage, lack of biological equipment designed for high temperatures, and measurement of cell growth. For the isolation of specific gene products, recombinant DNA technology has provided a means of circumventing the need to cultivate extreme thermophiles, i.e., by the direct cloning and expression of genes derived from DNA present in environmental samples (107). However, this approach is not suitable for gene products difficult to express in an active form in mesophilic systems or for which screening assays are not available. Hence, any comprehensive evaluation of the scientific or technological potential of an extremely thermophilic microorganism necessitates its cultivation under laboratory conditions, in many cases on a scale that provides sufficient cellular material for subsequent evaluation of a particular biomolecule.

### 2.1 Availability and Handling of Extremely Thermophilic Microorganisms

A wide variety of geothermal sites have been examined for the presence of culturable extreme thermophiles. These range from continental hot springs, such as those found in Yellowstone National Park (23), to deep sea hydrothermal vent systems, such as those associated with mid-ocean ridges (12,119). Initially, the general availability of these

organisms was limited, forcing those interested in their study to visit geothermal sites and isolate their own. Fortunately, an ever-growing number of these microorganisms are now available from culture collections. In particular, DSMZ (Braunschweig, Germany) has an extensive stock of extreme thermophiles, including many hyperthermophiles, available for a modest fee with accompanying recommendations for growth media.

Methodology for isolation and handling of extreme thermophiles has been developed to some extent and is based on techniques used previously for less thermophilic microorganisms (12,106). Storage and handling techniques can be straightforward. Some anaerobic hyperthermophiles, for example, can be maintained in liquid growth media at room temperature for extended periods, although storage at 4°C limits the possibility of mesophilic contaminants arising from the handling procedures. While oxygen is usually toxic to anaerobic hyperthermophiles if exposed at or near growth temperatures (127), there are cases where such isolates have been recovered from oxygenated, refrigerated samples stored for five years (53). Lyophilization has been used successfully for some hyperthermophilic archaea, although there are also reports of limited storage times for organisms stored in this way (39). *Pyrococcus furiosus*, a hyperthermophile that grows optimally at 100°C (44), can be stored in dimethyl sulphoxide (DMSO) for extended periods in glass capillary tubes over liquid nitrogen. Plastic cryotubes have been found to be too permeable to oxygen and, hence, ineffective for extended storage (32,39).

Plating techniques have been reported for *Thermotoga* species (35,51), *Aquifex pyrophilus* (59), *P. furiosus* (103), and *Sulfolobus* strains (48), presenting the possibility of genetic manipulation of these organisms. Methodology to carry out mutagenesis experiments for hyperthermophilic pyrococci has been developed and used to produce a uracil auxotrophic mutant of *Pyrococcus abyssi* (136). It should be mentioned that, in many cases, extreme thermophiles can be very difficult to develop into a pure, laboratory

culture, whether from isolation efforts or even from storage. This is especially true for some of the hyperthermophilic, sulfur-reducing chemolithotrophs, such as *Pyrodictium occultum* (93), which grow to very low cell densities, and are highly sensitive both to shearing forces and various metals (Kelly, unpublished observation). The pioneering contributions of Karl Stetter of the University of Regensburg, Germany, Holger Jannasch of the Woods Hole Marine Biology Laboratory, Thomas Brock of the University of Wisconsin, and others who have isolated a number of extreme thermophiles and developed media and protocols for their laboratory cultivation, are best appreciated by those who endeavor to grow and study these often fastidious microorganisms.

## 2.2 Media Development

Considerable effort has been expended into the development of growth media for the isolation of extreme thermophiles, and subsequently for their cultivation in laboratory settings (12,23,106). For aerobic thermoacidophiles belonging to the order *Sulfolobales*, media containing complex carbon sources, such as yeast extract and tryptone, and chemolithotrophic substrates, such as elemental sulfur, ferrous iron or iron pyrite, have been used in the isolation efforts (21,22). Although there has been much debate concerning the direct or indirect dissolution of iron pyrite by thermoacidophiles (34,133,138), it is clear that these cells attach to such solid substrates (138). The importance of organic as compared to inorganic carbon and energy sources for these organisms is often unclear, with some isolates appearing to grow as mixotrophs (96,97).

There are variations within the extreme thermoacidophiles in terms of nutritional requirements and growth physiology. Although most are aerobes, members of the genus *Acidianus* are facultative anaerobes that grow anaerobically by reducing elemental sulfur with $H_2$ (118). Several members of the *Sulfolobales* are facultative or obligate

heterotrophs that grow on sugars, yeast extract, and peptone (48,49,126,128), and are known to have a modified (non-phosphorylated) Entner-Doudoroff pathway (41). However, species of *Metallosphaera (M. sedula* and *M. prunae*) do not grow on sugars (46,58), and *M. prunae* does not utilize ferrous sulfate, which is somewhat unusual among the extreme thermoacidophiles (46). Members of the *Sulfolobus, Acidianus,* and *Metallosphaera* genera also grow chemolithoautotrophically by aerobic oxidation of $S^o$ (and $S^{2-}$) and $H_2$ (48,58,118). In particular, *S. metallicus* grows strictly by chemolithoautotrophy; no growth occurs on beef extract, casamino acids, peptone, tryptone, yeast extract, arabinose, fructose, or other sugars (56). *Desulfurolobus ambivalens,* an obligate chemolithoautotroph, has been found to contain several plasmids (143). In our laboratory, many of the extreme thermoacidophiles (*A. brierleyi, A. infernus, S. acidocaldarius, S. solfataricus, S. shibatae, M. sedula*) grow on the same medium, with some minor differences: *Acidianus* strains and *S. acidocaldarius* grow better when trace elements are added. We have found *M. sedula*'s nutritional requirements (see section 4) to be the simplest, which may explain why it is one of most effective leaching organisms (37).

As indicated in Table A.2.2, virtually all of the currently known hyperthermophiles are obligate anaerobes, including methanogens, sulfur-respiring chemolithotrophs, and both sulfur-respiring and fermentative heterotrophs. In contrast to their mesophilic cousins, only hydrogenotrophic hyperthermophilic methanogens have been identified with no evidence for acetate utilization within this group. Sulfur-reducing chemolithotrophs, such as *Pyrodictium brockii*, grow by the reduction of sulfur with molecular hydrogen. Small amounts of complex substrates, e.g., yeast extract, may be required or stimulate growth of these organisms (98, 121, 124). For unknown reasons, efforts to eliminate organic carbon sources entirely from these chemolithotrophs often result in very poor

growth (Schicho and Kelly, unpublished data). Electron acceptors other than sulfur, such as sulfate (63,125) and nitrate (134), can be used by some hyperthermophiles, however, such physiologies have been encountered to only a limited extent.

By far most attention among the extreme thermophiles has been directed toward hyperthermophilic, fermentative anaerobes. These organisms have been isolated from various marine hydrothermal sources using peptides and/or sugars (oligosaccharides) as carbon and energy sources. While there have been several successful efforts to develop defined media for members of this group (16, 52,53,62,75,99,103,104,135), the fact that most also require sulfur or polysulfides (15) to grow complicates interpretation of bioenergetic patterns.

Of the fermentative hyperthermophiles, the archaea *P. furiosus* (44) and *Thermococcus litoralis* (90), and the bacterium *Thermotoga maritima* (55) are the best studied, probably because they were among the first in this group to be isolated and they grow well in the absence of reducible sulfur compounds (65,68,117). *T. maritima* grows with glucose as a carbon and energy source, apparently carrying out glycolysis by a conventional Embden-Meyerhoff pathway (117). *P. furiosus* (103) and *T. litoralis* (104) grow on sulfur-free defined media containing a number of single amino acids. However, *P. furiosus* requires an oligosaccharide in defined medium, while *T. litoralis* grows without the addition of any sugars. In contrast to *T. maritima*, *P. furiosus* cannot utilize a monosaccharide as the carbon and energy source. Disaccharides such as maltose enhance growth (27) and appear necessary in the absence of reducible sulfur compounds (18,114). *T. litoralis* possesses a high affinity maltose transport system (140) and *P. furiosus* shows a regulatory response induced by maltose (108). Both organisms contain several glycosyl hydrolases capable of breaking down starch to glucose (26,27). The inability of these

organisms to grow on simple sugars, such as glucose, is interesting in light of reports that monosaccharides are taken up by resting cells of *T. litoralis* and *P. furiosus* (132).

Media development must also take into account some additional features of hyperthermophilic growth physiology. For example, the growth of *P. furiosus* and several hyperthermophiles examined to date is stimulated by tungsten, an unusual requirement for biological systems (70). *P. furiosus* increases in cell density three-fold in continuous culture by low concentrations of tungsten (115). Hyperthermophilic heterotrophs typically accumulate acetate as the predominant liquid phase product (127), thereby reducing pH and, hence, growth yields. The adverse effects of acetate build-up may be partly due to uncoupling reactions, in addition to the resulting drop in pH, as has been seen in *Clostridium thermoaceticum* (11). High levels of acetate inhibit growth due to the drop in the internal pH of the cell brought about by the influx of acid. However, this may not be a significant problem in all cases since *P. furiosus* grows well in filtered spent media, suggesting a lack of product inhibition (16). Recent work has also revealed the presence of compatible solutes in hyperthermophiles, whose formation may be induced by environmental conditions (79).

In addition to acetate, alanine is a significant by-product of heterotrophic hyperthermophiles. This is especially true if molecular hydrogen is allowed to accumulate in the medium, i.e., if it is not removed by sparging with an inert gas. Significant alanine excretion in the presence of ammonia has been found for *Thermococcus profundus* when grown on pyruvate- or maltose-based media (71,72). In *P. furiosus*, alanine production increases with the $NH_4Cl$ concentration and decreases in the presence of sulfur or in co-culture with *Methanococcus jannaschii*, a hyperthermophilic methanogen (66). Propionate, isobutyrate, isovalerate, and butyrate have also been detected by gas chromatography at

low levels in *P. furiosus* cultures (114). The presence of a methanogen stimulates organic acid production in deep-sea heterotrophic isolates growing at 85°C (31).

Central metabolic pathways have been well studied in *P. furiosus* and the available evidence suggests that the same metabolic routes are also present in *T. litoralis* and related organisms (4). So far these studies have revealed some surprising results. In contrast to the conventional glycolytic pathway in the bacterium *T. maritima,* the heterotrophic archaea metabolize sugars via a modified Embden-Meyerhof pathway. This route contains ADP- rather than ATP-dependent hexose kinases (67) and the novel enzyme glyceraldyde-3-phosphate ferredoxin oxidoreductase (GAPOR) replaces the expected NAD-dependent dehydrogenase (87). Interestingly, GAPOR contains at its catalytic site the metal tungsten, an element rarely used in biological systems, and this, at least in part, explains why the addition of tungsten stimulates growth (70). The main product of carbohydrate catabolism is acetate. This is generated by acyl coenzyme A synthetase (ACS), which converts acetyl CoA, ADP and phosphate into ATP and acetate in a single step. ACS is yet another unusual enzyme, which has so far been found only in archaea (112).

The heterotrophic archaea also contain unusual pathways for peptide metabolism. The first step involves various transaminases which convert peptide-derived amino acids to their corresponding 2-ketoacid derivatives. These are oxidized to the corresponding coenzyme A ester by four different types of 2-ketoacid oxidoreductase which are specific for pyruvate, 2-ketoglutarate, branched chain or aromatic 2-ketoacids (4). Interestingly, no known microorganism contains more than two enzymes of this type, with the notable exception of species of *Pyrococcus* and *Thermococcus*. The various coenzyme A esters that these enzymes generate are used to conserve energy in the form of ATP by two different isoenzymes of ACS which differ in their substrate specificities (77). Between them, they produce various branched chain and aromatic acids, as well as acetate, and

these, like acetate, are excreted into the medium. This explains the presence of organic acids, such as isovalerate, isobutyrate, and phenyl acetate, in spent media after growth of various *Pyrococcus* and *Thermococcus* species.

As mentioned above, an unique aspect of metabolism by these hyperthermophilic archaea is their requirement for tungsten. Virtually all other life forms utilize the analogous element, molybdenum, with only a few microorganisms additionally able to use tungsten (70). Yet, *Pyrococcus* and *Thermococcus* species are obligatorily dependent upon tungsten and cannot utilize molybdenum (88). They uniquely contain three different types of tungstoenzyme; GAPOR (involved in glycolysis), aldehyde oxidoreductase (AOR; 85), and formaldehyde oxidoreductase (FOR; 86). The latter two enzymes are involved in oxidizing various aldehydes, which are generated as side-products by the four 2-ketoacid oxidoreductases, to the corresponding acid. Hence, two tungstoenzymes, AOR and FOR, produce acetate and various organic acids in spent growth media, in addition to those generated by the ACS isoenzymes (4).

## 2.3 Cultivation Equipment and Strategies

The growth of microorganisms at elevated temperatures has both advantages and disadvantages when compared to the cultivation of mesophiles. One potential advantage is the reduced risk of contaminant growth at high temperatures, although care must be taken to maintain media (sometimes containing high levels of yeast extract and oligosaccharides) under sterile conditions prior to utilization. Other favorable aspects of high temperature cultivation include reduced viscosity of fermentation media and increased rates of gas-liquid mass transfer. The latter advantage may however be offset in the growth of aerobic extreme thermoacidophiles by the concomitant reduced solubility of oxygen at elevated temperatures. It well may be that the reduction in dissolved $O_2$ levels with increasing

temperature is consistent with the growth requirements of extreme thermoacidophiles. For example, growth of the thermoacidophilic archaeon *Sulfolobus acidocaldarius* is inhibited at enhanced dissolved oxygen levels applied by over-pressurization with air (129).

For anaerobic hyperthermophiles, only autotrophic species utilize gaseous substrates (e.g., $H_2$ and $CO_2$), while heterotrophs in this group typically produce $H_2S$, $H_2$ and $CO_2$ as by-products (127). Since many hyperthermophiles have relatively high growth rates, gas-liquid or liquid-gas mass transfer may be a factor under certain circumstances. The growth of *Pyrodictium brockii*, a chemolithotrophic hyperthermophile growing by $H_2$-S° autotrophy, is limited by gas-liquid mass transfer under quiescent conditions (94). This cannot be overcome by increased agitation or high rates of gas sparging, since *P. brockii* is sensitive to even moderate shear rates (98). Other hyperthermophiles, such as members of the genus *Pyrococcus*, are also shear-sensitive (75,111). The release of inhibitory by-product gases, especially $H_2$, is thought to be important in the growth of hyperthermophilic, heterotrophic anaerobes (127), and has been dealt with by using high inert gas sparging rates (102) and use of dialysis reactors (75). Alternatively, since these heterotrophs typically reduce polysulfide to $H_2S$ during growth, the presence of sulfur may serve as a mechanism by which $H_2$ inhibition is reduced or averted. The consequence, of course, is the formation of large amounts of corrosive and toxic $H_2S$, creating a new set of problems involving the durability of cultivation equipment and safety of laboratory personnel.

Key considerations in the cultivation of aerobic and anaerobic extreme thermophiles are low cell density and biomass yields (64). While these may be related to, in general, a poor understanding of nutritional requirements, it may be characteristic of this group of microorganisms. There have been reports of cell densities in excess of $10^9$ cells/ml being obtained (75,102,103, Rinker and Kelly, unpublished data), but this level

of growth is not typical and usually comes after an extensive effort at optimizing media, cultivation strategy, and equipment. In fact, even the most experienced hands at cultivating extremely thermophilic organisms have witnessed problems in maintaining cultures over a prolonged period of time, in addition to observing erratic patterns in growth rates and cell density levels within a particular set of experiments.

The earliest efforts to cultivate extreme thermophiles attempted to mimic the characteristics of their peculiar habitats. In some cases, this involved sophisticated high pressure, high temperature bioreactors that were used to simulate and explore the effects of deep sea hydrostatic pressure on growth (e.g., 36,64,83,100). At pressures of several hundred atmospheres, growth was stimulated for certain organisms optimal growth temperatures were increased in some cases by several degrees (83). Ceramic-lined fermentors designed for operation with slight overpressures to prevent boiling at temperatures above 100°C were also utilized (57). Such systems were built to withstand the potentially corrosive attack by $H_2S$, a product of anaerobic growth, and by $H_2SO_4$, which is produced by thermoacidophiles.

The apparent need for bioreactors capable of high pressure, high temperature operation, as well as being resistant to corrosion, initially limited the number of laboratories with the capability to study extreme thermophiles. This was especially true where significant amounts of biomass were needed for protein purification. However, strategies to minimize equipment damage by corrosion have been developed and high pressure has not been found to be essential for the growth of many high temperature, deep sea isolates. Now, fermentations of up to 500 liters (working volume) have been performed for various hyperthermophilic heterotrophs isolated from both shallow and deep sea environments, both in the presence and absence of elemental sulfur. Yields of over 1 kg (wet weight) per 600 liter run have been reported, facilitating the purification of

a growing list of thermostable proteins (1). Stainless steel fermentors have been used over long periods of time without significant corrosive damage, if proper care was taken with welds during equipment fabrication and the system was meticulously cleaned after a particular run. Alternatively, biomass can be generated using continuous culture. This approach is also useful for determining growth yields and providing a steady-state environment to investigate the effect of changes in the cellular environment (in nutrients, temperature, etc.) on cell physiology (102,113-116). For example, a continuous culture of the hyperthermophile *P. furiosus* with a 1 liter liquid volume operating at a dilution rate of 0.4 $hr^{-1}$ produces almost 10 liters of biomass per day from which approximately 5 g (wet weight) can be harvested. Under some conditions, *P. furiosus* can be cultivated at dilution rates up to 0.8 $hr^{-1}$ (25). In a gas-lift reactor, *P. furiosus* has been found to have an optimal biomass yield of 1.5 g-cells/h-liter at a dilution rate of 0.4 $hr^{-1}$ (102). Dialysis reactors have also been utilized for biomass production with reported yields of 2.6 g/l for *P. furiosus* and 114 g/l for *S. shibatae* (75).

In some cases, increases in cell densities can be achieved by co-culturing hyperthermophilic heterotrophs and methanogens (17). For a rapidly growing hyperthermophilic methanogen, *M. jannaschii*, and a hydrogen-inhibited heterotroph, *T. maritima*, very close association of the two species has been noted, with a 5-fold enhancement of final cell densities for the heterotroph (89). Since the methanogen is often at a 50- to 100-fold lower concentration than the heterotroph, this may be a convenient route for obtaining cell mass for protein purification purposes. A possible problem is the significant amount of methane formed during cell growth, which could be hazardous.

While improving biomass yield is an on-going effort with many extreme thermophiles, it is also important to be able to create physiological environments that lead to optimal production of an enzyme or other metabolites. This can only be achieved with

some understanding of the connection between growth physiology and the regulation of genes encoding proteins of interest. Unfortunately, this type of cause and effect relationship is not yet well understood for many extreme thermophiles. As this information becomes available, better strategies for producing biomolecules of interest in particular extreme thermophiles will evolve.

## 3.0 Growth of Hyperthermophilic, Heterotrophic Anaerobes: *Thermococcus litoralis*

To illustrate cultivation procedures and protocols for hyperthermophilic, heterotrophic anaerobes, *T. litoralis* (13,90), with an optimal growth temperature of 88°C, will be used as an example. *T. litoralis* was isolated from shallow geothermal waters near Vulcano Island, Italy. It is a coccus with an approximate diameter of 1 mm. Unlike many heterotrophic hyperthermophiles, *T. litoralis* grows well in the presence or absence of elemental sulfur (104). Several proteins have been purified and characterized from *T. litoralis*, including DNA polymerase (73), amylopullulanase (27), ferredoxin (30) and formaldehyde ferredoxin oxidoreductase (86).

### 3.1 Media Formulation

Several different growth media have been used for cultivation of *T. litoralis*. These range from complex media, such as that used in its isolation, to a sulfur-free, defined medium, RDM (104). Tables A.2.3 through 5 summarize the composition of media commonly used. In development of the RDM media (104), alanine, asparagine, glutamine, and glutamate are the only naturally occurring amino acids that could be eliminated without adverse affects to either the cell density or growth rate of the organism. Glycine, isoleucine, threonine, and valine have the strongest adverse effect on growth

rates and cell densities when omitted from a medium containing the other 19 common amino acids at 0.1 g/l each, maltose at 2 g/l, and 0.05 g/l yeast extract. However, only glycine increases cell densities, when added to a defined medium containing 16 amino acids (minus Ala, Asn, Gln, and Glu) at 0.05 g/l. The concentration of amino acids significantly affected cell densities. In media containing 5 g/l maltose, an increase in the concentrations of the 16 amino acids from 0.05 to 0.1 g/l each results in stimulation of cell densities by more than two-fold, while a further increase to 0.2 g/l increases cell densities 3-fold. The yeast extract requirement can be eliminated when 0.01 g/l adenine and uracil are included, with cytosine able to replace adenine.

**Media containing sulfur**. When sulfur is needed in the media, one of several forms of sulfur may be chosen. Most simply, elemental sulfur or flowers of sulfur can be added directly to the culture, after sterilizing at 100°C for 24 hours. Since it has been established that several hyperthermophiles grow on soluble polysulfides (15,105), this form can be used as well. Sulfur can also be added as colloidal suspensions (113,114,116); this is particularly useful if sulfur is being fed continuously to a fed-batch or continuous culture. Since colloidal sulfur preparations require the use of concentrated acid and generate toxic fumes, gloves and safety glasses should be worn and work should be performed in a chemical fume hood. Briefly, the method for generating colloidal sulfur is as follows:

1. Prepare two solutions: solution A (500 ml) contains 64 g of $Na_2S \cdot 9H_2O$ in $H_2O$, and solution B (500 ml) contains 36 g of $Na_2SO_3$ in $H_2O$.
2. Solution B (15 ml) is added to solution A and 30 ml concentrated $H_2SO_4$ is added to the remaining solution B.
3. 80-100 ml dilute $H_2SO_4$ (add 15 ml acid to 100 ml H2O) is slowly added to solution A until the solution remains turbid.

4. Over a period of 0.5 to 3 minutes, solution A is poured into solution B with vigorous stirring, after which the mixture is left to stand for 1 hr. The volume is brought to 2 liters with water and settling of the sulfur is promoted by the addition of 20 g NaCl.
5. After the suspension stands for at least four hours, the solution above the colloid is removed by aspiration and water and NaCl are again added, thereby initiating a washing procedure which is repeated twice more.
6. After the washing, the colloid should be resuspended in 1 liter of $H_2O$. This suspension is stable for several months. Before use, the colloid is diluted 10 to 20 times in $H_2O$ to a turbidity of $A_{850}$ = 1.25, which decreases its storage time to 1 month. This absorbance corresponds to approximately 85 mg elemental sulfur per liter (113).

## 3.2 Culture Preservation

The most common storage procedure is to leave liquid cultures at room temperature or at 4°C, where they may be viable for several months or longer. Many hyperthermophiles have been found to tolerate oxygen better when maintained well below growth temperatures. For longer storage, lyophilization can be used; however, the equipment for doing this is not available to all laboratories. An alternative method is to grow 10 ml of the culture in a Hungate tube and add sterile, anaerobic glycerol to 10% (v/v). The cultures can then be stored at -20°C or –70°C for years. This procedure has worked successfully in our laboratory for *T. litoralis*, *P. furiosus*, and *Thermotoga neapolitana*.

### 3.3 Batch Cultivation in Serum Bottles

For media development, culture maintenance, culture storage and scale-up, *T. litoralis* (and other heterotrophic hyperthermophiles) can be grown in 125 ml serum bottles, with 50 to 100 ml liquid volume. The procedure used has been adapted from the Hungate technique (61,82) for cultivation of anaerobic microorganisms. Although ovens can be used to maintain culture temperatures, an alternative is a static or shaking bath filled with a heat transfer medium, such as silicon oil. This provides easy access to culture bottles for sampling at intermediate times, without affecting temperature control. The recommended procedure is as follows:

1. Prepare media and sterilize by filtration or autoclaving. Some components that cannot be autoclaved, such as saccharides and vitamins, must be filter-sterilized and added separately to the sterile media.
2. Autoclave serum bottles and butyl rubber stoppers (Fisher Scientific, Pittsburg, PA).
3. Prepare a reducing agent, such as $Na_2S \cdot 9H_2O$, filter-sterilize, and store under $N_2$. This solution should remain stable as long as it is kept under an $O_2$-free atmosphere.
4. Add appropriate amount of media to the serum bottle using sterile technique. Add sulfur at this point if required.
5. Incubate the bottle at 98°C for 30 minutes.
6. Remove the bottle from the incubator and immediately sparge with high purity $N_2$ to remove oxygen. If the stopper is slotted, it will rest on the top of the bottle with the pipet in the slot. If a plug stopper is used, place it to the side for the sparging procedure. Masterflex tubing size 15 (Cole-Parmer, Vernon Hills, IL) is used to connect the cylinder regulator to a sterile glass pipet which is inserted into the bottle.
7. Add $Na_2S \cdot 9H_2O$ and continue to sparge until the rezasurin turns clear. The inoculum can be added at this point if desired, or added through a syringe at a later time. Place a

rubber stopper on the bottle. Quickly remove the pipet, affix the stopper, and secure an aluminum seal using a hand crimper (Fisher Scientific, Pittsburgh, PA). Seals can be removed with a hand decapper (Fisher Scientific, Pittsburgh, PA).

It is strongly recommended to use butyl rubber stoppers due to the oxygen permeability of other types of rubber. Sterile glass syringes, preflushed with anaerobic liquid, are commonly used for inoculation. The amount of reducing agent necessary is dependent on the medium formulation and the organism to be cultivated. Typically, 500ml of 50 g/l $Na_2S \cdot 9H_2O$ is added to 50 ml of the medium, however, 100ml of 50 g/l $Na_2S \cdot 9H_2O$ is added to the medium RDM, used for cultivation of *T. litoralis*. In some media, addition of reducing agent may cause significant precipitation of the medium components. If precipitation occurs, reduce the amount of reducing agent added.

Epifluorescence microscopy is typically used to quantify cell growth, especially in cultures containing precipitates (54, 142). The following procedure is recommended:

1. A cell sample (1 ml) is fixed in 100 ml 2.5% glutaraldehyde for at least 5 minutes.
2. An appropriate volume of sample or dilution is added to 200 ml of 1 g/l acridine orange and sterile water to make 5 ml.
3. A black, 0.2 mm, 25mm, polycarbonate filter (Poretics, Livermore, CA) is soaked in water for 2-5 minutes. The filter is then placed on a pad on a 15 ml vacuum tower (Fisher Scientific, Pittsburgh, PA) and well-mixed samples are vacuum-filtered.
4. The filter is then removed, placed on a glass slide, covered with a drop of Type A immersion oil (Cargille Laboratories, Inc., Cedar Grove, NJ), a No. 1 cover slip, and another drop of oil. This allows viewing under a microscope adapted for epifluorescence (HBO 100W, 440-490 nm excitation) with a 100X oil-immersion objective.

5. Count the cells in the 10x10 grid composed of 1x1mm squares in the eye-piece of the microscope. Ten randomly chosen grids with approximately 30 cells per grid should be counted which gives a cell density (cell density [cells/ml]= [{average # of cells/grid}{Dilution factor}{1.2E4}]/volume of cells) with an approximate standard deviation of 10% for a 95% confidence interval (104).

## 3.4 Continuous Cultivation

An advantage of *T. litoralis* cultivation, and for most extremophiles, is the lowered risk of contaminant growth at elevated temperatures. This allows utilization of continuous and semi-continuous methods for cultivation over extended periods of time (123). Details for this approach were first discussed for *P. furiosus* growth at 98°C (25), and methodology has been extended in recent years (75, 102). The design of a continuous culture is somewhat dependent on the organism, the type of samples needed for analysis, sterility requirements, and the level of operating complexity desired. One particular system that we have used for culturing hyperthermophiles continuously is shown in Figure A.2.1. The procedure for start-up of continuous culture is as follows:

1. Heat 1 liter of medium to 98°C in a 2 liter, 5-neck round-bottom flask (Ace Glass, Vineland, NJ) using a J thermocouple (Cole-Parmer, Vernon Hills, IL), a Glas-Col heating mantle with splash guard (Glas-Col, Terre Haute, IN), and a temperature controller (Cole-Parmer, Vernon Hills, IL).
2. Grow up a batch culture in a serum bottle to late exponential phase for use as the seed culture.
3. A stir plate and an egg-shaped stir-bar or a stirrer provide adequate mixing. Sparge the medium with high purity $N_2$ through a gas dispersion tube. Add reducing agent and adjust temperature to the growth temperature of the organism. A Graham condenser

(Ace Glass, Vineland, NJ) is connected to the reactor to limit water loss. A gas manifold can be attached to the exit of the condenser to send gas to a mass spectrometer, gas chromatograph, or the exhaust. If sulfur is included in the media, a solution of NaOH should be used to scrub out the $H_2S$ from the exhaust.

4. Inoculate the continuous culture with the seed culture, grow the cells to late log-phase, and start the feed and product pumps (peristaltic) to begin continuous operation. Liquid and gas lines are made of norprene Masterflex tubing, chosen because of its low oxygen permeability, autoclavability, and durability. Masterflex tubing in the pump head decreases in resilience with time. This will alter flow rates, necessitating an in-line flowmeter for flow rate measurement and adjustment during long operation (123). Polypropylene luer-lock fittings are used for connectors. The product tank should have quick-connects to allow for rapid exchanging of tanks. Media for feed (10 liters) should be autoclaved for at least 105 minutes (74), if filter sterilization is not included in the continuous culture setup. The medium should be sparged and reduced before use. The feed and product tanks are continuously sparged with $N_2$. The product tank should also be kept on ice.
5. The pH is controlled using a pH controller and autoclavable electrode. Teflon adapters with Viton O-rings (Cole-Parmer, Vernon Hills, IL) can be used for pH, temperature and tubing adapters.
6. After the product is collected, filter the culture broth if elemental sulfur is used, centrifuge to harvest the cells at 10,000 x g for 30 min at 4°C, and wash the cells with sterile medium or in pH 7.0 buffer containing 10 mM phosphate and 100 mM NaCl. If processing samples for anaerobic assays (oxygen-sensitive), more stringent anaerobic protocols are required (115).

### 3.5 Large Scale Cultivation

*T. litoralis* has been successfully grown in conventional stainless steel fermentors up to the 600 liter scale (86). The procedure is based on scale up from the batch cultivation method described above with the following modifications. The organism is grown at 85°C under all conditions.

1. The basic medium is summarized in Table A.2.3. This is typically supplemented with maltose (1.25 g/l), yeast extract (5 g/l) and tryptone (5 g/l) and the pH is adjusted to 5.5 prior to autoclaving. For the 100 ml to 16 liter cultures, elemental sulfur (7 g/l), pre-sterilized by incubation at 100°C for 24 h, is added after autoclaving. After degassing and flushing with Ar, the culture medium is made anaerobic by the addition of a titanium chloride/nitriloacetic acid mixture (1 ml/liter of medium) which contains $TiCl_3$ (10 g/l) and nitriloacetic acid (32 g/l) adjusted to pH 7.5.
2. Cultures are successively transferred from the 100 ml to the 1 liter to the 16 liter scale using 10(v/v)% inocula. The 16 liter cultures are grown in 20 liter glass carboys (# 06-406J, Fisher Scientific, Pittsburgh, PA) sealed with butyl rubber stoppers (#06-447H, Fisher Scientific, Pittsburgh, PA). Two 16 liter cultures are used to inoculate a large scale fermentor such as the 600-liter (500-liter working volume) fermentor from W.B. Moore, Easton, PA. The fermentor medium, lacking elemental sulfur, is stirred at 75 rpm and is continuously sparged with Ar (5 l/min).
3. Cell growth is routinely monitored by optical density at 600 nm. After approximately 16 h when the $A_{600}$ reaches ~ 1.0 (late exponential phase), the fermentor is chilled to 20°C (this takes ~ 30 min) and the cells are harvested using a continuous centrifuge (# AS-16 equipped with stainless steel cooling coils, Sharples-Strakes, Warminster, PA) at 4°C with a flow rate of 2 l/min.

4. The cell paste is quickly transferred to a metal pan and the cells are rapidly frozen by immersion in liquid $N_2$. Approximately 500 g (frozen wet weight) are obtained from 500 l of medium. The frozen cells are stored at - 80°C until needed.

## 3.6 Product Formation

The primary gaseous products formed by *T. litoralis* are $CO_2$ and $H_2$, along with $H_2S$ formed from $H_2$ when sulfur is included in the medium. Acetate is the primary liquid phase product, with smaller amounts of propionate, isobutyrate and isovalerate also produced (18). Alanine production has been noted in other thermococci (72) and *P. furiosus* (66), and presumably is formed by *T. litoralis*. Growth inhibition by H2 has been reported (18), although this may not be true in all cases (104). The inhibitory effects of $H_2$ can be reduced or eliminated by the presence of $S^o$ or a methanogen (17,18,31,66,78,114) leading to the production of $H_2S$ or methane, respectively.

## 3.7 Exopolysaccharide Production and Biofilm Formation

Exocellular polymeric substances, EPSs (47), can be simple chains containing one sugar or complex polymers containing several different sugars linked together with acetate, pyruvate, formate, sulfate, phosphate and/or other ester or N-linked side groups (137). Several different functions have been proposed for EPSs including a role in cell protection, cell to cell recognition, excess reducing equivalent elimination, and biofilm formation (137). EPSs have been investigated for hydrothermal vent bacteria (50,101), halobacteria (7), methanogens (122), autotrophic acidophiles (91), and heterotrophic hyperthermophiles (104). A common feature is the presence of sulfate groups in the exopolymer, which is a characteristic found in all domains of life. The EPS from the mesophilic archaeon *Haloferax mediterranei* contains 6-6.8% sulfate (7), from *Sulfolobus*

*solfataricus*, 5-12% (91), and from *T. litoralis*, 1-2% (104). A more uncommon feature is the predominance of mannose in the EPS from *T. litoralis* (104), a feature usually confined to eukarya (81).

Many techniques have been utilized for EPS extraction including high-speed centrifugation, steaming, sodium hydroxide treatment, ultrasonication, and precipitation with ethanol or acetone (24,109,110). The EPS from *T. litoralis* can be precipitated from the culture supernatant with 2 volumes of 95% ethanol at 4$^{o}$C (104). After three successive $H_2O$ washes and ethanol precipitations, the material can be dried in a speed-vac, lyophilizer, or a vacuum oven. The stringency of acid-hydrolysis of the polymer depends on its constituents. Typically, 2-3 N HCl is added to 2-5 mg of the polysaccharide, which is then incubated at 100$^{o}$C for 2-5 hours (95). A more suitable hydrolyzing agent is hydrofluoric acid that produces fewer by-products, but is more hazardous (141).

Characterization of EPS ranges from simple, qualitative observations to in-depth analysis. Physical assessment includes gelling behavior, precipitation with certain salts, melting temperature, and color. The molecular weight of the polymer can be determined by gel permeation chromatography (45,80). Many techniques are available to identify saccharidic components in the acid-hydrolyzed polymer, such as thin-layer chromatography (33) or high performance liquid chromatography (HPLC), which provides more accurate quantification (38,43,76,131). Composition and linkage information can be obtained using NMR with or without acetolysis of the polymer (19,45,80,120) or by derivitization of the polymer followed by gas liquid chromatography and mass spectrometry (95). Amino acid, acetyl, pyruvate, phosphorous, and sulfate groups may also be components of the polymer and should be quantified (80,120,137).

Biofilms, which typically contain large amounts of EPS, can be analyzed by a variety of microscopic techniques, each with their own pros and cons (130). Many

procedures can be performed with little, if any, modifications for hyperthermophilic biofilms as compared to mesophilic samples. A representation of three techniques, acridine orange staining of cells, congo red staining of EPS, and electron microscopy of cells in a biofilm, is shown in Figure A.2.2. The polymeric substances involved in the attachment of cells to each other and the support are typically not visible in electron microscopy techniques due to the dehydration steps involved in the sample processing. One method, confocal scanning laser microscopy, allows a more quantitative analysis of biofilms *in vivo* with few artifacts (42,130). This technique has allowed much progress to be made in the analysis of biofilms, no longer thought of as simply flat sheets of polymer and cells (40,42). Two methods are presented here for visualizing the *T. litoralis* biofilm. They are recommended due to the common availability of the light and fluorescent microscope and ease of use in determining the extent of cell adhesion.

Acridine orange staining of *T. litoralis* cells in biofilm:

1. Autoclave a 125 ml serum bottle containing a black polycarbonate filter (25mm, Poretics, Livermore, CA) and a butyl rubber stopper.
2. Prepare the bottle and the medium as described in section 3.3.
3. Grow the culture well into stationary phase. The time point of optimal biofilm development will vary for different organisms and growth conditions.
4. Remove culture from the incubator and allow it to cool to room temperature.
5. Open the bottle and carefully remove the filter.
6. Rinse the filter twice with the sterile medium.
7. Incubate the filter in 2.5% glutaraldehyde for 30 minutes.
8. Stain nucleic acids in the cells by immersing the filter in 0.04 g/L acridine orange for 5 min.

9. Dry the filter and place it on a glass slide with a cover slip. If an oil-immersion objective is used, add a drop of oil between the filter and the cover slip and on top of the cover slip.

Congo Red staining of the polysaccharide component of the biofilm (6):

1. Insert a chemically clean glass slide into a 100 ml large mouth bottle and autoclave.
2. Follow steps 2-6 in the above procedure, except that the filter is replaced by the glass slide.
3. Cover slide in 10 mM cetyl pyridinium chloride to precipitate the polysaccharide, and allow to dry for 30 min.
4. Gently heat the slide over a Bunsen burner to fix slide and let it cool to room temperature.
5. Stain the polysaccharide in the biofilm for 15 min. in a 2:1 solution of saturated Congo Red and 10% (v/v) Tween 80. Tween 80 intensifies the polysaccharide stain.
6. Rinse slide in sterile media twice.
7. Stain cells with 10% (v/v) Ziehl carbol fuchsin.
8. Rinse the slide in the sterile medium twice and dry the slide at 37°C.

Quantification of polysaccharide in the biofilm can be obtained by the total sugar assay (6), while the biomass content can be estimated by the protein assay.

## 4.0 Growth of Extremely Thermoacidophilic Archaea: *Metallosphaera sedula*

All thermoacidophiles of the archaeal crenarchaeota branch belong to the Sulfolobaceae (126,139). They are members of the genera *Sulfolobus, Acidianus, Desulfurolobus,* and *Metallosphaera* (22,58,118,143). *Metallosphaera sedula*, the type species of the genus *Metallosphaera,* will be used as an example of general cultivation

procedures for thermoacidophiles. *M. sedula* was isolated from a solfataric field in Italy (58) and can grow at temperatures between 50°C and 79°C (optimum around 75°C) and pH 1.0 to 4.0 (optimum around 2.0). This organism has a highly efficient ore-leaching (metal-extracting) capability and has a potential to be used for *in-situ* leaching of geothermally heated ore deposits (37,58,96,97). Thermoacidophiles can also be utilized for investigating unusual bioenergetic features (84), since these organisms must maintain a large transmembrane pH gradient under strongly acidic as well as thermal growth parameters.

.

## 4.1 Media Formulation

Most thermoacidophiles can be cultivated with the basal medium of Allen (5) and Brock et al. (22), which is similar in composition to the water (of hot springs or solfataric field) from which the organisms were isolated. Unlike other thermoacidophiles, the media for *M. sedula* is somewhat less complicated, with no need for trace elements. The composition of this modified medium is (in grams per liter): 0.4 $K_2HPO_4$, 0.4 $(NH_4)_2SO_4$, 0.4 $MgSO_4 \cdot 7H_2O$, and 0.2 acid-hydrolyzed casein or enzymatically hydrolyzed casein. The medium is adjusted to pH 2.0 with 10 N $H_2SO_4$ and autoclaved for at least 20 min. Since *M. sedula* is a chemolithotroph or a mixotroph, either sulfur or iron can be used as energy source. Flowers of elemental sulfur or colloidal sulfur can be directly added to the basal medium (see section 3.1 for details in preparation of sulfur). In the case of iron, 10 grams of $FeSO_4 \cdot 7H_2O$ in 100 ml of deionized water is filter-sterilized using a 0.2 µm filter and acidified to pH 2.0 by adding 10 N $H_2SO_4$. The iron solution (10 ml) is mixed with 80 ml of the basal medium for batch cultures. Alternatively, iron pyrite ($FeS_2$) is used as an energy source instead of sulfur or $FeSO_4 \cdot 7H_2O$ to study *M. sedula*'s leaching capacity. Iron pyrite (2 g of NIST standard reference pyrite [92]) is added to 100

ml of the basal medium before inoculation. No extra autoclaving or filter-sterilization is required when pyrite is added to the sterilized medium.

## 4.2 Culture Preservation

For long-term storage, lyophilization is the most often used method in our laboratory for extreme thermoacidophiles. Alternatively, cultures can be stored over liquid nitrogen. Both methods keep the culture viable for at least one year. For short-term storage, a late-log phase sample of active culture can be placed at room temperature in a serum bottle or test tube with a foam stopper; such cultures remain viable for up to two months. After this period, the culture can be transferred and will remain viable for a similar period.

## 4.3 Batch Cultivation in Flasks

*M. sedula* can be grown in 250 ml flasks with 100 ml total liquid volume, using either a silicon oil bath or an air shaker (with capability to operate at 75-80$^{o}$C) for temperature control and agitation. Periodically, samples can be withdrawn, fixed with glutaraldehyde, diluted and stained with acridine orange for enumeration of the cell population by epifluorescence microscopy ([142]; see also section 3.3). One particular protocol that has been used in our laboratory is as follows:

1. Autoclave flasks with foam stoppers.
2. Prepare the medium by autoclaving the basal medium and by filter-sterilizing the ferrous sulfate medium. The pH of media should be pre-adjusted to pH 2.0.
3. Add an appropriate amount of the sterile basal medium (e.g., 90 ml or 90% of the total culture volume) to the autoclaved flask, supplemented with the organic substrates

(e.g., acid-hydrolyzed casein) and inorganic substrates (e.g., ferrous sulfate, sulfur, or pyrite), using the sterile technique.

4. The flask is heated to the culture temperature (~75°C) and then inoculated with an appropriate amount of the *M. sedula* cells (e.g., 10 ml or 10 % of the total culture volume). The cell density of the inoculum used is typically between 8 x $10^5$ cells per ml and 2 x $10^6$ cells per ml.
5. Check the cell density of the culture periodically. As ferrous sulfide is oxidized to ferric hydroxide and other oxidation products as a consequence of growth, orange discoloration of the medium and walls of the culture vessel can be seen.

## 4.4 Continuous Cultivation

Figure A.2.3 shows a schematic drawing of the 10 liter continuous culture apparatus developed for continuous cultivation of extreme thermoacidophiles; this system is somewhat simpler to operate than that used for anaerobic cultures. Basal medium is first autoclaved and then fed to a 12 liter (5-neck) round bottom flask (Ace Glass, Vineland, NJ). The inorganic feed is filter-sterilized through a 0.2 μm filter to avoid contamination of the culture, although contamination problems are rare due to the highly thermal, and strongly acidic, growth conditions. However, due to the acidic environment, all internal parts of the system should be glass- or Teflon-coated to avoid corrosion problems. The culture is agitated at 150 rpm with an impeller and aerated by filtered (0.2 μm filter) house air at a rate of 100 cc/min. A condenser is used to decrease loss of water vapor in exit gas stream. A Digi-Sense temperature controller (Cole Parmer, Vernon Hills, IL), connected to a Teflon-coated type-J probe, is used to monitor and adjust culture temperature. System operation is initiated by pumping the sterilized medium (pH 2.0) into the round-bottom vessel containing cells at mid-log phase (5 x $10^7$ cells/ml). Dilution rate can be varied but

should be chosen with regard to the demand for and, thus preparation of, the feed medium. In routine cases, we use a dilution rate of 0.04 $h^{-1}$ (corresponding to a 17 h doubling time). Cells and the spent medium are collected in sterile polypropylene vessels, with the cells stored at 4°C for later analysis or processed immediately for enzyme purification. Samples are taken regularly to check cell density.

The suggested protocol for operation of the continuous culture set-up shown in Figure A.2.3 is as follows:

1. Grow *M. sedula* (500 ml) in flasks as the seed culture.
2. Assemble the sterile connections (ports, aerator, etc.). Add 10 liters of water and boil the water to sterilize the reactor. Cooling water should be turned on to the condenser.
3. After the reactor sterilization, the water is drained out carefully. Immediately add the autoclaved basal salts medium and the sterilized inorganic medium (e.g. ferrous-sulfate, pyrite) through the port into the reactor.
4. Heat the reactor to the *M. sedula* culture temperature (~75°C) and aerate before inoculation.
5. Using sterile technique, inoculate the vessel with the prepared active *M. sedula* batch culture. Start the continuous culture by pumping the sterilized medium (pH 2.0) at an appropriate dilution rate.
6. Maintain and monitor the culture periodically.

Caution: The culture vessel and other parts should be acid-cleaned to remove the scale which builds up inside the reactor, usually after a few months of continuous operation. For the vessel clean-up, carefully add 2 liters of concentrated HCl (through a funnel) into the top of the reactor and close the port immediately. Be extremely aware of toxic acid fumes and wear proper protection all the time.

## 5. New Developments in the Isolation and Characterization of High Temperature Microorganisms

There have been several interesting developments that impact the discovery and isolation of new extremely thermophilic microorganisms. Based on the work of Woese and co-workers with 16S rRNA phylogeny (139), RNA from numerous extreme thermophiles has been sequenced and compared on this basis. While 16S rRNA analysis does not directly reveal physiological information, it is useful for placing a newly discovered microorganism within a framework, which provides a starting point for metabolic and physiological characterization. It has also been used to reveal the yet untapped biodiversity of geothermal environments. Estimates on the fraction of total microorganisms of all types that have been isolated and characterized are usually on the order of 1%. This implies that the fraction of extreme thermophiles that have been identified to date is also likely to be small, a point that is supported by some recent investigations using 16S rRNA signatures from samples taken from hot springs (10).

Recently methods for isolation of extreme thermophiles using specific 16S rRNA sequence information have been described. Pure cultures of archaea containing 16S rRNA signatures previously observed from *in situ* analysis of samples from hot springs were obtained by the use of "optical tweezers" (8,9). This technique combines the use of an inverse microscope and an infrared laser to pull a single cell with a particular 16S rRNA signature into a separate section of a capillary and then put it into cultivation media, where it can reproduce and subsequently be characterized (60). This approach short-circuits the often laborious methods of serial dilution, from which the result might be an organism that has previously been isolated and characterized.

Recombinant DNA technology has provided another powerful avenue for recovering enzymes of potential commercial importance from geothermal environments, in

some cases bypassing the need to culture organisms. The difficulty in culturing extreme thermophiles in the laboratory and the untapped potential represented by the many difficult to culture and, perhaps, uncultivatable extreme thermophiles present in natural samples have been the motivation for expression cloning and robotic screening (107). DNA is extracted from geothermal waters or sediments and subsequently placed in expression libraries from which enzyme activities of interest can be determined by automated screening techniques. Although the source of an interesting biocatalyst may never be known, this approach ensures its availability as a recombinant product (20).

The physiological and metabolic biodiversity of extremely thermophilic microorganisms that have yet to be isolated and characterized is difficult to assess. Not only is much more to be learned about the ecology of geothermal environments, but individual organisms must be studied more completely. Emerging tools to accomplish the latter include the availability of genome sequence information, recently reported for the hyperthermophilic methanogen, *Methanococcus jannaschii* (28), and currently being sought for several other extreme thermophiles. The relationship between gene sequence and function will have to be established through a combination of approaches ranging from classical microbiology to molecular biology.

## 6. Literature Cited


1. **Adams, M.W.W.** 1996. Enzymes and proteins from hyperthermophilic microorganisms. Adv. Prot. Chem., vol. 48, 509 pp., Academic Press, New York.
2. **Adams, M.W.W., F.B. Perler, and R.M. Kelly.** 1995. Extremozymes: expanding the limits of biocatalysis. Bio/technology **13**:662-668.
3. **Adams, M.W.W., and R.M. Kelly.** 1995. Extremozymes. Chem. Eng. News. **73**:32-42.
4. **Adams, M. W. W., and A. Kletzin.** 1996. Oxidoreductase-type enzymes and redox proteins involved in the fermentative metabolisms of hyperthermophilic archaea. Adv. Prot. Chem. **48:** 101-180.
5. **Allen, M. B.** 1959. Studies with *Cyanidium caldarium*, an anomalously pigmented chlorophyte. Arch. Mikrobiol. **32:** 270-277.
6. **Allison, D.G., and I.W. Sutherland.** 1984. A staining technique for attached bacteria and its correlation to extracellular carbohydrate production. J. Microbiol. Meth. **2**:93-99.
7. **Antón, J., I. Meseguer, and F. Rodríguez-Valera.** 1988. Production of an extracellular polysaccharide by *Haloferax mediterranei*. Appl. Environ. Microbiol. **54**:2381-2386.
8. **Ashkin, A., J.M. Dziedzic, and T. Yamane**. 1987. Optical trapping and manipulation of single cells using infrared laser beams. Nature **330**:769-771.
9. **Ashkin, A., and J.M. Dziedzic**. 1987. Optical trapping and manipulation of viruses and bacteria. Science **235**:1517-1520.

10. **Barns, S.M., R.E. Fundyga, M.W. Jeffries, and N.R. Pace.** 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc. Natl. Acad. Sci. U.S.A. **91**:1609-1613.
11. **Baronofsky, J.J., W.J.A. Schreurs, and E.R. Kashket.** 1984. Uncoupling by acetic acid limits growth of and acetogenesis by *Clostridium thermoaceticum*. Appl. Environ. Microbiol. **48**:1134-1139.
12. **Baross, J.A., and J.W. Deming**. 1995. Growth at high temperatures: Isolation and taxonomy, physiology, and ecology, p. 169-217 . *In* D. M. Karl (ed.), The microbiology of deep-sea hydrothermal vents. CRC Press, Boca Raton.
13. **Belkin, S., and H.W. Jannasch.** 1985. A new extremely thermophilic, sulfur-reducing heterotrophic, marine bacterium. Arch. Microbiol. **141**:181-186.
14. **Belkin, S., C.O. Wirsen, and H.W. Jannasch.** 1986. A new sulfur-reducing, extremely thermophilic eubacterium from a submarine thermal vent. Appl. Environ. Microbiol. **51**:1180-1185.
15. **Blumentals, I.I., M. Itoh, G.J. Olson, and R.M. Kelly**. 1990. Role of polysulfides in reduction of elemental sulfur by the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Appl. Environ. Microbiol. **56**:1255-1262.
16. **Blumentals, I.I., S.H. Brown, R.N. Schicho, A.K. Skaja, H.R. Constantino, and R.M. Kelly.** 1990. The hyperthermophilic archaebacterium, *Pyrococcus furiosus*: development of culturing protocols, perspectives on scaleup, and potential applications. Ann. N.Y. Acad. Sci. **589**:301-314.
17. **Bonch-Osmolovskaya, E.A., and K.O. Stetter.** 1991. Interspecies hydrogen transfer in cocultures of thermophilic *Archaea*. Syst. Appl. Microbiol. **14**:205-208.

18. **Bonch-Osmolovskaya, E.A., and M.L. Miroshnichenko**. 1994. Effect of molecular hydrogen and elemental sulfur on metabolism of extremely thermophilic archaebacteria of the genus *Thermococcus.* Microbiology. **63**:433-436.

19. **Bradbury, J.H., and J.G. Collins.** 1979. An approach to the structural analysis of oligosaccharides by NMR spectroscopy. Carb. Res. **71**:15-24.

20. **Brennan, M.B**. 1996. Enzyme discovery heats up. Chem. Eng. News. **74**:31-33.

21. **Brierley, C.L., and J.A. Brierley**. 1973. A chemoautotrophic and thermophilic microorganism isolated from an acid hot spring. Can J. Microbiol. **19**:183-188.

22. **Brock, T. D., K. M. Brock, R. T. Belly, and R. L. Weiss.** 1972. *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. Arch. Microbiol. **84:** 54-68.

23. **Brock, T.D.** 1978. Thermophilic microorganisms and life at high temperatures. Springer-Verlag, New York.

24. **Brown, M.J., and J.N. Lester.** 1980. Comparison of bacterial extracellular polymer extraction methods. Appl. Environ. Microbiol. **40**:179-185.

25. **Brown, S.H., and R.M. Kelly**. 1989. Cultivation techniques for hyperthermophilic archaebacteria: continuous culture of *Pyrococcus furiosus* at temperatures near 100°C. Appl. Environ. Microbiol. **55**:2086-2088.

26. **Brown, S.H., H.R. Costantino, and R.M. Kelly.** 1990. Characterization of amylolytic enzyme activities associated with the hyperthermophilic archaebacterium, *Pyrococcus furiosus*. Appl. Environ. Microbiol. **56**:1985-1991.

27. **Brown, S.H. and R.M. Kelly.** 1993. Characterization of amylolytic enzymes, having both a-1,4 and a-1,6 hydrolytic activity, from the thermophilic

archaea *Pyrococcus furiosus* and *Thermococcus litoralis*. Appl. Environ. Microbiol. **59**:2614-2621.

28. **Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, G. G. Sutton, J. A. Blake, L. M. FitzGerald, R. A. Clayton, J. D. Gocayne, A. R. Kerlavage, B. A. Gougherty, J.-F. Tomb, M. D. Adams, C. I. Reich, R. Overbeek, E. F. Kirkness, K. G. Weinstock, J. M. Merrick, A. Glodek, J. L. Scott, N. S. M. Geoghagen, J. F. Weidman, J. L. Fuhrmann, D. Nguyen, T. R. Utterback, J. M. Kelley, J. D. Peterson, P. W. Sadow, M. C. Hanna, M. D. Cotton, K. M. Roberts, M. A. Hurst, B. P. Kaine, M. Borodovsky, H.-P. Klenk, C. M. Fraser, H. O. Smith, C. R. Woese, and J. C. Venter.** 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science **273**:1058-1073

29. **Burggraf, S., G.J. Olsen, K.O. Stetter, and C.R. Woese.** 1992. A phylogenetic analysis of *Aquifex pyrophilus*. Syst. Appl. Microbiol. **15**:352-356.

30. **Busse, S. A., G.N. La Mar, L.P. Yu, J.B. Howard, E.T. Smith, Z.H. Zhou, and M.W.W. Adams.** 1992. Proton NMR investigation of the oxidized three-iron clusters in the ferredoxins from the hyperthermophilic archaea, *Pyrococcus furiosus* and *Thermococcus litoralis.* Biochemistry **31:** 11952-11962.

31. **Canganella, F., and W.J. Jones.** 1994. Fermentation studies with thermophilic Archaea in pure culture and in syntrophy with a thermophilic methanogen. Curr. Microbiol. **28**:293-298.

32. **Carlsson, J., G.P.D. Granberg, G.K. Nyberg, and M.B.K. Edlund.** 1979. Bactericidal effect of cysteine exposed to atmospheric oxygen. Appl. Environ. Microbiol. **37**:383-390.

33. **Chaplin, M.F.** 1994. Monosaccharides, p. 1-42. *In* M. F. Chaplin & J. F. Kennedy (ed.), Carbohydrate analysis: a practical approach (2nd edn). IRL Press, Oxford.

34. **Chen, C. Y., and D. R. Skidmore.** 1988. Attachment of *Sulfolobus acidocaldarius* cells on coal particles. Biotechnol. Prog. **4:** 25-30.

35. **Childers, S.E., M. Vargas, and K.M. Noll**. 1992. Improved methods for cultivation of the extremely thermophilic bacterium *Thermotoga neapolitana*. Appl. Environ. Microbiol. **58**:3949-3953.

36. **Clark, D.S., and R.M. Kelly.** 1990. Microorganisms at extreme temperatures and pressures: engineering insights. Chemtech **20**:641-648.

37. **Clark, T.R., F. Baldi, and G.J. Olson.** 1993. Coal depyritization by the thermophilic archaeon *Metallosphaera sedula*. Appl. Environ. Microbiol. **59**:2375-2379.

38. **Clarke, A.J., V. Sarabia, W. Keenleyside, P.R. MacLachlan, and C. Whitfield.** 1991. The compositional analysis of bacterial extracellular polysaccharides by high-performance anion-exchange chromatography. Anal. Biochem. **199**:68-74.

39. **Connaris, H., D. Cowan, M. Ruffett, and R.J. Sharp**. 1991. Preservation of the hyperthermophile *Pyrococcus furiosus*. Lett. Appl. Microbiol. **13**:25-27.

40. **Costerton, J.W.** 1995. Overview of microbial biofilms. J. Ind. Microbiol. **15**:137-140.

41. **Danson, M.J.** 1989. Central metabolism of the archaebacteria: an overview. Can. J. Microbiol. **35**:58-64.

42. **De Beer, D., P. Stoodley, F. Roe, and Z. Lewandowski.** 1994. Effects of biofilm structures on oxygen distribution and mass transport. Biotech. Bioeng. **43**:1131-1138.

43. **Farnsworth, V., and K. Steinberg.** 1993. The generation of phenlythiocarbamyl or anilinothiazolinone amino acids from the postcleavage products of the Edman degradation. Anal. Biochem. **251**:200-210.

44. **Fiala, G., and K. O. Stetter.** 1986. *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. Arch. Microbiol. **145**: 56-61.

45. **Flatt, J.H., R.S. Hardin, J.M. Gonzalez, D.E. Dogger, E.N. Lightfoot, and D.C. Cameron.** 1992. An anionic galactomannan polysaccharide gum from a newly-isolated lactose-utilizing bacterium. I. Strain description and gum characterization. Biotechnol. Prog. **8**:327-334.

46. **Fuchs, T., H. Huber, K. Teiner, S. Burggraf, and K.O. Stetter.** 1995. *Metallosphaera prunae*, sp. nov., a novel metal-mobilizing, thermoacidophilic archaeum, isolated from a uranium mine in Germany. Syst. Appl. Microbiol. **18**:560-566.

47. **Geesey, G.G.** 1982. Microbial exopolymers: ecological and economic considerations. ASM News **48**:9-14.

48. **Grogan, D.W.** 1989. Phenotypic characterization of the archaebacterial genus *Sulfolobus*: comparison of five wild-type strains. J. Bacteriol. **171**:6710-6719.

49. **Grogan, D., P. Palm, and W. Zillig**. 1990. Isolate B-12, which harbors a virus-like element, represents a new species of the archaebacterial genus *Sulfolobus*, *Sulfolobus shibatae*, sp. nov. Arch. Microbiol. **154**:594-599.

50. **Guezennec, J.G., P. Pignet, G. Raguenes, E. Deslandes, Y. Lijour, and E. Gentric.** 1994. Preliminary chemical characterization of unusual eubacterial exopolysaccharides of deep-sea origin. Carb. Polym. **24**:287-294.

51. **Harriott, O. T., R. Huber, K.O. Stetter, P.W. Betts, and K.M. Noll.** 1994. A cryptic miniplasmid from the hyperthermophilic bacterium *Thermotoga* Sp Strain Rq7. J. Bacteriol. **176:** 2759-2762.

52. **Hoaki, T., C.O. Wirsen, S. Hanzawa, T. Maruyama, and H.W. Jannasch.** 1993. Amino acid requirements of two hyperthermophilic archaeal isolates from deep-sea vents, *Desulfurococcus* Strain SY and *Pyrococcus* Strain GB-D. Appl. Environ. Microbiol. **59**:610-613.

53. **Hoaki, T., M. Nishijima, M. Kato, K. Adachi, S. Mizobuchi, N. Hanzawa, and T. Maruyama.** 1994. Growth requirements of hyperthermophilic sulfur-dependent heterotrophic archaea isolated from a shallow submarine geothermal system with reference to their essential amino acids. Appl. Environ. Microbiol. **60**:2898-2904.

54. **Hobbie, J.E., R.J. Daley, and S. Jasper.** 1977. Use of nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225-1228.

55. **Huber, R, T.A. Langworthy, H. König, M. Thomm, C.R. Woese, U.B. Sleytr, and K.O. Stetter.** 1986. *Thermotoga maritima* sp. nov. represent a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch. Microbiol. **144:** 324-333.

56. **Huber, G., and K.O. Stetter.** 1986. *Sulfolobus metallicus*, sp. nov., a novel strictly chemolithoautotrophic thermophilic archaeal species of metal-mobilizers. Syst. Appl. Microbiol. **14**:372-378.

57. **Huber, R., J.K. Kristjannson, and K.O. Stetter.** 1987. *Pyrobaculum* gen. nov., a new genus of neutrophilic, rod-shaped archaebacteria from continental solfataras growing optimally at 100°C. Arch. Microbiol. **149**:95-101.

58. **Huber, G., C. Spinnler, A. Gambacorta, and K. O. Stetter.** 1989. *Metallosphaera sedula* gen. and sp. nov. represents a new genus of aerobic, metal-mobilizing, thermophilic archaebacteria. Syst. Appl. Microbiol. **12:** 38-47.

59. **Huber, R., T. Wilharm, D. Huber, A. Trincone, S. Burggraf, H. König, R. Rachel, I. Rockinger, H. Fricke, and K.O. Stetter.** 1992. *Aquifex pyrophilus* gen. nov. sp. nov., represents a novel group of marine hyperthermophilic hydrogen oxidizing bacteria. Arch. Microbiol. **15:** 340-351.

60. **Huber, R., S. Burggraf, T. Mayer, S.M. Barns, P. Rossnagel, and K.O. Stetter.** 1995. Isolation of a hyperthermophilic archaeum predicted by *in situ* RNA analysis. Nature **376**:57-58.

61. **Hungate, R.E**. 1969. A roll tube method for cultivation of strict anaerobes, p. 117-132. *In* J.R. Norris and D.W. Ribbons (ed.), *Methods in microbiology*, Vol. 3B. Academic Press Inc., New York.

62. **Jannasch, H.W., C.O. Wirsen, S.J. Molyneaux, and T.A. Langworthy.** 1992. Comparative physiological studies on hyperthermophilic archaea isolated from deep-sea hot vents with emphasis on *Pyrococcus* strain GB-D. Appl. Environ. Microbiol. **58**:3472-3481.

63. **Jørgensen, B.B., M.F. Isaksen, and H.W. Jannasch.** 1992. Bacterial sulfate reduction above 100°C in deep-sea hydrothermal vent sediments. Science **258:**1756-1757.

64. **Kelly, R. M., and J. W. Deming.** 1988. Extremely thermophilic archaebacteria: biological and engineering considerations. Biotechnol. Prog. **4**:47-62.

65. **Kelly, R.M., and M.W.W. Adams**. 1994. Metabolism in hyperthermophilic microorganisms. Antonie van Leeuwenhoek. **66**:247-270.

66. **Kengen, S.W.M., and J.M. Stams.** 1994. Formation of L-alanine as a reduced end product in carbohydrate fermentation by the hyperthermophilic archaeon *Pyrococcus furiosus*. Arch. Microbiol. **161**:168-175.

67. **Kengen, S.W.M., F.A.M. Debok, N.D. Vanloo, C. Dijkema, A.J.M. Stams, and W.M. deVos.** 1994. Evidence for the operation of a novel Embden-Meyerhof pathway that involves ADP-dependent kinases during sugar fermentation by *Pyrococcus furiosus.* J. Biol. Chem. **269:** 17537-17541.

68. **Kengen, S.W.M., A.J.M. Stams, and W.M. deVos.** 1996. Sugar metabolism of hyperthermophiles. FEMS Microbiol. Rev. **18**:119-137.

69. **Klages, K.U., and H.W. Morgan.** 1994. Characterization of an extremely thermophilic sulphur-metabolizing archaebacterium belonging to the Thermococcales. Arch. Microbiol. **162**:261-266.

70. **Kletzin, A., and M.W.W. Adams.** 1996. Tungsten in biological systems. FEMS Microbiol. Rev. **18**:5-63.

71. **Kobayashi, T., S. Higuchi, K. Kimura, T. Kudo, and K. Horikoshi.** 1995. Properties of glutamate dehydrogenase and its involvement in alanine production in a hyperthermophilic archaeon, *Thermococcus profundus*. J. Biochem. **118**:587-592.

72. **Kobayashi, T., Y.S. Kwak, T. Akiba, T. Kudo, and K. Horikoshi.** 1994. *Thermococcus profundus* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. Syst. Appl. Microbiol. **17**:232-236.

73. **Kong, H., R.B. Kucera, and W.E. Jack.** 1993. Characterization of a DNA polymerase from the hyperthermophile archaea *Thermococcus litoralis*. J. Bacteriol. **268**:1965-1975.

74. **Korczynski, M.S.** 1981. Sterilization, p. 476-486. *In* P. Gerhardt (ed.), Manual of methods in general bacteriology. American Society for Microbiology, Washington, D.C.

75. **Krahe, M., G. Antranikian, and H. Märkl**. 1996. Fermentation of extremophilic microorganisms. FEMS Microbiol. Rev. **18**:271-285.

76. **Lu, H-S., and P-H. Lai.** 1986. Use of narrow-bore high-performance liquid chromatography for microanalysis of protein structure. J. Chromat. **368**:215-231.

77. **Mai, X., and M.W.W. Adams.** 1996. Characterization of a fourth type of 2-keto acid oxidizing enzyme from hyperthermophilic archaea: 2-ketoglutarate ferredoxin oxidoreductase from *Thermococcus litoralis*. J. Bacteriol **178:** 5890-5896.

78. **Malik, B., W.-W. Su, H.L. Wald, I.I. Blumentals, and R.M. Kelly.** 1989. Growth and gas production for hyperthermophilic archaebacterium, *Pyrococcus furiosus*. Biotechnol. Bioeng. **34**:1050-1057.

79. **Martins, L.O., and H. Santos.** 1995. Accumulation of mannosylglycerate and di-myo-inositol-phosphate by *Pyrococcus furiosus* in response to salinity and temperature. Appl. Environ. Microbiol. **61**:3299-3303.

80. **Mbawala, A., S. A. Mahmood, V. Loppinet, and R. Bonaly.** 1990. Acetolysis and $^{1}$H NMR studies on mannans isolated from very flocculent and

weakly flocculent cells of *Pichia pastoris* IFP 206. J. Gen. Microbiol.**136**:1279-1284.

81. **Millane, R.P., and T.L. Hendrixson.** 1994. Crystal structures of mannan and glucomannans. Carb. Polym. **25**:245-251.

82. **Miller, T.L., and M.J. Wolin.** 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl. Microbiol. **27**:985-987.

83. **Miller, J.F., E.L. Almond, N.N. Shah, J.M. Ludlow, J.A. Zollweg, W.B. Streett, S.H. Zinder, and D.S. Clark**. 1988. High-pressure-temperature bioreactor for studying pressure-temperature relationships in bacterial growth and productivity. Biotech. Bioeng. **31**:407-413.

84. **Mitchell, P.** 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev. **41:** 445-504.

85. **Mukund, S., and M.W.W. Adams.** 1991. The novel tungsten-iron-sulfur protein of the hyperthermophilic archaebacterium, *Pyrococcus furiosus*, is an aldehyde ferredoxin oxidoreductase: evidence for its participation in a unique glycolytic pathway. J. Biol. Chem. **266:** 14208-14216.

86. **Mukund, S., and M.W.W. Adams.** 1993. Characterization of a novel tungsten-containing formaldehyde ferredoxin oxidoreductase from the extremely thermophilic archaeon, *Thermococcus litoralis*: a role for tungsten in peptide catabolism. J. Biol. Chem. **268:** 13592-13600.

87. **Mukund, S., and M.W.W. Adams.** 1995. Glyceraldehyde-3-phosphate ferredoxin oxidoreductase, a novel tungsten-containing enzyme with a potential glycolytic role in the hyperthermophilic archaeon, *Pyrococcus furiosus*. J. Biol. Chem. **270:** 8389-8392.

88. **Mukund, S., and M.W.W. Adams.** 1996. Molybdenum and vanadium do not replace tungsten in the three tungstoenzymes of the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Bacteriol. **178:** 163-167.

89. **Muralidharan, V., K.D. Rinker, I.S. Hirsh, E.J. Bouwer, and R.M. Kelly.** 1997. Hydrogen transfer between methanogens and fermentative heterotrophs in hyperthermophilic cocultures. Biotech. Bioeng. **56**: 268-278.

90. **Neuner, A., H.W. Jannasch, S. Belkin, and K.O. Stetter.** 1990. *Thermococcus litoralis* sp. nov.: a new species of extremely thermophilic marine archaebacteria. Arch. Microbiol. **153**:205-207.

91. **Nicolaus, B., M.C. Manca, I. Romano, and L. Lama.** 1993. Production of an exopolysaccharide from two thermophilic archaea belonging to the genus *Sulfolobus*. FEMS Microbiol. Lett. **109**:203-206.

92. **Olson, G.J**. 1991. Rate of pyrite bioleaching by *Thiobacillus ferrooxidans*: results of an inter-laboratory comparison. Appl. Environ. Microbiol. **57**:642-644.

93. **Parameswaran, A.K., C.N. Provan, F.J. Sturm, and R.M. Kelly.** 1987. Sulfur reduction by the extremely thermophilic archaebacterium *Pyrodictium occultum*. Appl. Environ. Microbiol. **53**:1690-1693.

94. **Parameswaran, A.K., R.N. Schicho, J.P. Soisson, and R.M. Kelly**. 1988. Effect of hydrogen and carbon dioxide partial pressures on growth and sulfide production of the extremely thermophilic archaebacterium *Pyrodictium brockii*. Biotech. Bioeng. **32**:438-443.

95. **Pazur, J.H.** 1994. Neutral Polysaccharides, p. 73-124. *In* M. F. Chaplin & J. F. Kennedy (ed.), Carbohydrate analysis: a practical approach (2nd edn). IRL Press, Oxford.

96. **Peeples, T. L., and R. M. Kelly.** 1993. Bioenergetics of the metal/sulfur oxidizing extreme thermoacidophile, *Metallosphaera sedula*. Fuel **72:** 1619-1624.

97. **Peeples, T. L., and R. M. Kelly.** 1995. Bioenergetic response of the extreme thermoacidophile *Metallosphaera sedula* to thermal and nutritional stress. Appl. Environ. Microbiol. **61:** 2314-2321.

98. **Pihl, T. D., R.N. Schicho, L.K. Black, B.A. Schulman, R.J. Maier, and R. M. Kelly.** 1990. Hydrogen sulfur autotrophy in the hyperthermophilic archaebacterium *Pyrodictium brockii.* Biotechnol. Genet. Eng. Rev. **8:** 345-377.

99. **Pledger, R.J., and J.A. Baross.** 1991. Preliminary description and nutritional characterization of a chemoorganotrophic archaeobacterium growing at temperatures of up to 110°C isolated from a submarine hydrothermal vent environment. J. Gen. Microbiol. **137**:203-211.

100. **Pledger, R.J., B.C. Crump, and J.A. Baross**. 1994. A barophilic response by two hyperthermophilic hydrothermal vent Archaea: acceleration of growth rate at supra-optimal temperature by elevated pressure. FEMS Microbiol. Ecol. **14**:233-241.

101. **Raguenes, G., P. Pignet, G. Gauthier, A. Peres, R. Christen, H. Gougeaux, G. Barbier, and J. Guezennec.** 1996. Description of a new polymer-secreting bacterium from a deep-sea hydrothermal vent, *Alteromonas macleodii* subsp. *fijiensis*, and preliminary characterization of the polymer. Appl. Environ. Microbiol. **62**:67-73.

102. **Raven, N., N. Ladwa, D. Cossar, and R. Sharp.** 1992. Continuous culture of the hyperthermophilic archaeum *Pyrococcus furiosus*. Appl. Microbiol. Biotechnol. **38**:263-267.

103. **Raven, N., and R. Sharp.** 1997. Development of defined and minimal media for the growth of the hyperthermophilic archaeon *Pyrococcus furiosus* Vc1. FEMS Microbiol. Lett. **146**:135-141.

104. **Rinker, K.D., and R.M. Kelly**. 1996. Growth physiology of the hyperthermophilic archaeon *Thermococcus litoralis*: development of a sulfur-free defined medium, characterization of an exopolysaccharide, and evidence of biofilm formation. Appl. Environ. Microbiol. **62**:4478-4485.

105. **Ritzau, M., M. Keller, P. Wessels, K.O. Stetter, and A. Zeeck.** 1993. Secondary metabolites by chemical screening, 25-new cyclic polysulfides from hyperthermophilic archaea of the genus *Thermococcus*. Liebigs Ann. Chem. **1993**:871-876.

106. **Robb, F.J.** Archaea: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

107. **Robertson, D.E., E.J. Mathur, R.V. Swanson, B.L. Marrs and J.M. Short**. 1996. The discovery of new biocatalysts from microbial diversity. SIM News **46**: 3-8.

108. **Robinson, K. A., and H.J. Schreier** 1994. Isolation, sequence and characterization of the maltose-regulated *mlr*A gene from the hyperthermophilic archaeum *Pyrococcus furiosus*. Gene **151**:1173-176.

109. **Rudd, T., R.M. Sterritt, and J.N. Lester.** 1982. The use of extraction methods for the quantification of extracellular polymer production by *Klebsiella aerogenes* under varying cultural conditions. Eur. J. Appl. Microbiol. Biotechnol. **16**:23-27.

110. **Rudd, T., R.M. Sterritt, and J.N. Lester.** 1983. Extraction of extracellular polymers from activated sludge. Biotechnol. Lett. **5**:327-332.

111. **Rüdiger, A., J.C. Ogbonna, H. Märkl, and G. Antranikian**. 1992. Effect of gassing, agitation, substrate supplementation and dialysis on the growth of an extremely thermophilic archaeon *Pyrococcus woesei*. Appl. Microbiol. Biotechnol. **37**:501-504.

112. **Schäfer, T, M. Selig, and P. Schönheit.** 1993. Acetyl-CoA synthetase (ADP-forming) in archaea, a novel enzyme involved in acetate formation and ATP synthesis. Arch. Microbiol. **159:** 72-83.

113. **Schicho, R.N**. 1992. Ph.D. Thesis. Johns Hopkins University, Baltimore.

114. **Schicho, R.N., K. Ma, M.W.W. Adams, and R.M. Kelly.** 1993. Bioenergetics of sulfur reduction in the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Bacteriol. **175**:1823-1830.

115. **Schicho, R.N., Snowden, L.J., S. Mukund, J.B. Park, M.W.W. Adams, and R.M. Kelly.** 1993. Influence of tungsten on metabolic patterns in the hyperthermophile *Pyrococcus furiosus*. Arch. Microbiol. **159**:380-385.

116. **Schicho, R.N., S.H. Brown, I.I. Blumentals, T.L. Peeples, G.D. Duffaud, and R.M. Kelly**. 1995. Continuous culture techniques for thermophilic and hyperthermophilic archaea, p. 31-36. *In* F.T. Robb (ed.), Archaea: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

117. **Schönheit, P., and T. Schäfer**. 1995. Metabolism of hyperthermophiles. World. J. Microbiol. Biotechnol. **11:** 26-57.

118. **Segerer, A., A. Neuner, J. K. Kristjansson, and K. O. Stetter.** 1986. *Acidianus infernus* gen. nov., sp. nov. and *Acidianus brierleyi* comb. nov.: facultatively aerobic, extremely acidophilic thermophilic sulfur-metabolizing archaebacteria. Int. J. Syst. Bacteriol. **36:** 559-564.

119. **Seyfried, W.E., and M.J. Mottl**. 1995. Geologic setting and chemistry of deep-sea hydrothermal vents. p. 1-34. *In* D. M. Karl (ed.), The microbiology of deep-sea hydrothermal vents. CRC Press, Boca Raton.

120. **Skjåk-Bræk, G., H. Grasdalen, and B. Larsen.** 1986. Monomer sequence and acetylation pattern in some bacterial alginates. Carb. Res. **154**:239-250.

121. **Smith, P.F., T.A. Langworthy, and M.R. Smith.** 1975. Polypeptide nature of growth requirement in yeast extract for *Thermoplasma acidophilum*. J. Bacteriol. **124**:884-892.

122. **Sowers, K.R., and R.P. Gunsalus.** 1988. Adaptation for growth at various saline concentrations by the archaebacterium *Methanosarcina thermophila*. J. Bacteriol. **170**:998-1002.

123. **Stafford, K**. 1986. Continuous fermentation. p. 137-151. *In*: A. L. Demain and N.A. Solomon (ed.), Manual of industrial microbiology and biotechnology. American Society for Microbiology, Washington, D.C.

124. **Stetter, K.O., H. König, and E. Stackebrandt**. 1983. *Pyrodictium* gen. nov., a new genus of submarine disc-shaped sulfur reducing archaebacteria growing optimally at 105°C. Syst. Appl. Microbiol. **4**:535-551.

125. **Stetter, K.O., G. Lauerer, M. Thomm, and A.-M. Neuner**. 1987. Isolation of extremely thermophilic sulfate reducers: evidence for a novel branch of archaebacteria. Science **236**:822-824.

126. **Stetter, K. O.** 1989. Order III. *Sulfolobales* ord. nov. p. 2250-2253. *In* J.G. Holt (ed), *Bergey's manual of systematic bacteriology*. Vol. 3, Williams and Wilkins, Baltimore.

127. **Stetter, K. O., G. Fiala, G. Huber, R. Huber, and G. Segerer.** 1990. Hyperthermophilic microorganisms. FEMS Microbiol. Rev. **75:** 117-124.

128. **Stetter, K.O.** 1995. Microbial life in extreme environments. ASM News **61**:285-290.

129. **Su, Wei-Wen, and R.M. Kelly.** 1988. Effect of hyperbaric oxygen and carbon dioxide on the heterotrophic growth of the extreme thermophile, *Sulfolobus acidocaldarius*. Biotechnol. Bioeng. **31**:750-754.

130. **Surman, S.B., J.T. Walker, D.T. Goddard, L.H.G. Morton, C.W. Keevil, W. Weaver, A. Skinner, K. Hanson, D. Caldwell, and J. Kurtz**. 1996. Comparison of microscope techniques for the examination of biofilms. J. Microbiol. Meth. **25**:57-70.

131. **Tarr, G.E.** 1981. Rapid separation of amino acid phenylthiohydantoins by isocratic high-performance liquid chromatography. Anal. Biochem. **111**:27-32.

132. **Usenko, I.A., L.O. Severina, and V.K. Plakunov.** 1993. Uptake of sugars and amino acids by extremely thermophilic archae- and eubacteria. Microbiology **62**:272-277.

133. **Vitaya, V. B., and K. Toda.** 1991. Physiological adsorption of *Sulfolobus acidocaldarius* on coal surfaces. Appl. Microbiol. Biotechnol. **35:** 690-695.

134. **Völkl, P., R. Huber, E. Brobner, R. Rachel, S. Burggraf, A. Trincone, and K. O. Stetter.** 1993. *Pyrobaculum aerophilum* sp. nov., a novel nitrate-reducing hyperthermophilic archaeum. Appl. Environ. Microbiol. **59:** 2918-2926

135. **Watrin, L., V. Martin-Jezequel, and D. Prieur.** 1995. Minimal amino acid requirements of the hyperthermophilic archaeon *Pyrococcus abyssi*, isolated from deep-sea hydrothermal vents. Appl. Environ. Microbiol. **61**:1138-1140.

136. **Watrin, L., and D. Prieur**. 1996. UV and ethyl methanesulfonate effects in hyperthermophilic archaea and isolation of auxotrophic mutants of *Pyrococcus* strains. Curr. Microbiol. **33**:377-382.

137. **Weiner, R., S. Langille, and E. Quintero.** 1995. Structure, function, and immunochemistry of bacterial exopolysaccharides. J. Ind. Microbiol. **15**:339-346.

138. **Weiss, B.L.** 1973. Attachment of bacteria to sulphur in extreme environments. J. Gen. Microbiol. **77**:501-507.

139. **Woese, C. R., O. Kandler, and M. L. Wheelis.** 1990. Towards a natural system of organisms: proposal for the domains archae, bacteria and eucarya. Proc. Natl. Acad. Sci. USA. **87:** 4576-4579.

140. **Xavier, K.B., L.O. Martins, R. Peist, M. Kossmann, W. Boos, and H. Santos.** 1996. High-affinity maltose/trehalose transport system in the hyperthermophilic archaeon *Thermococcus litoralis.* J. Bacteriol. **178**:4773-4777.

141. **Yadav, M.P., J.N. BeMiller, and M.E. Embuscado.** 1994. Compositional analysis of polysaccharides via solvolysis with liquid hydrogen fluoride. Carb. Polym. **25**:315-318.

142. **Yeh, T. Y., J. R. Godshalk, G. J. Olson, and R. M. Kelly.** 1987. Use of epifluorescence microscopy for characterizing the activity of *Thiobacillus ferrooxidans* on iron pyrite. Biotechnol. Bioeng. **30:** 138-146.

143. **Zillig, W., S. Yeats, I. Holz, A. Böck, M. Rettenberger, F. Gropp, and G. Simon.** 1986. *Desulfurolobus ambivalens*, gen. nov., sp. nov., an autotrophic archaebacterium facultatively oxidizing or reducing sulfur. Syst. Appl. Microbiol. **8:** 197-203.

**Table A.2.1. Biodiversity of Extreme Environments**

| Extreme Condition | Microorganism<br><br>Archaea*<br>Bacteria | Habitat | Extreme growth conditions | Metabolic characteristics |
|---|---|---|---|---|
| **High Temperature** | *Pyrococcus furiosus** | Geothermal marine sediments | 100°C | Anaerobic heterotroph; facultative S° reducer |
| | *Thermotoga neapolitana* | Geothermal marine sediments | 85°C | Anaerobic heterotroph; facultative sulfur reducer |
| **Cold Temperature** | *Bacillus TA41* | Antarctic sea water | 4°C | Aerobic heterotroph |
| **High Pressure + High Temperature** | *Methanococcus janaschii** | Deep sea hydrothermal vent | 250 atm<br><br>85°C | Growth/methane production stimulated by pressure |
| **High pH** | *Clostridium paradoxum* | Sewage sludge | pH 10.1<br><br>56°C | Anaerobic heterotroph |
| **Low pH + High Temperature** | *Metallosphaera sedula** | Thermal, acid pools | pH 2.0<br><br>75°C | Facultative chemolitho-troph |
| **High Salt** | *Halobacterium halobium** | Hypersaline waters | 4-5 M NaCl | Aerobic heterotroph |

**Table A.2.2. The Extremely Thermophilic Genera (Topt 75°C)**

| Order/Genus[a] | Tmax[b] | Metabolism[c] | Substrates[d] | Acceptors[e] |
|---|---|---|---|---|
| **ARCHAEA** | | | | |
| THERMOPROTEALES | | | | |
| *Thermoproteus* | 92° | Het (Auto) | PEP, CBH (H2) | S° |
| *Thermofilum* | 100° | Het | PEP | S° |
| *Staphylothermus* | 98° | Het | PEP | S° |
| *Desulfurococcus* | 90° | Het | PEP | S° |
| *Pyrolobus* | 113° | Het | PEP | ? |
| *Pyrobaculum* | 102° | Het (Auto) | PEP ($H_2$) | -, S°, m$O_2$ |
| SULFOLOBALES | | | | |
| *Acidianus* | 96° | Auto | S°, $H_2$ | S°, $O_2$ |
| *Desulfurolobus* | 87° | Auto | S°, $H_2$ | $O_2$ |
| *Metallosphaera* | 81° | Auto (Het) | S°, PEP | $O_2$ |
| *Sulfolobus* | 90° | Auto (Het) | S°, PEP, CBH | $O_2$ |
| PYRODICTIALES | | | | |
| *Pyrodictium* | 110° | Het (Auto) | PEP, CBH (H2) | S° |
| *Thermodiscus* | 98° | Het | PEP | S° |
| THERMOCOCCALES | | | | |
| *Pyrococcus* | 105° | Ferm | PEP, CBH | -, S° |
| *Thermococcus* | 97° | Ferm | PEP, CBH | -, S° |
| *Hyperthermus* | 110° | Ferm | PEP (H2) | -, S° |
| ARCHAEOGLOBALES | | | | |
| *Archaeoglobus* | 95° | Het (Auto) | CBH ($H_2$) | $SO_4$, $S_2O_3$ |
| METHANOCOCCALES | | | | |
| *Methanococcus* | 91° | Auto | $H_2$ | $CO_2$ |
| METHANOBACTERIALES | | | | |
| *Methanothermus* | 97° | Auto | $H_2$ | $CO_2$ |
| METHANOPYRALES | | | | |
| *Methanopyrus* | 110° | Auto | $H_2$ | $CO_2$ |

**Table A.2.2.cont.**

| Order/Genus[a] | Tmax[b] | Metabolism[c] | Substrates[d] | Acceptors[e] |
|---|---|---|---|---|
| **BACTERIA** | | | | |
| THERMOTOGALES | | | | |
| *Thermotoga* | 90° | Ferm | PEP, CBH | -, S° |
| AQUIFICALES | | | | |
| *Aquifex* | 95° | Auto | S° ($H_2$) | $mO_2$, $NO_3$ |

[a] Modified from Kelly and Adams (65).
[b] Tmax, maximum growth temperture within genus.
[c] Het, heterotrophic; Auto, autotrophic; Ferm, fermentative.
[d] PEP, peptides; CBH, carbohydates.
[e] -, growth in the absence of an added electron acceptor; $mO_2$, microaerophilic.

**Table A.2.3. Hyperthermophile Media**

| Compound | concentration (g/l) | | |
|---|---|---|---|
| | RDM[a] | ASW[b] | Adams (Tlit)[c] |
| Initial pH | 6.0 | 6.5 | 5.5 |
| NaCl | 25 | 23.9 | 38 |
| $MgCl_2 \cdot 6H_2O$ | 1 | 1.8 | 2.75 |
| $MgSO_4 \cdot 7H_2O$ | --- | --- | 3.5 |
| $NH_4Cl$ | --- | --- | 1.2 |
| $Na_2SO_4$ | 1 | 4.0 | --- |
| $NaHCO_3$ | --- | 0.2 | --- |
| $CaCl_2 \cdot 2H_2O$ | 0.075 | 1.5 | 0.75 |
| KCl | 0.35 | 0.7 | 0.325 |
| KBr | --- | 0.1 | --- |
| NaBr | 0.05 | --- | 0.05 |
| $H_3BO_3$ | 0.02 | 0.03 | 0.015 |
| KI | 0.02 | | 0.05 |
| $SrCl_2 \cdot 6H_2O$ | 0.01 | 0.025 | 0.0075 |
| $Na_2WO_4 \cdot 2H_2O$ | 0.003 | --- | --- |
| $Na_2SeO_4$ | 0.001 | --- | --- |
| Rezasurin | 0.001 | 0.001 | 0.0025 |
| Trace elements | 10 ml | --- | 10 ml |
| Vitamin solution | 10 ml | --- | --- |
| $K_2HPO_4$ | 0.14 | --- | --- |
| $KH_2PO_4$ | — | --- | 0.5 |
| MES | 2 | --- | --- |
| Citric Acid | — | --- | 0.005 |
| $Na_2S \cdot 9H_2O$ | 0.2 | 0.25 | 0.25 |

[a] 0.2 g/l 16 amino acids (-Ala, Asn, Gln, Glu) and 0.01 g/l adenine and uracil are added

[b] 1 g/l yeast extract and 5 g/l tryptone are typically added

[c] 5 g/l yeast extract, 5 g/l tryptone, and 1.25 g/l maltose are typically added

**Table A.2.4. Trace Mineral Solutions[a]**

| Component | RDM | Adams' |
|---|---|---|
| nitrilotriacetate | 1.5 | 1 |
| $MnSO_4 \cdot H_2O$ | 0.5 | 0.5 |
| $FeSO_4 \cdot 7H_2O$ | 1.4 | --- |
| $FeCl_3$ | --- | 1.1 |
| $NiCl_2 \cdot 6H_2O$ | 0.2 | 0.2 |
| $CoSO_4 \cdot 7H_2O$ | 0.362 | 0.1 |
| $ZnSO_4 \cdot 7H_2O$ | 0.1 | 0.1 |
| $CuSO_4 \cdot 5H_2O$ | 0.01 | 0.01 |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.01 | 0.01 |
| NiCl2•$6H_2O$ | __ | 0.2 |
| $Na_2WO_4 \cdot 2H_2O$ | __ | 0.3 |

[a] protect from light; store at 4°C

**Table A.2.5. Vitamin Solution[a]**

| Compound | Concentration (mg/l) |
|---|---|
| folic acid | 2 |
| pyridoxine-HCl | 10 |
| thiamine-HCl | 5 |
| riboflavin | 4 |
| nicotinic acid | 5 |
| biotin | 2 |
| DL-Ca-pantothenate | 5 |
| vitamin B-12 | 0.1 |
| p-aminobenzoic acid | 5 |
| DL-6,8-thioctic acid | 5 |

[a] protect from light; store at 4°C

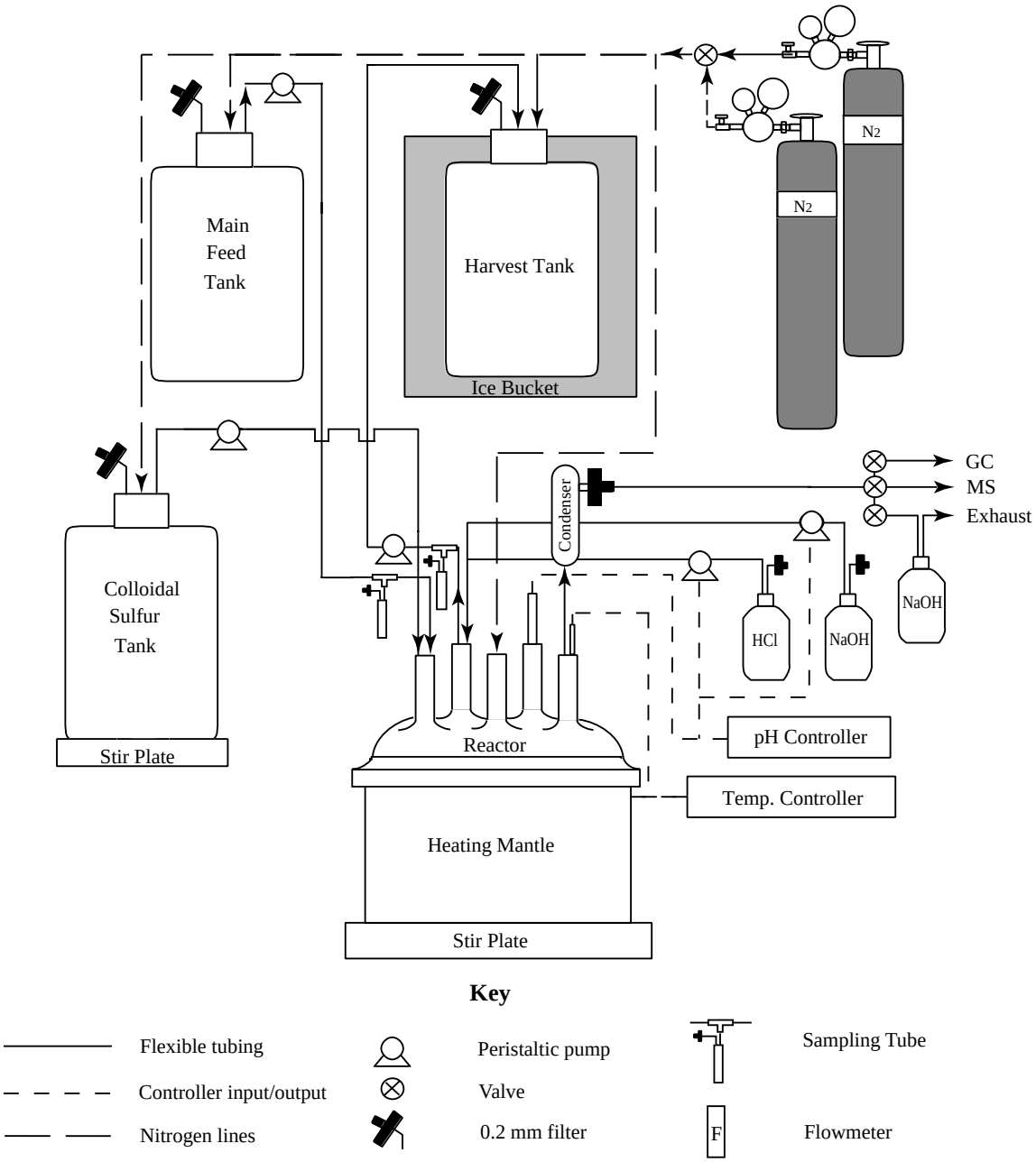



**Figure A.2.1.** Chemostat for continuous cultivation of anaerobic extreme thermophiles.

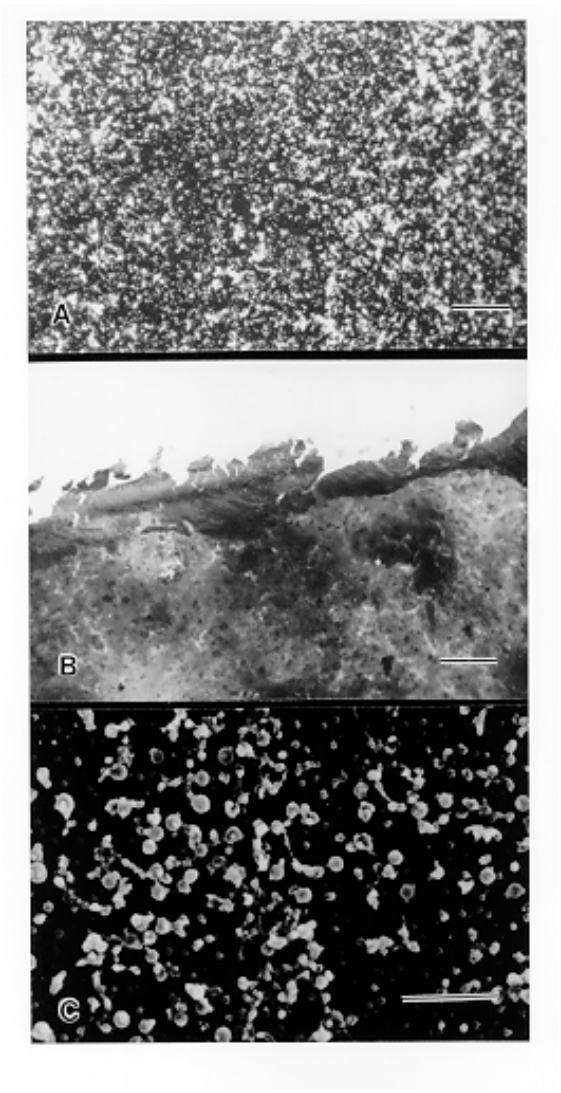



**Figure A.2.2.** *T. litoralis* biofilm formation: (A) epifluorescent microscopy of cells attached to polycarbonate filter, bar equals 60 μm; (B) Congo Red stained polysaccharide coating of biofilm formed on glass slide, (torn with tweezers at top), bar equals 10 μm; (C) SEM of cells in biofilm on a polycarbonate filter, bar equals 5 μm (104).

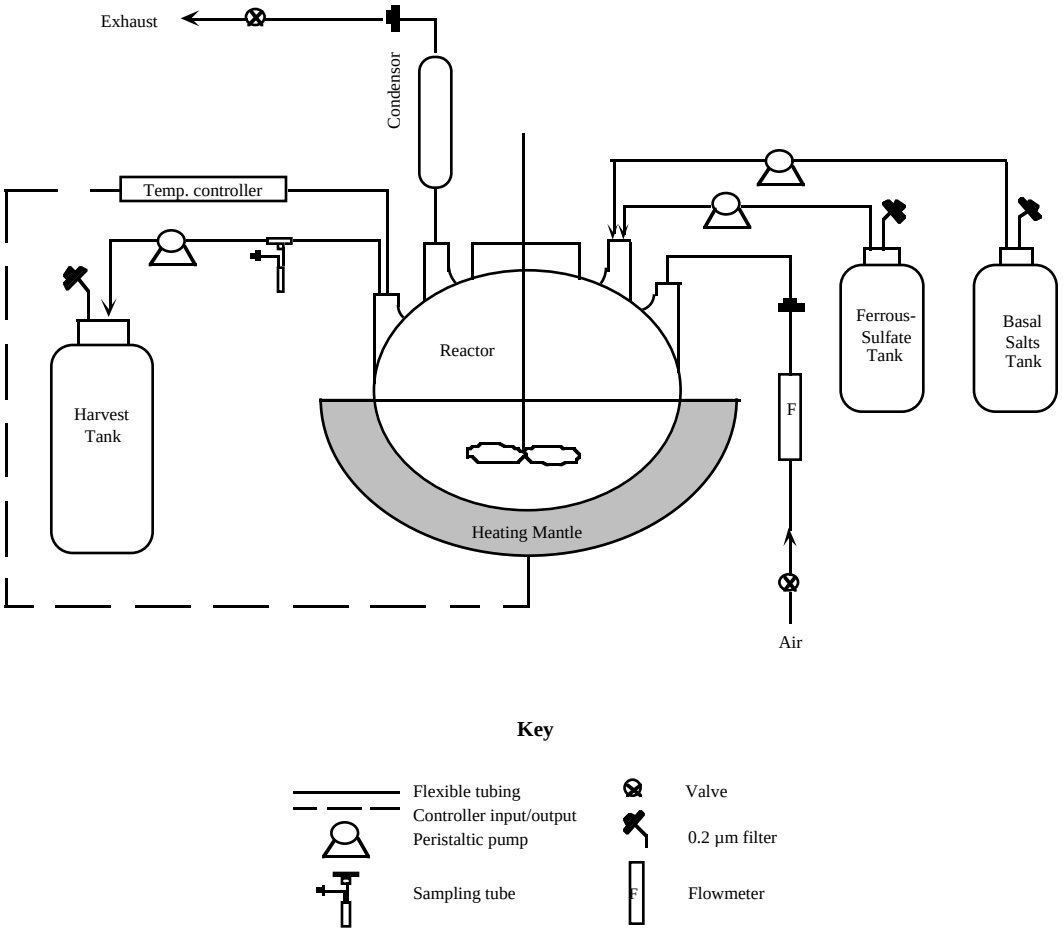



**Figure A.2.3.** Chemostat for continuous cultivation of extreme thermoacidophiles.