Abstract

PYSZ, MARYBETH ANNE. Transcriptional analysis of biofilm formation and stress response in hyperthermophilic microorganisms. (Under the direction of Robert M. Kelly.)

The significance of surface colonization and changing thermal conditions in hydrothermal environments motivated examination of biofilm formation and thermal stress response in the model heterotrophic hyperthermophilic microorganisms, *Thermotoga maritima* and *Pyrococcus furiosus*. Continuous culture, using maltose-based media and anaerobic conditions at 80°C for *T. maritima* and 95°C for *P. furiosus*, was used to generate dense biofilms on nylon mesh and polycarbonate filters; significant amounts of wall growth were observed in the chemostats for both organisms. Transcriptional analysis of biofilm-bound cells showed that genetic mechanisms observed for biofilm formation in less thermophilic bacteria applied to *T. maritima*. L-lactate dehydrogenase (TM1867), NADH oxidase (TM0379), sensor histidine kinase (TM0187), and TetR family transcriptional regulator (TM0823) were among the genes induced in *T. maritima* biofilms with mesophilic counterparts. Also consistent with cells in mesophilic biofilms was the differential expression of stress-related genes. Thermal stress genes, *hrcA* (TM0850), *grpE* (TM0851), and *dnaK* (TM0373) were up-regulated, indicating that elements of stress response are operational in hyperthermophilic biofilm environments.

Expression of stress-related genes in the *T. maritima* biofilm prompted a study of stress response during heat shock at 90°C. A 407-gene targeted cDNA microarray was used to study the genetic differences between cells at 80°C and cells at 90°C after 0, 5, 10, 20, 30, 60, and 90 minutes. The two major heat shock operons *dnaJ-grpE-hrcA* (TM0849-TM0850-

TM0851) and *groEL-groES* (TM0505-TM0506), as well as the genes encoding DnaK (TM0373) and heat shock protein class I (TM0374), exhibited maximal induction at early times (~5 minutes), subsequently decreasing to a steady-state level. This expression pattern has also been observed during heat shock of the mesophilic bacteria *Escherichia coli* and *Bacillus subtilis*. Also observed was the stress-related response of the SOS regulon involving *usrB* (TM1761) and *recA* (TM1859), and the down-regulation of this operon's repressor *lexA* (TM1082). Atypical of heat shock response, the majority of genes encoding ATP-dependent proteases, including ClpP (TM0695), ClpQ (TM0521), ClpY (TM0522), LonA (TM1633), and LonB (TM1869), were down-regulated. ATPase Clp C subunits 1 (TM0198) and 2 (TM0873) were both up-regulated, along with ClpX (TM0146) and FtsH (TM0580). The ATP-independent heat shock serine protease HtrA (TM0571) was also induced. A number of other genes not related to stress response also showed significant changes in expression levels. These include transcriptional regulators, genes within the gluconate metabolic pathway, sugar transporters and glycosidases, and sigma factors. Homologs to σ^E and σ^A were induced during heat shock at 90°C, and suggesting that they are implicated in stress response regulation in *T. maritima*, although they have not been characterized to date.

This work led to the development of chemostat-based methods for generating RNA from hyperthermophiles embedded in anaerobic biofilms that could be used for transcriptional analysis. Such analysis indicated possible connections between the genetic response of biofilm-bound cells and thermal stress response. The results here point to the significance of surface colonization and modification of cellular function arising from thermal changes in the microbial ecology of hydrothermal environments.

Transcriptional Analysis of Biofilm Formation and Stress Response in Hyperthermophilic Microorganisms

By

MARYBETH ANNE PYSZ

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, NC

2003

APPROVED BY:

James W. Brown	Steven W. Peretti
David F. Ollis	Robert M. Kelly

Chair of Advisory Committee

Biography

Marybeth Anne Pysz was born on December 20, 1974 to Sylvia Frances and John Edward Pysz in Buffalo, New York. She attended Fourteen Holy Helpers elementary school and Villa Maria Academy for high school education. She pursued a Bachelors of Science degree in chemical engineering at the State University of New York at Buffalo, where she graduated in May, 1997. She also received two minors in mathematics and computer science. In the fall of 1997, she moved to Raleigh, North Carolina to pursue a Ph.D. in chemical engineering at North Carolina State University, where she has also received her Masters of Science in chemical engineering (December, 1999) and a minor in biotechnology.

Acknowledgments

I would like to thank Robert M. Kelly for providing financial and technical support for this research, as well as all of the lab members for their expertise: Keith Shockley, Clemente Montero, Shannon Burns, Kristina Rinker, Jun Gao, Lara Chang, Swapnil Chhabra, Dave Sehgal, Donald Ward, Joshua Michel, Matthew Johnson, Donald Comfort, Kevin Epting, Hank Chou, and Sarah Hsu.

I would like to express my gratitude to my family and friends, especially my parents (John and Sylvia Pysz) and grandmother (Estelle Kuwik) for love and support throughout my academic career.

Table of Contents

List of Figures

List of Tables

Chapter I. Microbial Ecology of Hyperthermophilic Environments

I.1. Hyperthermophilic Microorganisms

Life at high temperatures intrigued microbial ecologists like Thomas Brock who began studying thermophiles (i.e., organisms that grow between 45 and 70°C) in the 1960s at Octopus Spring in Yellowstone National Park (41, 248). In 1965, the thermophile *Thermus aquaticus* was isolated and in 1968, microscope slides immersed in the boiling water of Yellowstone springs revealed a plethora of microbial diversity, later confirmed by radioisotope studies (41). Communities of diverse microbial species have also been identified in cyanobacterial mats within Octopus Spring (227). Ecological and evolutionary studies in a wide range of hot environments arose from intrests in Brock's discovery of high-temperature organisms (41). In addition to thermophilic microorganisms, other extreme environments (e.g., with broad pH ranges (0 to 12), high pressures (above 200 atm), and high salt concentrations) contain life resident there in, and led to the name "extremophiles" for these unusual microorganisms (2, 3, 124, 150, 247-250).

Higher temperature systems with hyperthermophiles were discovered in shallow and deep-sea hydrothermal vent systems, and provided an even more diverse and "exotic" set of microorganisms (41, 249, 250). The most thermophilic of these so far is *Pyrolobus fumarii*, an archaeum that was isolated from a black smoker wall at the Mid Atlantic Ridge, and grows up to 113°C (33). Hydrothermal vents over 6000 meters below the surface of the ocean offer a wide variety of chemistries (78, 191, 240), which can support metabolic reactions for a variety of microbial inhabitants (21, 78). These high-temperature areas are characterized by the anaerobic conditions, reducing gases, and nutrient fluxes necessary for an extensive diversity of extremophilic inhabitants to populate and survive (138, 247, 249). Baross

and co-workers have postulated the existence of thermophiles and hyperthermophiles within the subseafloor where thermal and chemical gradients would promote growth (124). Analysis of sulfide samples show evidence of microbial lipids within the porous sediments, therefore, suggesting diverse microbial communities living beneath the subsurface (124). Huber et al. (2002) (126) identified a diverse community of mesophilic and thermophilic microorganisms in a subseafloor habitat at Axial Seamount. More biodiversity was found in particle-attached fractions, as opposed to the free-living population, which suggests that the microorganisms may prefer to live in biofilms (126).

Life on pyrite surfaces such as hydrothermal vent chimneys has been postulated to be the precursor of all life to date. In 1990, Wachterhauser (277) suggested that the chemical reaction for the oxidative formation of pyrite (FeS_2) from metal sulfides and hydrogen sulfide (H_2S) yielded enough energy (ΔG = -38.4 kJ/mol) to support anabolic pathways (i.e., citric acid cycle). Furthermore, volcanic gases may be representative of early atmospheres; thus, CO_2 and N_2 could combine with water to make amine and organic biomolecules (179). Interestingly, several hyperthermophiles are anaerobic chemolithoautotrophs, which utilize CO_2 as a carbon source. Phylogenetic comparisons of 16S rRNA sequences have aligned sequences and constructed trees with the deepest and shortest lineages belonging to hyperthermophilic chemolithoautrophs such as *Pyrodictium, Methanopyrus,* or *Aquifex* (249). The long lineages are probably due to higher G+C content of the 16S rRNA (compared to Eukarya and Bacteria), and indicate that these prokaryotes were able to adapt readily to changing environments whereas organisms that grow between 15 and 45°C had a faster rate of evolution (248). Thus, it appears that the first living organism on earth was a sessile hyperthermophilic autotroph.

Hyperthermophiles are divided into two main classifications: bacteria and archaea. The main chemical difference between these two groups is the lipid structure, whereby archaea possess ether-linked lipids and bacteria have ester-linkages. Another distinctive feature of prokaryotic archaea is observed in the cell envelope structure. While the mesophilic Bacteria contain cell walls and/or peptidoglycan layers, most archaea possess layer(s) of proteins, called S-layers, outside the cellular membrane apparently for maintenance of cell shape (25, 249). Several reviews (3, 4, 236, 249) provide an extensive description of physiological characteristics of hyperthermophiles. Various metabolic utilization pathways are discussed in further detail in Chapter II.

I.2. Biofilm Physiology

The prevalent form of microbial growth has been proposed to involve cells adherent to surfaces that exhibit a unique phenotype (30, 63-65, 130). This complex phenomenon, involving cellular communities attached to surfaces, is called a biofilm (65, 149). Several reviews have described the physiological characteristics and industrial significance of biofilms (30, 56, 62, 63, 65, 149, 156, 253).

Understanding of this natural microbial habitat has significant ramifications in many other fields, including microbiology, agricultural, industrial processes, environmental systems, the food industry, and medicine (63, 130). The exopolysaccharide matrix offers a stable and protected microenvironment for the cells to inhabit, as they resist outside attacks from potential harmful sources in the environment such as ultraviolet radiation, bacteriophages, excessive heat, and anti-microbial agents (50, 63). Extracellular polysaccharides secreted during biofilm formation contain various proteins and sulfated sugars allowing the biopoly-

mer to bind to various metals, such as lead, cadmium, and zinc for processes, including waste-water treatment and toxic metal ingestion (130, 166). On the other hand, this strong binding affinity to metals can also cause serious harm in many industrial processes where biofilms may cause damage to equipment, such as heat exchangers and piping systems (130). Metal corrosion is also a concern in oil platforms, where sulfates and low pH environments are created by microbial biofilms (130, 163). As the need for biomaterials, the population, and industrial demands increase exponentially, the importance of understanding biofilm growth cannot be underestimated for controlling the undesirable biofilms and welcoming the beneficial biofilms for the appropriate application (63, 130).

Mechanisms of biofilm formation are complex, although the fundamentals of the matrix formation are controlled by the cell's ability to self-replicate and produce exopolysaccharide (47, 64). Three mechanisms of biofilm formation have been proposed: (i) redistribution of attached cells; (ii) cellular division of attached cells; or (iii) recruitment of planktonic cells into the biofilm; thus, mature biofilm structures may take some time to develop (253). Continuous culture has been used to study biofilm formation in *Pseudomonas aeruginosa*, a Gram-negative bacterium that causes many medical infections, including cystic fibrosis (73). Flow cells and reporter gene technology have also been used to demonstrate genetic responses to biofilm formation. Initially, cells utilize flagellar motion to swim towards the surface, where they use pili for attachment and sensing (80, 193). Once they are near the surface, the genes for exopolysaccharide production are activated (72). Other cells are drawn towards the surface by sensing the attached population through autoinducer molecules such as homoserine lactones (i.e., quorum sensing) (74). The quorum sensing cascade has been found to turn on a variety of genes, including those involved in virulence (e.g., chitinase, en-

dotoxin, etc.) for resistance mechanisms common to biofilm bacteria (117). The maturation phase of biofilm development results in the formation of complex "mushroom-like" communities of cells encased in exopolysaccharide. These cellular communities have been shown to vary phenotypically from planktonic cells (253). In some cases, changes were observed in 50% of the proteome (229), while others have reported only 1% difference in transcriptional levels (283).

The specific roles of genes differentially expressed in biofilms are difficult to identify. They depend on the organism being studied, what biofilm stage is sampled, and the analysis being performed, be it translational or transcriptional, among others. Gene expression patterns in *P. aeruginosa* and *E. coli* has been studied at various stages of biofilm formation. Several key events have been identified. First, flagellar motion is needed at the initial stages to propel the bacterium towards the surface. Once at the surface, twitching motility via type IV pili is needed to traverse the surface and adhere (193, 194, 202, 204). Surfaces have also been found to induce exopolysaccharide synthetic genes, such as alginate synthetic genes (e.g., *algC, algD*) in *P. aeruginosa*, as demonstrated by Davies et al. (1993) (72). The complexity of the mature biofilm stage is a direct result of quorum sensing genetic expression. *P. aeruginosa* PAO1 requires the *lasI* gene to develop normal biofilms. The *lasI* gene product, $3OC_{12}$-HSL, is an autoinducer that informs the bacteria of cell density, and has been found to regulate many genes, including exopolysaccharide synthetic genes and virulence genes via signal transduction (i.e., through membrane-associated sensors such as sensor histidine kinases) (74). Another quorum sensing mechanism through the *rhlI* gene product, $3OC_4$-HSL, is also activated in *P. aeruginosa* mature biofilms, and is responsible for rhamnolipid biosynthesis (74, 229, 253). Yet, quorum sensing regulates only a small subset of the

differentially expressed genes in the biofilm. Other genes involved in metabolism, lipid biosynthesis, membrane transport, and protective mechanisms (e.g., stress-related genes and proteolysis) have been identified to be important for mature biofilm formation (229, 253). Some cells detach from the biofilm and revert to the planktonic phase, although the physiological basis for this event is unknown. Future efforts of gene expression analyses will provide a better understanding of the regulation of biofilm detachment (253).

Biofilm formation is largely dependent on the ability to form exopolysaccharide as an adhesin. The encapsulatation of the cell envelope initiates the attachment by providing an adhesive material to interact physico-chemically via Lifshitz-Van der Waals forces, electrostatic forces, hydrogen bonding, and Brownian motion forces (44, 79, 170). Exopolysaccharides are produced in various forms, ranging from simple homopolymers of a monosaccharide to highly decorated and branched heteropolymers. For example, the EPS of *P. aeruginosa*, alginate, is composed of mannuronic acid (40), whereas the structure of the EPS produced by the deep-sea vent bacterium *Alteromonas macleodii* subsp. *fijiensis* has a backbone containing →4)-β-D-glucose*p*-(1→4)-α-D-galactose*p*(A)-(1→4)-α-D-galactose*p*-(1→ with a branch of 4,6-Pyr-β-D-mannose*p*-(1→4)-β-D-glucose*p*A-(1→3)-α-D-glucose*p*A-(1→3)-connected to the α-D-galactose*p*A in the center of the backbone repeat (223). Sutherland (1997) (258) discusses some of the significant structural properties of polysaccharides. Specifically, an O-acetyl or pyruvate group on an oligosaccharide can greatly affect the properties of exopolysaccharides; for example, an increase in deacetylation increases the gel strength of the biopolymer (258). Highly sulfated polymers, on the other hand, have lower viscosities, making them especially useful for anticoagulants in the pharmaceutical industry (109, 111). High molecular weight polymers (e.g., xanthan gum) are used as thickeners in the

food industry (180, 207, 281, 282). The importance of polysaccharides in biofilm formation, the biosynthetic pathways, and the relevance of polysaccharides in deep-sea environments are further addressed in Chapter II.

The hyperthermophiles, *Archaeoglobus fulgidus, Methanococcus jannaschii, Thermococcus litoralis, Thermotoga maritima,* and *Pyrococcus furiosus,* have been shown to produce biofilms in laboratory settings (114, 163, 181, 214, 217). LaPaglia et al. (1997) (114, 163) reported evidence of *A. fulgidus* biofilms during stress conditions of heat shock, pH adjustment, addition of antibiotics, high metal concentration, and oxygen exposure. Rinker et al. (1998; 2000) (214, 216) also noted *T. litoralis* and *P. furiosus* biofilm formation increased with ammonium chloride concentrations. However, not much is known about the physiology of hyperthermophilic biofilms to determine why these stress conditions induce biofilm formation.

This work provides genetic analyses of *Thermotoga maritima* and *Pyrococcus furiosus* biofilms using cDNA microarrays to compare planktonic and biofilm phases. The goal is to understand more about hyperthermophilic biofilm formation to gain an ecological perspective of how they grow in their natural environments. Higher complex communities of mixed cultures, such as co-cultures of *T. maritima* and *Methanococcus jannaschii* (181), and natural samples can then be studied more in depth. The study of mixed culture biofilms can provide insight to metabolic relationships in a quantitative sense through population dynamic studies. Furthermore, identification of biofilm-specific genes may be used to probe surface-associated communities for new microorganisms (173). Fluorescent *in situ* hybridization (FISH) probes (i.e., 16S rRNA) may also be designed less specifically for general domains (e.g., Bacteria, Archaea, and Eukarya) and can be used in mixed cultures (173). For example,

Schramm *et al.* (1996) (237) used a 16S rRNA-targeted oligonucleotide probe to observe the distribution of *Nitrobacter* and *Nitrosomonas* in a nitrifying biofilm on a trickling filter of an aquaculture water recirculation system. The lithoautotrophic ammonia-oxidizers (*Nitrosomonas*) formed a dense microcolony layer in the top section of the biofilm, while the nitrite-reducing species (*Nitrobacter*) formed a thinner layer behind the ammonia-oxidizers (237).

Identification of biofilm and exopolysaccharide genes is also important for its potential in the biotechnology industry. Many hyperthermophilic enzymes have been used commercially because of their high thermostability. For example, DNA polymerase from *Thermus aquaticus* is used in polymerized chain reactions for gene amplification (2). Similarly, polysaccharide synthetic and degrading enzymes, such as glycosyl transferases and glycosidases, can be used commercially to make polysaccharides for the oil/gas well recovery process, drug delivery systems, and food industry. For example, cyclodextrans are used in the pharmaceutical industry to solubilize hydrophobic drugs, and dextran polymers are used in photographic emulsions, iron carriers, blood plasma substitutes, and chromatography supports (111, 180). Similarly, the large inventory of hyperthermophilic glycosidases (discussed in Chapter II) can be used for transglycosidation reactions. Fischer *et al.* (1996) (93) demonstrated the transglycosylation of cellobiose with the thermostable *P. furiosus* β-glucosidase (93). β-O-D-glucopyranoside synthesis was achieved using primary, secondary, and tertiary alcohols as aglycones accepted by the *P. furiosus* β-glucosidase (93).

I.3. Stress Response

Bacteria may experience a variety of stresses within laboratory and natural environments, including osmotic shock, temperature stress, oxidative stress, and pH shock, among others.

Specific defense mechanisms associated with recovering from various stresses have been studied using cDNA microarrays (120), proteomics (15), differential display (98), and genomic BLAST searches (158). The recovery process involves various molecular chaperones to refold proteins, proteases to promote protein turnover, transcriptional regulators for control of the expression of specific operons, and synthesis of compounds, such as compatible solutes. The cell responds to environmental changes very rapidly to initiate recovery (290), and in many cases, the genetic machinery for recovery is utilized for various stress responses (i.e., there are some general stress proteins and also proteins specific to a particular stress condition) (168).

Thermal stress response is the most widely studied stress condition, and heat shock proteins are generally well conserved in microorganisms (158). In hyperthermophiles, increased growth temperatures result in the synthesis of heat shock proteins, the accumulation of compatible solutes, and formation of biofilms, as reported for *Archaeoglobus fulgidus* (114, 163, 168). Other cellular changes include the increased fluidity of lipid membranes, increased protein flexibility (i.e. unraveling), and changes in DNA and RNA. Hyperthermophilic bacterial membranes are composed of glycerol fatty acyl esters, which combine with proteins to form a matrix in a liquid crystalline state for bioenergetic maintenance (168). Heat shock proteins control this liquid crystalline state during thermal stress and adjust the fluidity to maintain appropriate gradients of protons and solutes. Archaeal membranes are made up of tetraethers and cycles in isoprenoid chains. An increase in tetraether content is observed during thermal stress; however, studies of lipid changes in hyperthermophilic archaea due to heat shock have not been undertaken (168).

Alteration of RNA in response to temperature upshifts occurs by increasing GC content, fewer bulged nucleotides, and reduction of hairpins and other folded structures. In some organisms, the 2' OH of the ribose is methylated to prevent the breaking of the phosphodiester bond and further degradation. DNA is affected by thermal stress because higher temperatures cause the double helix to unwind. A range of structures has been reported in hyperthermophiles, from relaxed and positive supercoiled in archaea, to negative supercoil for bacteria. Positive and negative supercoiled structures are assembled by reverse gyrase and gyrase respectively. Furthermore, archaea such as *Pyrococcus furiosus*, have been found to have very efficient DNA repair mechanisms that may help the cells recover during thermal stress.

Hyperthermophilic proteins are attractive from a biotechnological standpoint for their stability at high temperatures, as well as resistance to other extreme conditions such as pH, pressure, and organic solvents. They are more resilient than mesophilic counterparts due to increased hydrophobicity, differing amino acids (more charged and less polar residues), increased packing efficiency, and better structural stabilization through ion pair networks, hydrogen bonding, and loop stabilization (168). Despite this inborn rigidity, hyperthermophilic proteins may be altered in terms of their flexibility due to temperature shifts; although, the intricacies of the regulatory mechanisms are unknown.

Heat shock proteins (Hsps) are a set of proteins that are induced during thermal stress response; although, their expression has also been observed during other stress responses. The most conserved genes are the molecular chaperones, HSP40, HSP60, HSP70, and HSP90 families. Interestingly, the archaea genomes sequenced to date indicate that HSP60 is the only chaperone present, but are more similar to bacterial orthologs than eukaryotic orthologs.

Macario *et al*. (1999) (169) provides a detailed review of stress response in archaea; however, the focus here is directed at the thermal stress response in the hyperthermophilic bacterium *Thermotoga maritima*. Sequence analysis reveals that the *T. maritima* genome contains the HrcA-controlled and CIRCE-element mediated heat shock operons *dnaK-grpE-hrcA* and *groEL-groES* respectively. These genes are well conserved throughout the bacterial kingdom (158). Stress response is also controlled by sigma factors in *E. coli* (σ^{32}) and *B. subtilis* (σ^{B}). Although *T. maritima* does possess sigma factors, it does not have homologues to these mesophilic regulators. Thermal stress response in hyperthermophiles has not been studied to a great extent and the examination of heat shock response in *T. maritima* is reported here.

I.4. Objectives

The goal of this thesis was to demonstrate biofilm formation in the hyperthermophilic bacterium *T. maritima* (T_{opt} 80°C) and archaeaon *P. furiosus* (T_{opt} 98-100°C), and to develop methods to further understand the physiological and genetic basis of biofilm formation mechanisms. The genomes sequences for these two organisms are available (www.tigr.org) and were utilized to identify potential genes involved in biofilm formation. Primers were designed for 500 bp fragments to be used on a targeted cDNA microarray for determination of differential gene expression patterns between planktonic and biofilm cells. The first objective was to establish a system to collect significant amounts of biofilm for RNA isolation. Continuous cultivation systems based on previous reactor designs (214, 232, 233) were used with Nylon mesh submerged in the culture as a biofilm substratum. Davies *et al*. (1993) (72) used a similar system with Teflon mesh to study gene expression in *Pseudomonas aeruginosa* biofilms with reporter gene technology. As expected, a variety of genes were differentially

expressed in sessile populations, which was also observed in many mesophilic biofilms including *E. coli* (200, 203), *B. cereus* (192), and *P. aeruginosa* (228, 229, 283).

The results from the biofilm gene expression studies indicated that there may be a stress response induced at the surface as several heat shock genes (DnaK, HrcA, heat shock protein class I) were up-regulated in sessile cells. However, the stress response mechanisms in hyperthermophiles have not been studied to date. Therefore, to gain a better perspective of biofilm gene events, the second objective was to study the stress response of *T. maritima* during heat shock at 90°C. A 14.0 L fermenter was used to grow the hyperthermophilic bacterium at 80°C for 8 hours, after which the temperature was increased to 90°C. Samples were withdrawn at various time points to observe the dynamic response of the cells to the heat shock. Indeed, several non-heat shock associated genes were expressed under biofilm and thermal stress conditions; thus, regulation patterns may be similar and that the biofilm microenvironment may induce a stress response. Full-genome expression analyses will yield more complete information regarding some of the initial reports described here.

Chapter II. Significance of Polysaccharides in Microbial Physiology and the Ecology of Hydrothermal Vent Environments

Accepted in: **AGU Monograph Series**

"The Subseafloor Biosphere at Mid-Ocean Ridges," (August, 2002)

Authors: Marybeth A. Pysz, Clemente I. Montero,

Swapnil R. Chhabra, Kristina D. Rinker, and Robert M. Kelly

Abstract

Hyperthermophilic microorganisms (those with maximum growth temperatures of 90°C and above) are known to inhabit deep-sea hydrothermal vent environments and are suspected of being present in the associated subsurface biosphere. One characteristic of the growth physiology of many heterotrophic hyperthermophiles is the capacity to use complex polysaccharides (e.g., α- and β-linked glucans as well as non-glucan hemicellulases) as carbon and energy sources. Polysaccharides may also play an important ecological role in the deep-sea subsurface biosphere as the structural elements of biofilms harboring both heterotrophic and chemolithotrophic microorganisms, representing a range of growth temperatures. Genome sequence analysis of several hyperthermophiles indicates that the enzymatic machinery to synthesize and hydrolyze polysaccharides is present in this group of microorganisms. This is supported by the biochemical characteristics of glycosidases from hyperthermophiles in addition to the observation that several hyperthermophiles form biofilms in pure and co-culture. It remains to be seen if biofilms form the basis for a subsurface biosphere but this possibility seems likely given the physiological characteristics of several hyperthermophiles and mesophiles, representative of microorganisms previously isolated from vent sites.

II.1. Introduction

Polysaccharides are complex carbohydrates that are produced by plants, animals, and microorganisms (10, 37, 56, 109, 256, 258, 281, 282). They are necessary for maintenance of cell structure (e.g., lipopolysaccharides and archaeal S-layers), and can also serve as a cellular adhesin for attaching to solid surfaces and other cells to form biofilms, matrices of cells and exopolysaccharides (EPS) arranged in mushroom-like communities on sur-

faces (65, 149, 156, 257, 258). Polysaccharides serve as the framework for biofilms, responsible for their stable structure and protection against harmful attacks by ultraviolet radiation, bacteriophages, excessive heat, and anti-microbial agents (50). Capsular polysaccharides, associated with the cell outer surface, are responsible for initiating cellular attachment through physico-chemical interactions (e.g., Van der Waals forces, hydrogen bonding, electrostatic forces) (44, 79, 170, 225, 226). In some cases, such as with *Pseudomonas aeruginosa*, a well-characterized, biofilm-producing microorganism, the production of polysaccharide (i.e., alginate) is induced by contact with a surface, which is promoted by cellular mechanisms, such as intracellular signaling, flagellar motility, and pili sensing (72-74, 193). Polysaccharides also determine the chemical characteristics of the biofilm microenvironment; physical characteristics dictate the mass transport of nutrients, redox compounds, pollutants, and other microorganisms within the biofilm (56, 65). Since biofilms have been proposed to be the prevalent form of microbial activity in natural environments (30, 63-65), polysaccharides by implication play an important ecological role in a diverse set of microbial communities. One such natural environment for which biofilms have not been studied as yet is a deep-sea biotope. This review assesses the microbiological importance of polysaccharides in deep-sea hydrothermal environments, and discusses the implications of polysaccharide utilization and synthesis in the ecology of microorganisms inhabiting these niches.

II.2. Hydrothermal Vent Microbiology

Deep-sea sediments and hydrothermal vent systems contain varying gradients of organic compounds, redox potentials, pressures, and temperatures suitable to host an extremely

diverse population of microorganisms (78, 174, 276, 287). Biological communities were first identified around the deep-sea ridge, Galapagos Rift, in 1977 (60, 240), and this discovery set the stage for exploring a new type of biotope (240, 276). The geochemical composition and convective transport of hydrothermal vent fluids has been well characterized at the Mid-Atlantic Ridges, 21°N, East Pacific Rise, and Southern Juan de Fuca Ridge (48, 66, 240, 245, 266, 267). The chemical, temperature, and pH variations determined from geochemical surveys of these sites are indicative of the potential for a wide range of microbial life that could be supported within the sediments, water column, smoker structures and sub-seafloor. In support of this, fermentation, sulfur and sulfate reduction, methanogenesis, nitrification, hydrogen oxidation, and iron oxidation, among other metabolic and redox processes, have been identified in anaerobic and aerobic deep-sea microbial communities. The interaction and simultaneous utilization (e.g., mixotrophy) of these metabolic processes determine the speciation of planktonic and sessile microbial communities in hydrothermal vent habitats (138). Yet, there have not been sufficient analyses of the population distribution to reach significant conclusions about the ecology of these environments. While there are numerous mesophiles and moderate thermophiles associated with deep-sea hydrothermal systems, the most thoroughly characterized microorganisms from these sites to date are the hyperthermophiles (21).

Hyperthermophilic genera are represented in both the bacteria (e.g., *Thermotoga* and *Aquifex*) and archaea (e.g., *Thermococcus, Pyrodictium,* methanogens) domains of life. Most deep-sea vent hyperthermophiles isolated thus far are anaerobic, archaeal cocci that utilize sulfur as an electron acceptor (3, 21). Consistent with deep-sea geochemistry, hyperthermophilic isolates represent a range of metabolic features. *Pyrococcus glycovorans*

(T_{opt} 95°C) (20), *Pyrococcus horikoshii* (T_{opt} 98°C) (106), *Thermococus profundus* (T_{opt} 80°C) (153), and *Pyrodictium abyssi* (T_{opt} 97°C) (251) are among the fermentative archaea isolated from deep-sea vent areas. Autotrophic deep-sea archaea have various bioenergetic modes, including sulfate and sulfite reduction (*Archaeoglobus veneficuss*, T_{opt} 80°C), and methanogenesis (*Methanococcus jannaschii,* T_{opt} 85°C; *Methanococcus* strain AG86, T_{opt} 85°C; *Methanopyrus kandleri*, T_{opt} 98°C) (21, 125, 127, 133, 293). Blochl et al. (1997) (33) isolated a novel facultative aerobic archaeon, *Pyrolobus fumarii* (T_{growth} 90-113°C), from a black smoker wall on the Mid Atlantic Ridge that is an H_2-oxidizing chemolithoautotroph. Yet, these isolates likely comprise only a small subset of the hyperthermophiles from hydrothermal systems, many of which may prove to be unculturable in laboratory settings. It has been shown that there is significant genetic and metabolic diversity of archaea in vent chimneys and sediments, inferred from 16S rRNA-based phylogenetic analysis and lipid signatures (261, 262, 276). At this point, work with complex hyperthermophilic consortia in laboratory settings is just beginning. However, clues to the microbial ecology of such systems available from the study of model hyperthermophiles.

II.3. Metabolism of Hyperthermophilic Microorganisms

The physiological, metabolic, and genetic characteristics of several hyperthermophiles, including *Pyrococcus furiosus, Thermotoga maritima,* and *Thermococcus* sp., have been studied extensively for the last 15 years. *P. furiosus* (T_{opt} 98-100°C), an anaerobic heterotroph that facultatively reduces sulfur, was isolated from shallow geothermal waters near Vulcano Island, Italy (92). Other members of this genus have been found in deep-sea

hydrothermal vent systems. A modified, ADP-dependent Embden-Meyerhof (EM) pathways is used by *P. furiosus* as the main metabolic pathway for the fermentation of various carbohydrates, including maltose, cellobiose, starch, and pyruvate among others. Primary metabolic products include acetate, L-alanine, hydrogen, and carbon dioxide (Figure II.1), as well as hydrogen sulfide, when *P. furiosus* is grown in the presence of sulfur. In the absence of sulfur, hydrogen accumulates and inhibits growth resulting in high alanine production; whereas H_2S is formed when *P. furiosus* is grown in the presence of sulfur (i.e., low partial pressures of H_2), causing diminutive amounts of alanine to be produced. In the absence of a citric acid cycle, fermentative hyperthermophiles, such as *P. furiosus*, convert pyruvate to acetyl-CoA and finally acetate. Hyperthermophilic archaea have the ability to use a single enzyme (ADP-dependent acetyl-CoA synthase) for this conversion, while bacteria require both a phosphate acetyltransferase and acetate kinase (75, 172). Pyruvate is also converted to alanine, an alternative electron sink, via an alanine aminotransferase and glutamate dehydrogenase (75, 147). Other electron sinks involve the formation of hydrogen from ferredoxin (growth in absence of sulfur) or polysulfides from nucleophilic attack of sulfur (18, 35). Ferredoxin is preferentially used over NAD(P) in hyperthermophiles which may be an adaptation related to coenzyme thermolability (69, 75). Similar metabolic pathways have been identified in *Thermococcus* species, such as *T. celer* and *T. litoralis*, suggesting that the EM pathway is also utilized in these heterotrophic hyperthermophiles (75, 144, 146-148, 231). In terms of peptide metabolism, *Pyrococcus* and *Thermococcus* species grow well on complex medium components (e.g., yeast extract, tryptone, peptone). However, there are reports of growth on defined media supplemented with amino acids (210, 211, 214, 217). Amino acid metabo-

lism in hyperthermophiles is not well studied. However, the first step is most likely catalyzed by aminotransferases, which convert the amino acids to their corresponding keto derivatives (144). Indeed, two different types of aminotransferases have been identified in *P. furiosus* and *T. litoralis* (13, 14).

Other fermentative hyperthermophiles utilize an Entner-Doudoroff (ED) pathway or a combination of ED and EM pathways, as in the case with the hypthermophilic bacterium, *T. maritima* (75). Thermophilic microorganisms that use the ED pathway include *Sulfolobus acidocaldarius* (uses ED) and *Thermoproteus tenax* (uses EM and ED) (70, 239).

Archaeal methanogens can utilize carbon dioxide, hydrogen, formate, methanol, methylamines, acetate, and carbon monoxide to produce methane as the main metabolic product. However, acetate utilization has not been noted in hyperthermophilic methanogens. Methanogenesis is coupled with energy generation via hydrogen oxidation. Methanogenic hydrogenases contain the usual iron-sulfur clusters as well as unique redox-active nickel centers, (134). *M. jannaschii* (T_{opt} 85°C), a hyperthermophilic methanogen isolated from a hydrothermal vent on the East Pacific Rise, uses H_2 (growing autotrophically) or formate (growth under N_2 atmosphere) as electron donors for methane production (133). Several reviews (12, 107, 134, 171, 195, 197, 230, 270, 280) discuss the characteristics of methanogenesis and related metabolic processes.

Insights into the microbial physiology of hyperthermophiles have been enhanced in recent years by the publication of several hyperthermophilic genome sequences, including those of *A. fulgidus* (151), *T. maritima* (183, 184), *A. aeolicus* (76), *M. jannaschii* (43), *P. horikoshii* (142), *P. abyssi* (http://www.genoscope.cns.fr/Pab/), and *P. furiosus* (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/framik?db=genome&gi=228). Putative en-

zymes encoded in these genomes can then be identified by amino acid sequence homology searches, from which the corresponding gene can be cloned and expressed in a foreign host for further biochemical characterization. This approach, coupled with growth physiology studies and biochemical characterization of specific enzymes, can be used to study the metabolic features of these organisms. Of interest here are the pathways by which hyperthermophiles form and utilize polysaccharides, the enzymes associated with these pathways, and the implications that polysaccharides have on the microbial ecology of deep-sea hydrothermal vent biotopes. Clearly, the aforementioned hyperthermophiles are the most commonly cultivated microorganisms from hydrothermal environments and only represent a small subset of this diverse microbial habitat. However, insights concerning their physiological characteristics obtained through laboratory studies will add to our understanding of how polysaccharides are produced and utilized in this natural environment.

II.4. Complex Carbohydrate Utilization by Hyperthermophiles

Many hyperthermophilic organisms utilize carbohydrates as carbon and energy sources (24, 86). Because of the lability of glucose at high temperatures, disaccharides and higher oligosaccharides are best suited as laboratory growth subtrates for hyperthermophiles (23). The degradation of oligo- and polysaccharides is usually achieved by multienzyme systems consisting of cell wall-associated or secreted endo-glycosidases, intracellular exo-glycosidases and in some cases, intra- or extra-cellular debranching glycosidases. Exo-acting glycosidases are retained within the cytoplasm and play essential roles in assimilation and catabolism of oligosaccharides produced by endo-glycosidases, and pro-

vide monosaccharides, e.g., glucose and galactose, to central glycolysis pathways (75, 148). For instance, *T. maritima*, which has been observed to grow on the α-glucan, starch (53), possesses all the necessary glycosidases for its degradation to the monosaccharide glucose. These include two amylases, Amy13A and Amy13B, both of which possess signal peptides for extracellular transport, the intracellular α-glucosidases Amy4A-4D, and a debranching pullulanase, Pul13 (183). Information from genome sequences has led to the identification of a number of such multi-enzyme systems for polysaccharide hydrolysis in hyperthermophiles. Glycosidases from hyperthermophilic organisms hydrolyze various glycosidic linkages including the α-(1,4)-, α-(1,6)-, β-(1,4)-, and β-(1,3)- bonds present in natural polysaccharides (255). The repertoire of glycosyl hydrolases from hyperthermophilic archaea is usually smaller than that of hyperthermophilic bacteria (122). Hyperthermophilic archaea of the order Thermococcales, including the genera *Pyrococcus* and *Thermococcus*, as well as certain thermophilic bacteria, can utilize starch (92, 153). *P. furiosus* readily grows on α-1,4- and α-1,6-linked glucans, as well as on certain β-linked glucans but not on non-glucan hemicelluloses (86). Hyperthermophilic bacteria (e.g., *Thermotoga* species) can also utilize β-linked polysaccharides (cellulose, xylan, mannan) as carbon and energy sources since they produce a number of endo-acting β-specific glycosyl hydrolases (23). On the other hand, certain hyperthermophilic archaea, like *Aeropyrum pernix* (142) and *Archaeoglobus fulgidus* (75, 151), appear to be totally devoid of glycosidases. Limited information is available on the utilization of exopolysaccharides as carbon and energy sources by hyperthermophiles. Most of the aforementioned studies involve the degradation of plant polysaccharides; however, attention needs to be directed towards utilization of microbial polysaccharides, as deep-sea environments do

not contain photosynthetic life (78, 138). For example, the exopolysaccharide from *T. litoralis* is a mannose-based polysaccharide (217). The excellent growth of both *T. maritima* and *T. neapolitana* on galactomannan and glucomannan (53, 196) may suggest that hyperthermophiles occurring in the vicinity of *T. litoralis* are capable of utilizing this polysaccharide. Thus, the polysaccharide synthesized in hyperthermophilic communities may be utilized by neighboring microorganisms as a carbon and energy source.

II.5. Exopolysaccharide Production and Biofilm Formation in Hyperthermophiles

The sources of complex carbohydrates for utilization by heterotrophic hyperthermophiles have not been identified in natural extreme environments. Several hyperthermophiles, including *Sulfolobus* (188), *Thermococcus* (216, 217), and *Thermotoga* (214), have been shown to produce polysaccharides, which could serve as a substrate of various glycosyl hydrolases they possess. Sulfated extracellular polysaccharides were produced in laboratory cultures of *Sulfolobus acidocaldarius,* an extreme thermoacidophilic aerobe, and the archaeon *Haloferax mediterranei,* a mesophilic halophilic aerobe. Monosaccharide constituents of these exopolymers include glucose, mannose, galactose, and glucosamine (Table II.1) (16, 188, 198). A capsular polysaccharide, which may aid in colonization, has also been identified in the moderately thermophilic *Methanosarcina thermophila* (219, 244). We have recently been able to establish growth conditions that induce exocellular and capsular polysaccharide formation by the hyperthermophile *P. furiosus* (Figure II.2) (Pysz and Kelly, unpublished). Intracellular polysaccharides (i.e., glycogen) have also been identified in various thermophilic Archaea, including members of the genera *Thermococcus* and *Sulfolobus* (157).

In connection with the production of hyperthermophilic exopolysaccharides, the formation of biofilms has also been observed. Hyperthermophilic biofilms have been formed in pure cultures of *A. fulgidus* (114, 163), *T. maritima* (214), *T. litoralis* (217), and *P. furiosus* (Pysz and Kelly, unpublished), and in co-culture of *T. maritima* and *M. jannaschii* (181, 214). The biofilms are formed under various conditions, but seem to be inducible. The *A. fulgidus* biofilm was formed under stress conditions, including elevated pH, decreased and increased growth temperature, high salt, and exposure to ultraviolet light, oxygen, or antibiotic (114, 163). Elevated levels of ammonium chloride seem to create a stress environment for *T. litoralis,* thus leading to biofilm formation and exopolysaccharide production (214, 217). *P. furiosus* produced a biofilm on a modified *A. fulgidus* medium (Figure II.2); however, the effects of the medium constituents are currently being assessed (Pysz and Kelly, unpublished).

Although exopolysaccharide production and biofilm formation by hyperthermophiles is a recent finding, these phenomena have been well characterized in several mesophilic microorganisms. *Pseudomonas aeruginosa* (40, 74), *Lactococcus lactis* (167, 209, 272), *Streptococcus thermophilus* (39, 90, 94, 96, 97, 155), and *Escherichia coli* (8, 202, 204), have become model organisms for studying the genes involved in exopolysaccharide (EPS) production and biofilm formation. EPS production in bacteria follows a basic biosynthetic pathway, which resembles the mechanism for synthesis of O-antigens and several types of capsular polysaccharides (CPS) (102, 218). This pathway is composed of five stages: (i) Synthesis of sugar nucleotides. Phosphates on monosaccharides are first added to the 6-carbon, and then transferred to the 1-carbon position (via mutases). Nucleotides are then added to the first carbon as a diphosphate to form Nucleotide DiPhos-

phate-sugars (NDPs) (via NDP transferases). This is followed by saccharide modification (i.e., change of hydroxyl groups) or reduction/oxidation (via epimerases and dehydrogenases) (263). (ii) Assembly of the polysaccharide repeating subunits. These units are formed by glycosyltransferases, which sequentially link sugars from the intracellular pools of NDP-sugars to a lipid carrier in the cell wall (102, 104, 141). (iii) Addition of other chemical groups (e.g., phosphate, sulfate, acetyl groups, pyruvate, succinate) to EPS repeating units. Many EPS are adorned with different compounds by phosphatases, succinyl, acetyl, or pyruvyl transferases (51, 57, 101). (iv) Translocation of the repeating units in the cytoplasmatic side of the cell membrane to the extracellular side. Not much is known regarding polysaccharide transporters on the cell wall. Some suggest that the repeating unit is "flipped", and some genes responsible for this mechanism have been identified in *P. aeruginosa* (102), *Yersinia enterocolitica* (243), and *Rhizobium* sp. (49), among others. (v) Polymerization of the repeating units. Polymerization and translocation of the EPS's repeating units are tightly coupled processes, which have been difficult to study independently (101, 102).

In all bacterial cases reported to date, the structural EPS genes *per se* (i.e., involved in steps ii through v) are grouped in clusters. The genes responsible for the formation of NDP-sugars (step i) may or may not lie within these clusters (31, 101, 141). In addition, it has been shown for the majority of microorganisms studied (except *Sphingomonas* (265)) that the availability of sugar nucleotides becomes the limiting factor for EPS production; thus, the first step is the limiting reaction (72, 77, 167, 209, 265). NDP-sugars for EPS biosynthesis come from two intermediates within the glycolysis pathway: glucose-6-phosphate and fructose-6-phosphate (Figure II.1) (http://www.genome.ad.jp/kegg/ me-

tabolism.html) (263). Precursor monosaccharides for polysaccharide synthesis (via conversion to NDP-sugars) may also be derived from oligosaccharides in the environment, a provided carbon source, or an independent pathway. Levander and Radstrom (2001) (165) showed that *Streptococcus thermophilus* could synthesize galactose from lactose (i.e., provided carbon source) without going through the glycolysis intermediates via phosphoglucomutase; however, more comprehensive studies on direct incorporation of monosaccharides for polysaccharide production need to be done.

The complexity of the EPS biosynthetic pathways is directly related to the EPS composition. As mentioned, the hyperthermophilic EPSs have not been fully characterized for their chemical composition or physical structure (i.e., sugar linkage). In addition, physiological studies of hyperthermophilic enzymatic pathways are limited due to the lack of a genetic system. Despite these limitations and the difficulties associated with the culture of hyperthermophiles, preliminary identification of EPS synthetic genes using the genomic sequences can be connected to what happens at the physiological level. Comparative sequence analysis of mesophilic genes related to EPS, CPS, and LPS production has allowed us to identify homologous genes in the hyperthermophiles *P. furiosus* and *T. maritima* (Montero and Kelly, unpublished). Inducible systems, such as stress-related biofilm formation, can provide a set of comparable conditions to see if these potential genes are indeed responsible for the EPS formation that is observed. Proteomics, RNA techniques, and DNA microarrays can be used to investigate the gene regulation that is associated with EPS production. These techniques can also compare the differential gene expression of planktonic and sessile cells to observe the effects of the bulk culture and biofilm microenvironment, respectively.

II.6. Deep-Sea Mesophilic Polysaccharides

Polysaccharides produced by mesophilic and moderately thermophilic microorganisms are a potential source of carbon and energy in hydrothermal vent systems, shallow geothermal springs, oil reservoirs, and acidic pools, among other extreme environments. There are many mesophiles that thrive in the vicinity of these unique environments, and have been found to form biofilms and produce various polysaccharides. Polysaccharides from the *Alteromonas, Pseudoalteromonas,* and *Vibrio* genera have been isolated from deep-sea hydrothermal vents, and represent novel bacterial exopolysaccharides (Table II.1) in terms of their saccharide composition and structure (109-111, 206, 207, 220-223).

The mesophilic habitats in extreme environments, such as hydrothermal vents, can also provide insights into the ecological relationships among extremophiles, mesophiles, and higher organisms, as well as planktonic and sessile organisms. Guezennec et al. (1998) (110) studied the bacterial colonization on various materials over time in the vicinity of a Mid Atlantic Ridge hydrothermal area. While the adhesion was surface-dependent, the types of adherent bacteria varied over time, suggesting a change in community structure. These communal changes can often occur in biofilms due to the unique microenviroment created from the various exopolysaccharides and bacterial members. The constituents within a biofilm can then become an attractant to other planktonic organisms, which can be recruited (62, 64, 65). For example, mannose is a common monosaccharide constituent of bacterial exopolysaccharides, as mentioned above. The attachment of *E. coli* cells to human tissue occurs by the binding of a cell-surface lectin specific to D-mannose (88, 259). Similar microbial attachment mechanisms (i.e., carbohydrate

specific lectins), in addition to others (e.g., chemotaxis (193), physicochemical forces (67), etc.), may take place in deep-sea hydrothermal vents. *Alteromonas* and *Vibrio* species have been isolated from the polychaete annelid (i.e., worm) *Alvinella pompejana* that inhabit hydrothermal vent systems (206, 207, 220-223). In addition, the exopolysaccharides of a biofilm may serve as a nutrient source for nearby microorganisms. Hyperthermophiles and mesophiles could use an array of glycosidases (e.g., endoglucanases, amylases, pullanases, and mannanases) for recruiting carbon and energy sources from environmental pools. While numerous studies have documented the diffuse flow of ions and metals in hydrothermal vent systems (266, 267), the flux of polysaccharides in these environments has not been studied. This is due to the difficulties associated with sampling vent systems and the recent identification of polysaccharides in these environments (112, 221). Thus, the ecological interaction of hyperthermophilic and mesophilic microorganisms and polysaccharides needs to be addressed. These interactions could provide insight into the metabolic, genetic, and evolutionary relationships within extreme environments.

II.7. Mixed Cultures and Implications on Natural Microbial Communities

The ecology of deep-sea environments is extremely diverse based on the variety of animal and microbial inhabitants and geochemical characteristics (21, 240). The colonization of these environments, as can happen in any natural setting, may be based around biofilms, where microorganisms form a matrix of cells and polysaccharide and adhere to a surface as a community (30, 78). Biofilms offer the distinct advantage of allowing microbial species to interact and coexist in an optimized microenvironment (i.e., the matrix), thus, limiting the adverse consequences of competition and selectivity (45). Micro-

bial relationships within hyperthermophilic communities can begin to be studied by establishing co-cultures of organisms exhibiting interacting metabolic characteristics. For example, a heterotrophic microorganism that produces hydrogen and a hydrogen-utilizing methanogen can benefit each other in co-cultures as has been demonstrated for a number of pairings (38). A very tight interaction between two hyperthermophiles, *T. maritima* and *M. jannaschii,* was observed in co-culture, as revealed by electron microscopy and cell sorting (Figure II.3). The close proximity of the different cells was related to the high growth rates of heterotrophs relative to the methanogens, causing an increased dependence of H_2 availability (181, 214). Co-cultures of interacting acetate-oxidizers and hydrogen-oxidizers have also been reported (118). With the observance of biofilm formation in hyperthermophilic pure cultures, biofilm-based consortia in hydrothermal environments represent an interesting ecosystem that cannot be overlooked. However, at present, little is known about mixed culture dynamics at high temperatures, especially with respect to intra- and inter-consortial processes. Other synergistic relationships (235) may exist between fermentative hyperthermophiles, which contain a diverse set of glycosyl hydrolases and glycosyl transferases, as discussed. Thus, biofilm consortia of polysaccharide-degrading and -utilizing microorganisms may play a central role in the ecology of deep-sea environments and be the key to what might be an extensive subsurface biosphere. In addition to the effects of biofilms on microbial ecology, dense communities of microorganisms and associated polysaccharides can also influence the chemical and physical properties of the subsurface environment. It may be that viscous polysaccharides with varying chemical properties (i.e., charges, ability to form gels, etc.) contribute to the

geochemistry and fluid rheology of hydrothermal vents. Further experiments, both in the laboratory and *in situ*, are needed to explore this prospect.

II.8. Acknowledgments

This work was supported in part by grants from the National Science Foundation and the Department of Energy. We also acknowledge helpful discussions with Dr. Robert Embley, NOAA/PMEL, Newport, Oregon.

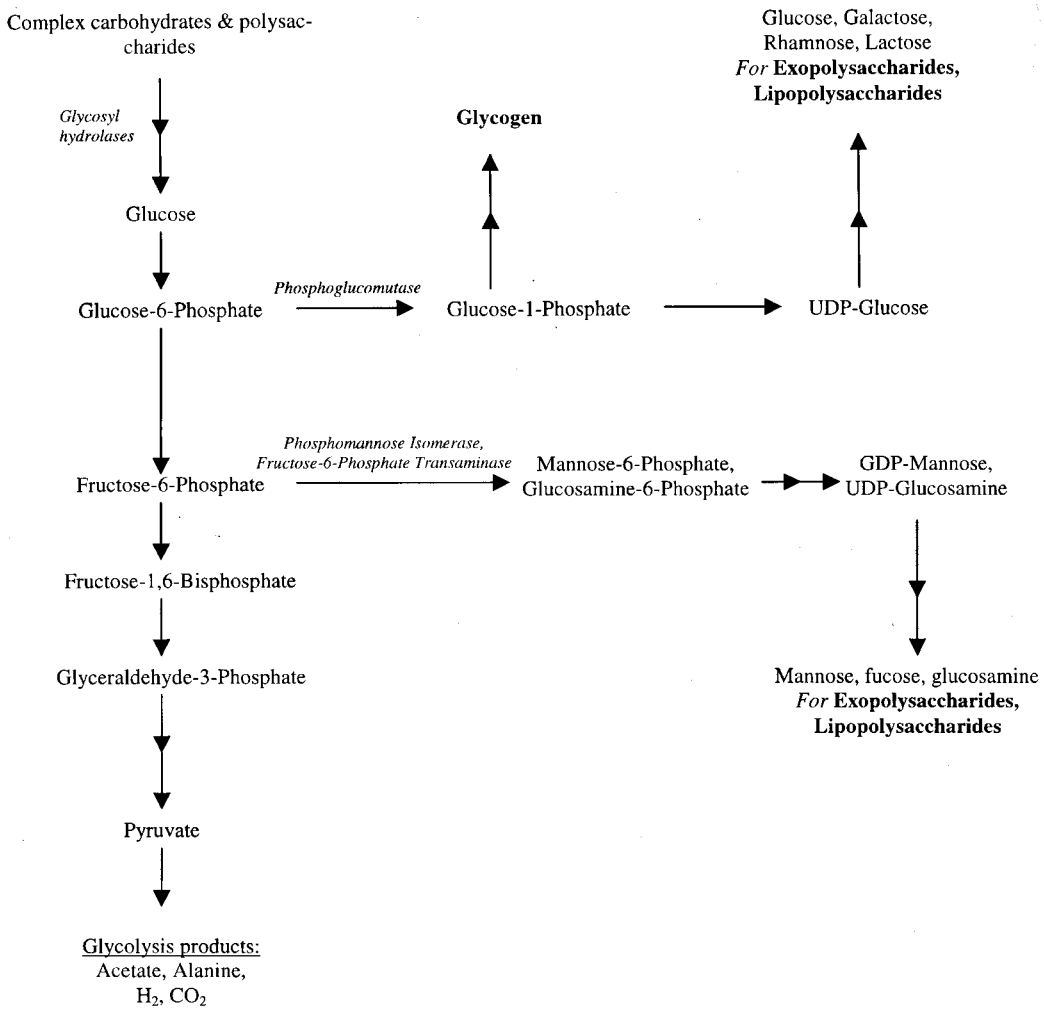


Figure II.1. Glycolysis pathway with branch point intermediates, glucose-6-phosphate and fructose-6-phosphate, for biopolymer synthesis (http://www.genome.ad.jp/kegg/ kegg2.html).

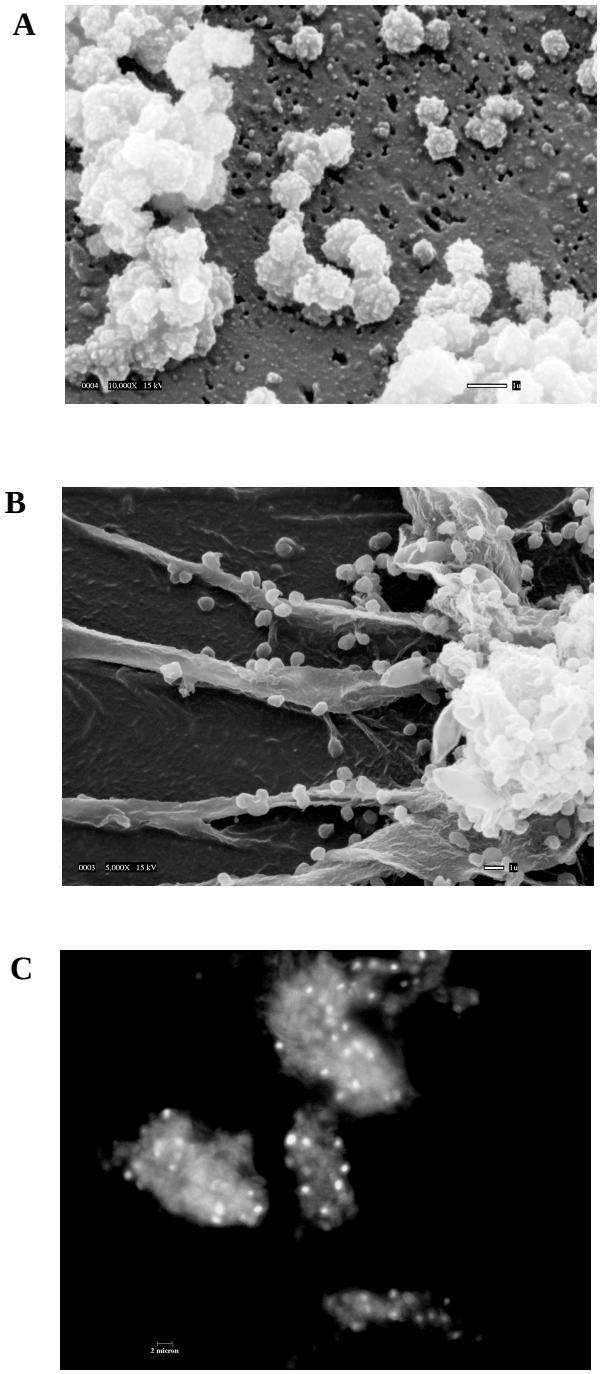


Figure II.2. Scanning electron (A, B) and epifluorescence (C) micrographs of *Pyrococcus furiosus* biofilms. Polycarbonate filters were used as a biofilm substratum in batch cultures growing on a complex medium with (A) cellobiose (5 g/l) or (B, C) maltose (5 g/l) as carbon sources. Note that a capsular material forms in (A) and a looser exocellular polymer appears in (B) (Pysz and Kelly, unpublished).

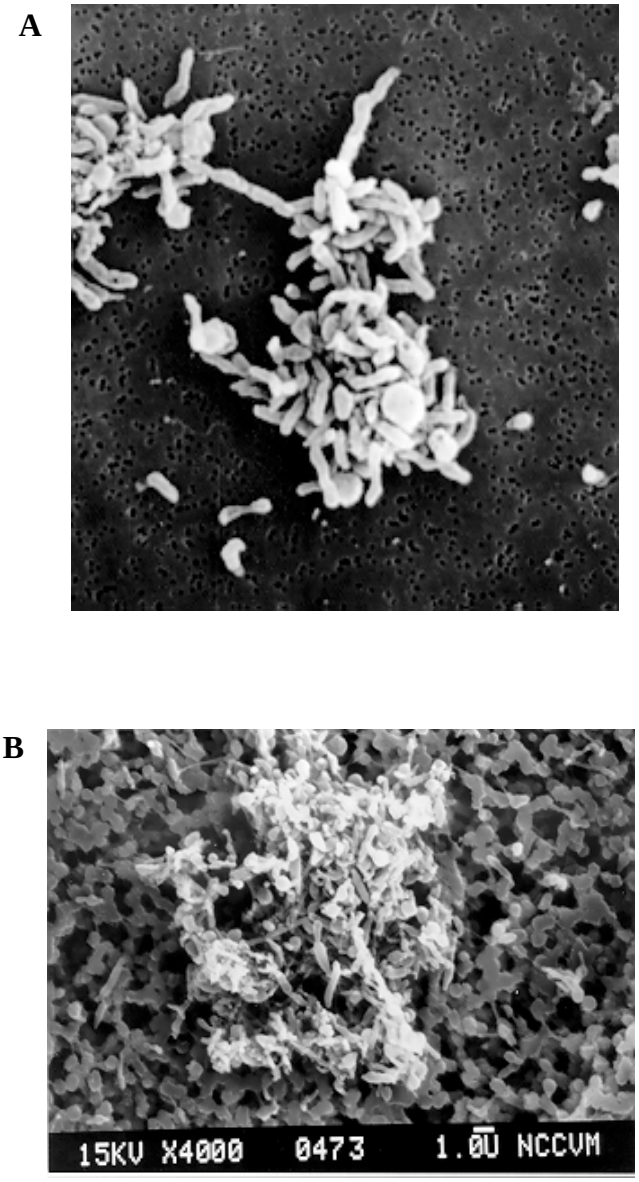


Figure II.3. Scanning electron micrographs of *Thermotoga maritima* and *Methanococcus jannaschii* co-culture. (A) planktonic cell aggregates; (B) biofilm formed on a polycarbonate filter inserted in culture (182, 215).

Microorganism	Characteristics	Polysaccharide structure	Polysaccharide repeat length	Saccharide composition	Reference
Alteromonas macleodii subsp *fijiensis*	hydrothermal vent mesophilic bacterium	branched	hexasaccharide	Glc/GalA/GlcA/4,6 PyrMan	(207, 222, 223)
Pseudoalteromonas sp.	hydrothermal vent mesophilic bacterium	branched	octasaccharide	(SO_3H)-Man/Rha/Glc/Gal/GlcA	(220, 222)
Vibrio diabolicus	hydrothermal vent mesophilic bacterium	linear	tetrasaccharide	NAcGlc/NAcGal/GalA/GlcA	(112, 206, 221)
Mastigocladus laminosus	thermophilic cyano-bacterium	branched	pentadecasaccharide	Glc/Fuc/Gal/GalA/GlcA/Man/Rha/ Xyl	(99)
Haloferax mediterranei	halophilic archaeon	linear	trisaccharide	GlcNAc/Man/GlcNAc-(O-SO_3)	(198)
Sulfolobus sp.	thermophilic ar-chaeon	n/a	n/a	Glc/Man/GlcN/Gal	(17)
Thermococcus litoralis	hyperthermophilic archaeon	n/a	n/a	Man	(217)
Pyrococcus furiosus	hyperthermophilic archaeon	n/a	n/a	Man/Glc/Rha/Xyl	(Pysz and Kelly, unpub-lished)
n/a: information not available; Glc – glucose; Gal – galactose; Man – mannose; Rha – rhamnose; Fuc – fucose; Xyl – xylose; GlcN – glucosamine; A – uronic acid form; Pyr – pyruvated ; NAc – amino deoxy-sugar					

Table II.1. Structural, chemical, and physical characteristics of various polysaccharides from mesophilic vent bacteria and thermophilic microorganisms.

Chapter III. Continuous Cultivation of Hyperthermophiles

Published in: **Methods in Enzymology**. (2001). 330A:31-39.

Authors: Marybeth A. Pysz, Kristina D. Rinker,

Keith R. Shockley, and Robert M. Kelly

III.1. Introduction

In general, a significant amount of biomass must be generated from pure cultures of microorganisms in order to purify sufficient quantities of enzymes for detailed analyses. A number of cultivation techniques have been used for hyperthermophilic organisms ranging from glass serum bottles to large-scale fermentors constructed of ceramic or stainless steel to dialysis membrane reactors (123). Even if the gene encoding a given hyperthermophilic enzyme can be successfully expressed in a mesophilic host (5), often there is the need to examine the native version. There is always the question of whether the recombinant and native versions have precisely the same properties, and native biomass is the only source of enzymes whose genes are not readily expressed in mesophilic hosts. An additional motivation is that a large fraction of open reading frames identified in the genomes of hyperthermophiles have not yet been assigned a biochemical function (3, 128). Thus, the ability to cultivate hyperthermophiles remains an important element of research efforts focusing on the biology and biotechnology of these organisms.

A continuous culture system is described here that can generate biomass from hyperthermophiles on a scale suitable for enzyme purification. High temperature chemostats have several advantages over large-scale batch systems. Long-term, stable, steady state operation (arising from minimal problems with contamination) can provide biomass generated from exponential growth phase, i.e., balanced growth (32). Because of the smaller operating volumes, continuous systems are inexpensive to construct and minimize problems with handling toxic and explosive gas substrates and products (e.g., H_2S, H_2, CH_4). Small (i.e., 1-10 liter) operating volumes also minimize problems associated with growth of sulfide producing an-

aerobes and thermoacidophiles in terms of chosing a proper material for reactor construction (e.g., glass, gold). Continuous cultivation has also been useful for studying the bioenergetics and physiology of hyperthermophiles, and for developing media formulations that induce enzyme expression (145, 214, 215, 217, 233, 234).

High temperature continuous cultivation systems were originally used to grow *Pyrococcus furiosus* (a fermentative anerobe and facultative sulfur-reducer, T_{opt} 98°C) (42), but other hyperthermophiles, including *Thermococcus litoralis* (a fermentative anerobe and facultative sulfur-reducer, T_{opt} 88°C), *Methanococcus jannaschii* (methanogen, T_{opt} 85°C), and *Thermotoga maritima* (a fermentative anerobe and facultative sulfur-reducer, T_{opt} 80°C), have also been successfully grown (34, 42, 113, 211, 214, 215, 217, 232, 233).

III.2. Materials

Reactor System

A schematic of a typical configuration for the high temperature continuous culture system is shown in Figure III.1. Although operating volumes up to 10 liters have been used, usually a 2-liter, 5-neck round-bottom flask (Ace Glass, Vineland, NJ) is chosen to process a 1-liter (operating volume) culture. A Graham condenser (Ace Glass, Vineland, NJ) is used to reduce water content in the reactor's headspace effluent gas, which may then be analyzed by gas chromatography (GC), mass spectrometry (MS), or vented (after scrubbing with 1 N NaOH for reduction of odors from sulfidic gases). The culture vessel is heated by an insulated mantle (Glas-Col, Terre Haute, IN) that is connected to a temperature controller (Cole-Parmer, Vernon Hills, IL) and J thermocouple (Cole-Parmer, Vernon Hills, IL). The pH of the culture

can be monitored and adjusted with a pH controller (Cole-Parmer, Vernon Hills, IL) and an autoclavable, double-junction pH electrode (Cole-Parmer, Vernon Hills, IL). Fixed rate peristaltic pumps (Cole-Parmer, Vernon Hills, IL) with Norprene® Masterflex tubing (Cole-Parmer, Vernon Hills, IL) are used with the pH controller to add 1 N HCl or NaOH as needed. Norprene® Masterflex tubing is used since it is autoclavable, durable, and has low O_2 permeability (an important feature of an anaerobic system). Teflon adapters with Viton O-rings (Cole-Parmer, Vernon Hills, IL) are used for insertion of the pH electrode, thermocouple, and other tubing into the culture vessel.

There are several ways to agitate the culture, including a mechanical impeller in the center of the flask, a magnetic egg-shaped stir bar placed in the center of the culture, or by sparging with gas directly into the culture volume. A mechanical impeller (Model SL 300, Fisher Scientific, Pittsburgh, PA) works well, but care must be taken to avoid excessive shearing. A magnetic stir bar may be difficult to control through the heating mantle, although it is less expensive than an impeller. Sparging with an inert gas (e.g., argon, nitrogen) also sufficiently mixes reactor contents, and also maintains anaerobic conditions. Gas may be sparged into the culture (as well as into the culture medium, cell collection, and colloidal sulfur feed tanks) with glass tubing connections (inserted into rubber stoppers) and controlled with a flowmeter.

The continuous feed (i.e., culture media and sulfur) and harvest collection (i.e., reactor effluent) are pumped to and from the reactor with Masterflex tubing and a Masterflex peristaltic pump (Cole-Parmer, Vernon Hills, IL). Polypropylene carboys (Nalge, available through Cole-Parmer, Vernon Hills, IL) are used to store the feed and harvest liquids. Pump

heads (Masterflex, Cole-Parmer, Vernon Hills, IL) varying in size from 14, 15, and 16 can be used for the colloidal-sulfur feed, product stream, and media feed, respectively. A single peristaltic pump (Masterflex, Cole-Parmer, Vernon Hills, IL) should be used for both inlet streams (i.e., the medium and colloidal sulfur, if used), and the outlet stream, such that any constant dilution rate will prevent the total feed from exceeding the amount of harvest removed. The media feed, sulfur feed, and harvest tanks should be sparged with an inert gas (monitored by a flowmeter) to maintain the anaerobic conditions (where applicable), and also have 0.2 μm filters (Gelman Acro 50, Fisher Scientific, Pittsburgh, PA) attached to rubber stoppers to relieve excess pressure from the carboys.

Gas Sparging

The choice of gas for the headspace depends on the hyperthermophile that is being grown. Methanogens (e.g., *Methanococcus jannaschii*) and other autotrophs (e.g., *Archaeoglobus fulgidus*) use an 80:20 mixture of H_2:CO_2. Typically, N_2 is used for heterotrophs (e.g., *Pyrococcus furiosus*).

Media for Growth of Hyperthermophiles

Table III.1 lists several types of medium that have been used to grow various hyperthermophiles (for a more complete listing of hyperthermophiles isolated to date, see (128)). If adding a carbohydrate source (e.g., maltose, cellobiose, etc.), the medium should be 1.2-fold concentrated. Prepare 1 liter of the desired concentration of carbohydrate and filter-sterilize through 0.2 μm filters (Whatman; Fisher Scientific, Pittsburgh, PA) in vacuum-filtration units. The medium (10 liters) should be autoclaved just prior to starting the continuous feed. Sterilized carbohydrate solutions should then be added to the hot medium, if required. For

anaerobic conditions, reduce the hot medium using 8 to 10 ml of reducing agent (e.g., 100 g/liter each of $Na_2S \cdot 9\ H_2O$ and L-cysteine prepared in a chemical hood and kept under N_2 gas). The medium should be sparged until oxygen levels are reduced (e.g., if resazarin is used as an oxygen indicator, the medium should turn from a pink to a golden color). The anaerobic feed is then ready to be pumped into the reactor system. Aerobic medium should be aerated while heating, and any inorganic substrates (e.g., 10 g/liter $FeSO_4 \cdot 7\ H_2O$, elemental sulfur, or FeS_2) should be passed through a 0.2 μm filter (Falcon bottle-top filters, Fisher Scientific, Pittsburgh, PA) to avoid contamination.

Sulfur Feed

Many hyperthermophiles, either obligately and facultatively, reduce sulfur (3, 128). Sulfur may be fed to a continuous cultivation system as periodic additions of elemental sulfur (87), or by a continuous feed of either a colloidal sulfur suspension (232, 233) or of a soluble polysulfide (34). The colloidal sulfur must be added separately from the medium to avoid precipitation in the presence of 10 g/l or more of NaCl (232). Polysulfides may accumulate in the reactor under sulfur-limiting conditions from the presence of H_2S, a product of sulfur metabolism (34, 201).

The colloidal sulfur feed is prepared as follows (232, 233). All work should be performed in a chemical fume hood, and gloves and safety glasses should be worn for protection from toxic sulfides. For a sterilized feed, autoclave the feed bottle or tank and enough water to dilute the colloid (see below). Prepare two solutions: *A*, containing 64 g of $Na_2S \cdot 9\ H_2O$ in 500 ml of H_2O, and *B*, containing 36 g of Na_2SO_3 in 500 ml of distilled H_2O. Add 15 ml of

solution *B* to solution *A*. Slowly add 80-100 ml of diluted H_2SO_4 (15 ml of concentrated acid with 100 ml of distilled H_2O) to solution *A* until turbidity persists, and add 30 ml of concentrated H_2SO_4 to solution *B*. Add solution *A* to solution *B* (over a time period of 30 to 160 seconds) with vigorous stirring, and let stand for 1 hour. Add H_2O to give a total volume of 2 liters, and then add 20 g of NaCl (which promotes settling) with stirring until it dissolves. Let the solution stand for at least 4 hours, during which time the colloid should settle to less than a 50 ml volume. Remove the cloudy solution by aspiration and repeat the settling procedure three times, using 2 liters of H_2O and 20 g of NaCl each time. The colloid is resuspended in the solution (using a magnetic stirrer) each time. Finally, resuspend the colloid in 1 liter of H_2O and store at room temperature. The suspension should be diluted with H_2O to a final turbidity of A_{850}=1.25 for use as a continuous feed. If the H_2O was autoclaved prior to dilution, it must be cooled to room temperature before the concentrated colloid is added. The colloid can be stored for up to a month without particle agglomeration. It is sparged with gas and stirred prior to addition.

III.3. Cultivation Methods

Pre-startup

Prior to operation, it is important to make sure that the pumps, pH meter, temperature controller are working properly. The pump speed needs to be correlated with the dilution rate, D, which is equal to the flow rate divided by the working culture volume. The pH probe is best calibrated just before startup, recognizing that pH measurement is sensitive and temperature-dependent.

A growth curve should be prepared for the inoculum to be used. Approximately 50 ml of a dense, late log phase culture (10^8 cell/ml) is used to inoculate the reactor. The batch inoculum is prepared in 125-ml serum bottles (Fisher Scientific, Pittsburgh, PA), containing 50 ml of medium (and carbohydrate source and/or sulfur, if applicable). Heat the medium at 98°C, and the starter culture at its optimum growth temperature (e.g., *P. furiosus* at 98°C, or *A. fulgidus* at 83°C), in oil baths for an hour prior to inoculation. For anaerobic cultures, reduce the medium by adding 2 to 3 drops of $Na_2S{\cdot}9\ H_2O$ (or another reducing agent such as L-Cysteine-HCl) with a syringe while sparging the system with gas. Inoculate the medium (while continuously sparging with gas) with enough stock to yield a final culture density of about 10^8 cells/ml. Quickly seal the serum bottle with butyl rubber stoppers and aluminum crimp seals (Fisher Scientific, Pittsburgh, PA). Place the culture bottle in an oil bath at the appropriate growth temperature for the organism, and grow to late log phase. The batch culture bottles for many hyperthermophiles can be stored at room temperature for several months and at 4°C for several years.

Startup

Prior to continuous operation, the reactor should be operated as a batch culture. Fill the culture vessel with medium 50% of its volume and heat to 98°C. Add the carbohydrate source (e.g., maltose) and/or sulfur (e.g., elemental form), if necessary. Agitate the medium using the mechanical stirrer, sparging gas, or magnetic stir bar. Reduce the medium by adding the reducing agent, e.g., $Na_2S{\cdot}9\ H_2O$, dropwise while sparging until the medium is deoxygenated. Adjust the heat of the culture vessel to the appropriate growth temperature of the organ-

ism (e.g., *A. fulgidus* 83°C), and inoculate the reactor using the 50 ml batch culture that was grown as described above. Be sure all reactor ports are effectively sealed with rubber stoppers. Grow the batch culture until it reaches late log phase (e.g., for *Pyrococcus furiosus* about 8 or 9 hours), and then turn on the pump for the feed/harvest (e.g., for *P. furiosus*, an optimal dilution rate is 0.24). The culture stability should be periodically checked. This can be done in a variety of ways, including measurements of cell density, cell dry/wet weights, total protein assays, sugar assays of the medium and harvest, gas production profile etc. These measurements can be used to determine when the reactor reaches a biological steady state, i.e., the cells are in the state of balanced growth.

III.4. Enzyme Collection

Processing Harvest

Collection volumes for a culture volume of 1 liter in a 2-liter vessel are typically on the order of 20 liters in a couple of days, depending on the dilution rate. Higher dilution rates (i.e., greater than the growth rate; however, be careful not to wash out the cells) lead to higher biomass productivities but require more frequent media preparation and cell processing. Large volumes of biomass can be difficult and time consuming to centrifuge directly unless appropriate equipment (e.g., a continuous centrifuge) is available. Alternatively, harvested cells can be concentrated into smaller volumes (e.g., about 2 liters for a 20 liter harvest) with a cross-flow membrane filter concentrator. For example, a Millipore concentrator (Millipore, Bedford, MA) with Durapore Microporous, GVPP/GVLP pellicon cassette membranes (filter pore size of 0.22 μm; Millipore, Bedford, MA) has been used successfully for a variety of

hyperthermophiles such as *P. furiosus, T. maritima,* and *T. litoralis*. The concentrated cells from the cross-flow step are of a volume such that typical lab-scale units at 8,000 rpm for 20 minutes can be used to collect the cell pellet.

Extracellular Enzyme Collection

The extracellular proteins in the culture supernatant can also be concentrated by filtration using polyethersulfone, PTGC 10 kDa membranes (Millipore, Bedford, MA). The Millipore concentrator (Millipore, Bedford, MA) will concentrate 20 liter down to 1 liter in a few hours. The extracellular proteins and material can then be processed in a smaller concentrator to about 200 ml for purification efforts.

Intracellular Enzyme Collection

Centrifuged cell pellets can be lysed using a French-pressure cell (18,000 psi, SLM Aminco, Urbana, IL). If working with bacteria (e.g., *Thermotoga maritima*, or *Aquifex aeolicus*), lysosyme can also be used in conjunction with French-press or sonication (on ice for 5 minutes of 30 s pulse, 30 s rest) to break open the cells (177). The lysed cell material can then be spun down at 10,000 × g for 30 minutes at 4°C. The resulting crude extract can be processed using small concentrators, in addition to separation (e.g., DEAE chromatography, hydrophobic interaction chromatography, hydroxylapatite chromatography, etc.), for enzyme purification. Verhagen *et al.* (2001) (275) describe typical enzyme purification protocols for *P. furiosus*.

III.5. Concluding Comments

Continuous culture systems have proved to be useful for hyperthermophiles and producing biomass in part because they can be operated for long periods of time with low potential for

contamination and can be constructed with inexpensive equipment. In addition, continuous cultures have distinct advantages over batch cultures in exploring transcriptional and translational phenomena. For example, the effects of specific medium components can be studied to observe changes in protein and mRNA expression levels (e.g., NH_4Cl on *T. litoralis* biofilms) (215).

III.6. Acknowledgments

This work was supported in part by grants from the National Science Foundation and the Department of Energy.

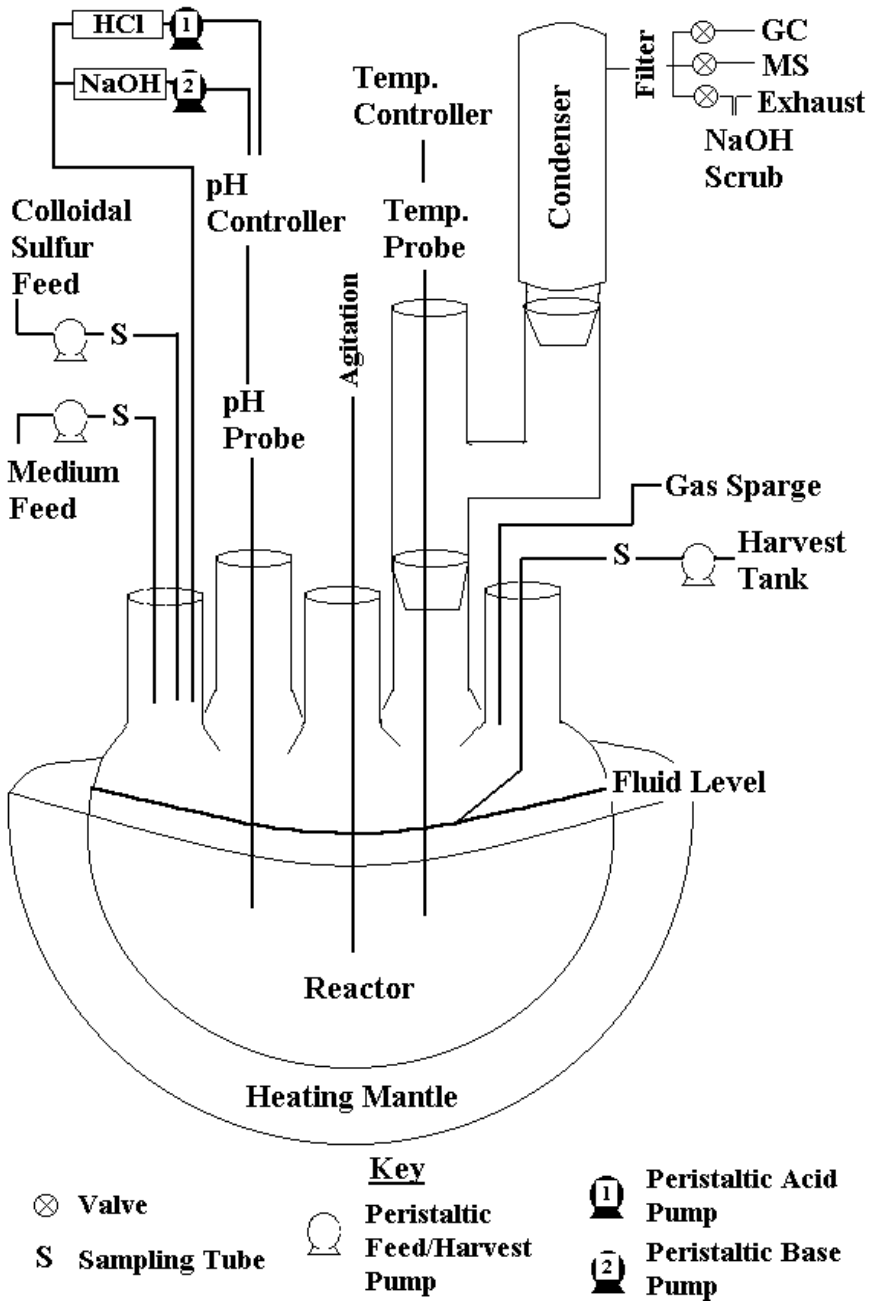


Figure III.1. Schematic of continuous culture vessel for growth of hyper-thermophiles.

Hyperthermophile	T_{opt} (°C)	Physiological and Growth Characteristics	Suggested Growth Medium and Conditions
Archaeoglobus fulgidus	83	• Archaeon • Obligate chemolithoautotroph (H_2/CO_2 or N_2 in precense of lactic acid) • Sulfate/thiosulfate reducer (19, 163)	STL medium (19, 163) Modified marine medium (28, 252) Medium (19, 68)
Methanococcus jannaschii	85	• Archaeon • Methanogen (H_2/CO_2) • Higher pressures enhanced growth (176, 269)	Medium (91, 160, 176, 178, 246, 291)
Pyrococcus furiosus	100	• Archaeon • Anaerobic chemoorganotroph • Facultative S° reducer • Enhanced growth on S°(146, 233, 251, 271)	ASW medium (135, 146) Modified ASW medium (146, 251) Modified SME medium (135, 147) Defined and minimal media (211) Modified M. jannaschii medium (214) Modified A. fulgidus medium (28), (Chapter VI)
Thermococcus barossii	83	• Archaeon • Anaerobic chemolithoorganotroph • Obligate S° reducer	Medium (87)
Thermococcus litoralis	88	• Archaeon • Anaerobic chemolithoorganotroph • Facultative S° reducer • Enhanced growth energetics on S° (29)	Marine medium (185, 284) 2216 marine broth (29, 224) ASW medium (29, 105)
Thermotoga maritima	80	• Bacterium • Anaerobic chemoorganotroph • Facultative S° reducer • S° has neutral effect on growth energetics (153)	MMS medium (153) Modified SME medium (135, 199) Medium (54, 153)
Thermotoga neapolitana	80	• Bacterium • Anaerobic chemoorganotroph • Facultative S° reducer • S° enhances growth (274)	TB medium (54, 274) Modified TB medium (54, 95) Common marine medium (54, 291) Medium (54, 153)

Table III.1. Hyperthermophiles and extreme thermoacidophiles grown in continuous culture.

Chapter IV. Chemostat-Based Approach to Examine Biofilm-Induced Differential Gene Expression Patterns in the Hyperthermophilic Bacterium *Thermotoga Maritima*

Manuscript to Submit to: **Applied and Environmental Microbiology** (March, 2003)

Authors: Marybeth A. Pysz, Keith R. Shockley, Clemente I. Montero,

Shannon B. Conner, Donald E. Ward, and Robert M. Kelly

Abstract

Thermotoga maritima, a fermentative anaerobic hyperthermophilic bacterium, was found to form exopolysaccharides (EPS) that served as the basis for biofilm formation on nylon mesh and polycarbonate filters in chemostat cultures grown on maltose-based media at 80°C. In addition to visual evidence of significant wall growth in the culture vessel, mesh and filter samples collected after 180 hours of operation showed substantial *T. maritima* colonization. Biofilm-bound cells from the mesh could be recovered and processed such that RNA could be extracted for transcriptional analysis. A targeted cDNA microarray, containing genes identified in the *T. maritima* genome suspected to be involved in polysaccharide synthesis and biofilm formation, was used to examine differential expression patterns of sessile and planktonic cells. Mixed model statistical analysis of differential expression data revealed the induction of several ORFs in the sessile cells that were related to biofilm-formation genes previously identified in less thermophilic bacteria. Up-regulated in biofilm-bound cells were several transcriptional regulators (TetR-, LytR- and CRP-related) as well as a sensor histidine kinase, homologs to all of which have been implicated in the regulation of biofilm formation in mesophilic bacteria (i.e., *Pseudomonas aeruginosa, Escherichia coli,* and *Staphylococcus epidermidis*). Biofilm-bound cells also exhibited features of thermal and oxidative stress response, as evidenced by the induction of genes encoding GrpE, DnaK, HrcA, and heat shock protein class I as well as NADH oxidases, glucose 6-phosphate 1-dehydrogenase, and L-lactate dehydrogenase. In addition to providing information on transcriptional mechanisms underlying biofilm formation in *T. maritima*, the results reported here show that this phenomenon in a hyperthermophilic bacterium related to similar processes in less thermophilic bacteria.

Furthermore, the methodology used here involving a high temperature, anaerobic chemostat to generate samples of attached cells yielding RNA for transcriptional analysis can be extended to examine biofilm formation in other hyperthermophilic microorganisms.

IV.1. Introduction

Mesophilic bacteria proliferating in natural and pathogenic environments are often associated with biofilms (30, 63, 65, 156, 253). Biofilms allow microbial species to interact and coexist in an optimized microenvironment (i.e., the matrix), thus, limiting the adverse consequences of competition and selectivity (46). The establishment of a sessile community of cells encapsulated by a polysaccharide matrix on a surface involves a complex series of steps: initial attachment, production of exopolysaccharides (EPS), early biofilm development, mature biofilm formation, and detachment of cells and/or communities (156, 253). These stages for biofilm formation have been investigated for mesophilic bacteria, including *Pseudomonas aeruginosa* (40, 72, 73, 268), *Bacillus cereus* (192), *Vibrio cholerae* (289), and a *Streptococcus* sp. (260). Biofilm formation appears to require the expression of a distinct set of genes that differentiate sessile from planktonic cells (192, 200, 228, 229, 253, 283); these include genes related to chemotaxis and motility, exopolysaccharide biosynthesis, and stress response (89, 102, 159, 193, 202, 228, 229, 283). However, this subset of genes may only comprise about 1% of the total genome, such that the phenotypic differences between planktonic and sessile cells may be subtle (283). In fact, cell populations, which have the capacity to form biofilms, likely consist of a continuous distribution of phenotypes. For example, biofilm-bound populations likely include newly recruited cells that have a planktonic phenotype (8, 229, 279). Interactions

between biofilm and planktonic populations can also directly affect gene expression. For *Bacillus cereus*, planktonic cells grown in the presence of biofilm substratum (glass wool) shared common differentially expressed genes with biofilm cells (192). These genes did not appear to be expressed in planktonic cells grown in the absence of glass wool. YhbH, a stress response protein in *B. cereus* related to sigma factor 54, was found to be induced in both biofilm-bound cells as well as in planktonic cells grown in cultures containing glass wool (192).

Biofilm formation is not limited to mesophilic bacteria. Biofilms are also evident in higher temperature environments, such as geothermal settings and hydrothermal vents (213). Several hyperthermophiles have been shown to produce exopolysaccharides (16, 109, 110, 188). These exopolysaccharides form the basis of biofilms, which have been observed in pure cultures of *Archaeglobus fulgidus* (114, 163), *Thermotoga maritima* (214), and *Thermococcus litoralis* (217), as well as in co-cultures of *T. maritima* and *Methanococcus jannaschii* (181, 214). Biofilm formation by these hyperthermophiles appeared to be inducible. In *A. fulgidus* cultures, biofilm was formed under stress conditions, generated through elevated pH, decreased and increased growth temperature, high salt, and exposure to ultraviolet light, oxygen, or antibiotic (114, 163). Elevated levels of ammonium chloride led to biofilm formation for *T. litoralis* (214, 216, 217).

Among the issues to be considered for biofilm formation by hyperthermophilic microorganisms is determining the genetic basis of this phenotype and how this relates to what has been found for less thermophilic microorganisms. Reported here is a high temperature, anaerobic chemostat-based approach for collecting biofilm material from cultures of *T. maritima*, from which extracted RNA can be used, in conjunction with cDNA

microarrays, to examine differential gene expression patterns comparing planktonic to sessile cells.

IV.2. Materials and Methods

Microorganism and Growth Conditions

Thermotoga maritima (DSM 3109) was grown anaerobically on Sea Salts Medium (SSM) containing 40 g/l sea salts (Sigma, St. Louis, MO), 1 g/l yeast extract (Fisher Scientific, Pittsburgh, PA), 3.1 g/l PIPES buffer (Sigma Chemical, St. Louis, MO), 2 g/l tryptone, 2 ml/l 0.05% Resazurin, and 10 ml/l 10X trace element solution (114). Media was adjusted to pH 6.8 with KOH (Fisher Scientific, Pittsburgh, PA), and autoclaved prior to use. Batch cultures (50 ml) were inoculated under N_2 (high purity nitrogen; National Welders, Raleigh, NC) headspace as previously described (205, 214, 215), and were grown at 80°C for 8 to 10 hours in oil baths. Maltose (Sigma Chemical, St. Louis) was added to SSM (final concentration 5 g/l) as a carbon source prior to inoculation. Continuous cultivation of *T. maritima* was performed in a 2-L five-neck, round-bottom flask as previously described (205, 215). A 50 ml batch culture was used to inoculate 1 L of SSM medium supplemented with 5 g/l maltose in the flask. This seed culture was grown at 80°C for 8.5 hours under continuous nitrogen sparging, after which medium was fed at a dilution rate of 0.25 h^{-1}. Media for continuous cultivation was prepared in 9 L batches at 1.2 X concentration as mentioned above, to which 1 L of a filter-sterilized maltose solution (50 g) was added immediately after autoclaving. The pH of the culture was continuously monitored with a Chemcadet pH controller (Cole Parmer, Vernon Hills, IL) by the addition of 1 M NaOH. Temperature control was effected using a Digi-Sense

controller (Cole-Parmer, Vernon Hills, IL) such that variations were typically ± 0.8°C, and verified by a mercury glass thermometer inserted into the culture. Steady-state conditions were monitored by constant cell densities (see below) and optical densities at 600 nm. Samples (25 ml) were also collected at specific intervals for subsequent cell density enumeration by epifluorescence microscopy and protein content (see below). All planktonic cell samples were collected in sterile pyrex bottles from the outlet line and immediately refrigerated; samples for RNA and protein extractions were collected on ice.

After a week of operation, the continuous culture was subjected to a temperature rise to 85°C. Cell densities were measured every hour immediately following the temperature stabilization at 85°C to determine the new steady-state, which occurred after approximately 4 volume changes. Planktonic and biofilm cells and biofilm samples (see below) were then collected for protein and RNA extractions at the new steady-state.

Biofilm Substrata and Collection

Nylon mesh (Sefar America, Hamden, CT) and polycarbonate filters (Poretics 0.22 μm, Fisher Scientific, Pittsburgh, PA) were used as substrata for biofilm formation. Twelve squares of mesh (13.3 cm × 9.8 cm) were cut, rolled tightly, and tied with polycarbonate string. Three rolls were tied to one another at the ends, such that 4 strings of 3 tubes were constructed. Polycarbonate filters were tied to the center of each tube to be used for imaging the biofilms, while the mesh was used for protein and RNA samples within the biofilm. The mesh strings, as well as loose polycarbonate filters, were placed in the reactor and autoclaved prior to startup. The mesh tube strings were suspended in the growing culture until the sample was collected, whereby the string was pulled quickly through one of the 5 necks of the reactor. One string (3 tubes) was used for protein and another for

RNA isolations. The tubes were rinsed twice in sterile media on ice. The polycarbonate filters were removed from each tube and placed in 2.5% glutaraldehyde (Sigma, St. Louis, MO) to fix the biofilm cells and subsequent imaging. The tubes were separated from their strings and were submerged in 50 mL conical tubes containing 300 mM NaCl (Fisher Scientific, Pittsburgh, PA). The conical tubes were vortexed vigorously to remove the biofilm from the mesh, after which the mesh tube was removed and the suspension was centrifuged (10000 × g, 20 minutes) to pellet the biofilm cell material. Protein and RNA were extracted as described below. As mentioned above, biofilm samples were taken at the steady-state regions during growth at 80°C and 85°C. Thus, two strings were removed at each temperature, using one for protein and one for RNA extractions.

Imaging and Microscopy Methods

Epifluorescent micrographs were taken with a SPOT digital camera (Southern Micro Instruments, Atlanta, GA) attached to a Nikon (Labophot-2) microscope (Southern Micro Instruments, Atlanta, GA) with 100 X oil-immersion lens. Planktonic cell suspensions were fixed in 2.5% glutaraldehyde and stained with acridine orange (1 g/l; Fisher Scientific, Pittsburgh, PA) to determine cell densities (205, 215). Biofilm cells on polycarbonate filters were fixed as described above, stained in acridine orange (1 g/l), and dried briefly under vacuum prior to imaging. A scanning electron microscope (JEOL JSM-35CF Microscope, North Carolina State University, Department of Veterinary Medicine) was also used to image biofilm cells on polcarbonate filters. Filters were fixed in 2.5% glutaraldehyde, and critically point dried in CO_2. Images of the continuous culture were also taken regularly with a Nikon Coolpix 950 digital camera.

Protein and RNA sample collection

Approximately 200 ml samples of planktonic cells were collected for RNA and protein extractions, and centrifuged at 10000×g for 20 minutes. Pellets were rinsed twice with 300 mM NaCl (Fisher Scientific, Pittsburgh, PA) and spun at 13,000 rpm for 5 minutes. Biofilm pellets were rinsed once after being extracted in 300 mM NaCl. Pellets for protein extractions were frozen at -20°C for later extractions, and those for RNA isolation were used immediately. RNA was isolated by ethanol precipitation, and purified using Promega total RNA kits (Promega, Madison WI). Concentrations and degree of purity were determined by optical density at 260 nm and 280 nm, as well as gel electrophoresis (1% agarose gel, 60 V).

Pellets for protein extractions were thawed and re-suspended in 40 mM Tris (Fisher Scientific, Pittsburgh, PA). Protease inhibitor cocktail (1 ml/4 g wet weight; Calbiochem, San Diego, CA) was added and the suspension was sonicated for 5 minutes with a 10 second on/off time. Cell debris was removed by centrifugation at 13000 rpm for 30 minutes, and the resulting supernatant was used for assays and gel electrophoresis. Total protein was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

Construction of the Targeted cDNA Microarray

Open reading frames of known and putative genes related to biofilm formation and other related cellular functions (269 total or about 15% of the total genome) were identified through BLAST (11) comparisons of protein sequences from the *T. maritima* MSB8 genome available at http://www.tigr.org/tigrscripts/CMR2/GenomePage3.spl?database=btm. DNA primers were designed with similar annealing temperatures and minimal hairpin formation using Vector NTI 7.0 (Informax, Bethesda, MD). The selected probes were

PCR-amplified in a PTC-100 Thermocycler (MJ Research, Inc., Waltham, MA) using *Taq* polymerase (Boehringer, Indianapolis, IN) and *T. maritima* genomic DNA, isolated as described previously (52). The integrity and concentration of the PCR products were verified on 1% agarose gels. PCR products were purified to 100 ng/µl using 96-well QIAquick PCR purification kits (Qiagen, Valencia, CA), re-suspended in 50% DMSO, and printed onto CMT-GAPS aminosilane-coated microscope slides (Corning, Corning, NY) using a 417 Arrayer (Affymetrix, Santa Clara, CA) in the NCSU Genome Research Laboratory (Raleigh, NC). Eight replicates of each gene fragment were printed onto each slide. The DNA was then attached to the slides by UV crosslinking using a GS GeneLinker UV Chamber (BioRad, Hercules, CA) set at 250 mJ and baked at 75^{o}C for 2 hours.

Labeling and Hybridization

First-strand cDNA was prepared from *T. maritima* total RNA using Stratascript (Stratagene, La Jolla, CA) and random hexamer primers (Invitrogen Life Technologies, Carlsbad, CA) by the incorporation of 5-[3-Aminoallyl]-2'-deoxyuridine-5'-triphosphate (aa-dUTP) (Sigma), as described elsewhere (115). The slides were scanned using a Scanarray 4000 scanner (GSI Lumonics, Canada Billerica, MA) in the NCSU Genome Research Lab. Signal intensity data was obtained using Quantarray (GSI Lumonics).

Statistical Analyses and Determination of Differential Gene Expression

A loop design was constructed (see Figure IV.1) to ensure reciprocal labeling for all four different experimental conditions. Replication of treatments, arrays, dyes, and cDNA spots allowed the use of analysis of variance (ANOVA) models for data analysis. Jin et al. (132) have described the use of ANOVAs for loop designs.

The statistical procedures and Perl code reported by Chhabra (2002) (52) were used to analyse the intensity data generated by the Quantarray files. Briefly, a linear normalization ANOVA model (285) was used to estimate global variation in the form of fixed (dye, treatment) and random (array, pin) effects and random error using the model $y_{ijkl} = D_i + T_k + Pj + \varepsilon_{ijk}$. The average effects calculated from this model were used to predict an expected intensity value for each replicate spot on each array, which can be viewed as the normalized intensity for that spot. A residual was calculated as the difference between a replicate's observed and predicted intensity to capture variation attributable to gene-specific effects after accounting for global variation. A gene-specific ANOVA model was then used to partition gene-specific variation into treatment effects, gene-specific dye effects, and pin effects using the model $y_{ijkl} = D_i + T_k + Pj + \varepsilon_{ijk}$. Volcano plots were used to visualize interesting contrasts, or comparisons between two treatments or two groups of treatments (285). These plots display the difference in residuals for a set of genes under two different conditions plotted against the significance of the gene-treatment interaction from the gene ANOVA model. The significance measure is transformed by taking the $-\log_{10}$(p-value) and the difference between residuals is represented in $\log_2$ units. By these measures, genes with a difference of residuals greater than 1 are equivalent to a 2-fold change in expression and a $-\log_{10}$(p-value) greater than 4 is equivalent to a p-value of 0.0001. A Bonferroni correction was used to adjust for the expected increase in false positives due to multiple comparisons (285). Genes meeting the Bonferroni significance criteria, and showing fold changes of ± 1.5 or greater were selected for further study, ensuring that genes with high but inconsistent fold changes would be eliminated from further analysis. In support of this, Jin et al. (132) used

ANOVA model analysis with biological replicates to demonstrate that transcriptional changes as small as 1.2-fold can be physiologically significant.

For complete information on signal intensity, significance of expression changes, fold changes, pairwise volcano plots, and hierarchical clustering for all of the genes included on the array, see Website (to be launched upon acceptance of the manuscript).

IV.3. Results

T. maritima Growth and Biofilm Formation

T. maritima was grown in continuous culture (D=0.25 hr^{-1}) for 574 hours. After 310 hours, the culture was shifted from 80 to 85°C. Planktonic cells grew very well in SSM media, reaching a mean cell density of 2.1×10^8 cells/ml at 80°C and 3.2×10^8 cells/ml at 85°C (Figure IV.2). Previous continuous culture studies had provided evidence that *T. maritima* formed biofilms on glass reactor walls, leading to substantial wall growth which in turn impacted washout behavior (Rinker and Kelly, 2000). Here, because efforts to produce biofilm material in batch culture were unsuccessful in generating sufficient cellular material for transcriptional analysis, a high temperature anaerobic chemostat was operated to collect significant amounts of biofilm formed on removable substrata. A similar approach was previously reported for the study of *P. aeruginosa* biofilm formation on Teflon mesh (73). Here, nylon mesh was rolled and inserted into the reactor prior to inoculation; polycarbonate microfilters were also included to be used for biofilm imaging analysis. Figure IV.3 shows the biofilms on the mesh rolls within the reactor. Substantial wall growth was also observed during operation.

After 301.5 hours at 80°C, the temperature was shifted to 85°C. A slight increase in cell density was observed as mentioned above. This may be due to sloughing of sessile cells on the mesh and reactor wall due to the experience of thermal stress (6). Biofilm cell morphologies were similar at the higher temperature, despite the appearance of browned material on the mesh (Figure IV.3), perhaps due to carmelization of EPS. Epifluorescent micrographs of polycarbonate filters showed widespread biofilm formation at both temperatures (Figure IV.4). Interestingly, the biofilm cells appear to stand on the surface (Figure IV.5) and are connected by a rope-like material, commonly seen in mesophilic biofilms (83, 84, 136). Conventional preparation methods for SEM cause severe dehydration and a collapse of the biofilm and exopolysaccharide (136), which can be seen in Figure IV.5.

Targeted cDNA Microarray Analysis of Gene Expression in 80°C Sessile and Planktonic Cells

Total RNA was extracted from 3 different time points during the steady-state operation of the continuous culture at 80°C (see Figure IV.2). Approximately 1 mg of RNA was obtained from each sampling time of which 100 μg was used to create a pooled sample to reduce the effects of biological variability (132). Similarly, the total RNA from the 3 rolls of mesh was pooled for to produce a biofilm sample, yielding similar amounts of RNA to that obtained from planktonic cells. The cDNA generated from the planktonic and biofilm cells was hybridized to glass slides containing the targeted microarray, scanned and analyzed. The volcano plot resulting from the mixed model analysis (Figure IV.6) shows the differential expression of genes at 80°C, along with statistical significance. Genes that were differentially expressed 1.5-fold or greater with a p-value of 0.0001 are

listed in Tables IV.1 (up-regulated) and IV.2 (down-regulated). Cellobiose phosphorylase (TM1848) (183) and L-lactate dehydrogenase (TM1867) (183) exhibited the highest fold-changes of 7.1-fold and 5.8-fold, respectively. Alkyl hydroperoxide reductase (TM0807) was down-regulated 5.6-fold. Other genes changed by approximately 3-fold or less.

Differentially expressed genes represented a variety of cell functions, including glycolysis, stress response, proteolysis, two-component regulatory systems, and energy metabolism, among others. Similar patterns been observed in biofilms of *B. cereus* (192), *Pseudomonas* sp. (228, 229, 283), *E. coli* (203), *S. aereus* (27), and *S. epididermis* (59). In most cases, the basis for observed differential expression patterns in mesophilic bacteria is unknown and appears to vary depending on the stage of biofilm being studied. For example, Sauer et al. (2002) (229) have observed changes in phenotype within the maturation stage for *Pseudomonas* sp., thus separating it into sub-stages.

Targeted cDNA Microarray Analysis of Gene Expression in 85°C Sessile and Planktonic Cells

At 80°C, biofilm cells indicated aspects of stress response as evidenced by the induction of thermal stress genes, including DnaK, heat shock operon repressor HrcA, and heat shock class I protein. This may relate to previous work, which has shown that deeply embedded biofilm cells are in stationary phase (8, 286, 294). Stress genes were also up-regulated in biofilms formed by *S. mutans* (260) and *P. aeruginosa* (229, 283). The second question addressed by the loop design (Figure IV.1) is what happens to biofilm cells exposed to supraoptimal temperatures. To investigate this, the culture temperature was raised 5°C to 85°C. Although thermal stress response for *T. maritima* has not yet been studied, Han *et al.* (1997) (113) demonstrated that an extreme thermoacidophile, *Metal-*

losphaera sedula, exhibited heat shock response less than 5°C above the optimal growth temperature of 74°C. Indeed, several heat shock response genes were up-regulated in the 85°C planktonic cells as compared to the 80°C planktonic cells (Table IV.3). There were only small differences between biofilm cells at the two temperatures (Figure IV.7). Most notable was the down-regulation of genes encoding cellobiose phosphorylase (TM1848, -3.3-fold), a putative esterase (TM0053, -2.8-fold), and L-lactate dehydrogenase (TM1867, -2.4 fold) (see website).

Identification of gene-specific roles in biofilms and thermal stress

All of the genes on the array were clustered according to least square means (Figure IV.8A) and standardized least square means (Figure IV.8B). Figure IV.9 shows functional groupings from the standardized least square means cluster (Figure IV.8.B) for biofilm-specific and thermal stress-specific genes. Biofilm-specific genes are mostly response regulators. As expected, heat shock genes, including GroEL (TM0505) and GroES (TM0506), clustered under thermal stress. Other heat shock genes (e.g., DnaK (TM0373), heat shock protein class I (TM0374), DnaJ (TM0849), and GrpE (TM0850)) also clustered with similar patterns. It was interesting that the heat shock operon repressor, HrcA (TM0851) clustered with the biofilm-specific genes. Other genes that were up-regulated included α-glucosidase (TM0434), α-glucosidase Amy4B (TM1068), and transcriptional regulator DeoR family (TM1069). Further details regarding the thermal stress response of *T. maritima* are reported in Chapter V.

IV.4. Discussion

The only previous report for cDNA microarray-based analysis of differential gene expression examined *P. aeruginosa* and found that only about 1% of the genome (about 75 genes) was differentially expressed 2-fold or more in the biofilm compared to planktonic cells (283). The relatively small number of genes differentiating seemingly dissimilar phenotypes likely reflected the fact that biofilm communities are physiologically heterogeneous and dynamic, such that a single temporal and spatial sample may not be representative.

Sessile cells presumably exhibit different phenotypic characteristics than planktonic cells, due to the unique microenvironment created within the biofilm. Here, differential gene response in biofilm-bound *T. maritima* indicated elements of oxidative and thermal stress (50). The connection between oxidative stress response and biofilm formation is not clear but this relationship has been observed in the hyperthermophilic archaeon, *A. fulgidus* (163), as well as in certain mesophiles (103, 116). Aspects of oxidative stress response (6) in sessile *T. maritima* were evidenced by the up-regulation of genes encoding L-lactate dehydrogenase, NADH oxidases, lipoamide dehydrogenase, and glucose-6-phosphate 1-dehydrogenase. L-lactate dehydrogenase (TM1867) induction has also been observed in *B. cereus* biofilms (192), and may be involved in regenerating NAD^+ (186) in conjunction with NADH oxidase (TM0379), an enzyme which has been implicated in microbial antioxidant defense systems (131, 278). In addition to oxidative stress, elements of heat shock response were also induced in sessile *T. maritima* cells. Planktonic cells from 85°C chemostat operation serve as a reference point. HrcA (TM0851), known to be involved in heat shock in mesophiles (290), was up-regulated 2.4-fold in biofilm cells at 80°C. GrpE (TM0850), another known mesophilic heat shock protein, occurs in

the same operon (5 bases downstream of *hrcA*) in the *T. maritima* genome (Nelson et al., 1999); this gene was up-regulated 2.4-fold in biofilm cells. *Dna*K (TM0373) and heat shock protein class I (TM0374) were also up-regulated in the 80°C biofilm cells (1.7- and 2.2-fold, respectively). Thermal stress genes were up-regulated in biofilms of *P. aeruginosa* (*Gro*ES, *dna*K) (283) and *S. mutans* (*Grp*E and *dna*K) (260).

Cellobiose phosphorylase was the most differentially expressed gene in *T. maritima* biofilms. This enzyme from *Thermotoga neopolitana* was shown to have sole substrate specificity for cellobiose (288), acting to convert cellobiose to D-glucose and glucose-1-phosphate (189). The up-regulation of this gene is interesting since maltose (5 g/l) and not cellobiose was used as the primary carbon source in the growth medium. One possibility is that biofilm cells may be recruiting β-glucans present in yeast extract (1 g/l), which can contain approximately 10% glucans (Difco Laboratories Manual, Detroit, MI). This is also consistent with the up-regulation of β-endoglucanases Cel12B (TM1524) and Cel12A (TM1525) (53). It remains unclear what role these glycosidases may have in biofilm-bound cells.

Several other common features between mesophilic and *T. maritima* biofilm cells were observed. Sensor histidine kinases (e.g., TM0187) and other response regulators have been implicated in biofilm formation (9, 26, 27, 36, 59, 61, 71, 74, 108, 121, 137, 200, 203, 204, 228, 229). Some are responsible for detecting environmental chemical signals, such as autoinducers, which initiate a cascade of reactions involved in cell-to-cell communication (74, 103, 253). Quorum sensing mechanisms are responsible for regulation of a large variety of genes, including virulence factors that enable biofilms to be resistant to environmental stresses (117, 200). Although a homolog to a virulence factor

(MviN-related) gene (TM0086) was induced 1.5-fold in *T. maritima* biofilm cells, quorum sensing and protective mechanisms have not been identified in this hyperthermophilic bacterium, nor is much known about two-component regulatory systems.

Although the need for twitching motility in biofilm environments has been noted (193, 202, 228, 229), Whiteley *et al*. (2001) (283) reported a decrease in type IV pili transcription in *P. aeruginosa* biofilms; this is consistent with the results here for a homolog to this gene in *T. maritima* (see Table V.2). However, twitching motility experiments have used translationally-based approaches, and there may be differences in regulation between the transcriptional and translational processes (273).

A variety of transcriptional regulators were induced in sessile *T. maritima* cells. These include members of various families: LytR (TM1866), CRP (TM1171), XylR (TM0411), RpiR (TM0326), biotin repressor (TM1602), GntR (TM0275) and TetR (TM0823). The *T. maritima* membrane-bound LytR transcriptional regulator (TM1866) is most closely related to LytR from *Bacillus subtilis*, based on COG recognition patterns (182, 264). LytR represses the transcription of the *lytABC* and *lytR* operons, the former of which encodes for N-acetylmuramoyl-L-alanine amidase (164). Lazarevic *et al.* (1992) (164) reported that it belongs to the flagellar regulon and its activity is co-regulated with motility. However, there appears to be no homologous lytABC proteins in the *T. maritima* genome based on BLAST search or COG similarity. CpsA, a protein which encodes for capsular polysaccharide synthesis in *Streptococcus agalactiae*, is also a member of the same family (i.e., pfam03816.2) as *B. subtilis* LytR and TM 1866. It is a positive regulator of the cpsABCD operon, which encodes a putative phosphoesterase (CpsB) and putative chain length regulators (CpsC and CpsD). Again, no homologous proteins could be identified in

the *T. maritima* genome by BLAST search. Therefore, the target of the LytR regulator in *T. maritima* is not yet known.

The CRP family has been found to be involved in repressing flagellar synthesis in *Salmonella typhimurium* (161), *E. coli* (162), and *P. aeruginosa* (71). As mentioned above, flagella are needed at the initial stages of biofilm formation when the cells are swimming towards the surface, after which twitching motility via type IV pili becomes the dominant mode of transportation across a surface (193, 202). In *E. coli*, it was also found that CRP suppresses lipopolysaccharide biosynthesis (162); this gene was down-regulated 1.6-fold in *T. maritima* biofilms (TM0622).

TetR family regulators are important in biofilm formation. For example, IcaR regulator was found to repress the *ica* operon in *S. aereus* (59). The *icaADBC* operon encodes for intracellular capsule adhesin necessary for surface attachment (55, 59). However, there was no related operon or genes identifiable in *T. maritima* by BLAST search. *Pseudomonas* biofilms also involve a TetR regulator, PsrA, which initiates *rpoS* transcription (154). The gene product, RpoS (σ^{38}) (154), is involved in regulating a variety of genes in biofilm formation, as well as at least 50 genes in *E. coli* (61). RpoS, on the other hand, has been shown to play a significant role in cell survival strategies during stationary phase and biofilm formation (286). In contrast, Whiteley et al. (2001) (283) observed a down-regulation of RpoS in *P. aeruginosa* biofilms. BLAST sequence analysis indicated that TM0823 is 37% identical to *P. putida* PsrA (CAC17801). However, while there may be some connection between the two genes, the RpoS sigma factor does not exist in *T. maritima*.

Although a variety of transcriptional regulators were induced during biofilm formation by *T. maritima,* it is difficult to assign these to regulatory elements since related proteins seem to involve operons not present in *T. maritima.* Further characterization of these regulators, as well as a full genome cDNA microarray, may provide clues as to the functions of these genes, as well as transcriptional properties (e.g., co-transcription). Other functional categories of genes were also differentially expressed in *T. maritima* biofilms. This was expected due to the similar gene expression patterns observed in well-characterized mesophilic biofilms. Furthermore, transcriptional levels related to several of these, including L-lactate dehydrogenase, NADH oxidase, GrpE, and DnaK, among others, were similar to various mesophilic biofilms, including *P. aeruginosa, E. coli, B. cereus,* and *S. epidermidis* (59, 203, 283).

While there is still much to be understood about biofilm formation and dynamics for *T. maritima*, this work provides a glimpse into the transcriptional mechanisms involved and how they relate to this phenomenon in better-studied but less thermophilic bacteria. There are clearly some common features between hyperthermophiles and mesophiles with respect to biofilms, although it is not clear whether the differences observed are related to growth temperature. In any case, the chemostat-based approach used here should be useful for more comprehensive studies that based on a whole genome microarray; such efforts are in progress.

IV.5. Acknowledgments

This was work was supported in part by grants from the National Science Foundation (LexEn Program) and the Department of Energy (Energy Biosciences Program). The au-

thors wish to thank Dr. Michael Dykstra at the Electron Microscopy Center, NCSU School of Veterinary Medicine, for assistance with electron microscopy.

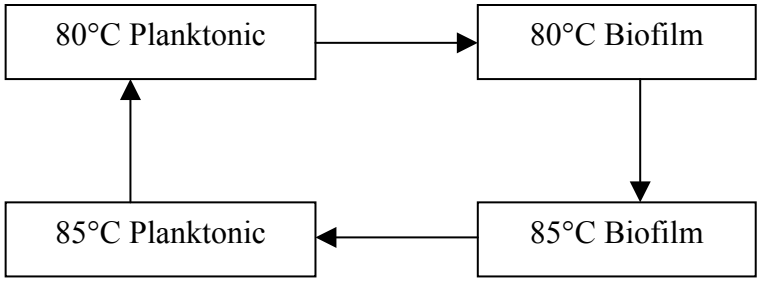


Figure IV.1. Loop design of RNA samples used for mixed model ANOVA analysis.

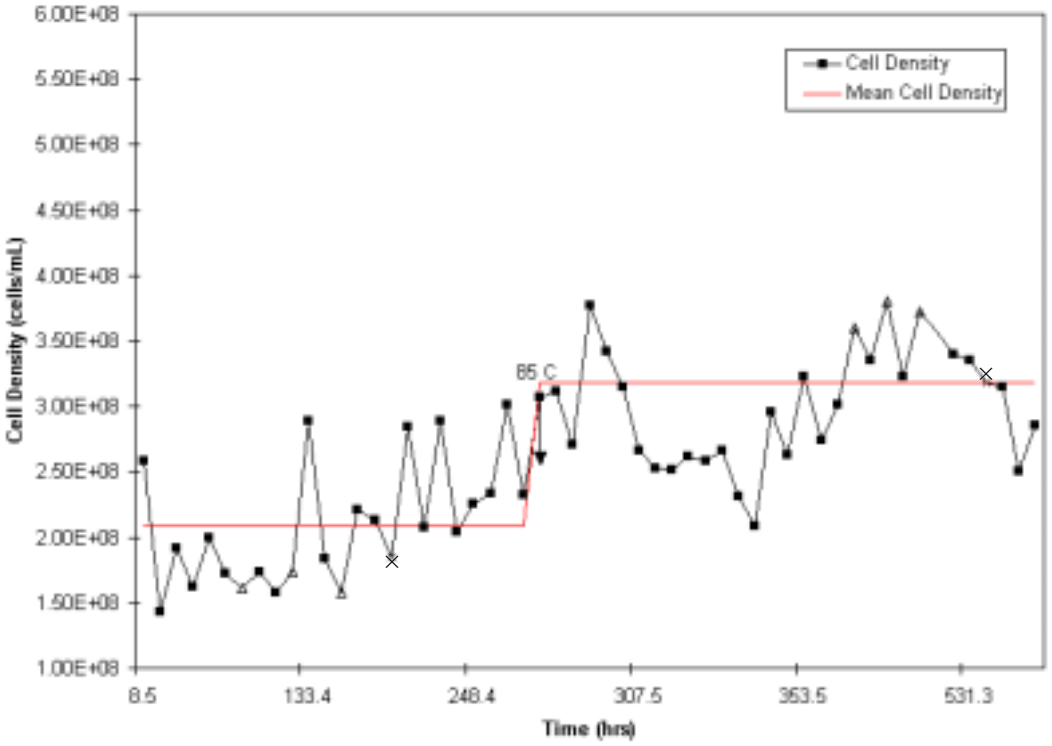
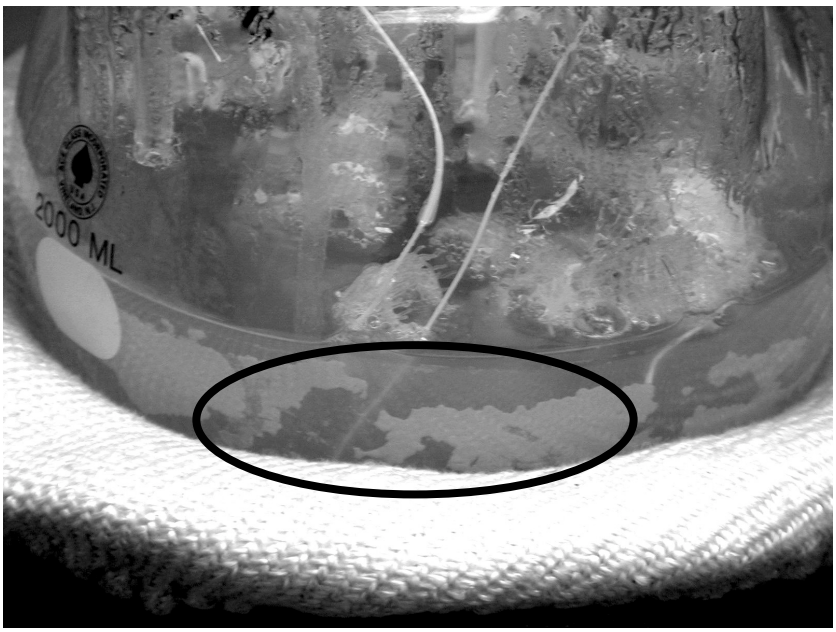


Figure IV.2. *T. maritima* cell densities were monitored over time of growth in 1.5-L continuous culture. Cell density levels at 80°C (0 to 301.5 hrs) and 85°C (301.5 to 574 hrs) are shown. RNA and protein samples were collected at points designated by clear triangles (Δ), and biofilm samples were collected at points designated by crosses (×).

A



B

Figure IV.3. Wall growth and biofilm formation on nylon mesh in *T. maritima* chemostat cultures grown on SSM medium supplemented with 5 g/l maltose. A more dense film occurred on the wall at (A) 80°C, whereas the film exhibited more void spaces at (B) 85°C. Note the biofilm mesh became discolored at 85°C, which may be due to carmelization of polysaccharides.

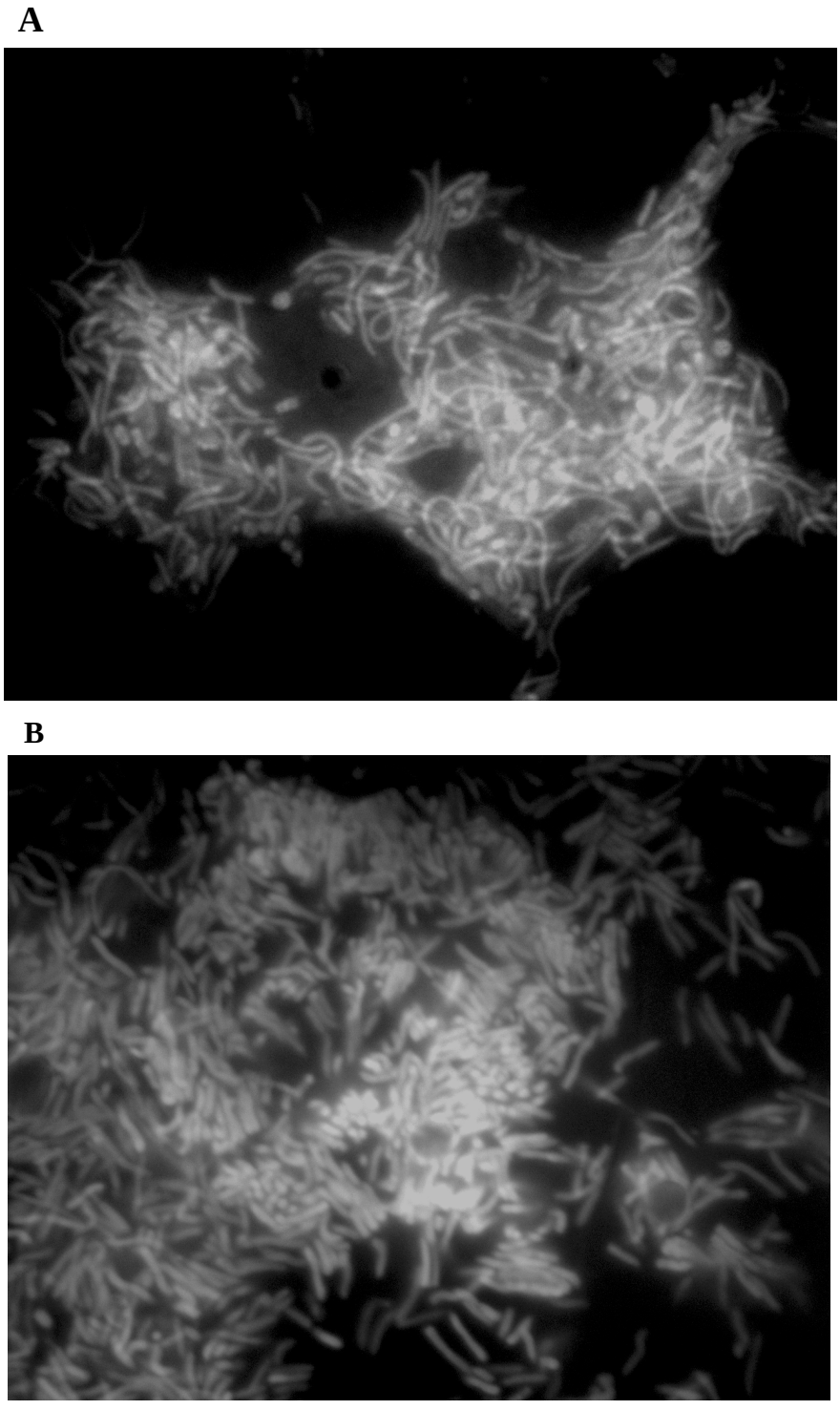


Figure IV.4. There were no differences observed between *T. maritima* biofilms at (A) 80°C and (B) 85°C under the epifluorescent microscope.

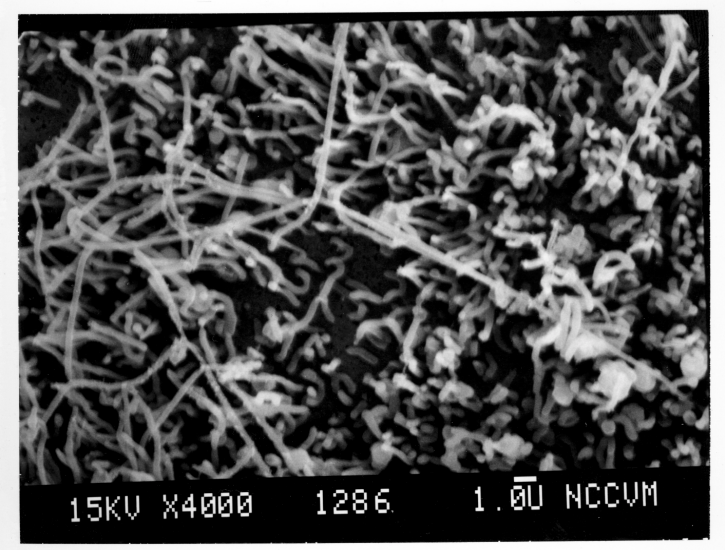


Figure IV.5. Scanning electron micrograph of *T. maritima* biofilm on polycarbonate filter at 85°C.

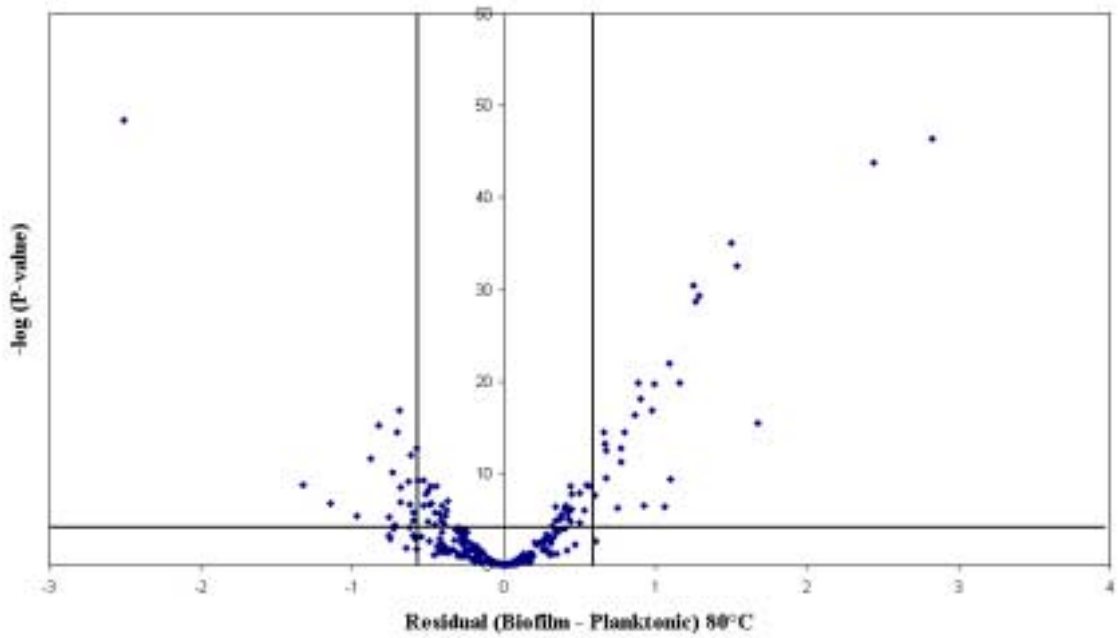


Figure IV.6. Volcano plot comparing biofilm and planktonic genes expressed at 80°C. Horizontal line indicates cutoff for significance criteria (P-value ≥ 4.0) and vertical lines indicate cutoff for fold-changes above (up regulated genes) or below (down regulated genes) 1.5-fold.

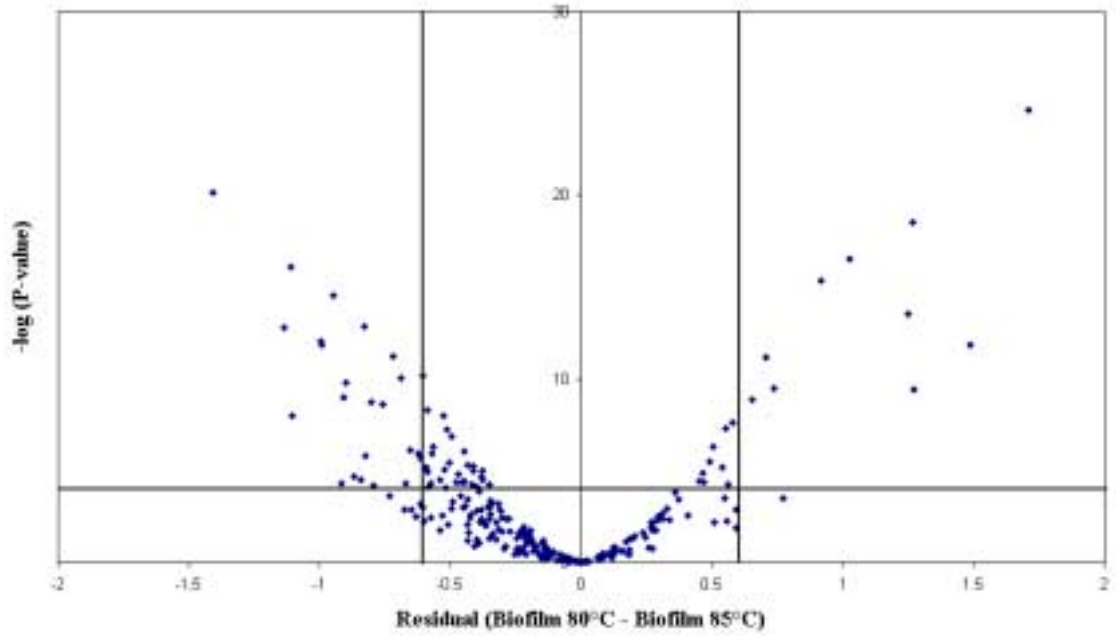


Figure IV.7. Volcano plot comparing biofilm genes expressed at 80°C and 85°C. Horizontal line indicates cutoff for significance criteria (P-value ≥ 4.0) and vertical lines indicate cutoff for fold-changes above (up regulated genes) or below (down regulated genes) 1.5-fold. Note that there are only a few genes that fit within the imposed criteria.

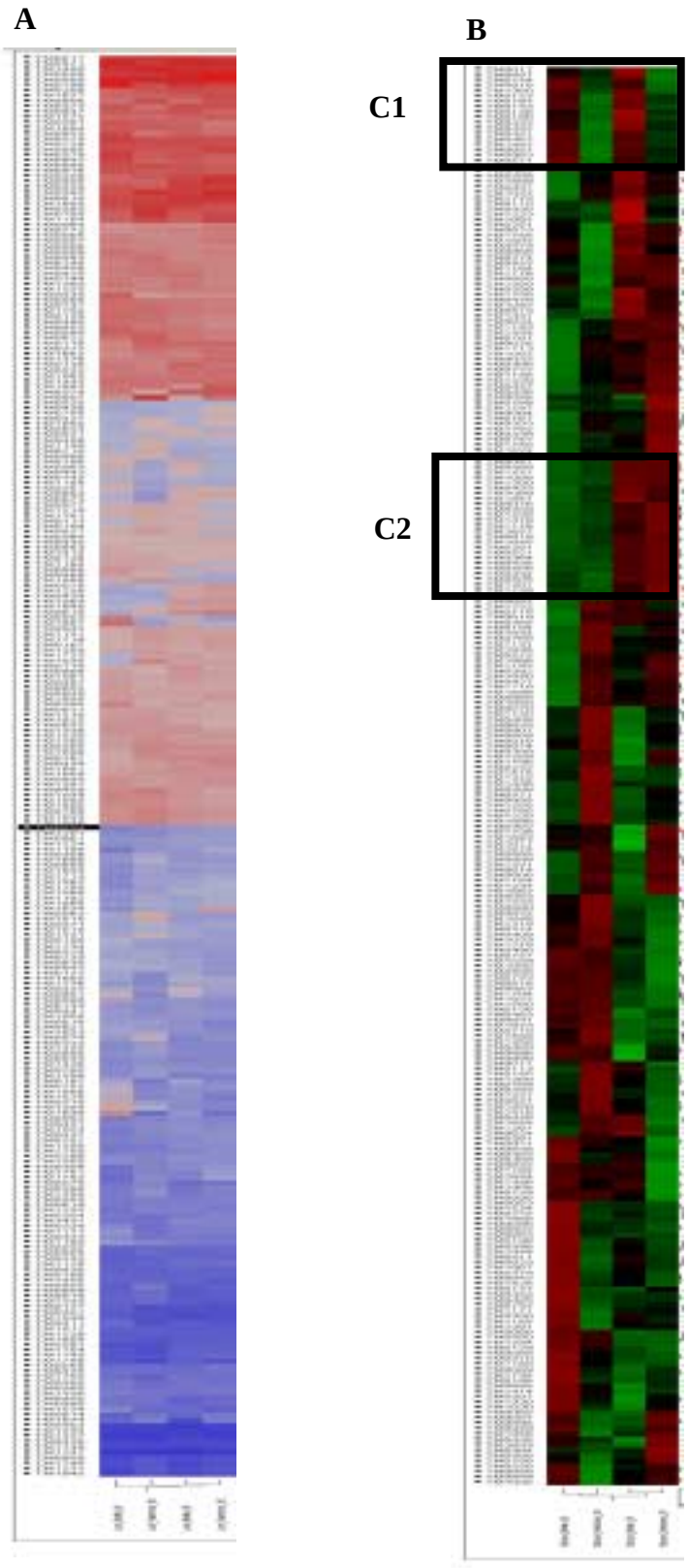
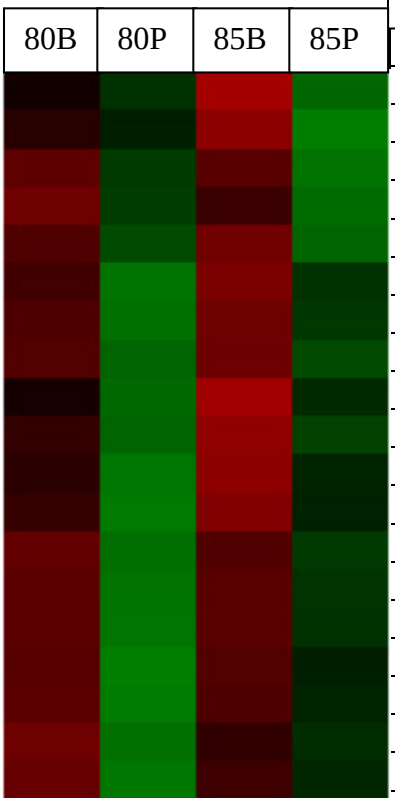
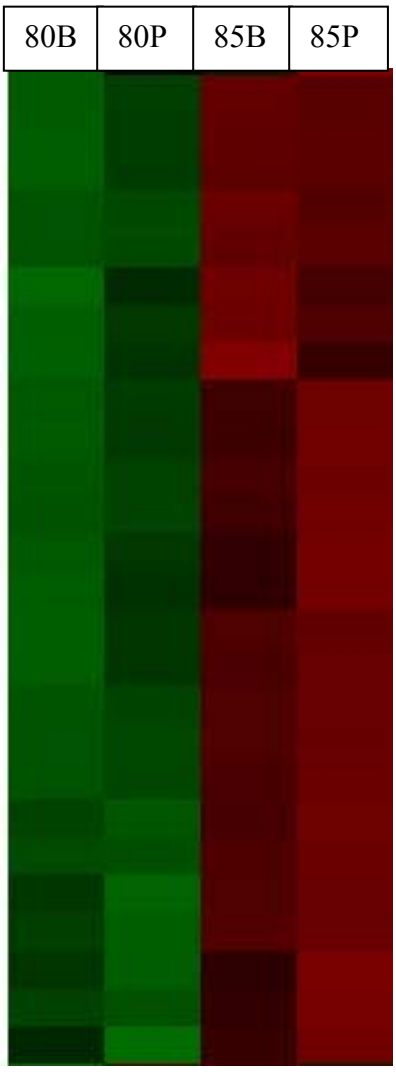


Figure IV.8. Hierarchal clustering of least square means (A) and standardized least square means (B) from mixed model analysis of 80°C Biofilm (80B), 80°C Planktonic (80P), 85°C Biofilm (85B), and 85°C Planktonic (85P).



Cluster C1: Biofilm specific genes	
Locus	**Function**
TM0017	Pyruvate ferredoxin oxidoreductase, alpha subunit
TM0847	Conserved hypothetical protein
TM0744	Conserved hypothetical protein
TM0916	Periplasmic serine protease, putative
TM1360	Response regulator
TM0122	Ferric uptake regulation protein
TM0187	Response regulator
TM0143	Response regulator
TM0146	ATP-dependent protease/ATPase ClpX
TM0186	Response regulator
TM0432	Sugar ABC transporter, periplasmic
TM0442	Conserved hypothetical protein
TM0441	DKI metabolism (kduD)
TM0467	Regulatory protein, putative
TM1442	Anti-sigma factor antagonist, putative
TM0851	Heat-shock operon repressor, HrcA
TM0963	Oligoendopeptidase, putative
TM0571	Heat-shock serine protease, periplasmic (htrA)
TM0742	Serine/threonine protein phosphatase

Figure IV.9. Functional clusters from Figure IV.8.B demonstrate up-regulation of biofilm specific and thermal stress specific gene clusters.



Cluster C2: Thermal stress specific genes	
Locus	**Function**
TM0061	Endo-1,4-beta-xylanase A
TM0440	Hypothetical protein
TM1227	β-mannanase, Man5
TM1068	α-glucosidase, Amy4B
TM1200	Transcriptional regulator, LacI family
TM0433	Pectate lyase
TM1069	Transcriptional regulator, DeoR family
TM1851	Putative α-mannosidase, Man38B
TM0076	β-xylosidase, Xyl13
TM0209	6-phosphofructokinase (pfkA)
TM1228	Transcriptional regulator, RpiR family
TM1478	Methionine aminopeptidase
TM1176	Transcriptional regulator, metal-sensing
TM1627	General stress protein Ctc (ctc)
TM0818	Lipopolysaccharide biosynthesis protein, putative
TM1845	Pullulanase, Pul13
TM0281	α-L-arabinofuranosidase
TM0521	Heat-shock protein HslV protease ClpQ
TM0364	4-α-glucanotransferase
TM0436	Alcohol dehydrogenase, zinc-containing
TM0506	GroEL
TM0434	α-glucosidase, putative
TM0505	GroES
TM1201	Arabinogalactan endo-1,4-beta-galactosidase, putative
TM1749	Oligopeptide transport system ATP-binding protein
TM1572	Signal peptidase I, putative

Figure IV.9. continued.

Gene Description	Gene ID	Fold-Change	$-\log_{10}P$ value	Similarity to other biofilms
Transcriptional regulator, GntR family	TM0275	1.5	9.7	
Transcriptional regulator, RpiR family	TM0326	1.5	8.6	
ATP-dependent protease/ATPase ClpC-2 frameshift	TM0873	1.5	7.5	
Multiple sugar transport system substrate-binding protein	TM0432	1.6	14.4	
Transcriptional regulator, XylR-related	TM0411	1.6	13.2	
Nitrogen regulatory protein P-II	TM0403	1.6	9.5	
Heat-shock serine protease, periplasmic (htrA)	TM0571	1.6	12.5	
Transcriptional regulator, biotin repressor family	TM1602	1.7	6.3	
Glucose-6-phosphate 1-dehydrogenase	TM1155	1.7	11.2	
GrpE	TM0850	1.7	12.7	*S. mutans* (260)
DnaK	TM0373	1.7	14.5	*P. aeruginosa* (283), *S. mutans* (260)
Response regulator	TM0842	1.8	1.8	*P. aeruginosa* (283)
Transcriptional Regulator CRP family	TM1171	1.8	19.8	
Endoglucanase cel12A	TM1524	1.9	18.1	
β-D-galactosidase	TM0310	1.9	6.5	
Endoglucanase cel12B	TM1525	2.0	16.8	
NADH oxidase	TM0379	2.0	19.7	Downregulated *S. mutans* (260)
α-amylase, Amy4C	TM0752	2.1	6.4	
Sensor histidine kinase	TM0187	2.1	21.9	*P. aeruginosa* (283)
NADH oxidase, putative	TM0395	2.1	9.3	Downregulated *S. mutans* (260)
Heat shock protein class I	TM0374	2.2	19.8	
Heat Shock Repressor HrcA	TM0851	2.4	30.4	
Transcriptional Regulator TetR	TM0823	2.4	28.7	*S. epidermis* (59)
Membrane bound LytR	TM1866	2.4	29.3	*P. aeruginosa* (190)
ATPase ClpC1	TM0198	2.8	35.0	*P. aeruginosa* (283)
Conserved hypothetical protein (probable GTPase)*	TM0445	2.9	32.6	
Esterase, putative	TM0053	3.2	15.5	
L-lactate dehydrogenase	TM1867	5.4	43.7	*B. cereus* (192); Downregulated *S. mutans* (260)
Cellobiose phosphorylase	TM1848	7.1	46.4	

*Probable function determined by BLASTnr at http://www.genome.ad.jp/kegg/kegg2.html

--- not within significance criteria of $-\log_{10}P$ value > 3

Table IV.1. Genes significantly up-regulated in 80°C biofilm compared to 80°C planktonic cells.

Gene Description	Gene ID	Fold-Change	$-\log_{10}P$ value	Similarity to other biofilms
Alkyl-hydroperoxide reductase	TM0807	-5.7	48.4	*Streptococci* (187)
Cyclomaltodextrinase	TM1835	-2.5	8.8	
Bacteriocin	TM0785	-2.2	6.7	
Endoglucanase	TM1752	-2.0	5.4	
Conserved hypothetical protein (related to trehalose synthase)*	TM0392	-1.8	11.7	
Hypothetical protein**	TM0606	-1.8	15.3	
Transcriptional regulator, XylR related	TM0393	-1.7	3.2	
Ferric uptake regulation protein	TM1515	-1.7	5.3	
β-glucouronidase	TM1062	-1.7	10.0	
β-galactosidase	TM1195	-1.7	4.0	
Conserved hypothetical protein**	TM0874	-1.6	4.4	
Type IV prepilin peptidase (pilD)	TM1696	-1.6	14.4	*P. aeruginosa* (283)
Aspartate aminotransferase	TM1698	-1.6	16.8	
Cold shock protein (cspL)	TM1874	-1.6	6.9	
Maltodextrin glycosyltransferase	TM0767	-1.6	8.4	
β-mannosidase, Man2	TM1624	-1.5	9.1	
Putative α-L-fucosidase, GH29	TM0306	-1.5	4.2	
Clostripain-related protein	TM1589	-1.5	6.6	
α-galactosidase, Gal36	TM1192	-1.5	11.9	
α-amylase, Amy13A	TM1840	-1.5	4.9	
Lipopolysaccharide biosynthesis protein, putative	TM0622	-1.5	5.7	
Aminopeptidase, putative	TM0365	-1.5	5.7	
Ribulose-P 3-epimerase	TM1718	-1.5	12.7	
2-oxoacid ferredoxin oxidoreductase, alpha subunit	TM1164	-1.5	9.2	

*Probable function determined by BLASTnr at http://www.genome.ad.jp/kegg/kegg2.html
**No sequence identity based on BLASTnr at http://www.genome.ad.jp/kegg/kegg2.html

Table IV.2. Genes significantly down-regulated in 80°C biofilm compared to 80°C planktonic cells.

Gene Description	Gene ID	Fold Change	$-\log_{10}$(P-value)
Heat shock protein class I	TM0374	4.1	28.3
GroES	TM0505	2.6	21.1
GrpE	TM0850	2.4	17.8
DnaK	TM0373	1.7	9.5
GroEL	TM0506	1.7	11.7
Heat shock operon repressor HrcA	TM0851	1.6	11.9

Table IV.3. Thermal stress-related genes were up regulated in planktonic cells due to temperature shift from 80°C to 85°C, indicating a heat shock response.

Chapter V. Dynamic Changes in Gene Expression of *Thermotoga maritima* During Thermal Stress

Manuscript to Submit to: **Applied and Environmental Microbiology**, (March, 2003)

Authors: Marybeth A. Pysz, Donald E. Ward, Keith R. Shockley,

Clemente I. Montero, Shannon C. Burns, Matthew R. Johnson, and Robert M. Kelly

Abstract

The thermal stress response of *T. maritima* was characterized using a targeted cDNA microarray. Gene expression levels at 7 time points (0, 5, 10, 20, 30, 60, and 90 minutes) after reaching 90°C were compared to the expression levels at the optimum growth temperature (80°C) with mixed model ANOVA analysis in a loop design. Least square means clustering of the data revealed that the two major heat shock operons *dnaJ-grpE-hrcA* (TM0849-TM0850-TM0851) and *groEL-groES* (TM0505-TM0506), as well as the genes encoding DnaK (TM0373) and heat shock protein class I (TM0374), exhibited maximal induction at early times (~5 minutes), subsequently decreasing to a steady-state level (~4-fold higher than at 80°C). Other stress-related responses were identified, including the induction of the SOS regulon involving UsrB (TM1761) and RecA (TM1859), and the down-regulation of this operon's repressor lexA (TM1082). Unlike heat shock response in *Escherichia coli* (290), the majority of ATP-dependent proteases, including ClpP (TM0695), ClpQ (TM0521), ClpY (TM0522), LonA (TM1633), and LonB (TM1869), were down-regulated. On the contrary, ATPase Clp C subunits 1 (TM0198) and 2 (TM0873) were both up-regulated, along with ClpX (TM0146) and FtsH (TM0580). The ATP-independent heat shock serine protease HtrA (TM0571) was also induced. The functions of sigma factors in *T. maritima* remain unknown, but the observed induction of homologs to σ^E and σ^A during heat shock at 90°C suggest that they are implicated in stress response regulation in this hyperthermophile. A number of other genes not related to stress response also showed significant changes in expression levels for reasons that are not yet clear.

V.1. Introduction

Hyperthermophiles (i.e., microorganisms that optimally grow at 80°C or higher) encounter environmental variations in their natural habitats, which include terrestrial solfataras, geothermal springs, deep-sea hydrothermal vents, and deep subsurfaces theermal niches. Dynamic aspects of geothermal systems likely result in cellular exposure to a variety of perturbations, such as heat shock, cold shock, osmotic shock, starvation, and oxidative stress (168). Once the stress is detected by sensory mechanisms, the cell responds by altering gene expression and changing its molecular composition (168). These changes include DNA repair, membrane repair, protein refolding via chaperones, and protein degradation via proteases (168, 290). Often the stress responses overlap such that common gene expression patterns are observed (6, 120, 168, 290). Helmann *et al*. (2001) (120) demonstrated the use of cDNA microarray technology to separate general stress response from the stress-specific response of *Bacillus subtilis* to heat shock at 48°C. Transcriptional profiles revealed that the induction of class I heat shock genes (HrcA-dependent) reached maximal levels after 10 minutes of thermal stress and decreased to basal levels at 20 minutes (120). Although only three time points were examined in the *B. subtilis* studies (120), it is clear that the transcriptional response of bacteria to thermal stress occurs within minutes. This phenomenon has also been shown in *Escherichia coli*, where the major heat shock regulator σ^{32} increased 15- to 20-fold within 5 minutes and then decreased to approximately 2-fold higher than the levels at the optimal growth temperature (254, 290).

Adaptation to thermal stress by hyperthermophiles has been studied but only to a limited extent. Various heat shock genes have been identified (158, 168) and, in some

cases characterized, at the protein level (168). Koonin *et al.* (2000) (158) provides an extensive review of stress response genes in various sequenced genomes, including the hyperthermophiles *Aquifex aeolicus, Thermotoga maritima, Pyrococcus horikoshii, Aeropyrum pernix, Archaeoglobus fulgidus, Methanococcus jannaschii,* and *Methanobacterium thermoautotrophicum*. However, the mechanisms underlying regulation of thermal stress response and other affected genes in hyperthermophiles are largely unknown. As such, this issue was investigated using dynamic differential expression analysis during exposure of the hyperthermophilic bacterium *Thermotoga maritima* to supraoptimal temperatures.

V.2. Materials and Methods

Microorganism and growth conditions

Thermotoga maritima (DSM 3109) was grown anaerobically on Sea Salts Medium (Chapter II), as previously described (205, 214, 215), at 80°C for 8 to 10 hours in oil baths. Maltose (Sigma, St. Louis) was added to SSM (final concentration 5 g/l) as a carbon source prior to inoculation. A 50 mL batch culture was used to inoculate 500 mL of SSM medium supplemented with 5 g/L maltose in a 1 L pyrex bottle. Two hundred fifty ml of this culture was used to inoculate 13 L of SSM medium (1.08× concentrated) and 1 L of maltose (i.e., the final concentrations were 1× SSM medium and 5 g/L maltose in 14.0 L culture) in a 14.0-L fermento (New Brunswick, Edison, NJ). The steam-jacketed fermentor contained an internal temperature controller, and the pH was controlled by a Chemcadet controller (Cole Parmer, Vernon Hills, IL). High purity N_2 was used to reduce the medium and sparge during inoculation. The culture was grown for 8 hours at 80°C,

after which a sample was collected and then the temperature was set to 90°C. It took 2 minutes to reach 90°C. Once at 90°C, samples for 0 minutes were withdrawn. Approximately 20 mL of liquid was collected prior to sampling at each time point to eliminate pre-existing fluid in the tubing. Samples for 5 minutes, 10 minutes, 20 minutes, 30 minutes, 60 minutes, and 90 minutes from the time the fermenter reached 90°C were withdrawn. Five hundred mL of culture were withdrawn at each time point and immediately put on ice until processed for RNA extractions (see below). One mL of sample was removed for cell counting by epifluorescent microscopy (Chapter II). Figure V.1 shows the complete list of samples withdrawn and the loop used for mixed model analysis with microarrays.

RNA extractions and hybridizations. RNA was extracted as previously described (Chapter II). Briefly, the 500 mL samples were centrifuged for 20 minutes (10,000 × g, 4°C) and the pellets were rinsed twice in 300 mM NaCl (Fisher Scientific, Pittsburgh, PA). Once the pellets were lysed in RNA lysis buffer, the samples were stored at –80°C so that the later time interval samples could be processed. Extractions proceeded with ethanol precipitation and purification with Promega Total SV RNA kits (Promega, Madison, WI). Concentrations and degree of purity were determined by optical density at 260 nm and 280 nm, as well as gel electrophoresis (1% agarose gel, 60 V). Procedures for reverse transcription reactions, aminoallyl-labeling with Cy3 and Cy5, and hybridization reactions are reported elsewhere (Chapter II, (52)).

Targeted cDNA microarray analysis.

The targeted cDNA microarray was constructed with 407 genes (Appendix II). Open reading frames were identified and slides were printed, as described previously (Chapter II, (52)). The loop design in Figure V.1 was used with the mixed model analysis to generate hierarchal clustering of least square means and standardized least square means estimates of expression levels of all genes under all conditions. Fold-changes in gene expression between each of the time points and the baseline (80°C) were calculated by raising 2 to the power of the difference of the residuals between two conditions. A $-\log_{10}$ P-value > 4.0 was used as a standard for statistical significance (see Chapter II).

V.3. Results and Discussion

Dynamic changes in gene expression of *T. maritima* during heat shock in a 14.0 L fermenter were investigated using a targeted cDNA microarray consisting of 407 genes (about 20% of the genome) chosen for their relevance to stress response and related physiological phenomena. Helmann et al. (2001) (120) performed a similar study on *B. subtilis* and demonstrated that transcriptional responses to heat shock occur within minutes after an increase in temperature. Figure V.2 shows the decline of *T. maritima* cell density from 3.4×10^8 cells/ml to 1.6×10^8 cells/ml during the heat shock from 80°C to 90°C, respectively. Transcriptional responses of *T. maritima* were analyzed for each of seven time points over the period of 0 to 90 minutes following shift from 80 to 90°C.

Mixed model analysis and array design

Loop experimental designs were implemented by Jin et al. (2001) (132) to compare gene expression from multiple conditions in the same analysis. Figure V.1 shows the chrono-

logical loop design used to compare the baseline (80°C), 0 minutes (90°C), 5 minutes (90°C), 10 minutes (90°C), 20 minutes (90°C), 30 minutes (90°C), 60 minutes (90°C), and 90 minutes (90°C). The data generated from the mixed model ANOVA analysis were examined using two complimentary approaches: (1) clustering analysis; and (2) fold-change of gene expression using the baseline as a reference point. The two clusters, least square means and standardized least square means, are shown in Figure V.3. Clusters C1 and C2 showed dynamic trends in heat shock response (Figures V.3, V.4, and V.5) and are discussed below. The pairwise comparisons that represent fold-changes in gene expression, compared to the 80°C reference point, facilitated dynamic analysis by following the expression of a single gene over time (Figure V.5 and Tables V.1, V.2, and V.3).

Approximately 140 genes were added to the targeted *T. maritima* cDNA microarray previously used for the biofilm studies (Chapter II). These genes included the sigma factors: σ^H (TM0534), σ^A (TM1451), σ^E (TM1598) and σ^{27} (TM0902), anti-sigma factors RsbW (TM0733), FlgM (TM0085), anti-sigma regulatory factor (Ser/Thr protein kinase (TM1354), σ^E-regulator (TM1356), as well as the anti-sigma factor antagonists belonging to the SpoIIAA family (TM1081 and TM1442). The regulation of stress response by various sigma factors has been well documented (158, 290); however, the expression and regulation patterns of sigma factors in *T. maritima* have not been studied. It is noteworthy that none of the sigma factors in *T. maritima* are closely related to rpoH of *E. coli* or sigB of *B. subtilis*, which control well-documented heat shock regulons (158, 290).

Stimulation of heat shock genes

Expression of the various heat shock regulons was stimulated to a great extent during initial stages of heat shock and then leveled off after about 30 minutes (Figure V.4). Cluster C1 shows that the temporal expression of profiles of known heat shock genes clustered together showing a similar trend. At 80°C, heat shock genes were expressed at low levels and then increased significantly after the growth temperature was raised to 90°C. This phenomenon was observed for genes encoding DnaK (TM0373), ATP-dependent protease ClpC-2 frameshift (TM0873), DnaJ (TM0849), heat shock protein class I (TM0374), GroES (TM0505), heat shock operon repressor, HrcA (TM0851), and GrpE (TM0850) (Figure V.4). Although not appearing within this cluster, GroEL (TM0506), a ClpA-type ATP-binding subunit (TM0198), and a MalK-family ATP binding protein (TM1276) also displayed a similar expression pattern (Figure V.4) to a lesser extent. The observed trends in clusters C1 and C2 are quantitatively represented in Figures V.5 and V.6, respectively, where the fold-change with respect to the reference point (baseline at 80°C) is plotted as a function of time. Expression of most heat shock genes stabilized after about 30 minutes, with the exception of heat shock protein class I (TM0374) and ClpC-2 protease (TM0873), which increased slightly over the 30 to 90 minute period.

Several of the heat shock genes described above belong to the class I heat shock class, which are regulated by the HrcA repressor. In *B. subtilis*, HrcA regulates both the *hrcA* and *groEL-groES* operons, binding a well-conserved operator, the CIRCE element (TTAGCACTC (N9) GAGTGCTAA) (290). The CIRCE element is conserved upstream of the *T. maritima hrcA-grpE-dnaJ* and *groEL-groES* operons (Conners and Kelly, unpublished). CIRCE-mediated transcription is also accompanied by regulation by sigma

factors; however, the regulatory mechanisms differ for various microorganisms (158). The sharp increase and decline of heat shock gene expression to a steady-state level has been observed to correlate with σ^{32} activity in *E. coli* (254, 290), whereas, the σ^{32} and HrcA regulation occur in concert in *Bradyrhizobium japonicum* (290). In Gram-positive bacteria, such as *B. subtilis*, the transcription of genes encoding the DnaK-DnaJ-GrpE and GroEL-GroES chaperones are also dependent on the housekeeping sigma factor σ^{A} (σ^{70}) (290). In addition, a general stress regulator, σ^{B}, is responsible for activation of over 200 genes during various stress conditions, including heat shock, osmotic stress, and energy stress in *B. subtilis* (119, 120).

As previously noted, the *T. maritima* genome contains sigma factor homologs that are most similar to the *E. coli* flagellar sigma factor FliA (35% identity over 212 aa), *E. coli* stress sigma factor RpoE (34% identity over 183 aa), and *B. subtilis* SigH, a sporulation-related sigma factor (30% identiry over 188aa). The protein encoded by TM1451 is presumed to act as the vegetative sigma factor for *T. maritima* because it shares stronger identity with *B. subtilis* sigA (57% identity over 263 aa) and *E. coli* RpoD (53% identity over 272 aa) than with *E.coli* RpoH (28% identity over 286 aa) or *B. subtilis* SigB (23% identity over 231 aa). Analysis of putative promoters for CIRCE-regulated class I heat shock genes revealed the presence of –a sigA-like promoter located upstream of TM0505 and several sigA-like promoters located upstream of TM0851.

Analysis of sigma factors due to thermal stress

Response of putative sigma factors during the thermal stress time course for *T. maritima* is listed in Table V.1. The fold-changes of expression relative to the baseline at 80°C are

reported. Increased expression of two of the four major sigma factors was observed during heat shock: σ^A (TM1451) and σ^E (TM1598) were up-regulated by an average of 2.0-fold and 2.3-fold, respectively. An anti-sigma factor (TM0733), related to the σ^B regulator RsbW, was also observed to be up-regulated 2-fold. Expression of the putative flagellar sigma factor σ^F or FliA (TM0902) was found to decrease after 5 minutes of heat shock. The other major sigma factor, σ^H, did not show significant changes in expression during heat shock.

Sequence analyses were performed to gain a better understanding of how sigma factors may be involved in the regulatory mechanisms during heat shock of *T. maritima*. The anti-σ^B regulator (TM0733), a putative protein of 136 amino acids, was found by BLAST analysis that it is most similar to the RsbW negative regulator in *Listeria monocytogenes* (26% identical, 47% positives). Similar homology was observed to RsbW anti-sigma-B regulator found in other mesophilic microorganisms. In *B. subtilis*, RsbW prevents σ^B from incorporating into the RNA polymerase (158). In the absence of σ^B, it is possible that *T. maritima* RsbW regulates the incorporation of another sigma factor.

The vegetative sigma factor in *T. maritima,* σ^A (TM1451), is highly similar to σ^A in *Clostridium perfrigens* (61% identical, 73% positives over 399 aa). Other high scores were obtained with σ^A from *Enterococcus faecalis*, *Bacillus halodurans*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *B. subtilis*, among others, suggesting a highly conserved sequence. However, this is the first report of transcriptional changes of sigma factors in *T. maritima*, and further experiments need to be done to determine the regula-

tory mechanisms of σ^A as well as the other sigma factors. Yet, the increase in expression during thermal stress cannot be ignored.

Sigma-E (TM1598) is a putative protein of 189 amino acids and most closely resembles σ^E from *Yersinia pestis* (35% identical, 55% positives). However, this sequence seems to be fairly well conserved, and was also 35% identical with 55% positives to σ^E of *E. coli* O157:H7. This sigma factor protects against extracytoplasmic stress in *E.coli*, and controls the other sigma factor, σ^{32}, important for survival during heat stress (290). It is responsible for activating the Omp regulon, which controls outer membrane protein (OMP) folding as the membrane may lose shape due to environmental stresses such as heat shock (208). Interestingly, the *T. maritima* genome contains only three of the seven σ^E regulon members (*rpoH, degP, rpoE, fkpA, rseA, rseB,* and *rseC*) as identified in *E. coli* (208). These are σ^E (TM1598), anti-sigma factor RseC (TM1356), and σ^H (TM0534); however, σ^H did not show significant changes in expression as the σ^E did during heat shock. Sigma E is also involved in virulence properties of many pathogens, such as *Pseudomonas aeruginosa*, where activation of AlgU (σ^E homologue) is responsible for producing the mucoid capsule (208). There have not been many studies related to the possible virulence of *T. maritima*; however, MviN virulence-related protein (TM0086) was observed to increase both under heat shock and biofilm formation (Chapter IV). In addition to the σ^E stimulation of virulence properties, the Mar family of transcriptional regulators is implicated in the antibiotic resistance mechanisms of *E. coli* (7, 129, 175). A *T. maritima* Mar family transcription regulator (TM0816) was up-regulated 108.0-fold after 90 minutes at 90°C; though the physiological role of this protein remains unknown.

Sigma F (TM0902) showed 35% identity (210/220 aa, 59% positives) to flagellar sigma factor FliA in *Vibrio parahaemolyticus*. Although not much is known about this sigma factor in *T. maritima*, other flagellar- and chemotaxis- related proteins showed decreased expression levels. These include CheY (TM0700), CheW (TM701), flagellar biosynthesis protein FliP, and to a lesser extent, flagellar assembly proteins (TM0218-TM0221). This is an interesting result as it has been shown that *T. maritima* responds to increased temperature by increasing the rate of tumbles (100).

Response of proteases and SOS genes to thermal stress

Table V.2 shows the fold-changes of protease expression levels during the time intervals of thermal stress. These results are quite interesting as it is well-documented that proteases are induced under thermal stress (158, 290). In *E. coli,* most of the ATP-dependent proteases including Lon, ClpQ, ClpP, ClpX, ClpY, ClpB, and membrane-bound FtsH, are up-regulated under control of σ^{32} (290). However, this was not the case for thermal stress response observed in *T. maritima.* Only the ATPase ClpC-1 (TM0198), ATPase ClpC-2 (TM0873), FtsH (TM0580), heat shock serine protease HtrA (TM0571), carboxy terminal protease (TM0747), and a hypothetical protein with homology to protease maturation (TM1704), were up-regulated during heat shock at 90°C. The Lon proteases (TM1633 and TM1869) were down-regulated along with the bacteriocin (TM0785), which was also observed during thermal stress of *Pyrococcus furiosus* at 105°C (241). Overall expression levels were in the middle range of the least square means clusters, except for ClpX (TM0146) and ClpP proteolytic subunit (TM0695), which were highly expressed at 80°C as well as 90°C. In *E.coli*, Clp and FtsH proteases identify targets based on an 11-residue tag at the protein C-terminus; SsrA RNA acts as a tRNA and adds the target to the end of

the polypeptide. SmpB is a protein that binds to SsrA to form complex and is required for SsrA activity (139, 140, 143). *T. maritima* contains a SmpB ortholog (TM0254) with a frameshift; despite the loss of 25 C-terminal amino acid residues, it seems evident that SmpB protein is still produced (238, 290). Although this gene is not on the targeted microarray used here, a full genome analysis may yield interesting results with respect to SmpB (TM0254) in *T. maritima*. Another frameshifted gene encoding the ATP-dependent protease/ATPase ClpC-2 was observed to increase 42.6-fold after 5 minutes at 90°C. Genes with frameshifts are deleted from the genome (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/framik?db=Genome&gi=141); however, it is apparent that protein products may still be made. There may also be issues of cross-hybridization from similar products such as ClpC-1 or ClpC-3, which is currently being investigated.

The *T. maritima* genome contains several orthologous genes to the SOS regulon in *E. coli* (158). RecA is responsible for DNA repair mechanisms (242), and *T. maritima* RecA (TM0254) was found to be up-regulated slightly during heat shock. The LexA repressor (TM1082) was down-regulated by an average of 2.3-fold at 90°C. Although the regulation mechanism of the SOS regulon in *T. maritima* is unknown, our results are consistent with the reports of RecA regulation by a LexA-dependent promoter in other mesophilic species (158). RecA expression is also dependent on guanosine tetraphosphate (ppGpp) starvation signals (82). *T. maritima* ppGpp synthase (TM0729) was up-regulated during heat shock an average of 1.7-fold, and was expressed maximally 2.5-fold at 90 minutes. UvrABD is another well-conserved DNA damage recognition system in bacteria (290). UvrB (TM1761) was up-regulated an average of 2.5-fold in *T. maritima*

cells at 90°C; UvrD (TM1238) did not show significant changes. Unfortunately, UvrA (TM0480) was not on this targeted array (Appendix I), but will be investigated using a whole genome chip. Finally, the RuvABC genes repair Holliday junctions during DNA repair in *E. coli* (81). The *T. maritima* genome contains a RuvA (TM0165) and RuvB (TM1730); RuvB did show significant changes during thermal stress and RuvA was not on the array. Nonetheless, it is apparent that the heat shock of *T. maritima* at 90°C induced DNA repair mechanisms through the induction of the SOS regulon. Shockley et al. (2003) (241) also observed an induction of the RecA-like gene *radA* in *P. furiosus* during heat shock at 105°C.

Other identified stress response genes

In addition to known heat shock genes, two genes thought to be involved in glucuronic acid metabolism showed temperature-dependent expression profiles. The IclR/KdgR-type repressor (TM0065) (292) and the divergently transcribed uronate isomerase (TM0064) were both up-regulated 7.0-fold after 10 minutes at 90°C and remained at least 4.0-fold higher than the baseline throughout the 90 minutes (Table V.3). Other genes involved in this metabolic pathway showed different responses. Another hypothetical protein (TM0063) downstream of the uronate isomerase was also up-regulated an average of 3.9-fold over the thermal stress period, while TM0066-TM0069 (see Table V.3) showed no significant changes. The role of these genes in the *T. maritima* stress response is currently unknown, but these operons have been previously observed to be highly expressed under biofilm and planktonic growth in continuous culture on maltose (Chapter IV) and during batch growth on glucuronate-substituted β-xylan (52). These genes are also involved in pectin degradation in *Erwinia chrysanthemi*, where kdgR negatively controls the pectin

lyase genes (58, 212). *T. maritima* can grow on pectin as a sole carbon source and the pectin lyase PelA (TM0433) has been characterized (152). Although no pectin was added to the medium, the down-regulation of PelA is consistent with the up-regulation of the KdgR negative regulatory element (data not shown). Other sources of glucuronic acid may be present to induce this metabolic pathway. Although it is inconclusive in regards to *T. maritima* at this time, other mesophilic microorganisms, such as *Pseudomonas putida* (136), have shown to produce glucuronic acid-containing polysaccharides under stress response.

Several transcriptional regulators, including XylR (TM0032, TM0110), TetR (TM0823, TM1030), Mar (TM0816), LacI (TM0300, TM0949, TM1218, TM1856), GntR (TM0275) and DeoR (TM1069) showed significant expression during heat shock at 90°C. TetR, efflux pump related regulators (e.g., Mar family), and DeoR transcriptional regulators were also induced in the *B. subtilis* thermal stress response (120). GntR is a negative regulator of the glucuronic acid pathway, which was turned on as discussed above. This was also observed in the *T. maritima* biofilm gene expression patterns. In addition, the NADH oxidase (TM0379, TM0395) and GTPase (TM0445) were also up-regulated in heat shocked and biofilm populations. In contrast to the sessile cells, thermally stressed *T. maritima* showed decreased expression of the esterase (TM0053) and increased expression of the alkyl hydroperoxide reductase (TM0807).

V.4. Summary

Examination of thermal stress response of the hyperthermophilic bacterium *T. maritima* showed that, similar to less thermophilic bacteria, genetic reaction to supraoptimal temperatures is very rapid and widespread. While elements of heat shock response in *T. maritima* resembles what has been discerned from the study of mesophilic bacteria, there are apparently significant differences, especially with respect to roles of sigma factors and ATP-dependent proteases and chaperones. Additional work utilizing a whole genome array as well as biochemical and genetic analysis of specific genes and gene products will be needed so that these differences can be explored further and the role of growth temperature in this phenomenon can be determined.

V.5. Acknowledgments

This was work was supported in part by grants from the National Science Foundation and the Department of Energy (Energy Biosciences Program).

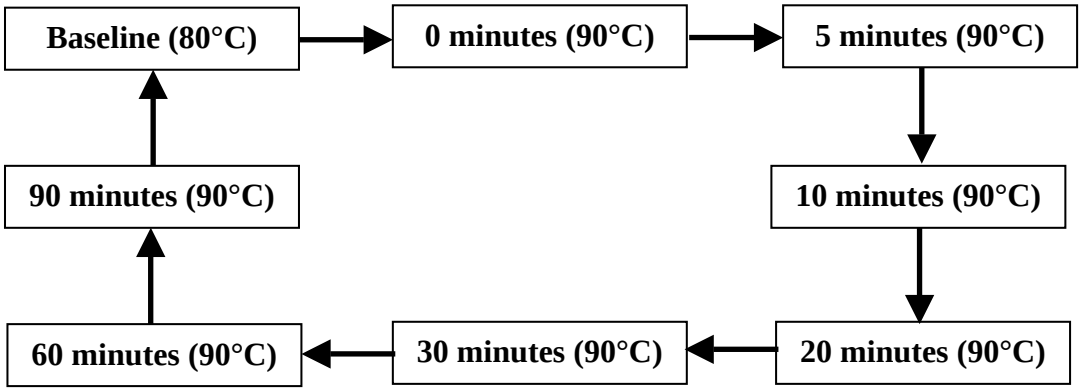


Figure V.1. Loop design for the mixed model analysis of gene expression for *T. maritima* during heat shock from 80°C to 90°C. Arrow tails and heads correspond to aminoallyl labels Cy3 and Cy5 respectively: Cy3 → Cy5.

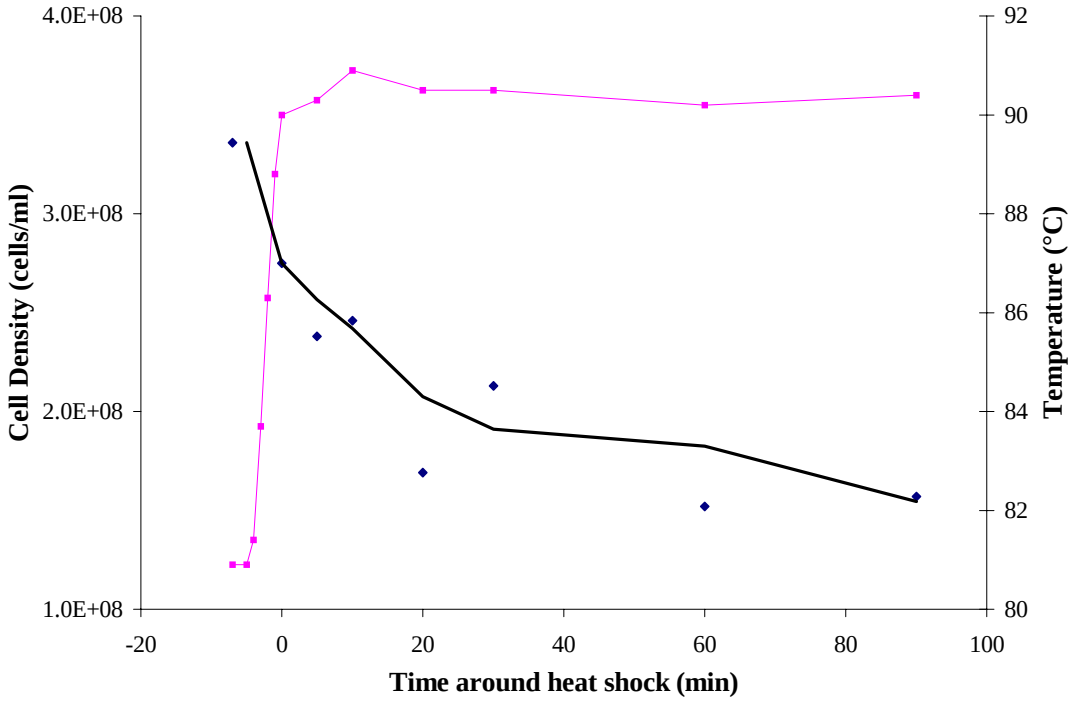


Figure V.2. Cell densities decreased sharply after the temperature was shifted from 3.4 x10^8 cells/ml at 80°C to 1.6 x10^8 cells/ml after 90 minutes at 90°C. Temperature profile is represented by solid pink line.

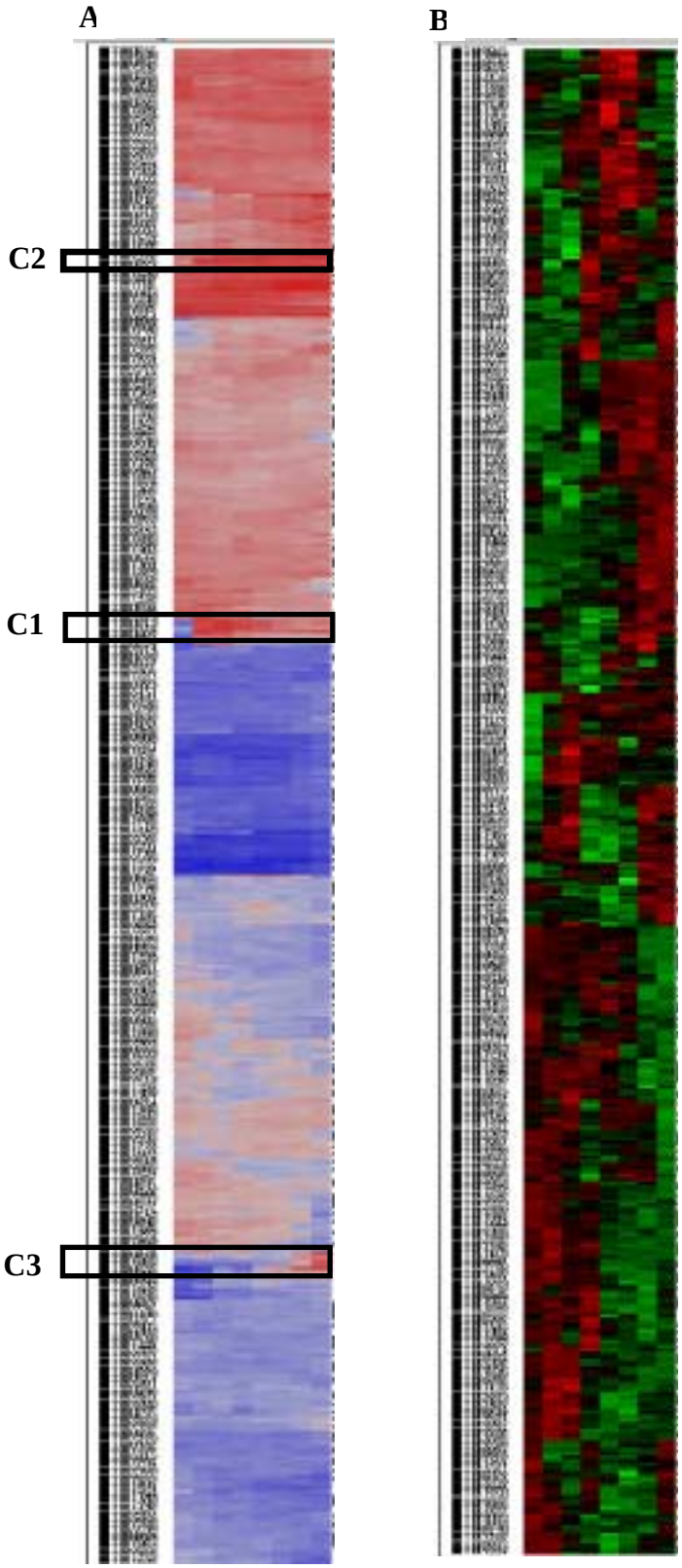
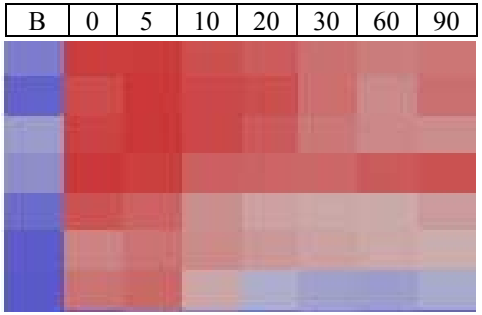


Figure V.3. Hierarchal clusters using least square means (A) and standardized values (B). Columns go in order of time course from left to right: Baseline (80°C); 0 min (90°C); 5 min (90°C); 10 min (90°C); 20 min (90°C); 30 min (90°C); 60 min (90°C); and 90 min (90°C).

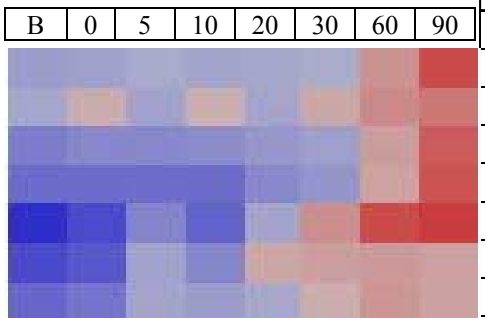


Cluster C1

Locus	Function	Max Fold-Change	Time
TM0373	DnaK	24.9	0
TM0873	ATP-dependent protease ClpC-2 frameshift	42.6	5
TM0849	DnaJ	14.7	5
TM0374	Heat shock protein, Class I	20.5	0
TM0505	GroES	22.3	0
TM0851	Heat shock operon repressor	14.8	5
TM0850	GrpE	19.3	5

Cluster C2

Locus	Function	Max Fold-Change	Time
TM0198	ATP-dependent protease/ATPase ClpC-1	3.8	90
TM1276	sugar ABC transporter, ATP-binding	3.6	20
TM0506	GroEL	3.9	90



Cluster C3

Locus	Function	Max Fold-Change	Time
TM0434	α-glucosidase	10.0	90
TM0616	ATPase	3.5	90
TM1068	α-glucosidase, Amy4B	13.0	90
TM1069	Transcriptional regulator, DeoR family	20.7	90
TM0816	Transcriptional regulator, Mar family	108.0	90
TM0032	Transcriptional regulator, XylR related	10.0	60
TM0300	Transcriptional regulator, LacI family	6.2	60

Figure V.4. Expanded least square means clusters C1, C2, and C3 from Figure V.3.A. Time at which maximal fold-change of expression with respect to the reference point (Baseline at 80°C) and fold-change are reported. Columns of cluster are chronological from B (baseline, 80°C), 0 (0 min), 5 (5 min), etc. Clusters C1 and C2 displayed maximal expression within minutes after the temperature was shifted from 80°C to 90°C, while cluster C3 displayed maximal fold-change at later times. Fold-changes had high significance with negative $\log_{10}$ p-values ≥ 27.3.

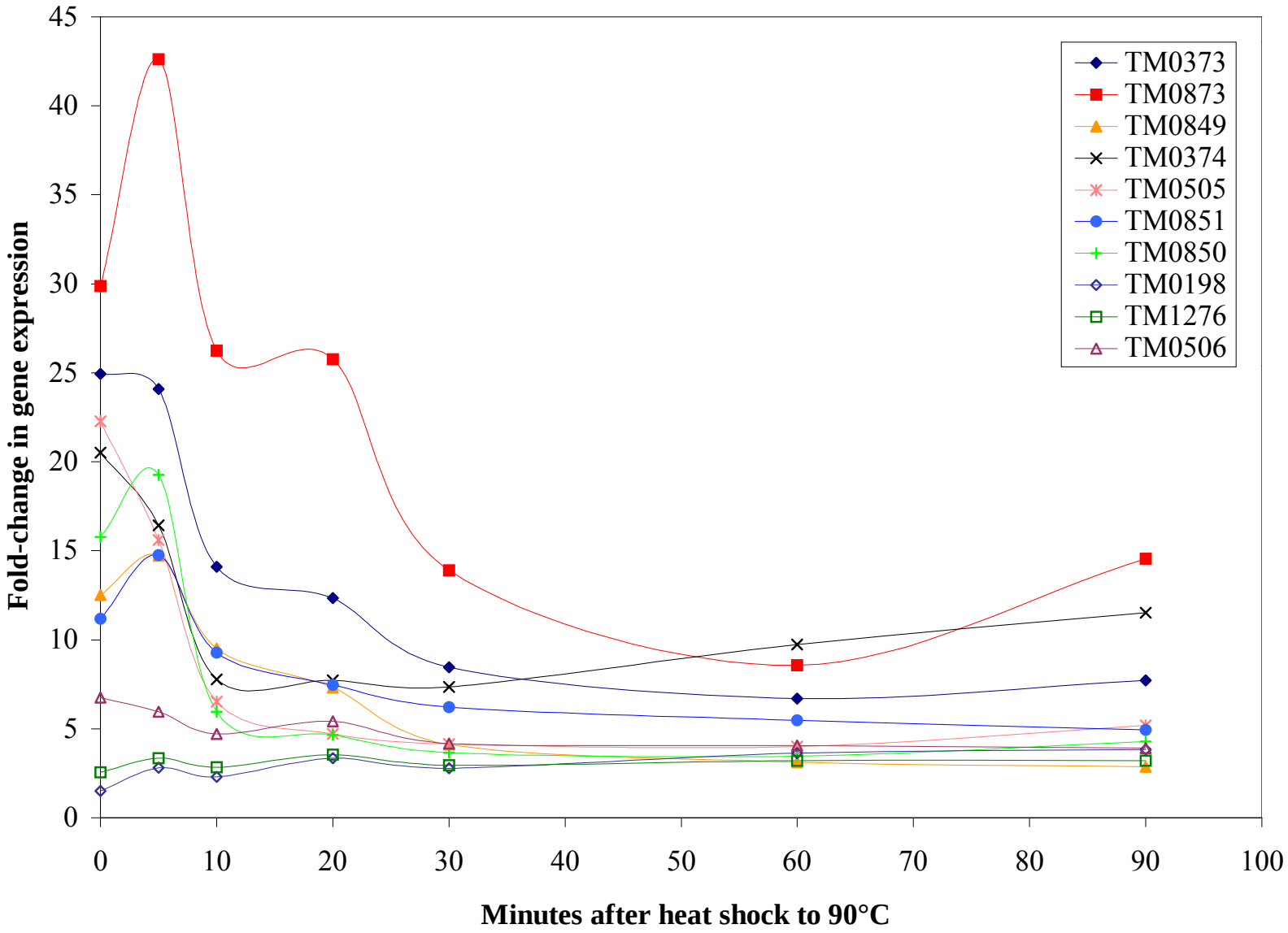


Figure V.5. Fold-changes at each time point compared to the reference point (baseline 80°C) are plotted over time. Genes from clusters C1 and C2 (Figure V.4) are represented and exhibit the same trend where they are highly expressed at early times and level off after approximately 30 minutes. Fold-changes were highly significant with $-\log_{10}$ (P-value) ≥ 4.0 for all of the data points.

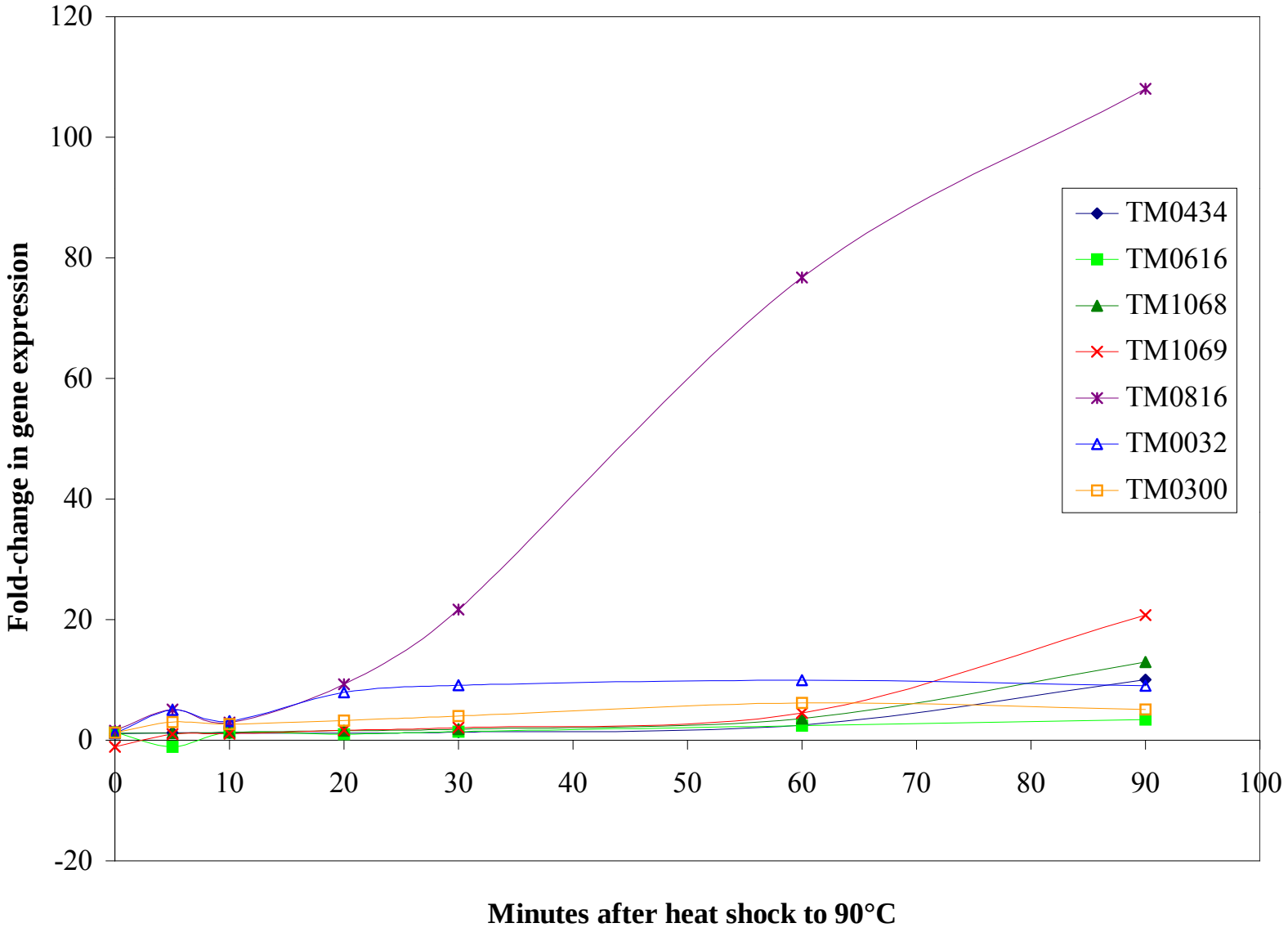


Figure V.6. Fold-changes at each time point compared to the reference point (baseline 80°C) are plotted over time. Genes from clusters C3 (Figure V.4) are represented and exhibit the same trend where the expression levels slowly ramp up to maximum levels at later times. Fold-changes were highly significant with $-\log_{10}$ (P-value) ≥ 4.0 for 85% of the data points.

Sigma Factors								
Locus	**Function**	**0**	**5**	**10**	**20**	**30**	**60**	**90**
TM0085	Anti-sigma 27	1.3	1.4	1.2	1.2	1.4	1.6	2.3
TM0534	Sigma H	-1.0	-1.1	1.0	1.2	1.2	1.3	1.9
TM0733	Anti-sigma factor sigma B, putative	1.1	1.8	1.9	2.8	1.8	1.7	1.1
TM0902	Sigma F, FliA	-1.1	-1.6	-2.2	-2.6	-2.1	-2.4	-2.7
TM1081	Anti-sigma factor antagonist, putative	-1.5	-1.9	-2.1	-1.9	-1.8	-2.2	-3.2
TM1354	Anti-sigma RsbW	-1.1	-1.3	-1.0	-1.2	-1.2	-1.4	-1.3
TM1356	Anti-sigma E factor RseC COG 3086	-1.2	-1.4	-1.0	1.0	-1.0	-1.2	1.1
TM1442	Anti-sigma factor antagonist, putative	1.3	-1.1	-1.3	-1.0	-1.0	1.2	-1.4
TM1451	Sigma A	1.6	2.8	1.2	1.8	2.2	2.5	4.2
TM1598	Sigma E	1.2	2.0	1.8	2.1	2.1	2.3	3.8

Table V.1. Fold-changes in expression of sigma factors in *T. maritima*. Values calculated from the residual differences between each time point (0 minutes, 5 minutes, 10 minutes, 20 minutes, 30 minutes, 60 minutes, and 90 minutes) and the baseline (80°C); thus, the baseline is used as a reference condition here. Positive fold-changes reflect up-regulation and negative fold-changes reflect down-regulation. Grey-shaded values are not statistically significant as $-\log_{10}$ P-value is ≤ 4.0.

ATP-dependent proteases								
Locus	**Function**	**0**	**5**	**10**	**20**	**30**	**60**	**90**
TM0146	ATP-dependent protease/ATPase ClpX	-1.2	-1.1	1.1	1.4	1.4	1.5	2.8
TM0198	ATP-dependent protease/ATPase ClpC-1	1.5	2.8	2.3	3.3	2.8	3.6	3.8
TM0521	Heat-shock protein HslV protease ClpQ	-1.3	-1.8	-1.2	-1.8	-1.7	-1.8	-1.3
TM0522	Heat-shock protein HslU ATPase ClpY	-1.2	-1.5	-1.3	-1.6	-1.6	-1.5	-1.1
TM0580	Cell division protein FtsH	1.5	1.8	1.6	2.4	2.0	2.2	1.4
TM0695	ATP-dependent Clp protease, proteolytic subunit	1.1	-1.1	-1.3	-1.3	-1.5	-1.5	-2.0
TM1391	ATP-dependent protease/ATPase ClpC-3	1.2	1.1	-1.2	-1.4	-1.1	1.1	-1.0
TM0873	ATP-dependent protease/ATPase ClpC-2 frameshift[+]	29.9	42.6	26.2	25.8	13.9	8.6	14.6
TM1633	ATP-dependent protease LonA	-1.2	-1.6	-1.5	-1.4	-1.2	-1.2	-1.1
TM1869	ATP-dependent protease LonB, putative	1.0	-1.2	-1.1	-1.6	-1.4	-1.3	1.0

[+]TM0873 was recently removed from the genome annotation due to a frameshift in sequence

ATP-independent proteases								
Locus	**Function**	**0**	**5**	**10**	**20**	**30**	**60**	**90**
TM0145	Secreted metalloendopeptidase Gcp, putative	-1.3	-1.3	-1.3	-1.0	1.1	1.1	1.7
TM0409	Conserved hypothetical, probable secreted proteinase	-1.5	-1.8	-1.1	-1.2	-1.3	-1.6	-1.7
TM0516	Clostripain-related protein	-1.1	-1.2	1.2	1.3	1.1	1.0	1.4
TM0571	Heat-shock serine protease, periplasmic (htrA)	1.1	1.7	1.3	1.9	2.1	2.7	2.5
TM0643	Clostripain-related protein	-1.2	-1.1	-1.0	1.1	-1.4	-1.4	-1.9
TM0747	Carboxy-terminal protease	1.0	1.3	1.3	1.8	1.7	1.9	2.5
TM0785	Protease, bacteriocin	1.1	-1.6	-1.3	-2.5	-2.5	-3.1	-2.3
TM0890	Hypothetical zinc metalloprotease	-1.2	-1.3	-1.0	-1.0	-1.1	-1.2	1.1
TM0916	Periplasmic serine protease, putative	-1.1	-1.2	-1.2	-1.2	-1.1	1.2	-1.2
TM1346	Processing protease, putative	-1.3	-1.9	-1.5	-2.0	-1.8	-2.1	-3.0
TM1589	Clostripain-related protein	-1.8	-2.6	-2.2	-3.8	-3.7	-4.5	-4.3
TM1704	Hypothetical protein (homology to protease maturation)	1.3	1.7	1.6	2.4	2.5	2.0	1.4
TM1822	FtsH protease activity modulator HflK	-1.2	-1.4	-1.2	1.1	1.1	1.1	-2.1
TM1823	FtsH protease activity modulator HflC	-1.6	-2.1	-1.8	-2.0	-2.3	-2.3	-3.1

[*] Values are not statistically significant as $-\log_{10}$ P-value is ≤ 4.0

Table V.2. Fold-changes in expression of proteases in *T. maritima*. Values calculated from the residual differences between each time point (0 minutes, 5 minutes, 10 minutes, 20 minutes, 30 minutes, 60 minutes, and 90 minutes) and the baseline (80°C); thus, the baseline is used as a reference condition here. Positive fold-changes reflect up-regulation and negative fold-changes reflect down-regulation. Grey-shaded values are not statistically significant as $-\log_{10}$ P-value is ≤ 4.0.

Non-Heat Shock Associated Genes								
Locus	**Function**	**0**	**5**	**10**	**20**	**30**	**60**	**90**
TM0024	Laminarinase	1.0	1.8	2.1	1.6	2.2	1.5	1.1
TM0025	β-glucosidase	-1.0	2.3	2.4	3.6	3.0	2.1	-1.1
TM0032	Transcriptional regulator, XylR-related	1.2	5.0	3.2	8.0	9.1	10.0	9.1
TM0042	Aminopeptidase P	-1.6	-1.8	-1.6	-1.9	-1.9	-2.4	-3.1
TM0053	Esterase, putative	-1.3	-1.5	-1.2	-1.8	-1.8	-1.5	-2.3
TM0063	Hypothetical protein: Downstream uronate isomerase	1.3	4.4	4.0	4.5	4.1	4.2	4.9
TM0064	Uronate isomerase, putative	1.7	6.7	5.3	5.7	5.1	5.8	7.1
TM0065	Transcriptional regulator, IclR family (kdgR)	1.8	7.1	5.8	5.3	5.8	6.1	5.9
TM0110	Transcriptional regulator, XylR-related	-2.3	-2.3	-2.0	-2.1	-1.8	-2.2	-2.9
TM0122	Ferric uptake regulation protein	1.7	2.7	2.2	2.5	2.3	2.0	4.2
TM0275	Transcriptional regulator, GntR family	-1.2	-1.8	-2.1	-2.4	-2.4	-2.0	-5.2
TM0299	Transcriptional regulator, LacI family	1.0	2.5	2.5	3.8	3.6	2.5	3.0
TM0300	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein, putative	1.2	3.0	2.7	3.3	4.0	6.2	5.1
TM0301	Oligopeptide ABC transporter, permease protein	-1.1	1.3	1.5	1.7	1.8	1.8	2.0
TM0302	Oligopeptide ABC transporter, permease protein	-2.1	-1.4	-1.1	-1.2	-1.1	-1.0	1.3
TM0365	Aminopeptidase, putative	-1.5	-1.7	-1.5	-1.3	-1.5	-2.0	-2.0
TM0379	NADH oxidase	1.1	1.2	1.3	1.8	2.0	2.5	3.2
TM0395	NADH oxidase, putative	-1.9	-1.8	-2.4	-1.4	1.4	2.3	1.1
TM0408	Protein-glutamate methylesterase	-1.1	-1.1	-1.0	1.0	-1.3	-1.6	-2.5
TM0434	α-glucosidase, putative	1.1	1.3	1.2	1.2	1.3	2.5	10.0
TM0445	Conserved hypothetical protein, COG1160: Predicted GTPase	1.4	1.6	1.6	2.1	2.6	3.8	4.8
TM0508	Conserved hypothetical protein, COG2110: Uncharacterized ACR related to the C-terminal domain of histone macroH2A1	1.1	1.7	1.7	2.1	2.0	2.3	3.3

Table V.3. Fold-changes in expression of non-heat shock associated genes in *T. maritima* during thermal stress at 90°C. Values calculated from the residual differences between each time point (0 minutes, 5 minutes, 10 minutes, 20 minutes, 30 minutes, 60 minutes, and 90 minutes) and the baseline (80°C); thus, the baseline is used as a reference condition here. Positive fold-changes reflect up-regulation and negative fold-changes reflect down-regulation. Grey-shaded values did not have a $-\log_{10}$ (P-value) ≥ 4, and grey-shaded genes were also observed by Helmann *et al*. (120) in heat shock of *B. subtilis*.

Non-Heat Shock Associated Genes								
TM0510	Iron-dependent transcriptional repressor, putative	1.6	1.9	1.8	2.1	2.2	2.8	4.6
TM0619	Conserved hypothetical protein, COG0438: Predicted glycosyl-transferases RfaG	-1.6	-1.4	-1.3	-1.7	-1.6	-2.2	-1.6
TM0624	N-acetylglucosaminyl-phosphatidylinositol biosynthesis-related protein	1.2	1.5	1.0	-1.0	-1.1	-1.8	-2.9
TM0630	Nucleotide sugar epimerase, putative	-1.5	-1.7	-1.6	-1.7	-1.8	-2.5	-3.5
TM0631	Lipopolysaccharide biosynthesis protein	-1.6	-1.5	-1.7	-2.1	-1.7	-1.7	-2.2
TM0668	Pleiotropic regulatory protein	-1.4	-2.0	-1.8	-2.0	-2.1	-2.3	-1.9
TM0689	Phosphoglycerate kinase, triose phosphate isomerase	-1.5	-1.9	-2.2	-1.5	-1.2	-1.2	-1.3
TM0694	Trigger factor, putative	-1.1	-1.3	-2.8	-2.8	-2.3	-2.3	-2.2
TM0696	Ray-related protein	-1.5	-2.0	-1.5	-1.6	-2.1	-3.2	-4.0
TM0698	Flagellar biosynthesis protein FliP	-1.6	-1.9	-1.9	-1.9	-2.1	-3.2	-4.8
TM0700	Chemotaxis response regulator CheY	-1.2	-1.6	-1.6	-1.8	-1.7	-2.3	-2.2
TM0701	Purine-binding chemotaxis protein CheW	-1.4	-1.8	-1.9	-2.4	-2.2	-2.4	-2.2
TM0729	(p)ppGpp synthetase	1.2	1.3	1.6	1.6	1.7	1.7	2.5
TM0767	Maltodextrin glycosyltransferase	-1.3	-1.9	1.1	-1.9	-2.2	-3.1	-3.3
TM0807	Alkyl hydroperoxide reductase, putative	1.1	1.4	1.1	1.4	1.2	2.3	2.1
TM0816	Transcriptional regulator, putative, Mar family	1.6	5.1	3.0	9.3	21.7	76.8	108.0
TM0823	Transcriptional regulator, TetR family	1.2	4.6	2.0	8.0	8.3	12.4	19.0
TM0897	SpoVS-related protein	-1.1	-1.6	-1.5	-2.1	-2.1	-2.1	-2.0
TM0949	Transcriptional regulator, LacI family	-1.3	-1.4	-1.7	-1.8	-1.5	-1.4	-2.0
TM1005	Transcriptional regulator, putative, COG2207: AraC-type DNA-binding domain-containing proteins	1.2	1.6	2.1	2.8	3.4	3.9	4.5
TM1030	Transcriptional regulator, TetR family	1.2	3.9	3.6	5.5	4.7	5.9	7.5
TM1048	Endoglucanase	-1.3	-1.7	-1.3	-1.7	-1.7	-2.0	-1.9
TM1068	α-glucosidase, putative	1.2	1.2	1.3	1.6	1.8	3.6	13.0
TM1069	Transcriptional regulator, DeoR family	-1.1	1.0	1.1	1.7	2.1	4.5	20.7
TM1082	LexA repressor	-1.5	-2.1	-2.7	-2.5	-2.3	-2.2	-2.5
TM1168	α-glucan phosphorylase, authentic frameshift	1.2	-1.2	1.2	-1.5	-1.7	-2.9	-4.7
TM1176	Transcriptional regulator, metal-sensing	-2.5	-2.6	-2.0	-2.2	-2.1	-2.1	-1.9

Table V.3. continued.

Non-Heat Shock Associated Genes								
TM1218	Transcriptional regulator, LacI family	-1.3	-1.6	-1.6	-2.1	-2.3	-2.7	-3.8
TM1219	Oligopeptide ABC transporter, ATP-binding protein	-1.4	-1.6	-1.3	-1.4	-2.0	-2.3	-3.4
TM1223	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein	-1.3	-1.9	-1.7	-1.7	-2.0	-1.8	-2.0
TM1232	Sugar ABC transporter, ATP-binding protein	2.9	3.2	3.2	4.5	3.4	2.6	2.9
TM1254	β-phosphoglucomutase, putative	-3.0	-3.9	-2.5	-3.5	-3.1	-3.6	-2.7
TM1255	Aspartate aminotransferase	-1.4	-2.3	-1.5	-2.3	-1.9	-1.9	-1.5
TM1259	Phosphate regulon transcriptional regulatory protein PhoB	-1.0	-1.6	-1.8	-2.3	-2.7	-2.5	-3.3
TM1260	Phosphate transport system regulator PhoU	-1.1	-1.9	-1.8	-2.5	-2.5	-2.9	-3.5
TM1275	Hypothetical protein	1.4	2.2	1.6	2.4	2.3	2.1	1.9
TM1276	Sugar ABC transporter, ATP-binding protein	2.6	3.4	2.8	3.6	2.9	3.2	3.2
TM1334	Conserved hypothetical protein, COG1032: Fe-S oxidoreductases family 2	-1.4	-1.7	-1.5	-1.7	-2.0	-2.6	-2.4
TM1363	Peptide chain release factor RF-1	-1.4	-2.1	-2.3	-2.3	-2.2	-2.2	-4.1
TM1368	ABC transporter, ATP-binding protein	1.1	1.5	1.8	2.5	2.2	2.4	2.7
TM1375	Spermidine/putrescine ABC transporter, periplasmic spermidine	1.0	1.1	1.3	1.1	1.2	2.5	3.8
TM1400	Aminotransferase	-2.6	-3.1	-1.9	-1.7	-1.2	1.1	-1.7
TM1405	Lipopolysaccharide biosynthesis protein-related protein	-1.0	-1.2	-1.5	-2.2	-2.0	-1.6	-1.8
TM1422	RnfB-related protein	1.1	1.7	1.3	1.9	2.1	2.0	2.0
TM1436	Glycerol uptake operon antiterminator-related protein	1.1	-1.0	-1.0	-1.3	-1.5	-2.4	-1.9
TM1450	Transcription repair coupling factor	-1.1	1.6	1.4	2.4	2.3	2.7	3.7
TM1468	Conserved hypothetical protein, COG1307: Uncharacterized BCR, DegV	-1.1	-1.9	-1.8	-2.5	-2.9	-3.8	-3.3
TM1469	Glucokinase	1.2	-1.4	-1.2	-1.6	-1.9	-2.1	-2.5
TM1478	Methionine aminopeptidase	1.7	-1.4	-1.1	-2.5	-2.4	-2.6	-3.5
TM1515	Ferric uptake regulation protein	-1.7	-1.9	1.8	-1.9	-1.9	-2.4	-2.3
TM1601	Conserved hypothetical protein, COG1031: Uncharacterized Fe-S oxidoreductases	1.1	1.7	1.7	2.4	2.0	1.7	1.6
TM1624	β-mannosidase, putative	1.1	-1.4	-1.9	-2.4	-2.9	-2.4	-2.3
TM1683	Cold-shock protein	2.0	-1.2	-1.4	-3.5	-2.5	-2.2	-2.0

Table V.3. continued.

Non-Heat Shock Associated Genes								
TM1696	Type IV prepilin peptidase	-1.2	-1.3	-1.2	-1.5	-1.6	-1.8	-2.3
TM1698	Aspartate aminotransferase	1.1	-1.2	-1.2	-1.3	-2.1	-3.0	-3.9
TM1706	Transcription elongation factor, GreA/GreB family	1.4	1.8	1.4	1.6	2.4	2.7	2.4
TM1707	Conserved hypothetical protein, COG1327: Predicted transcriptional regulator, consists of a Zn-ribbon and ATP-cone domains	1.4	1.8	1.4	1.7	2.3	2.7	2.4
TM1719	DNA mismatch repair protein, MutS	1.1	1.7	1.9	2.6	2.6	2.8	3.1
TM1761	Excinuclease ABC, subunit B, UvrB	1.2	2.0	1.5	2.3	2.6	3.3	4.6
TM1776	Ferric uptake regulation protein	2.0	3.4	2.3	2.4	2.4	2.5	4.0
TM1834	α-glucosidase	-1.1	-1.6	-1.5	-1.9	-2.4	-2.8	-2.3
TM1835	Cyclomaltodextrinase, putative	-1.3	-1.5	-1.3	-1.4	-2.2	-4.3	-4.3
TM1836	Maltose ABC transporter, permease protein	1.1	-1.0	-1.3	-1.5	-1.9	2.6	-2.7
TM1839	Maltose ABC transporter, periplasmic maltose-binding protein	1.1	1.1	1.0	-1.2	-1.5	-2.5	-2.7
TM1840	α-amylase, AmyA	-1.3	-1.3	1.1	-2.9	-3.3	-3.5	-2.4
TM1856	Transcriptional regulator, LacI family	1.1	1.5	1.6	1.7	1.6	2.3	2.4

Table V.3. continued.

Chapter VI. Biofilm Formation in the Hyperthermophilic Archaeon *Pyrococcus furiosus*

Manuscript to Submit to: **Applied and Environmental Microbiology** (August, 2003)

Authors: Marybeth A. Pysz, Keith R. Shockley, Clemente I. Montero,

Shannon C. Burns, and Robert M. Kelly

Abstract

Biofilm formation was induced in *Pyrococcus furiosus* batch cultures using a modified *Archaeoglobus fulgidus* medium (114, 163) supplemented with maltose as a carbon source. Other media formulas, including Raven and Sharp Defined Medium for *P. furiosus* (211), Rinker Defined Medium for *Thermococcus litoralis* (214, 217), and Artificial Sea Water for various hyperthermophiles including *P. furiosus* (22, 86), did not yield microcolony formation on polycarbonate filters as observed by epifluorescent, scanning electron, and confocal laser scanning microscopy methods. The establishment of biofilm formation proceeded to studies of gene expression using a whole genome cDNA microarray. A chemostat approach previously used for genetic expression analysis of *T. maritima* biofilms (Chapter IV) was applied to cultivate *P. furiosus* biofilms on Nylon mesh. Mixed model ANOVA analyis will be applied to determine the genes involved in biofilm formation by the hyperthermophilic archaeaon *P. furiosus*.

VI.1. Introduction

Hyperthermophilic microorganisms have the ability to form biofilms as a preferential microniche within their natural habitats, including geothermal settings, deep-sea hydrothermal vents, oil solfataras, and the subsurface (213). In addition to reports of natural hyperthermophilic biofilms (213), biofilm formation has also been observed in pure laboratory cultures of *Thermococcus litoralis* (214, 217), *Thermotoga maritima* (Chapter IV, (214)), *Archaeoglobus fulgidus* (114, 163), *Methanococcus jannaschii* (114, 163), and *Pyrococ-*

cus furiosus ((214), Chapter II). However, the genetic machinery responsible for sensing surfaces and environments, producing polysaccharides, and other biofilm-associated events is not known. Gene expression studies of various mesophilic biofilms, including *Pseudomonas aeruginosa* (228, 229, 283), *Escherichia coli* (200, 203, 204), and *Bacillus cereus* (192), among others, have identified quorum-sensing mechanisms, exopolysaccharide biosynthesis genes, chemotactic responses, and regulatory elements associated with biofilm formation. These results have contributed to the understanding the basis of microbial life in natural and artificial environments; thus, efforts to prevent or promote biofilm formation by these microorganisms can be successfully accomplished. Similarly, the biofilm phenotype in hyperthermophilic microorganisms must be studied to gain insights into the ecology of their natural habitats.

Chapter II reported a chemostat-based approach for collecting biofilm material from cultures of *T. maritima*, from which extracted RNA can be used, in conjunction with cDNA microarrays, to examine differential gene expression patterns comparing planktonic to sessile cells. The work presented here uses this system to study the biofilm phenotype of the hyperthermophilic archaeaon *Pyrococcus furiosus* (T_{opt} 98-100°C). Media was first formulated to induce biofilm formation on polycarbonate filters in batch culture. Epifluorescent, scanning electron, and confocal laser scanning micrographs revealed dense microcolony formation when *P. furiosus* was grown on a modified *A. fulgidus* medium supplemented with maltose (5 g/l). This medium was then used to cultivate *P. furiosus* biofilms on Nylon mesh in a chemostat for differential gene expression studies. Whole genome microarray analysis comparing the biofilm and planktonic populations is currently underway.

VI.2. Materials and Methods

Microorganism and growth conditions

Pyrococcus furiosus (DSM 3638) was grown on a modified *Archaeoglobus fulgidus* medium (114, 163), which we call Biofilm Complex Medium (BCM). All components were included except for the PIPES/Lactic Acid solution; 5 g/L maltose was used as a substitute carbon source. Yeast extract and tryptone were added at 1 g/L and 2 g/L concentrations respectively. Various media formulations, including Artificial Sea Water (ASW) (22, 85, 86), Rinker Defined Medium (RDM) (214, 217), and *Pyrococcus* Defined Medium (PDM) (211) were tried to induce biofilm formation by *Pyrococcus furiosus* in batch cultures. Fifty-milliliter batch cultivation and cell enumeration were performed as described in Chapter II (205, 215). Biofilm formation was determined visually with epifluorescent micrographs of polycarbonate filters inserted into the cultures (Chapter IV).

P. furiosus was grown in the chemostat similar to the *T. maritima* (Chapter IV), with Nylon mesh (Sefar America, Hamden, CT) as biofilm substrata. BCM supplemented with maltose (5 g/l) was fed continuously ($D=0.25\ h^{-1}$) to a 1.5-L culture growing anaerobically at 95°C. Cell densities were monitored as previously described (Chapter IV). Thirteen tubes of mesh were submersed in the culture for RNA, protein, and polysaccharide extractions. Three planktonic samples were collected and pooled for representative planktonic species; biofilm species were collected from six tubes and pooled. Total RNA was extracted by ethanol precipitation and Promega SV Total RNA extraction kits (Promega, location) for use with cDNA microarrays. All sample collections and extractions were performed as previously described (Chapter IV).

Imaging and microscopy methods

Epifluorescent micrographs were taken with a SPOT digital camera (Southern Micro Instruments, Atlanta, GA) attached to a Nikon (Labophot-2) microscope (Southern Micro Instruments, Atlanta, GA) with 100× oil-immersion lens. Biofilms on polycarbonate filters were stained with acridine orange (Chapter II) for epifluorescent micrographs. In addition, these filters were used for confocal laser scanning micrographs (North Carolina State University, Department of Botany). Confocal pictures were generated with an Argon laser and 100× oil-immersion lens. A scanning electron microscope (North Carolina State University, Department of Microbiology) was also used to image biofilm cells on polcarbonate filters. Filters were fixed in 2.5% glutaraldehyde, and critically point dried. Images of the continuous culture were also taken regularly with a Nikon Coolpix 950 digital camera.

VI.3. Results

Various medium formulations were used to induce biofilm formation in the hyperthermophilic archaeon *Pyrococcus furiosus*. Raven and Sharp (211) and Rinker Defined Medium (217) did not show formation of biofilms on polycarbonate filters inserted in 50 ml and 500 ml batch cultures. Although *P. furiosus* is a member of the *Thermococcales* genus, Rinker Defined Medium did not cause biofilms to form as it did with *Thermococcus litoralis* (217). Another medium used frequently in our lab is Artificial Sea Water (ASW) (22, 85, 86), which is a complex medium containing 1 g/L yeast extract and 5 g/L tryptone. Although complex medium enhances growth rates, yeast extract contains glucose, glucans, and mannans (< 0.2 g/l), which may interfere with polysaccharide extractions, as

well as influence glycosyl hydrolase expression due to changing carbon sources (167). Some cell attachment was observed; however, there was no evidence of microcolony formation (data not shown). Another archaeon, *Archeaoglobus fulgidus*, was shown to produce biofilms under various stress conditions (114, 163). *A. fulgidus* is a dissimulatory sulfate reducer and utilized lactate, H_2, or pyruvate as electron donors (1), whereas *P. furiosus* is a facultative sulfur reducer and utilizes various carbohydrates for glycolysis, where H_2, pyruvate, or acetate act as electron donors (148). Therefore, the lactic acid and PIPES buffer were removed from the *A. fulgidus* medium (114, 163) and replaced with a carbohydrate to account for these metabolic differences between *A. fulgidus* and *P. furiosus*. Yeast extract was also reduced to 1 g/L in the formulation of BCM to minimize interference mentioned above. Although no stress conditions were induced on the cultures, *P. furiosus* formed significant microcolonies during batch cultivation with maltose or cellobiose as a carbon source (Figure VI.1). Electron micrographs indicate that the exopolysaccharide (EPS) appears as a capsular material when grown on cellobiose, and as a loose pool of polymer during growth on maltose (Figure VI.1). The effect of carbon source on EPS composition or type is not known for *P. furiosus*; however, the type of carbon source should not influence the composition, based on EPS synthetic pathways (Chapter II). Nonetheless, the effect of medium components will be investigated at a later date. First, a genetic tool to identify and differentiate biofilm communities and planktonic species must be developed. One such tool is cDNA microarrays, which can be used to characterize populations based on specific genes expressed and quantitate their differences. Just as the biofilms of *T. maritima* were characterized by a targeted genechip in this work, *P. furiosus* biofilms will be analyzed for differential gene expression compared to the plank-

tonic species with a whole genome array available at the University of Georgia. Once genes involved in biofilm formation are identified, various studies with changes the medium components can be done to determine which components are most crucial for biofilm growth.

Genetic analyses of biofilm and planktonic populations require significant amounts of RNA. As demonstrated previously with *T. maritima*, the chemostat offers an advantageous method to collect substantial amounts of biofilm for RNA extractions (Chapter IV). Continuous cultivation of *P. furiosus* was performed to obtain biofilm on Nylon mesh for RNA extractions. *P. furiosus* was grown at 95°C for 7 days on BCM supplemented with maltose ($D=0.25\ h^{-1}$). The average cell density during this chemostat run was 1.8×10^{8} cells/ml. Although significant biofilms were not observed on the mesh by eye, epifluorescent and confocal laser scanning micrographs (CLSM) indicated that significant amounts of biofilm microcolonies were attached to polycarbonate filters (Figure VI.2). The biofilm height was approximately 15 μm as measured with an argon laser CSLM. RNA was extracted from the planktonic (i.e., reactor fluid) and biofilm (i.e., mesh) upon shutdown (Chapter IV). Efforts to determine the differential gene expression patterns between the planktonic and sessile *P. furiosus* populations are currently underway with collaboration of Dr. Michael W. W. Adams at the University of Georgia at Athens. The results will yield a complete genetic profile of an archaeal biofilm, and in conjunction with the bacterial analysis in Chapter IV, gene expression patterns may provide insights into mechanisms of biofilm formation in hyperthermophiles.

VI.4. Acknowledgments

This was work was supported in part by grants from the National Science Foundation (LexEn Program) and the Department of Energy (Energy Biosciences Program). The authors wish to thank Valerie Knowlton in the NCSU Department of Microbiology for use of the Scanning Electron Microscope, and Eva Johannes in the NCSU Department of Botany for assistance with the confocal laser scanning microscope.

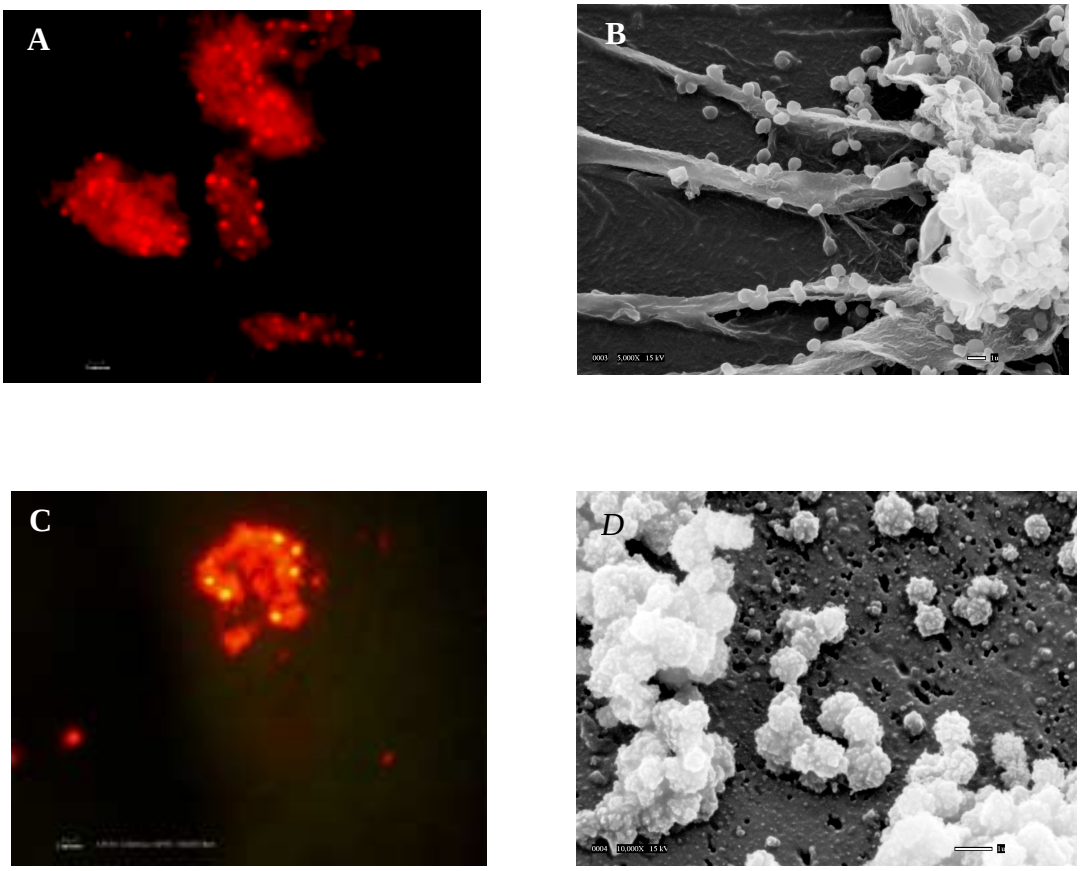
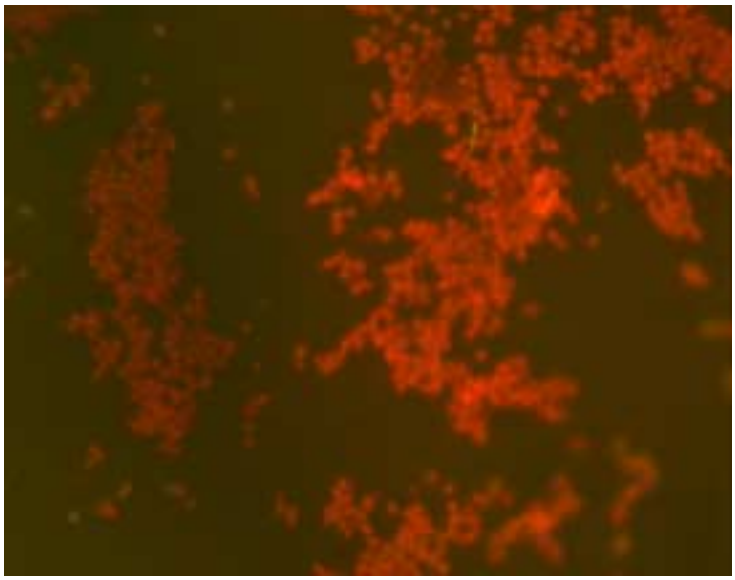


Figure VI.1. Epifluorescent (A and C) and scanning electron micrographs (B and D) of *P. furiosus* grown in batch culture on BCM and maltose (A and B) or cellobiose (C and D) as a carbon source. Polycarbonate filters were inserted in the 50 ml cultures grown at 98^{o}C and either stained with acridine orange (A and C) or critically point dried (B and D).

A



B

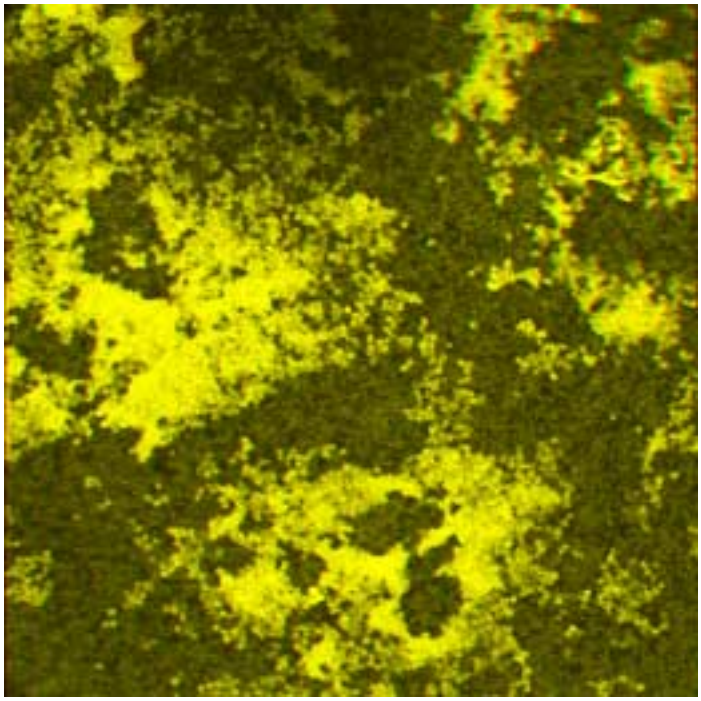


Figure VI.2. Dense biofilms of *P. furiosus* formed on polycarbonate filters in continuous culture, as shown by epifluorescent micrograph (A) and confocal scanning laser micrograph (CLSM), where the stereoview compiling z images is shown here (B).

Chapter VII. Concluding Remarks

The availability of genome sequences (www.tigr.org) and cDNA microarray technologies provide an immediate and comprehensive analysis of global gene expression at a given condition. Furthermore, experiments can be done using statistical loop designs (132) and mixed model ANOVA approaches (285), that allow for a variety of comparisons among numerous conditions. The analysis of various phenotypes can lead to assignment of gene function and an understanding of environmental response mechanisms within microorganisms. The work presented here combines engineering, microbiology, molecular biology, genetics and statistics, to better understand how hyperthermophiles live in their natural habitats:

- A chemostat system was developed to form substantial biofilms on Nylon mesh and polycarbonate filters for various types of imaging (e.g. epifluorescent, scanning electron, and confocal laser scanning micrographs) and RNA isolation for gene expression analysis with a targeted cDNA microarray.
- Mixed model ANOVA analysis was used to compare gene expression in planktonic and biofilm *T. maritima* cultures growing at 80°C and 85°C. Expression profiles revealed both similarities and differences to less thermophilic microorganisms that formed biofilms and were exposed to thermal stress. Response regulators, heat shock genes, and transcriptional regulators appear to be the key components in biofilm formation for this hyperthermophilic bacterium. Further analysis using a whole genome array will provide a better understanding of regulatory elements involved in hyperthermophilic biofilm formation.

- The dynamic stress response of *Thermotoga maritima* was investigated in a 14.0-L fermenter by exposure of an 80°C mid-exponential phase culture to the supra-optimal temperature of 90°C. A targeted cDNA microarray analysis revealed that the majority of the heat shock genes were maximally expressed after 5 minutes at 90°C. In addition, many other genes exhibited a temporal response indicating that bacterial stress response occurs within minutes.
- The chemostat system (this work) developed for the study of biofilm formation in hyperthermophiles was also used to study sessile populations of *Pyrococcus furiosus*. A whole genome cDNA microarray will be used to understand biofilm formation by archaeal species and for comparison to bacterial species, as part of an effort to understand mixed specie biofilms and natural population dynamics.

The continuous culture, cDNA microarray, and mixed model ANOVA techniques described here can be applied to study differential gene expression patterns between multiple conditions. Reactor systems can be further engineered to study the dynamic changes of genetic expression during the stages of biofilm development. Data can be compared to well-characterized mesophilic systems to assign functions to previously unknown genes. Furthermore, sequence analysis of these genes and promoter elements will provide insights into the regulatory mechanisms that underly response to environmental perturbations. Once the gene function is determined, proteins can be characterized and exploited for biotechnological potential. Hyperthermophiles are attractive in this respect because their enzymes maintain catalytic activity at high temperatures in which most industrial processes are operational. In addition to identifying new genes, functional genomics will

provide a better understanding of the ecology of hyperthermophiles in their natural environments such that new sampling methods can be developed to isolate various species.

References

1. **Achenbach-Richter, L., K. O. Stetter, and C. R. Woese.** 1987. A possible biochemical missing link among archaebacteria. Nature **327:**348-349.

2. **Adams, M. W., F. B. Perler, and R. M. Kelly.** 1995. Extremozymes: expanding the limits of biocatalysis. Biotechnol. **13:**662-668.

3. **Adams, M. W. W.** 1999. The biochemical diversity of life near and above 100^{o}C in marine environments. J. Appl. Microbiol. **85:**108S-117S.

4. **Adams, M. W. W., J. A. Baross, and R. M. Kelly.** 1998. Biological and chemical energetics of deep sea subsurface life: Evolutionary and exobiological implications, p. 3-26, (LExEn Proposal to the National Science Foundation).

5. **Adams, M. W. W., and R. M. Kelly.** 1998. Finding and using hyperthermophilic enzymes. Trends Biotechnol. **16:**329-332.

6. **Aldsworth, T. G., R. L. Sharman, and C. E. R. Dodd.** 1999. Bacterial suicide through stress. Cell. Mol. Life Sci. **56:**378-383.

7. **Alekshun, M. N., and S. B. Levy.** 1999. Alteration of the repressor activity of MarR, the negative regulator of the *Escherichia coli marRAB* locus, by multiple chemicals in vitro. J. Bacteriol. **181:**4669-4672.

8. **Allison, D. G., D. J. Evans, M. R. Brown, and P. Gilbert.** 1990. Possible involvement of the division cycle in dispersal of *Escherichia coli* from biofilms. J. Bacteriol. **172:**1667-1669.

9. **Allison, D. G., B. Ruiz, C. SanJose, A. Jaspe, and P. Gilbert.** 1998. Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms. FEMS Mirobiol. Lett. **167:**179-184.

10. **Allison, D. G., and I. W. Sutherland.** 1987. The role of exopolysaccharides in adhesion of fresh water bacteria. J. Gen. Microbiol. **133:**1319-1327.

11. **Altschul, S. F., and D. J. Lipman.** 1990. Protein database searches for multiple alignments. Proc. Natl. Acad. Sci. U S A **87:**5509-5513.

12. **Amend, J. P., and E. L. Shock.** 2001. Energetics of overall metabolic reactions of thermophilic and hyperthermophilic archaea and bacteria. FEMS Microbiol. Rev. **25:**175-243.

13. **Andreotti, G., M. V. Cubellis, G. Nitti, G. Sannia, X. Mai, M. W. W. Adams, and M. G.** 1995. An extremely thermostable aromatic amintransferase from the

hyperthermophilic archaeon *Pyrococcus furiosus*. BBA-Protein Structure M **1247:**90-96.

14. **Andreotti, G., M. V. Cubellis, G. Nitti, G. Sannia, X. Mai, G. Marino, and M. W. W. Adams.** 1994. Characterization of aromatic aminotransferases in the hyperthermophilic archaeon *Thermococcus litoralis*. Eur. J. Biochem. **220:**543-549.

15. **Antelmann, H., C. Scharf, and M. Hecker.** 2000. Phosphate starvation-inducible proteins of *Bacillus subtilis*: Proteomics and transcriptional analysis. J. Bacteriol. **182:**4478-4490.

16. **Anton, J., I. Meseguer, and F. Rodriguezvalera.** 1988. Production of an extracellular polysaccharide by *Haloferax mediterranei*. Appl. Environ. Microbiol. **54:**2381-2386.

17. **Anton, V., P. Rouge, and M. Daffe.** 1996. Identification of the sugars involved in mycobacterial cell aggregation. FEMS Mirobiol. Lett. **144:**167-170.

18. **Aono, S., F. O. Bryant, and M. W. W. Adams.** 1989. A novel and remarkably thermostable ferredoxin from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. J. Bacteriol. **171:**3433-3439.

19. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Worlfe.** 1979. Methanogens: Reevaluation of a unique biological group. Microbiol. Rev. **43:**260-296.

20. **Barbier, G., A. Godfroy, J. R. Meunier, J. Querellou, M. A. Cambon, F. Lesongeur, P. A. D. Grimont, and G. Raguenes.** 1999. *Pyrococcus glycovorans* sp. nov., a hyperthermophilic archaeon isolated from the East Pacific Rise. Int. J. Syst. Bact. **49:**1829-1837.

21. **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 Inc., Boca Raton.

22. **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.

23. **Bauer, M. W., E. J. Bylina, R. V. Swanson, and R. M. Kelly.** 1996. Comparison of a β-glucosidase and a β-mannosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. Purification, characterization, gene cloning, and sequence analysis. J. Biol. Chem. **271:**23749-23755.

24. **Bauer, M. W., L. E. Driskill, and R. M. Kelly.** 1998. Glycosyl hydrolases from hyperthermophilic microorganisms. Curr. Opin. Biotech. **9:**141-145.

25. **Baumeister, W., and G. Lembcke.** 1992. Structural features of archaebacterial cell envelopes. J. Bioenerg. Biomem. **24:**567-575.

26. **Becker, G., E. Klauck, and R. Hengge-Aronis.** 1999. Regulation of RpoS proteolysis in *Escherichia coli*: The response regulator RssB is a recognition factor that interacts with the turnover element in RpoS. Proc. Natl. Acad. Sci. U S A **96:**6439-6444.

27. **Becker, P., W. Hufnagle, G. Peters, and M. Herrmann.** 2001. Detection of differential gene expression in biofilm-forming versus planktonic populations of *Staphylococcus aureus* using micro-representational-difference analysis. Appl. Environ. Microbiol. **67:**2958-2965.

28. **Beeder, J., R. K. Nilsen, J. T. Rosnes, T. Torsvik, and T. Lien.** 1994. *Archaeoglobus fulgidus* isolated from hot North-Sea-oil field waters. Appl. Environ. Microbiol. **60:**1227-1231.

29. **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.

30. **Beveridge, T. J., S. A. Makin, J. L. Kadurugamuwa, and Z. Li.** 1997. Interactions between biofilms and the environment. FEMS Microbiol. Rev. **20:**291-303.

31. **Bik, E. M., A. E. Bunschoten, R. J. Willems, A. C. Chang, and F. R. Mooi.** 1996. Genetic organization and functional analysis of the otn DNA essential for cell-wall polysaccharide synthesis in *Vibrio cholerae* O139. Mol. Microbiol. **20:**799-811.

32. **Blanch, H. W., and D. S. Clark.** 1996. Biochemical Engineering. Marcel Dekker, Inc., New York.

33. **Blochl, E., R. Rachel, S. Burffraf, D. Hafenbradl, H. W. Jannasch, and K. O. Stetter.** 1997. *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113^{o}C. Extremophiles **1:**14-21.

34. **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 scale-up and potential applications. An. New York Ac. Sci. **589:**301-314.

35. **Blumentals, I. I., M. Itoh, G. J. Olson, and R. M. Kelly.** 1990. The role of polysulfides in the reduction of elemental sulfur by the hyperthermophile *Pyrococcus furiosus*. Appl. Environ. Microbiol. **56:**1255-1262.

36. **Bollinger, N., D. J. Hassett, B. H. Iglewski, J. W. Costerton, and T. R. McDermott.** 2001. Gene expression in *Pseudomonas aeruginosa*: Evidence of iron override effects on quorum sensing and biofilm-specific gene regulation. J. Bacteriol. **183:**1990-1996.

37. **Bolwell, G. P.** 2000. Biosynthesis of plant cell wall polysaccharides. Trends Glycosci. Glyc. **12:**143-160.

38. **Bonch-Osmolovskaya, E. A., and K. O. Stetter.** 1991. Interspecies hydrogen transfer in cocultures of thermophilic archaea. Syst. Appl. Microbiol. **14:**205-208.

39. **Bourgoin, F., A. Pluvinet, B. Gintz, B. Decaris, and G. Guedon.** 1999. Are horizontal transfers involved in the evolution of the *Streptococcus thermophilus* exopolysaccharide synthesis loci? Gene **233:**151-161.

40. **Boyd, A., and A. M. Chakrabarty.** 1995. *Pseudomonas aeruginosa* biofilms: role of the alginate exopolysaccharide. J. Indus. Microbiol. **15:**162-8.

41. **Brock, T. D.** 1998. Early days in Yellowstone microbiology. ASM News **64:**137-140.

42. **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.

43. **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. Geoghagen, and J. C. Venter.** 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science **273:**1058-73.

44. **Busscher, H. J., and H. C. van der Mei.** 1997. Physico-chemical interactions in initial microbial adhesion and relevance for biofilm formation. Adv. Dent. Res. **11:**24-32.

45. **Caldwell, D. E., E. Atuku, D. C. Wilkie, K. P. Wivcharuk, S. Karthikeyan, D. R. Korber, D. F. Schmid, and G. M. Wolfaardt.** 1997. Germ theory *vs.* community theory in understanding and controlling the proliferation of biofilms. Adv. Dent. Res. **11:**4-13.

46. **Caldwell, D. E., and J. W. Costerton.** 1996. Are bacterial biofilms constrained to Darwin's concept of evolution through natural selection? Microbiologia SEM **12:**347-358.

47. **Cammarota, M. C., and J. Sant'Anna, G. L.** 1998. Metabolic blocking of exopolysaccharides synthesis: effects on microbial adhesion and biofilm accumulation. Biotechnol. Lett. **20:**1-4.

48. **Campbell, A. C., T. S. Bowers, and J. M. Edmond.** 1988. A time-series of vent fluid compositions from 21^{o}N, EPR (1979, 1981, and 1985) and the Guaymas Basin, Gulf of California (1982, 1985). J. Geophys. Res.-Solid **93:**4537-4549.

49. **Carlson, R. W., B. L. Reuhs, S. Forsberg, and E. L. Kannenberg.** 1999. *Rhizobial* cell surface carbohydrates: Their structures, biosynthesis, and functions, p. 52-90. *In* J. B. Goldberg (ed.), Genetics of Bacterial Polysaccharides. CRC Press, Boca Raton.

50. **Carpentier, B., and O. Cerf.** 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. J. Appl. Bacteriol. **75:**499-511.

51. **Cheng, H. P., and G. C. Walker.** 1998. Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. J. Bacteriol. **180:**5183-5191.

52. **Chhabra, S. R., K. R. Shockley, S. B. Conners, K. Scott, R. Wolfinger, and R. M. Kelly.** 2002. Mixed model analysis of carbohydrate-induced differential expression patterns in the hyperthermophilic bacterium *Thermotoga maritima*. J. Biol. Chem. **epub: Dec 9**.

53. **Chhabra, S. R., K. R. Shockley, D. E. Ward, and R. M. Kelly.** 2002. Regulation of endo-acting glycosyl hydrolases in the hyperthermophilic bacterium Thermotoga maritima grown on glucan- and mannan-based polysaccharides. Appl. Environ. Microbiol. **68:**545-554.

54. **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.

55. **Cho, S. H., K. Naber, J. Hacker, and W. Ziebuhr.** 2002. Detection of the icaADBC gene cluster and biofilm formation in Staphylococcus epidermidis isolates from catheter-related urinary tract infections. Int. J. Antimicrob. Agents **19:**570-575.

56. **Christensen, B. E.** 1989. The Role of Extracellular Polysaccharides in Biofilms. J. Biotechnol. **10:**181-201.

57. **Clarke, A., H. Stating, and N. T. Blackburn.** 2000. Pathways of O-acetylation of bacterial cell wall polysaccharides, p. 187-223. *In* R. J. Doyle (ed.), Glycomicrobiology. Kluwer Academic/Plenum Publishers, New York.

58. **Condemine, G., and J. Robert-Baudouy.** 1991. Analysis of an Erwinia chrysanthemi gene cluster involved in pectin degradation. Mol. Microbiol. **5:**2191-202.

59. **Conlon, K. M., H. Humphreys, and J. P. O'Gara.** 2002. *icaR* encodes a transcriptional repressor involved in environmental regulation of *ica* operon expression and biofilm formation in *Staphylococcus epidermidis*. J. Bacteriol. **184:**4400-4408.

60. **Corliss, J. B., J. Dymond, L. I. Gordon, J. M. Edmond, R. P. Von Herzen, R. D. Ballard, G. K., D. Williams, A. Bainbridge, K. Crane, and T. J. H. Van Andel.** 1979. Submarine thermal springs on the Galapagos Rift. Science **203:**1073-1083.

61. **Corona-Izquierdo, F. P., and J. Membrillo-Hernandez.** 2002. A mutation in rpoS enhances biofilm formation in *Escherichia coli* during exponential phase of growth. FEMS Microbiol. Lett. **211:**105-110.

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

63. **Costerton, J. W., K. J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Ann. Rev. Microbiol. **41:**435-464.

64. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Ann. Rev. Microbiol. **49:**711-745.

65. **Costerton, J. W., Z. Lewandowski, D. DeBeer, D. Caldwell, D. Korber, and G. James.** 1994. Biofilms, the customized microniche. J. Bacteriol. **176:**2137-2142.

66. **Craig, H., A. Welhan, K. Kim, R. Poreda, and J. E. Lupton.** 1980. Geochemical studies of the 21°N EPS hydrothermal fluids. EOS Trans. Am. Geophys. Union **61:**996.

67. **Cunliffe, D., C. A. Smart, C. Alexander, and E. N. Vulfson.** 1999. Bacterial adhesion at synthetic surfaces. Appl. Environ. Microbiol. **65:**4995-5002.

68. **Dahl, C., N. M. Kredich, R. Deutzmann, and H. G. Truper.** 1993. Dissimilatory sulfite reductase from *Archaeoglobus fulgidus* - Physico-chemical properties of the enzyme and cloning, sequencing and analysis of the reductase genes. J. Gen. Microbiol. **139:**1817-1828.

69. **Daniel, R. M., and M. J. Danson.** 1995. Did primitive microorganisms use nonheme iron proteins in place of NAD/P? J. Mol. Evol. **40:**559-563.

70. **Danson, M. J.** 1993. Central metabolism of the Archaea, p. 1-24. *In* M. Kates, D. J. Kushner, and A. T. Matheson (ed.), The Biochemistry of Archaea. Elsevier, Amsterdam.

71. **Dasgupta, N., E. P. Ferrell, K. J. Kanack, S. E. West, and R. Ramphal.** 2002. fleQ, the gene encoding the major flagellar regulator of *Pseudomonas aeruginosa*, is sigma70 dependent and is downregulated by Vfr, a homolog of *Escherichia coli* cyclic AMP receptor protein. J. Bacteriol. **184:**5240-5250.

72. **Davies, D. G., A. M. Chakrabarty, and G. G. Geesey.** 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **59:**1181-1186.

73. **Davies, D. G., and G. G. Geesey.** 1995. Regulation of the alginate biosynthesis gene *algC* in *Pseudomonas aeruginosa* during biofilm development in continuous culture. Appl. Environ. Microbiol. **61:**860-867.

74. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295-298.

75. **de Vos, W. M., S. W. M. Kengen, W. G. B. Voorhorst, and J. van der Oost.** 1998. Sugar utilization and its control in hyperthermophiles. Extremophiles **2:**201-205.

76. **Deckert, G., P. V. Warren, T. Gaasterland, W. G. Young, A. L. Lenox, D. E. Graham, R. Overbeek, M. A. Snead, M. Keller, M. Aujay, R. Huber, R. A. Feldman, J. M. Short, G. J. Olsen, and R. V. Swanson.** 1998. The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. Nature **392:**353-358.

77. **Degeest, B., and L. De Vuyst.** 2000. Correlation of activities of the enzymes a-phosphoglucomutase, UDP-galactose 4-epimerase, and UDP-glucose pyrophophorylase with exopolysaccharide biosynthesis by *Streptococcus thermophilus* LY03. Appl. Environ. Microbiol. **66:**3519-3527.

78. **Deming, J. W.** 1998. Deep ocean environmental biotechnology. Curr. Opin. Biotech. **9:**283-287.

79. **Denyer, S. P., G. W. Hanlon, and M. C. Davies.** 1993. Mechanisms of Microbial Adherence, p. 13-27. *In* S. P. Denyer, S. P. Gorman, and M. Sussman (ed.), Microbial Biofilms: Formation and Control, 30 ed. Blackwell Scientific Publications, London.

80. **Deziel, R., C. Y., and R. Villemur.** 2001. Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and

highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. J. Bacteriol. **183:**1195-1204.

81. **Dickman, M. J., S. M. Ingleston, S. E. Sedelnikova, J. B. Rafferty, R. G. Lloyd, J. A. Grasby, and D. P. Hornby.** 2002. The RuvABC resolvasome. Eur J Biochem **269:**5492-5501.

82. **Diez, A., N. Gustavsson, and T. Nystrom.** 2000. The universal stress protein A of Escherichia coli is required for resistance to DNA damaging agents and is regulated by a RecA/FtsK- dependent regulatory pathway. Mol Microbiol **36:**1494-1503.

83. **Domingues, M. R., J. C. Araujo, M. B. A. Varesche, and R. F. Vazoller.** 2002. Evaluation of thermophilic anaerobic microbial consortia using fluorescence *in situ* hybridization (FISH). Water Sci. Technol. **45:**27-33.

84. **Donlan, R. M.** 2000. Biofilm control in industrial water systems: Approaching an old problem in new ways, p. 333-360. *In* L. V. Evans (ed.), Biofilms: Recent advances in their study and control. Harwood Academic Publishers, Singapore.

85. **Driskill, L. E., M. W. Bauer, and R. M. Kelly.** 1999. Synergistic interactions among β-laminarinase, β-1,4- glucanase, and β-glucosidase from the hyperthermophilic archaeon Pyrococcus furiosus during hydrolysis of β-1,4-, β-1,3-, and mixed-linked polysaccharides. Biotechnol. Bioeng. **66:**51-60.

86. **Driskill, L. E., K. Kusy, M. W. Bauer, and R. M. Kelly.** 1999. Relationship between glycosyl hydrolase inventory and growth physiology of the hyperthermophile *Pyrococcus furiosus* on carbohydrate-based media. Appl. Environ. Microbiol. **65:**893-897.

87. **Duffaud, G. D., O. B. d'Hennezel, A. S. Peek, A. L. Reysenbach, and R. M. Kelly.** 1998. Isolation and characterization of *Thermococcus barossii*, sp. nov., a hyperthermophilic Archaeon isolated from a hydrothermal vent flange formation. Syst. Appl. Microbiol. **21:**40-49.

88. **Duguid, J. P., and R. R. Gillies.** 1975. Fimbrae and adhesive properties of dysentry bacilli. J. Pathol. Bacteriol. **74:**397-411.

89. **Edwards, K. J., and N. A. Saunders.** 2001. Real-time PCR used to measure stress-induced changes in the expression of the genes of the alginate pathway of *Pseudomonas aeruginosa*. J. Appl. Microbiol. **91:**29-37.

90. **Escalante, A., C. Wacher-Rodarte, M. Garcia-Garibay, and A. Farres.** 1998. Enzymes involved in carbohydrate metabolism and their role on exopolysaccharide production in Streptococcus thermophilus. J. Appl. Microbiol. **84:**108-114.

91. **Ferrante, G., J. C. Richards, and G. D. Sprott.** 1990. Structures of polar lipids from the thermophilic, deep-sea archaeobacterium *Methanococcus jannaschii*. Biochem. Cell. Biol. **68:**274-283.

92. **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.

93. **Fischer, L., R. Bromann, S. W. Kengen, W. M. de Vos, and F. Wagner.** 1996. Catalytical potency of β-glucosidase from the extremophile *Pyrococcus furiosus* in glucoconjugate synthesis. Biotechnol. **14:**88-91.

94. **Flint, S. H., L. J. Ward, and J. D. Brooks.** 1999. *Streptococcus waius* sp. nov., a thermophilic *Streptococcus* from a biofilm. Int. J. Syst. Bact. **49:**759-767.

95. **Galperin, M. Y., K. M. Noll, and A. H. Romano.** 1997. Coregulation of beta-galactoside uptake and hydrolysis by the hyperthermophilic bacterium Thermotoga neapolitana. Appl. Environ. Microbiol. **63:**969-972.

96. **Gancel, F., and G. Novel.** 1994. Exopolysaccharide production by *Streptococcus salivarius* ssp. *thermophilus* cultures. 1. conditions of production. J. Diary Sci. **77:**685-688.

97. **Gancel, F., and G. Novel.** 1994. Exopolysaccharide production by *Streptococcus salivarius* ssp. *thermophilus* cultures. 2. Distinct modes of polymer production and degradation among clonal variants. J. Diary Sci. **77:**689-693.

98. **Gill, R. T., J. J. Valdes, and W. E. Bentley.** 1999. Reverse transcription-PCR differential display analysis of *Escherichia coli* global gene regulation in response to heat shock. Appl. Env. Microbiol. **65:**5386-5393.

99. **Gloaguen, V., H. Morvan, L. Hoffmann, Y. Plancke, J.-M. Wieruszeski, G. Lippens, and G. Strecker.** 1999. Capsular polysaccharide produced by the thermophilic cyanobacterium *Mastigocladus laminosus*: Structural study of an undecasaccharide obtained by lithium degradation. Eur. J. Biochem. **266:**762-770.

100. **Gluch, M. F., D. Typke, and W. Baumeister.** 1995. Motility and thermotactic responses of *Thermotoga maritima*. J. Bacteriol. **177:**5473-5479.

101. **Glucksmann, M. A., T. L. Reuber, and G. C. Walker.** 1993. Genes needed for the modification, polymerization, export, and processing of succinoglycan by *Rhizobium meliloti*: a model for succinoglycan biosynthesis. J. Bacteriol. **175:**7045-7055.

102. **Goldberg, J. B.** 1999. Genetics of *Pseudomonas aeruginosa* polysaccharides, p. 1-22. *In* J. B. Goldberg (ed.), Genetics of Bacterial Polysaccharides. CRC Press, Boca Raton.

103. **Golovlev, E. L.** 2002. The mechanism of formation of *Pseudomonas aeruginosa* biofilm, a type of structured population. Microbiol. **71:**249-254.

104. **Gonzalez, J. E., G. M. York, and G. C. Walker.** 1996. *Rhizobium meliloti* exopolysaccharides: synthesis and symbiotic function. Gene **179:**141-146.

105. **Gonzalez, J. M., C. Kato, and K. Horikoshi.** 1995. *Thermococcus peptonophilus* sp. nov., a fast-growing, extremely thermophilic archaebacterium isolated from deep-sea hydrothermal vents. Arch. Microbiol. **164:**159-164.

106. **Gonzalez, J. M., Y. Masuchi, F. T. Robb, J. W. Ammerman, D. L. Maeder, M. Yanagibayashi, J. Tamaoka, and C. Kato.** 1998. *Pyrococcus horikoshii* sp. nov., a hyperthermophilic archaeaon isolated from a hydrothermal vent at the Okinawa Trough. Extremophiles **2:**123-130.

107. **Grabarse, W., F. Mahlert, E. C. Duin, M. Goubeaud, S. Shima, R. K. Thauer, V. Lamzin, and U. Ermler.** 2001. On the mechanism of biological methane formation: Structural evidence for conformational changes in methyl-coenzyme M reductase upon substrate binding. J. Mol. Biol. **309:**315-330.

108. **Griffin, A. M., V. J. Morris, and M. J. Gasso.** 1996. The *cpsABCDE* genes involved in polysaccharide production in *Streptococcus salivarius ssp thermophilus* strain NCBF 2393. Gene **183:**23-27.

109. **Guezennec, J.** 1999. Microbial exopolysaccharides from extreme environments. Agro Food Ind. Hi Tec. **10:**34-35.

110. **Guezennec, J., O. Ortega-Morales, G. Raguenes, and G. Geesey.** 1998. Bacterial colonization of artificial substrate in the vicinity of deep-sea hydrothermal vents. FEMS Microbiol. Ecol. **26:**89-99.

111. **Guezennec, J., P. L. Pignet, Y., E. Gentric, J. Ratiskol, and S. Colliec-Jouault.** 1998. Sulfation and depolymerization of a bacterial exopolysaccharide of hydrothermal origin. Carbohyd. Polym. **37:**19-24.

112. **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. Carbohyd. Polym. **24:**287-294.

113. **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.

114. **Hartzell, P. L., J. Millstein, and C. LaPaglia.** 1999. Biofilm formation in hyperthermophilic archaea, p. 335-349. *In* R. J. Doyle (ed.), Biofilms, vol. 310.

115. **Hasseman, J.** 2001. TIGR microarray protocols. [http:\\www.tigr.org]

116. **Hassett, D. J., and M. S. Cohen.** 1989. Bacterial adaptation to oxidative stress: implications for pathogenesis and interaction with phagocytic cells. FASEB J. **3:**2574-2482.

117. **Hassett, D. J., J. F. Ma, J. G. Elkins, T. R. McDermott, U. A. Ochsner, S. E. H. West, C. T. Huang, J. Fredericks, S. Burnett, P. S. Stewart, G. McFeters, L. Passador, and B. H. Iglewski.** 1999. Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. Mol. Microbiol. **34:**1082-1093.

118. **Hattori, S., H. W. Luo, H. Shoun, and Y. Kamagata.** 2001. Involvement of formate as an interspecies electron carrier in a syntrophic acetate-oxidizing anaerobic microorganism in coculture with methanogens. J. Biosci. Bioeng. **91:**294-298.

119. **Hecker, M., W. Scfhumann, and U. Volker.** 1996. Heat-shock and general stress response in *Bacillus subtilis*. Mol. Microbiol. **19:**417-428.

120. **Helmann, J. D., M. F. W. Wu, P. A. Kobel, F. J. Gamo, M. Wilson, M. M. Morshedi, M. Navre, and C. Paddon.** 2001. Global transcriptional response of *Bacillus subtilis* to heat shock. J. Bacteriol. **183:**7318-7328.

121. **Hengge-Aronis, R.** 2002. Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. Microbiol. Mol. Biol. Rev. **66:**373-379.

122. **Henrissat, B., and P. M. Coutinho.** 2001. Classification of glycoside hydrolases and glycosyltransferases from hyperthermophiles, p. 183-201, Hyperthermophilic Enzymes, Pt a, vol. 330.

123. **Hicks, P. M.** 1999. Thermophilic Microorganisms, p. 2536-2252. *In* M. C. Flickinger and S. W. Drew (ed.), Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation. John Wiley & Sons, Inc., New York.

124. **Holden, J. F., M. Summit, and J. A. Baross.** 1998. Thermophilic and hyperthermophilic microorganisms in 3-30 degrees C hydrothermal fluids following a deep-sea volcanic eruption. FEMS Microbiol. Ecol. **25:**33-41.

125. **Huber, H., H. Jannasch, R. Rachel, T. Fuchs, and K. O. Stetter.** 1997. *Archaeoglobus veneficus* sp nov, a novel facultative chemolithoautotrophic hyperthermophilic sulfite reducer, isolated from abyssal black smokers. Syst. Appl. Microbiol. **20:**374-380.

126. **Huber, J. A., D. A. Butterfield, and J. A. Baross.** 2002. Temporal changes in archaeal diversity and chemistry in a mid-ocean ridge subseafloor habitat. Appl. Environ. Microbiol. **68:**1585-1594.

127. **Huber, R., M. Kurr, H. W. Jannasch, and K. O. Stetter.** 1989. A novel group of abyssal methanogenic archaebacteria (*Methanopyrus*) Growing At 110°C. Nature **342:**833-834.

128. **Huber, R., and K. O. Stetter.** 2001. Discovery of hyperthermophilic microorganisms. Method. Enzymol. **330:**11-24.

129. **Jair, K. W., R. G. Martin, J. L. Rosner, N. Fujita, A. Ishihama, and R. E. Wolf, Jr.** 1995. Purification and regulatory properties of MarA protein, a transcriptional activator of *Escherichia coli* multiple antibiotic and superoxide resistance promoters. J. Bacteriol. **177:**7100-7104.

130. **Jass, J., and H. M. LappinScott.** 1997. Microbial biofilms in industry: wanted dead or alive? Chem. Ind.-London**:**682-685.

131. **Jenney, F., M. Verhage, X. Cui, and M. W. W. Adams.** 1999. Anaerobic microbes: oxygen detoxification without superoxide dismutase. Science **286:**306-309.

132. **Jin, W., R. M. Riley, R. D. Wolfinger, K. P. White, G. Passador-Gurgel, and G. Gibson.** 2001. The contributions of sex, genotype and age to transcriptional variance in *Drosophila melanogaster*. Nature Genetics **29:**389-395.

133. **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. **136:**254-261.

134. **Jones, W. J., J. Nagel, D. P., and W. B. Whitman.** 1987. Methanogens and the diversity of archaebacteria. Microbiol. Rev. **21:**135-177.

135. **Jones, W. J., M. J. B. Paynter, and R. Gupta.** 1983. Characterization of *Methanococcus maripaludis* sp-nov, a new methanogen isolated from salt-marsh sediment. Arch. Microbiol. **135:**91-97.

136. **Kacklany, S. C., S. B. Levery, J. S. Kim, B. L. Reuhs, L. W. Lion, and W. C. Ghiorse.** 2001. Structure and carbohydrate analysis of the exopolysaccharide capsule of *Pseudomonas putida* G7. Environ. Microbiol. **3:**774-784.

137. **Kallipolitis, B. H., and P. Valentin-Hansen.** 1998. Transcription of rpoH, encoding the *Escherichia coli* heat-shock regulator sigma(32), is negatively controlled by the cAMP- CRP/CytR nucleoprotein complex. Mol. Microbiol. **29:**1091-1099.

138. **Karl, D. M.** 1995. Ecology of free-living hydrothermal vent microbial communities, p. 35-124. *In* D. M. Karl (ed.), The Microbiology of Deep-Sea Hydrothermal Vents. CRC Press Inc., Boca Raton.

139. **Karzai, A. W., and R. T. Sauer.** 2001. Protein factors associated with the SsrA.SmpB tagging and ribosome rescue complex. Proc. Natl. Acad. Sci. U S A **98:**3040-3044.

140. **Karzai, A. W., M. M. Susskind, and R. T. Sauer.** 1999. SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). Embo J. **18:**3793-3799.

141. **Katzen, F., D. U. Ferreiro, C. G. Oddo, M. V. Ielmini, A. Becker, A. Puhler, and L. Ielpi.** 1998. *Xanthomonas campestris* pv. *campestris* gum mutants: effects on xanthan biosynthesis and plant virulence. J. Bacteriol. **180:**1607-1617.

142. **Kawarabayasi, Y., M. Sawada, H. Horikawa, Y. Haikawa, Y. Hino, S. Yamamoto, M. Sekine, S. Baba, H. Kosugi, A. Hosoyama, Y. Nagai, M. Sakai, K. Ogura, R. Otsuka, H. Nakazawa, M. Takamiya, Y. Ohfuku, T. Funahashi, T. Tanaka, Y. Kudoh, J. Yamazaki, N. Kushida, A. Oguchi, K. Aoki, and H. Kikuchi.** 1998. Complete sequence and gene organization of the genome of a hyperthermophilic archaebacterium, *Pyrococcus horikoshii* OT3. DNA Res. **5:**55-76.

143. **Keiler, K. C., P. R. Waller, and R. T. Sauer.** 1996. Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. Science **271:**990-993.

144. **Kelly, R. M., and M. W. W. Adams.** 1994. Metabolism in hyperthermophilic microorganisms. Ant. v. Leewen. Int. J. Gen. Mol. Microbiol. **66:**247-270.

145. **Kelly, R. M., I. I. Blumentals, L. J. Snowden, and M. W. W. Adams.** 1992. Physiological and biochemical characteristics of *Pyrococcus furiosus*, a hyperthermophilic archaebacterium. An. New York Ac. Sci. **665:**309-320.

146. **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.

147. **Kengen, S. W. M., and A. 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.

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

149. **Kinder, S. A., and S. C. Holt.** 1994. Coaggregation between bacterial species. Method. Enzymol. **236:**254-270.

150. **King, G. M.** 1988. Methanogenesis From Methylated Amines in a Hypersaline Algal Mat. Appl. Environ. Microbiol. **54:**130-136.

151. **Klenk, H. P., R. A. Clayton, J. F. Tomb, O. White, K. E. Nelson, K. A. Ketchum, R. J. Dodson, M. Gwinn, E. K. Hickey, J. D. Peterson, D. L. Richardson, A. R. Kerlavage, D. E. Graham, N. C. Kyrpides, R. D. Fleischmann, J. Quackenbush, N. H. Lee, G. G. Sutton, S. Gill, E. F. Kirkness, B. A. Dougherty, K. McKenney, M. D. Adams, B. Loftus, J. C. Venter, and et al.** 1997. The complete genome sequence of the hyperthermophilic, sulphate- reducing archaeon *Archaeoglobus fulgidus*. Nature **390:**364-370.

152. **Kluskens, L. D., G. J. Van Alebeek, A. G. Voragen, W. M. De Vos, and J. Van Der Oost.** 2003. Molecular and biochemical characterisation of the thermoactive family 1 pectate lyase from the hyperthermophilic bacterium *Thermotoga maritima*. Biochem. J. **20:**651-659.

153. **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.

154. **Kojic, M., and V. Venturi.** 2001. Regulation of *rpoS* gene expression in *Pseudomonas*: Involvement of a TetR family regulator. J. Bacteriol. **183:**3712-3720.

155. **Kolkman, M. A. B., D. A. Morrison, B. A. M. vanderZeijst, and P. J. M. Nuijten.** 1996. The capsule polysaccharide synthesis locus of Streptococcus pneumoniae serotype 14: Identification of the glycosyl transferase gene cps14E. J. Bacteriol. **178:**3736-3741.

156. **Kolter, R., and R. Losick.** 1998. One for all and all for one. Science **280:**226-227.

157. **Konig, H., R. Skorko, W. Zillig, and W. D. Reiter.** 1982. Glycogen in thermoacidophilic archaebacteria of the genera *Sulfolobus, Thermoproteus, Desulfurococcus,* and *Thermococcus*. Arch. Microbiol. **132:**297-303.

158. **Koonin, E. V., L. Aravind, and M. Y. Galperin.** 2000. A comparative-genomic view of the microbial stress response, p. 417-444. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. ASM Press, Washington D. C.

159. **Kuchma, S. L., and G. A. O'Toole.** 2000. Surface-induced and biofilm-induced changes in gene expression. Curr. Opin. Biotech. **11:**429-433.

160. **Kurr, M., R. Huber, H. Konig, H. W. Jannasch, H. Fricke, A. Trincone, J. K. Kristjansson, and K. O. Stetter.** 1991. *Methanopyrus kandleri*, gen and sp-nov represents a novel group of hyperthermophilic methanogens, growing at 110°C. Arch. Microbiol. **156:**239-247.

161. **Kutsukake, K.** 1997. Autogenous and global control of the flagellar master operon, flhD, in *Salmonella typhimurium*. Mol Gen Genet **254:**440-448.

162. **Landini, P., and A. J. Zehnder.** 2002. The global regulatory hns gene negatively affects adhesion to solid surfaces by anaerobically grown *Escherichia coli* by modulating expression of flagellar genes and lipopolysaccharide production. J. Bacteriol. **184:**1522-1529.

163. **LaPaglia, C., and P. L. Hartzell.** 1997. Stress-induced production of biofilm in the hyperthermophile *Archaeoglobus fulgidus*. Appl. Environ. Microbiol. **63:**3158-3163.

164. **Lazarevic, V., P. Margot, B. Soldo, and D. Karamata.** 1992. Sequencing and analysis of the *Bacillus subtilis* lytRABC divergon: a regulatory unit encompassing the structural genes of the N- acetylmuramoyl-L-alanine amidase and its modifier. J Gen Microbiol **138:**1949-1961.

165. **Levander, F., and P. Radstrom.** 2001. Requirement for phosphoglucomutase in exopolysaccharide biosynthesis in glucose- and lactose-utilizing *Streptococcus thermophilus*. Appl. Environ. Microbiol. **67:**2734-2738.

166. **Loaec, M., R. Olier, and J. Guezennec.** 1998. Chelating properties of bacterial exopolysaccharides from deep- sea hydrothermal vents. Carbohyd. Polym. **35:**65-70.

167. **Looijesteijn, P. J., I. C. Boels, M. Kleerebezem, and J. Hugenholtz.** 1999. Regulation of exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* by the sugar source. Appl. Environ. Microbiol. **65:**5003-5008.

168. **Lopez-Garcia, P., and P. Forterre.** 2000. Thermal stress in hyperthermophiles, p. 369-382. *In* G. Storz and R. Henggep-Aronis (ed.), Bacterial Stress Response. ASM Press, Washington, D.C.

169. **Macario, A. J., M. Lange, B. K. Ahring, and E. C. De Macario.** 1999. Stress genes and proteins in the archaea. Microbiol Mol Biol Rev **63:**923-967.

170. **Mack, D., W. Fischer, A. Krokotsch, K. Leopold, R. Hartmann, H. Egge, and R. Laufs.** 1996. The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear β-1,6-linked glucosaminoglycan: purification and structural analysis. J. Bacteriol. **178:**175-83.

171. **Mah, R. A., D. M. Ward, L. Baresi, and T. L. Glass.** 1977. Biogenesis of methane. Ann. Rev. Microbiol. **31:**309-341.

172. **Mai, X. H., and M. W. W. Adams.** 1996. Purification and characterization of two reversible and ADP- dependent acetyl coenzyme a synthetases from the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Bacteriol. **178:**5897-5903.

173. **Manz, W., U. Szewzyk, P. Ericsson, R. Amann, K. H. Schleifer, and T. A. Stenstrom.** 1993. *In situ* identification of bacteria in drinking-water and adjoining biofilms by hybridization with 16S rRNA- directed and 23S rRNA-directed fluorescent oligonucleotide probes. Appl. Environ. Microbiol. **59:**2293-2298.

174. **Marteinsson, V. T., J. L. Birrien, and D. Prieur.** 1997. In situ enrichment and isolation of thermophilic microorganisms from deep-sea vent environments. Can. J. Microbiol. **43:**694-697.

175. **Martin, R. G., K. W. Jair, R. E. Wolf, Jr., and J. L. Rosner.** 1996. Autoactivation of the marRAB multiple antibiotic resistance operon by the MarA transcriptional activator in *Escherichia coli*. J Bacteriol **178:**2216-2223.

176. **Michels, P. C., and D. S. Clark.** 1997. Pressure-enhanced activity and stability of a hyperthermophilic protease from a deep-sea methanogen. Appl. Environ. Microbiol. **63:**3985-3991.

177. **Miller, E. S., Jr., K. N. Parker, W. Liebl, and R. M. Kelly.** 2001. α-D-galactosidases from *Thermotoga spp.* Method. Enzymol. **330:**246-259.

178. **Miller, J. F., N. N. Shah, C. M. Nelson, J. M. Ludlow, and D. S. Clark.** 1988. Pressure and temperature effects on growth and methane production of the extreme thermophile *Methanococcus jannaschii*. Appl. Environ. Microbiol. **54:**3039-3042.

179. **Miyakawa, S., H. Yamanashi, K. Kobayashi, H. J. Cleaves, and S. L. Miller.** 2002. Prebiotic synthesis from CO atmosphers: Implications for the origins of life. Proc. Natl. Acad. Sci. U S A **99:**14628-14631.

180. **Monsan, P., and F. Paul.** 1995. Enzymatic synthesis of oligosaccharides. FEMS Microbiol. Rev. **16:**187-192.

181. **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. Biotechnol. Bioeng. **56:**268-278.

182. **Natale, D. A., M. Y. Galperin, R. L. Tatusov, and E. V. Koonin.** 2000. Using the COG database to improve gene recognition in complete genomes. Genetics **108:**9-17.

183. **Nelson, K. E., R. A. Clayton, S. R. Gill, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, L. D. Peterson, W. C. Nelson, K. A. Ketchum, L. McDonald, T. R. Utterback, J. A. Malek, K. D. Linher, M. M. Garrett, A. M. Stewart, M. D. Cotton, M. S. Pratt, C. A. Phillips, D. Richardson, J. Heidelberg, G. G. Sutton, R. D. Fleischmann, J. A. Eisen, O. White, S. L. Salzberg, H. O. Smith, J. C. Venter, and C. M. Fraser.** 1999. Evidence for lateral gene transfer between Archaea and Bacteria from genome sequence of *Thermotoga maritima*. Nature **399:**323-329.

184. **Nelson, K. E., J. A. Eisen, and C. M. Fraser.** 2001. Genome of *Thermotoga maritima* MSB8. Method. Enzymol. **330:**169-180.

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

186. **Neves, A. R., R. Ventura, N. Mansour, C. Shearman, M. J. Gasson, C. Maycock, A. Ramos, and H. Santos.** 2002. Is the glycolytic flux in *Lactococcus lactis* primarily controlled by the redox charge? Kinetics of NAD+ and NADH pools determined by 13C NMR. J. Biol. Chem. **277:**28088-28098.

187. **Nguyen, P. T. M., J. Abranches, T. N. Phan, and R. E. Marquis.** 2002. Repressed respiration of oral *Streptococci* grown in biofilms. Curr. Microbiol. **44:**262-266.

188. **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 Mirobiol. Lett. **109:**203-206.

189. **Nidetzky, B., C. Eis, and M. Albert.** 2000. Role of non-covalent enzyme-substrate interactions in the reaction catalysed by cellobiose phosphorylase from *Cellulomonas uda*. Biochem. J. **351:**649-659.

190. **Nikolskaya, A. N., and M. Y. Galperin.** 2002. A novel type of conserved DNA-binding domain in the transcriptional regulators of the AlgR/AgrA/LytR family. Nuc. Acids Res **30:**2453-2459.

191. **(NOAA)** 2003. National Oceanic and Atmospheric Administration Vents Program. [http://www.pmel.noaa.gov/vents/]

192. **Oosthuizen, M. C., B. Steyn, J. Theron, P. Cosette, D. Lindsay, A. von Holy, and V. S. Brozel.** 2002. Proteomic analysis reveals differential protein expression by *Bacillus cereus* during biofilm formation. Appl. Environ. Microbiol. **68:**2770-2780.

193. **O'Toole, G. A., and R. Kolter.** 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. **30:**295-304.

194. **Packer, N. H., and M. J. Harrison.** 1998. Glycobiology and proteomics: Is mass spectrometry the Holy Grail? Electrophoresis **19:**1872-1882.

195. **Paerl, H. W., and J. L. Pinckney.** 1996. A mini-review of microbial consortia: Their roles in aquatic production and biogeochemical cycling. Microbial Ecol. **31:**225-247.

196. **Parker, K. N., S. R. Chhabra, D. Lam, W. Callen, G. D. Duffaud, M. A. Snead, J. M. Short, E. J. Mathur, and R. M. Kelly.** 2001. Galactomannanases man2 and man5 from *Thermotoga* species: Growth physiology on galactomannans, gene sequence analysis, and biochemical properties of recombinant enzymes. Biotechnol. Bioeng. **75:**322-333.

197. **Parkes, R. J., B. A. Cragg, and P. Wellsbury.** 2000. Recent studies on bacterial populations and processes in subseafloor sediments: A review. Hydrogeol. J. **8:**11-28.

198. **Parolis, H., L. A. S. Parolis, I. F. Boan, F. RodriguezValera, G. Widmalm, M. C. Manca, P. E. Jansson, and I. W. Sutherland.** 1996. The structure of the exopolysaccharide produced by the halophilic archaeon *Haloferax mediterranei* strain R4 (ATCC 33500). Carbohyd. Res. **295:**147-156.

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

200. **Perrot, F., M. Hebraud, R. Charlionet, J. G. A., and T. Jouenne.** 2000. Protein patterns of gel-entrapped *Escherichia coli* cells differ from those of free-floating organisms. Electrophoresis **21:**645-653.

201. **Poulsen, L. K., G. Ballard, and D. A. Stahl.** 1993. Use of ribosomal-RNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59:**1354-1360.

202. **Pratt, L. A., and R. Kolter.** 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. **30:**285-293.

203. **Prigent-Combaret, C., E. Brombacher, O. Vidal, A. Ambert, P. Lejeune, P. Landini, and C. Dorel.** 2001. Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the csgD gene. J. Bacteriol. **183:**7213-7223.

204. **Prigent-Combaret, C., O. Vidal, C. Dorel, and P. Lejeune.** 1999. Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. J. Bacteriol. **181:**5993-6002.

205. **Pysz, M. A., K. D. Rinker, K. R. Shockley, and R. M. Kelly.** 2001. Continuous cultivation of hyperthermophiles. Method. Enzymol. **330A:**31-40.

206. **Raguenes, G., R. Christen, J. Guezennec, P. Pignet, and G. Barbier.** 1997. *Vibrio diabolicus* sp. nov., a new polysaccharide-secreting organism isolated from a deep-sea hydrothermal vent polychaete annelid, *Alvinella pompejana*. Int. J. Syst. Bact. **47:**989-995.

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

208. **Raivio, T. L., and T. J. Silhavy.** 2001. Periplasmic stress and ECF sigma factors. Ann. Rev. Microbiol. **55:**591-624.

209. **Ramos, A., E. C. Boels, W. M. de Vos, and H. Santos.** 2001. Relationship between glycolysis and exopolysaccharide biosynthesis in *Lactococcus lactis*. Appl. Environ. Microbiol. **67:**33-41.

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

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

212. **Reverchon, S., W. Nasser, and J. Robert-Baudouy.** 1991. Characterization of kdgR, a gene of *Erwinia chrysanthemi* that regulates pectin degradation. Mol Microbiol **5:**2203-2216.

213. **Reysenbach, A. L., and E. Shock.** 2002. Merging genomes with geochemistry in hydrothermal ecosystems. Science **296:**1077-1082.

214. **Rinker, K. D.** 1998. Growth Physiology and Bioenergetics of the Hyperthermophilic Archaeon *Thermococcus litoralis* and Bacterium *Thermotoga maritima*. PhD. North Carolina State University, Raleigh.

215. **Rinker, K. D., C. J. Han, and R. M. Kelly.** 1999. Continuous culture as a tool for investigating the growth physiology of heterotrophic hyperthermophiles and extreme thermoacidophiles. J. Appl. Microbiol. **85:**118-127.

216. **Rinker, K. D., and R. M. Kelly.** 2000. Effect of carbon and nitrogen sources on growth dynamics and exopolysaccharide production for the hyperthermophilic archaeon *Thermococcus litoralis* and bacterium *Thermotoga maritima*. Biotechnol. Bioeng. **69:**537-547.

217. **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.

218. **Roberts, I. S.** 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. Ann. Rev. Microbiol. **50:**285-315.

219. **Robinson, R. W., H. C. Aldrich, S. F. Hurst, and A. S. Bleiweis.** 1985. Role of the cell-surface of *Methanosarcina mazei* in cell aggregation. Appl. Environ. Microbiol. **49:**321-327.

220. **Rougeaux, H., J. Guezennec, R. W. Carlson, N. Kervarec, R. Pichon, and P. Talaga.** 1999. Structural determination of the exopolysaccharide of *Pseudoalteromonas* strain HYD 721 isolated from a deep-sea hydrothermal vent. Carbohyd. Res. **315:**273-285.

221. **Rougeaux, H., N. Kervarec, R. Pichon, and J. Guezennec.** 1999. Structure of the exopolysaccharide of *Vibrio diabolicus* isolated from a deep-sea hydrothermal vent. Carbohyd. Res. **322:**40-45.

222. **Rougeaux, H., R. Pichon, N. Kervarec, G. H. C. Raguenes, and J. G. Guezennec.** 1996. Novel bacterial exopolysaccharides from deep-sea hydrothermal vents. Carbohyd. Polym. **31:**237-242.

223. **Rougeaux, H., P. Talaga, R. W. Carlson, and J. Guezennec.** 1998. Structural studies of an exopolysaccharide produced by *Alteromonas macleodii* subsp. *fijiensis* originating from a deep-sea hydrothermal vent. Carbohyd. Res. **312:**53-59.

224. **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.

225. **Sandford, P. A.** 1979. Exocellular, microbial polysaccharides, p. 265-313. *In* R. S. Tipson and D. Horton (ed.), Advances in Carbohydrate Chemistry and Biochemistry, vol. 36. Academic Press, New York.

226. **Sanford, B. A., V. L. Thomas, S. J. Mattingly, M. A. Ramsay, and M. M. Miller.** 1995. Lectin-biotin assay for slime present in in-situ biofilm produced by *Staphylococcus epidermidis* using transmission electron microscopy (TEM). J. Indus. Microbiol. **15:**156-161.

227. **Santegoeds, C. M., S. C. Nold, and D. M. Ward.** 1996. Denaturing gradient gel electrophoresis used to monitor the enrichment culture of aerobic chemoorganotrophic bacteria from a hot spring cyanobacterial mat. Appl. Environ. Microbiol. **62:**3922-3928.

228. **Sauer, K., and A. K. Camper.** 2001. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. J. Bacteriol. **183:**6579-6589.

229. **Sauer, K., A. K. Camper, G. D. Ehrlich, J. W. Costerton, and D. G. Davies.** 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J. Bacteriol. **184:**1140-1154.

230. **Schafer, G., M. Engelhard, and V. Muller.** 1999. Bioenergetics of the archaea. Microbiol. Mol. Biol. Rev. **63:**570-620.

231. **Schafer, T., K. B. Xavier, H. Santos, and P. Schonheit.** 1994. Glucose fermentation to acetate and alanine in resting cell- suspensions of *Pyrococcus furiosus* - proposal of a novel glycolytic pathway based on C-13 labeling data and enzyme-activities. FEMS Mirobiol. Lett. **121:**107-114.

232. **Schicho, R. N., S. H. Brown, I. I. Blumentals, T. L. Peeples, G. D. Duffaud, and R. M. Kelly.** 1995. Protocol 4: Continuous Culture Techniques for Extremely Thermophilic and Hyperthermophilic Archaea, p. 31-36, 167-187. *In* F. T. Robb and A. R. Place (ed.), Archaea: A Laboratory Manual (Thermophiles). Cold Spring Harbor Laboratory Press, Plainview.

233. **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.

234. **Schicho, R. N., L. J. Snowden, 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.

235. **Schink, B.** 1992. Syntrophism among Prokaryotes, p. 276-299. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The Prokaryotes, vol. 1. Springer-Verlag, Berlin.

236. **Schonheit, P., and T. Schafer.** 1995. Metabolism of Hyperthermophiles. World J. Microb. Biot. **11:**26-57.

237. **Schramm, A., L. H. Larsen, N. P. Revsbech, N. B. Ramsing, R. Amann, and K. H. Schleifer.** 1996. Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. Appl. Environ. Microbiol. **62:**4641-4647.

238. **Schurig, H., N. Beaucamp, R. Ostendorp, R. Jaenicke, E. Adler, and J. R. Knowles.** 1995. Phosphoglycerate kinase and triosephosphate isomerase from the hyperthermophilic bacterium *Thermotoga maritima* form a covalent bifunctional enzyme complex. Embo J. **14:**442-51.

239. **Selig, M., K. B. Xavier, H. Santos, and P. Schonheit.** 1997. Comparative analysis of Embden-Meyerhof and Entner-Doudoroff glycolytic pathways in hyperthermophilic archaea and the bacterium *Thermotoga*. Arch. Microbiol. **167:**217-232.

240. **Seyfried, J., W. E., and M. J. Mottle.** 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 Inc., Boca Raton.

241. **Shockley, K. R., D. E. Ward, S. R. Chhabra, S. B. Conners, C. I. Montero, and R. M. Kelly.** In press. Heat shock response by the hyperthermophilic archaeon *Pyrococcus furiosus*. Appl. Environ. Microbiol.

242. **Singleton, S. F., R. A. Simonette, N. C. Sharma, and A. I. Roca.** 2002. Intein-mediated affinity-fusion purification of the Escherichia coli RecA protein. Protein Expr Purif **26:**476-488.

243. **Skurnik, M.** 1999. Molecular genetics of *Yersinia* lipopolysaccharide, p. 23-51. *In* J. B. Goldberg (ed.), Genetics of Bacterial Polysaccharides. CRC Press, Boca Raton.

244. **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.

245. **Spivack, A. J., and J. M. Edmond.** 1987. Boron isotope exchange between seawater and the oceanic crust. Geochim. Cosmochim. Acta **51:**1033-1043.

246. **Sprott, G. D., I. Ekiel, and G. B. Patel.** 1993. Metabolic pathways in *Methanococcus jannaschii* and other methanogenic bacteria. Appl. Environ. Microbiol. **59:**1092-1098.

247. **Stetter, K. O.** 1999. Extremophiles and their adaptation to hot environments. FEBS Lett. **452:**22-25.

248. **Stetter, K. O.** 1996. Hyperthermophiles in the history of life, p. 1-18, Evolution of hydrothermal ecosystems on Earth (and Mars?), vol. 202. John Wiley & Sons Ltd, Chichester.

249. **Stetter, K. O.** 1998. Hyperthermophiles: Isolation, Classification and Properties, p. 1-24. *In* K. Horikoshi and W. D. Grant (ed.), Extremophiles: Microbial Life in Extreme Environments. John Wiley & Sons, Inc., New York.

250. **Stetter, K. O.** 1996. Hyperthermophilic procaryotes. FEMS Microbiol. Rev. **18:**149-158.

251. **Stetter, K. O., H. Konig, 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.

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

253. **Stoodley, P., K. Sauer, D. G. Davies, and J. W. Costerton.** 2002. Biofilms as complex differentiated communities. Ann. Rev. Microbiol. **56:**187-209.

254. **Strauss, D. B., W. A. Walter, and C. A. Gross.** 1987. The heat shock response of *E. coli* is regulated by changes in the concentration of sigma-32. Nature **329:**348-351.

255. **Sunna, A., M. Moracci, M. Rossi, and G. Antranikian.** 1997. Glycosyl hydrolases form hyperthermophiles. Extremophiles **1:**2-13.

256. **Sutherland, I. W.** 1977. Bacterial exopolysaccharides - their nature and production, p. 27-96. *In* I. W. Sutherland (ed.), Surface Carbohydrates of the Prokaryotic Cell. Academic Press, London.

257. **Sutherland, I. W.** 1985. Biosynthesis and composition of gram-negative bacterial extracellular and wall polysaccharides. Ann. Rev. Microbiol. **39:**243-270.

258. **Sutherland, I. W.** 1997. Microbial exopolysaccharides - structural subtleties and their consequences. Pure Appl. Chem. **69:**1911-1917.

259. **Sutherland, I. W.** 1980. Polysaccharides in the adhesion of marine and freshwater bacteria, p. 329-338. *In* R. C. W. Berkely, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (ed.), Microbial Adhesion to Surfaces. Ellis Horwood Limited, London.

260. **Svensater, G., J. Welin, J. C. Wilkins, D. Beighton, and I. R. Hamilton.** 2001. Protein expression by planktonic and biofilm cells of *Streptococcus mutans*. FEMS Mirobiol. Lett. **205:**139-146.

261. **Takai, K., and K. Horikoshi.** 1999. Genetic diversity of Archaea in deep-sea hydrothermal vent systems. Genetics **152:**1285-1297.

262. **Takai, K., T. Komatsu, F. Inagaki, and K. Horikoshi.** 2001. Distribution of archaea in a black smoker chimney structure. Appl. Environ. Microbiol. **67:**3618-3629.

263. **Tanner, M. E.** 2001. Sugar nucleotide-modifying enzymes. Curr. Org. Chem. **5:**169-192.

264. **Tatusov, R. L., M. Y. Galperin, D. A. Natale, and E. V. Koonin.** 2000. The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res **28:**33-36.

265. **Thorne, L., M. J. Mikolajczak, R. W. Armentrout, and T. J. Pollock.** 2000. Increasing the yield and viscosity of exopolysaccharides secreted by Sphingomonas by augmentation of chromosomal genes with multiple copies of cloned biosynthetic genes. J. Ind. Microbiol. Biot. **25:**49-57.

266. **Tivey, M. K.** 1995. The influence of hydrothermal fluid composition and advection rates on black smoker chimney mieralogy: Insights from modeling transport and reaction. Geochim. Cosmochim. Acta **59:**1933-1949.

267. **Tivey, M. K., and R. E. McDuff.** 1990. Mineral precipitation in the walls of black smoker chimneys - a quantitative model of transport and chemical reaction. J. Geophys. Res.-Solid **95:**12617-12637.

268. **Tolker-Nielsen, T., U. C. Brinch, P. C. Ragas, J. B. Andersen, C. S. Jacobsen, and S. Molin.** 2000. Development and dynamics of *Pseudomonas* sp. biofilms. J. Bacteriol. **182:**6482-6489.

269. **Tsao, J. H., S. M. Kaneshiro, S. S. Yu, and D. S. Clark.** 1994. Continuous culture of *Methanococcus jannaschii*, an extremely thermophilic methanogen. Biotechnol. Bioeng. **43:**258-261.

270. **Valentine, D. L., and W. S. Reeburgh.** 2000. New perspectives on anaerobic methane oxidation. Environ. Microbiol. **2:**477-484.

271. **van der Oost, J., G. Schut, S. W. M. Kengen, W. R. Hagen, M. Thomm, and W. M. de Vos.** 1998. The Ferredoxin-dependent conversion of glyceraldehyde-3-phosphate in the hyperthermophilic archaeon *Pyrococcus furiosus* represents a novel site of glycosylation regulation. J. Biol. Chem. **273:**28149-28154.

272. **van Kranenburg, R., van Swam, II, J. D. Marugg, M. Kleerebezem, and W. M. de Vos.** 1999. Exopolysaccharide biosynthesis in *Lactococcus lactis NIZO B40*: Functional analysis of the glycosyltransferase genes involved in synthesis of the polysaccharide backbone. J. Bacteriol. **181:**338-340.

273. **VanBogelen, R. A., K. D. Greis, R. M. Blumenthal, T. H. Tani, and R. G. Matthews.** 1999. Mapping regulatory networks in microbial cells. Trends Microbiol. **7:**320-328.

274. **Vargas, M., and K. M. Noll.** 1994. Isolation of auxotrophic and antimetabolite-resistant mutants of the hyperthermophilic bacterium *Thermotoga maritima*. Arch. Microbiol. **162:**357-361.

275. **Verhagen, M. F. J. M., A. L. Menon, G. J. Schut, and M. W. W. Adams.** 2001. *Pyrococcus furiosus*: Large scale cultivation and enzyme purification. Method. Enzymol. **330:**25-30.

276. **Vetriani, C., H. W. Jannasch, B. J. MacGregor, D. A. Stahl, and A. L. Reysenbach.** 1999. Population structure and phylogenetic characterization of marine benthic archaea in deep-sea sediments. Appl. Environ. Microbiol. **65:**4375-4384.

277. **Wachterhauser, G.** 1990. Evolution of the first metabolic cycles. Proc. Natl. Acad. Sci. U S A **87:**200-204.

278. **Ward, D. E., C. J. Donnelly, M. E. Mullendore, J. van der Oost, W. M. de Vos, and E. J. Crane.** 2001. The NADH oxidase for the protection of anaerobic hyperthermophiles against oxidative stress. Eur. J. Biochem. **268:**5816-5823.

279. **Watnick, P., and R. Kolter.** 2000. Biofilm, city of microbes. J. Bacteriol. **182:**2675-2679.

280. **Weimer, P. J., and J. G. Zeikus.** 1978. One carbon metabolism in methanogenic bacteria - cellular characterization and growth of *Methanosarcina barkeri*. Arch. Microbiol. **119:**49-57.

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

282. **Weiner, R. M.** 1997. Biopolymers from marine prokaryotes. Trends Biotechnol. **15:**390-394.

283. **Whiteley, M., M. G. Bangera, R. E. Bumgarner, M. R. Parsek, G. M. Teitzel, S. Lory, and E. P. Greenberg.** 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. Nature **413:**860-864.

284. **Windholz, M.** 1976. The Merck Index. Merck and Co., Inc., Rahway.

285. **Wolfinger, R. D., G. Gibson, E. D. Wolfinger, L. Bennett, H. Hamadeh, P. Bushel, C. Afshari, and R. S. Paules.** 2001. Assessing gene significance from cDNA microarray expression data via mixed models. J. Comput. Biol. **8:**625-637.

286. **Xu, K. D., M. J. Franklin, C. H. Park, G. A. McFeters, and P. S. Stewart.** 2001. Gene expression and protein levels of the stationary phase sigma factor, RpoS, in continuously-fed *Pseudomonas aeruginosa* biofilms. FEMS Mirobiol. Lett. **199:**67-71.

287. **Yayanos, A. A.** 1995. Microbiology to 10,500 meters in the deep-sea. Ann. Rev. Microbiol. **49:**777-805.

288. **Yernool, D. A., J. K. McCarthy, D. E. Eveleigh, and J. D. Bok.** 2000. Cloning and characterization of the glucooligosaccharide catabolic pathway β-glucan glucohydrolase and cellobiose phosphorylase in the marine hyperthermophile *Thermotoga neapolitana*. J. Bacteriol. **182:**5172-5179.

289. **Yildiz, F. H., and G. K. Schoolnick.** 1999. *Vibrio cholerae* O1 El Tor: Identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistane, and biofilm formation. Proc. Natl. Acad. Sci. U S A **96:**4028-4033.

290. **Yura, T., M. Kanemori, and T. Morita.** 2000. The heat shock response: Regulation and function, p. 3-18. *In* G. Storz and R. Hennge-Aronis (ed.), Bacterial Stress Responses. ASM Press, Washington, D.C.

291. **Zellner, G., E. Stackebrandt, H. Kneifel, P. Messner, U. B. Sleytr, E. C. Demacario, H. P. Zabel, K. O. Stetter, and J. Winter.** 1989. Isolation and characterization of a thermophilic, sulfate reducing archaebacterium, *Archaeoglobus fulgidus* strain-Z. Syst. Appl. Microbiol. **11:**151-160.

292. **Zhang, R. G., Y. Kim, T. Skarina, S. Beasley, R. Laskowski, C. Arrowsmith, A. Edwards, A. Joachimiak, and A. Savchenko.** 2002. Crystal structure of *Thermotoga maritima* 0065, a member of the IclR transcriptional factor family. J Biol Chem **277:**19183-19190.

293. **Zhao, H. X., A. G. Wood, F. Widdel, and M. P. Bryant.** 1988. An extremely thermophilic *Methanococcus* from a deep-sea hydrothermal vent and its plasmid. Arch. Microbiol. **150:**178-183.

294. **Zhou, Y. N., and S. Gottesman.** 1998. Regulation of proteolysis of the stationary-phase sigma factor RpoS. J. Bacteriol. **180:**1154-1158.

Appendix I. ORFs on *Thermotoga maritima*

Targeted cDNA Microarray

The following is a list of the ORFs used to design primers and make PCR products for printing the *T. maritima* targeted genechip. The genes in grey were added at the second printing and used in conjunction with the white-shaded genes for the dynamic heat shock experiment (Chapter V). ORF annotation was retrieved from NCBI at ftp://ftp.ncbi.nih.gov/genomes/Bacteria/Thermotoga_maritima/NC_000853.ptt.

Locus	Function
TM0017	Pyruvate ferredoxin oxidoreductase, α subunit
TM0024	Laminarinase
TM0025	β-glucosidase
TM0032	Transcriptional regulator, XylR-related
TM0042	Aminopeptidase P
TM0053	Esterase, putative
TM0055	α-glucuronidase
TM0056	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein
TM0057	Oligopeptide ABC transporter, ATP-binding protein
TM0058	Oligopeptide ABC transporter, ATP-binding protein
TM0059	Oligopeptide ABC transporter, permease protein
TM0060	Oligopeptide ABC transporter, permease protein
TM0061	Endo-1,4-β-xylanase A
TM0062	Hypothetical protein
TM0063	Hypothetical protein
TM0064	Uronate isomerase, putative
TM0065	Transcriptional regulator, IclR family (kdgR)
TM0066	2-Dehydro-3-deoxyphosphogluconate aldolase / 4-hydroxy-2-oxoglutarate aldolase
TM0067	2-Keto-3-deoxygluconate kinase
TM0068	D-mannonate oxidoreductase, putative
TM0069	D-mannonate hydrolase
TM0070	Endo-1,4-β-xylanase B
TM0071	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein
TM0072	Oligopeptide ABC transporter, permease protein
TM0073	Oligopeptide ABC transporter, permease protein
TM0074	Oligopeptide ABC transporter, ATP-binding protein
TM0075	Oligopeptide ABC transporter, ATP-binding protein
TM0076	Xylosidase
TM0077	Acetylxylan esterase
TM0078	Iron(III) ABC transporter, ATP-binding protein
TM0079	Iron(III) ABC transporter, permease protein
TM0080	Iron(III) ABC transporter, periplasmic-binding protein, putative
TM0085	Hypothetical protein: COG2747 Anti sigma 28 FlgM

TM0086	Virulence factor MviN-related protein
TM0110	Transcriptional regulator, XylR-related
TM0113	XylU-related protein
TM0122	Ferric uptake regulation protein
TM0126	Response regulator
TM0127	Sensor histidine kinase
TM0129	Carboxypeptidase G2, putative
TM0143	Response regulator
TM0145	Secreted metalloendopeptidase Gcp, putative
TM0146	ATP-dependent Clp protease, ATPase subunit clpX
TM0167	Phosphopentomutase
TM0184	Phosphoglucomutase/phosphomannomutase family protein
TM0186	Response regulator
TM0187	Sensor histidine kinase
TM0195	Guanosine pentaphosphate phosphohydrolase, putative
TM0198	ATP-dependent Clp protease, ATPase subunit
TM0208	Pyruvate kinase
TM0209	6-Phosphofructokinase
TM0218	Flagellum-specific ATP synthase
TM0219	Flagellar export/assembly protein
TM0220	Flagellar motor switch protein FliG
TM0221	Flagellar M-ring protein
TM0222	ABC transporter, ATP-binding protein
TM0232	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase
TM0251	Carbon storage regulator
TM0272	Pyruvate,orthophosphate dikinase
TM0274	Acetate kinase
TM0275	Transcriptional regulator, GntR family
TM0281	α-L-arabinofuranosidase
TM0289	6-phosphofructokinase, pyrophosphate-dependent
TM0299	Transcriptional regulator, LacI family
TM0300	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein, putative
TM0301	Oligopeptide ABC transporter, permease protein
TM0302	Oligopeptide ABC transporter, permease protein
TM0303	Oligopeptide ABC transporter, ATP-binding protein
TM0304	Oligopeptide ABC transporter, ATP-binding protein
TM0305	Endoglucanase, putative
TM0306	α-L-fucosidase, putative
TM0308	α-xylosidase
TM0310	Putative β-galactosidase, Gal42B
TM0326	Transcriptional regulator, RpiR family
TM0364	4-α-glucanotransferase
TM0365	Aminopeptidase, putative
TM0371	Arginine repressor
TM0373	DnaK protein
TM0374	Heat shock protein, class I
TM0379	NADH oxidase
TM0381	Dihydrolipoamide dehydrogenase
TM0392	Conserved hypothetical protein COG0438: Predicted glycosyltransferases RfaG
TM0393	Transcriptional regulator, XylR-related
TM0395	NADH oxidase, putative
TM0399	Response regulator

TM0400	Sensor histidine kinase
TM0403	Nitrogen regulatory protein P-II
TM0408	Protein-glutamate methylesterase
TM0409	Conserved hypothetical protein COG0739: Membrane proteins related to metalloen-dopeptidases NlpD family
TM0411	Transcriptional regulator, XylR-related
TM0427	Oxidoreductase, putative
TM0428	Oxidoreductase, putative
TM0429	Methyl-accepting chemotaxis protein
TM0430	Sugar ABC transporter, permease protein
TM0431	Sugar ABC transporter, permease protein
TM0432	Sugar ABC transporter, periplasmic sugar-binding protein, putative
TM0433	Pectate lyase
TM0434	α-glucosidase, putative
TM0435	Acetyl xylan esterase-related protein
TM0436	Alcohol dehydrogenase, zinc-containing
TM0437	Exo-poly-α-D-galacturonosidase, putative
TM0438	6-phosphogluconate dehydrogenase, decarboxylating
TM0439	Transcriptional regulator, GntR family
TM0440	Hypothetical protein
TM0441	Oxidoreductase, short chain dehydrogenase/reductase family
TM0442	Conserved hypothetical protein COG3875:
TM0443	Gluconate kinase
TM0445	Conserved hypothetical protein, COG1160: Predicted GTPase
TM0463	Lipoprotein signal peptidase
TM0467	Regulatory protein, putative
TM0468	Response regulator
TM0490	Regulatory protein, SIR2 family
TM0505	GroES protein
TM0506	GroEL protein
TM0508	Conserved hypothetical protein, COG2110: Uncharacterized ACR related to the C-terminal domain of histone macroH2A1
TM0510	Iron-dependent transcriptional repressor, putative
TM0516	Clostripain-related protein
TM0521	Heat shock protein HslV
TM0522	Heat shock protein HslU
TM0527	Conserved hypothetical protein, COG2262: GTPase HflX
TM0530	Oligopeptide ABC transporter, ATP-binding protein
TM0531	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein
TM0532	Oligopeptide ABC transporter, permease protein
TM0533	Oligopeptide ABC transporter, permease protein
TM0534	RNA polymerase sigma-H factor, putative
TM0565	Sugar fermentation stimulation protein, putative
TM0571	Heat shock serine protease, periplasmic
TM0580	Cell division protein FtsH
TM0602	Iron-dependent transcriptional repressor, putative
TM0606	Hypothetical protein, COG3906
TM0610	Lipopolysaccharide biosynthesis protein
TM0614	Conserved hypothetical protein, COG1708: Predicted Nucleotidyltransferases
TM0616	Conserved hypothetical protein, COG1373: ATPases of the AAA superfamily
TM0619	Conserved hypothetical protein, COG0438: Predicted glycosyltransferases RfaG
TM0620	Lipopolysaccharide biosynthesis protein
TM0622	Lipopolysaccharide biosynthesis protein, putative

TM0624	N-acetylglucosaminyl-phosphatidylinositol biosynthesis-related protein
TM0627	Lipopolysaccharide biosynthesis protein
TM0630	Nucleotide sugar epimerase, putative
TM0631	Lipopolysaccharide biosynthesis protein
TM0632	Extracellular polysaccharide biosynthesis-related protein
TM0633	Flagellar-related protein
TM0638	Polysaccharide export protein, putative
TM0643	Clostripain-related protein
TM0644	Hypothetical protein, COG3206: Uncharacterized protein involved in exopolysaccharide biosynthesis GumC
TM0654	Spermidine synthase
TM0656	Conserved hypothetical protein, COG1396: Predicted transcriptional regulators HipB
TM0668	Pleiotropic regulatory protein
TM0685	Hypothetical protein, COG0741: Soluble lytic murein transglycosylase and related regulatory proteins (some contain LysM/invasin domains)
TM0688	Glyceraldehyde-3-phosphate dehydrogenase
TM0689	Phosphoglycerate kinase, triose phosphate isomerase
TM0694	Trigger factor, putative
TM0695	ATP-dependent ClpP protease, proteolytic subunit
TM0696	Ray-related protein
TM0698	Flagellar biosynthesis protein FliP
TM0700	Chemotaxis response regulator CheY
TM0701	Purine-binding chemotaxis protein CheW
TM0702	Chemotaxis sensor hist. kinase CheA
TM0703	Competence-damage inducible CinA
TM0710	Transcriptional regulator, MarR family
TM0729	(p)ppGpp synthetase
TM0733	Sigma-B regulator, putative, RsbW related
TM0742	Serine/threonine protein phosphatase
TM0744	Conserved hypothetical protein, COG0438: Predicted glycosyltransferases RfaG
TM0747	Carboxyl-terminal protease
TM0752	α-glucosidase, putative
TM0756	Galactosyltransferase-related protein
TM0757	Hypothetical protein, COG0463: Glycosyltransferases involved in cell wall biogenesis, WcaA
TM0760	Lipopolysaccharide biosynthesis protein, putative
TM0766	Transcriptional regulator, GntR family
TM0767	Maltodextrin glycosyltransferase
TM0769	Phosphomannomutase
TM0772	Conserved hypothetical protein, COG1774: Uncharacterized ACR, PSP1 homologs
TM0785	Bacteriocin
TM0805	Lipophilic protein, putative
TM0807	Alkyl hydroperoxide reductase
TM0808	Transcriptional regulator, XylR-related
TM0809	Hydrolase, putative
TM0814	N-acetylglucosamine-6-phosphate deacetylase
TM0816	Transcriptional regulator, putative, Mar family
TM0818	Lipopolysaccharide biosynthesis protein, putative
TM0823	Transcriptional regulator, TetR family
TM0842	Response regulator
TM0847	Conserved hypothetical protein, COG1159: GTPases, Era
TM0849	DnaJ protein
TM0850	GrpE protein, putative

TM0851	Heat shock operon repressor HrcA
TM0853	Sensor histidine kinase
TM0866	Conserved hypothetical protein, COG0330: Membrane protease subunits, stomatin/prohibitin homologs, HflC
TM0873	This region contains an authentic frame shift and is not the result of a sequencing artifact; similar to PID:1001492 percent identity: 73.99; identified by sequence similarity; putative;ATP-dependent Clp protease, ATPase subunit, authentic frameshift
TM0874	Conserved hypothetical protein, COG1214: Inactive homologs of metal-dependent proteases, putative molecular chaperones
TM0886	Penicillin-binding protein, class 1A
TM0887	Methylated-DNA-protein-cysteine methyltransferase
TM0890	Conserved hypothetical protein, COG0750: Predicted membrane-associated Zn-dependent proteases 1
TM0895	Glycogen synthase
TM0896	Galactose 1-phosphate uridyltransferase
TM0897	SpoVS-related protein
TM0902	RNA polymerase sigma-28 factor, putative (FliA)
TM0916	Conserved hypothetical protein, COG0616: Periplasmic serine proteases (ClpP class), SppA
TM0921	Hypothetical protein, COG0741: Soluble lytic murein transglycosylase and related regulatory proteins (some contain LysM/invasin domains), MltE
TM0949	Transcriptional regulator, LacI family
TM0963	Oligoendopeptidase
TM0998	Heavy metal resistance transcriptional regulator
TM1005	Transcriptional regulator, putative, COG2207: AraC-type DNA-binding domain-containing proteins
TM1017	Conserved hypothetical protein, COG0697: Permeases of the drug/metabolite transporter (DMT) superfamily, RhaT
TM1022	Esterase
TM1030	Transcriptional regulator, TetR family
TM1033	Mannose-1-phosphate guanylyltransferase
TM1048	Endoglucanase
TM1049	Endoglucanase
TM1050	Endoglucanase
TM1062	β-glucuronidase
TM1068	α-glucosidase, putative
TM1069	Transcriptional regulator, DeoR family
TM1081	Anti-sigma factor antagonist, putative, SpoIIAA
TM1082	LexA repressor
TM1130	Phosphate butyryltransferase
TM1131	Aminotransferase, putative
TM1155	Glucose-6-phosphate 1-dehydrogenase
TM1156	Conserved hypothetical protein, COG1983, Putative stress-responsive transcriptional regulator
TM1160	Esterase
TM1164	2-oxoglutarate ferredoxin oxidoreductase, α subunit
TM1168	α-glucan phosphorylase, authentic frameshift
TM1171	Transcriptional regulator, crp family
TM1176	Transcriptional regulator, metal-sensing
TM1184	PleD-related protein
TM1192	α-galactosidase, GalA
TM1193	β-galactosidase, LacZ
TM1195	β-galactosidase, LacA

TM1196	Oligopeptide ABC transporter, ATP-binding protein
TM1197	Oligopeptide ABC transporter, permease protein
TM1198	Oligopeptide ABC transporter, permease protein
TM1199	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein
TM1200	Transcriptional regulator, LacI family
TM1201	Arabinogalactan endo-1,4-β-galactosidase, putative
TM1202	Maltose ABC transporter, permease protein
TM1203	Maltose ABC transporter, permease protein
TM1204	Maltose ABC transporter, periplasmic maltose-binding protein
TM1218	Transcriptional regulator, LacI family
TM1219	Oligopeptide ABC transporter, ATP-binding protein
TM1220	Oligopeptide ABC transporter, ATP-binding protein
TM1221	Oligopeptide ABC transporter, permease protein
TM1222	Oligopeptide ABC transporter, permease protein
TM1223	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein
TM1224	Transcriptional regulator, XylR-related
TM1226	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein, putative
TM1227	Endo-1,4-β-mannosidase
TM1228	Transcriptional regulator, RpiR family
TM1229	Conserved hypothetical protein, COG0463: Glycosyltransferases involved in cell wall biogenesis, WcaA
TM1231	α-mannosidase-related protein
TM1232	Sugar ABC transporter, ATP-binding protein
TM1233	Sugar ABC transporter, permease protein, putative
TM1234	Sugar ABC transporter, permease protein
TM1235	Conserved hypothetical protein, COG2446: Predicted ABC-type transport system, periplasmic component
TM1238	ATP-dependent DNA helicase UrvD
TM1254	β-phosphoglucomutase, putative
TM1255	Aspartate aminotransferase
TM1258	Sensor histidine kinase, PhoR-related
TM1259	Phosphate regulon transcriptional regulatory protein PhoB
TM1260	Phosphate transport system regulatory protein
TM1261	Phosphate ABC transporter, ATP-binding protein
TM1262	Phosphate ABC transporter, permease protein
TM1263	Phosphate ABC transporter, permease protein
TM1264	Phosphate ABC transporter, periplasmic phosphate-binding protein
TM1271	Type IV pilin-related protein
TM1275	Hypothetical protein
TM1276	Sugar ABC transporter, ATP-binding protein
TM1281	6-phospho-β-glucosidase
TM1294	Conserved hypothetical protein, COG1033: Predicted exporters of the RND super-family
TM1295	Conserved hypothetical protein, COG0491: Zn-dependent hydrolases, including glyoxylases, GloB
TM1296	Ribonuclease H
TM1302	ABC transporter, ATP-binding protein
TM1303	Conserved hypothetical protein, COG0842: ABC-type multidrug transport system, permease component
TM1304	Conserved hypothetical protein, COG0842: ABC-type multidrug transport system, permease component
TM1306	Conserved hypothetical protein, COG0842: ABC-type multidrug transport system, permease component

TM1310	ABC transporter, ATP-binding protein
TM1319	ABC transporter, ATP-binding protein
TM1326	Conserved hypothetical protein, COG0842: ABC-type multidrug transport system, permease component
TM1328	ABC transporter, ATP-binding protein
TM1330	LacI family transcriptional regulator, putative
TM1334	Conserved hypothetical protein, COG1032: Fe-S oxidoreductases family 2
TM1335	Hypothetical protein
TM1346	Processing protease; putative
TM1354	2 domains: 1) inosine-5-monophosphate dehydrogenase-related protein; 2) COG2172 RsbW related protein (anti-sigma regulatory factor)
TM1356	Hypothetical protein: COG3086 RseC Anti sigma E regulator
TM1359	Sensor histidine kinase
TM1360	Response regulator
TM1363	Peptide chain release factor RF-1
TM1364	Flagellar basal-body rod protein FlgB
TM1365	Flagellar basal-body rod protein FlgC
TM1366	Flagellar basal-body rod protein FliE
TM1368	ABC transporter, ATP-binding protein
TM1375	Spermidine/putrescine ABC transporter, periplasmic spermidine/putrescine-binding protein
TM1376	Spermidine/putrescine ABC transporter, ATP-binding protein
TM1377	Spermidine/putrescine ABC transporter, permease protein
TM1378	Spermidine/putrescine ABC transporter, permease protein
TM1385	Glucose 6-phosphate isomerase
TM1391	ATP-dependent ClpA protease, ATPase subunit
TM1400	Aspartate aminotransferase, putative
TM1405	Lipopolysaccharide biosynthesis protein-related protein
TM1414	β-fructosidase
TM1415	Inositol monophosphatase family protein, putative
TM1416	Conserved hypothetical protein, COG0719: Predicted membrane components of an uncharacterized iron-regulated ABC-type transporter SufB
TM1417	ABC transporter, ATP-binding protein
TM1419	Myo-inositol-1-phosphate synthase-related protein
TM1422	RnfB-related protein
TM1431	Glycerol uptake operon antiterminator
TM1436	Glycerol uptake operon antiterminator-related protein
TM1442	Anti-sigma factor antagonist, putative SpoIIAA
TM1450	Transcription repair coupling factor
TM1451	RNA polymerase sigma-A factor
TM1458	Ribosomal protein L21
TM1463	Ribonuclease P protein component
TM1467	Conserved hypothetical protein, COG2206: HD-GYP domain
TM1468	Conserved hypothetical protein, COG1307: Uncharacterized BCR, DegV
TM1469	Glucokinase
TM1478	Methionine aminopeptidase
TM1515	Ferric uptake regulation protein
TM1516	Hydrolase, ama/hipO/hyuC family
TM1524	Endoglucanase
TM1525	Endoglucanase
TM1572	Signal peptidase I
TM1580	Transcriptional regulator, putative; Transcriptional regulators containing an AAA-type ATPase domain and a DNA-binding domain, PspF
TM1589	Clostripain-related protein

TM1598	RNA polymerase sigma-E factor
TM1599	Hypothetical protein
TM1600	Hypothetical protein
TM1601	Conserved hypothetical protein, COG1031: Uncharacterized Fe-S oxidoreductases
TM1602	Transcriptional regulator, biotin repressor family
TM1624	β-mannosidase, putative
TM1627	General stress protein Ctc
TM1633	ATP-dependent protease LA, Lon
TM1636	Conserved hypothetical protein, COG0419: ATPase involved in DNA repair, SbcC
TM1650	α-amylase, putative, AmyA
TM1654	Sensor histidine kinase HpkA
TM1655	Response regulator DrrA
TM1662	Stationary phase survival protein, SurE
TM1663	ABC transporter, ATP-binding protein
TM1664	Conserved hypothetical protein, COG2813: 16S RNA G1207 methylase RsmC
TM1665	Hypothetical protein, COG0642: Signal transduction histidine kinase
TM1666	Succinyl-diaminopimelate desuccinylase, putative
TM1667	XylA xylose isomerase
TM1682	Conserved hypothetical protein, COG2206: HD-GYP domain
TM1683	Cold shock protein
TM1696	Type IV prepilin peptidase
TM1698	Aspartate aminotransferase
TM1699	Conserved hypothetical protein, COG2206: HD-GYP domain
TM1701	Conserved hypothetical protein, COG0534: Na^+-driven multidrug efflux pump, NorM
TM1702	Hypothetical protein
TM1704	Hypothetical protein, COG0760: Parvulin-like peptidyl-prolyl isomerase, SurA
TM1706	Transcription elongation factor, GreA/GreB family
TM1707	Conserved hypothetical protein, COG1327: Predicted transcriptional regulator, consists of a Zn-ribbon and ATP-cone domains
TM1713	Proline dipeptidase, putative
TM1714	UDP-N-acetylenolpyruvoylglucosamine reductase
TM1718	Ribulose-phosphate 3-epimerase
TM1719	DNA mismatch repair protein, MutS
TM1720	Conserved hypothetical protein, COG0454: Histone acetyltransferase HPA2 and related acetyltransferases, WecD
TM1723	Conserved hypothetical protein, COG1228: Imidazolonepropionase and related amidohydrolases, HutI
TM1730	Holliday junction DNA helicase, RuvB
TM1734	Phosphate transport system regulator PhoU, putative
TM1746	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein
TM1747	Oligopeptide ABC transporter, permease protein
TM1748	Oligopeptide ABC transporter, permease protein
TM1749	Oligopeptide ABC transporter, ATP-binding protein
TM1750	Oligopeptide ABC transporter, ATP-binding protein
TM1751	Endoglucanase
TM1752	Endoglucanase
TM1754	Putative butyrate kinase
TM1755	Phosphate butyryltransferase
TM1756	Branched-chain-fatty-acid kinase, putative
TM1761	Excinuclease ABC, subunit B, UvrB
TM1765	N utilization substance protein B
TM1768	Exodeoxyribonuclease VII, large subunit, XseA
TM1775	Hypothetical protein, COG0658: Predicted multitransmembrane, metal-binding

	protein, ComEC
TM1776	Ferric uptake regulation protein
TM1777	N utilization substance protein A, NusA
TM1797	Conserved hypothetical protein, COG1518: Uncharacterized ACR
TM1818	Chromate transport protein, putative
TM1819	Chromate transport protein, putative
TM1820	GMP synthase
TM1822	FtsH protease activity modulator HflK
TM1823	FtsH protease activity modulator HflC
TM1834	α-glucosidase
TM1835	Cyclomaltodextrinase, putative
TM1836	Maltose ABC transporter, permease protein
TM1839	Maltose ABC transporter, periplasmic maltose-binding protein
TM1840	α-amylase, AmyA
TM1845	Pullulanase
TM1847	ROK family protein
TM1848	Cellobiose phosphorylase
TM1851	α-mannosidase
TM1852	Conserved hypothetical protein, COG2152: Predicted glycosylase
TM1853	Sugar ABC transporter, permease protein
TM1854	Sugar ABC transporter, permease protein
TM1855	Sugar ABC transporter, periplasmic sugar-binding protein, putative
TM1856	Transcriptional regulator, LacI family
TM1858	RecX protein, putative
TM1859	DNA repair protein, RecA
TM1865	Endonuclease V
TM1866	Membrane bound protein LytR, putative
TM1867	L-lactate dehydrogenase
TM1869	ATP-dependent protease LA, putative, LonB
TM1870	MinD septum site determining prot.
TM1874	Cold shock protein
TM1876	Conserved hypothetical protein, COG0639: Diadenosine tetraphosphatase and related serine/threonine protein phosphatases
TM1878	UDP-sugar hydrolase