EXPERIMENTAL AND BIOINFORMATIC ANALYSES OF AMINO ACID METABOLISM IN THE HYPERTHERMOPHILIC ARCHAEON *PYROCOCCUS FURIOSUS*

by

ANGELA KATHLEEN SNOW

(Under the Direction of Michael W. W. Adams)

ABSTRACT

Pyrococcus furiosus is a hyperthermophilic archaeon that grows well on complex media containing peptides and various carbohydrates. Its growth on defined media is less well characterized. In this study, the growth of *P. furiosus* was investigated on various defined media. *P. furiosus* grows well on a defined medium containing all 20 amino acids. It also shows consistent growth on a defined medium containing arginine, cysteine, glycine, lysine, proline, and serine as the only amino acids. *P. furiosus* grows inconsistently in defined media with cysteine as the only amino acid. The experimental results are compared with bioinformatics data to better understand the amino acid biosynthesis capabilities of this fascinating organism.

INDEX WORDS: archaea, hyperthermophile, *Thermococcales*, *Pyrococcus furiosus*, amino acid biosynthesis, amino acid anabolism, metabolism, defined medium

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DEDICATION

I dedicate this work to my undergraduate mentor, Dr. Dianne Newman. She provided me with my first opportunity to work in a microbiology lab. Her patience and encouragement made the learning process an absolute joy. More importantly, she introduced me to the wonderous diversity of prokaryotes. She instilled in me the desire to learn about every novel environment in which microorganisms thrive and every peculiar adaptation that allows them to flourish. My experience working in her lab is what inspired me to join graduate school and pursue a career in microbiology.

I also dedicate this work to our mutual friend, *Shewanella* sp. strain ANA-3, who taught me that when life gives you arsenic, sometimes you just have to adapt to it the best you can. Who knows? Maybe that element in your life that was once so toxic will end up being the only thing that sustains you.

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CHAPTER 1

INTRODUCTION

Pyrococcus furiosus Overview

An organism is considered a hyperthermophile if it has an optimal growth temperature ≥80 ^{o}C, and a maximum growth temperature ≥90 ^{o}C (7). With an optimal growth temperature of 106 ^{o}C and a growth temperature range of 90 ^{o}C to 113 ^{o}C, the hyperthermophile *Pyrolobus fumarii* has the highest growth temperature for any validly-published species (19). The one characteristic shared exclusively by all hyperthermophiles is the presence of the enzyme reverse gyrase, which produces positive supercoils in DNA (42). In most other aspects, hyperthermophiles are quite diverse. They occupy a wide variety of high-temperature habitats, including hot springs, marine and terrestrial solfataras, and deep sea vents (59). Hyperthermophiles use diverse metabolic strategies to survive in these habitats (59). There are examples of chemolithoautotrophs and heterotrophs among hyperthermophile species, as well as examples of aerobic, anaerobic, and fermentative metabolism (59, 133). There are no photosynthetic hyperthermophiles; the highest growth temperature reached by photosynthetic bacteria is 74 ^{o}C (24, 29). Three classes of *Bacteria* are known to contain hyperthermophiles - *Aquificae*, *Thermodesulfobacteria*, and *Thermotogae* - with optimal growth temperatures ranging from 80 ^{o}C to 90 ^{o}C (60, 61, 69, 79). The majority of described hyperthermophiles are *Archaea* (76, 79). The most extreme hyperthermophiles are also found within the *Archaea*, including the

record-holding *P. fumarii* and several other species with temperature optima ≥100 ^{o}C (19, 133). There are no hyperthermophilic *Eucayota* (24).

The domain *Archaea* contains two major recognized phyla: *Crenarchaeota* and *Euryarchaeota* (1, 58, 144). *Crenarchaeota* contains a single recognized class, *Thermoprotei*, which contains three known orders, *Desulfurococcales*, *Sulfolobales*, and *Thermoproteales* (1, 2, 5, 26, 150). All cultured species of *Crenarchaeota* are thermophilic or hyperthermophilic (26, 144). However, PCR-based surveys of microbial diversity in mesophilic and psychrophilic environments have detected numerous uncultured *Crenarchaeota* species, which suggests that the currently cultured members are not representative of true temperature range of the phylum as a whole (36). *Euryarchaeota* currently contains seven recognized classes: *Archaeoglobi*, *Halobacteria*, *Methanobacteria*, *Methanococci*, *Methanopyri*, *Thermococci*, and *Thermoplasmata* (1). *Euryarchaeota* are phenotypically diverse, containing methanogenic and halophilic archaea in addition to several classes of thermophiles and hyperthermophiles (144). "*Nanoarchaeota*" (containing only one known species – "*Nanoarchaeum equitans*") has been proposed as a third archaeal phylum (58), but this designation is not universally accepted (23). A fourth proposed phylum, "*Korarchaeota*," is known only from environmental sequences and contains no cultured members (13).

The order *Thermococcales*, the sole member of the class *Thermococci*, is part of the *Euryarchaeota* (1, 149). *Thermococcales* consists of obligately anaerobic, heterotrophic thermophiles and hyperthermophiles that can reduce elemental sulfur (15, 71, 149). Some are obligate sulfur-reducers, while some have other metabolic capabilities in addition to sulfur reduction (125). The cells are cocci between 0.5 to 2.5 μm in size, and most species have multiple monopolar flagella (15). They have a fast growth rate compared to the other *Archaea*,

with optimal doubling times ranging from 25 to 70 minutes (15). The vast majority of *Thermococcales* species have an optimal pH for growth that is close to neutral (15, 71). The only known exceptions are *Thermococcus alcaliphilus* and *Thermococcus acidaminovorans*, which have an optimal pH of 9 (37, 72). The genus *Pyrococcus*, along with genera *Thermococcus* and *Palaeococcus*, make up the order *Thermococcales* (4, 125, 148, 149). As of April 2008, there are genome sequences available for four *Thermococcales* species: *Pyrococcus abyssi*, *Pyrococcus furiosus*, *Pyrococcus horikoshii*, and *Thermococcus kodakarensis* (31, 43, 70, 106).

Pyrococcus species are obligate anaerobes characterized by heterotrophic growth, optimal growth temperatures above 90^{o}C, coccoid morphology, and multiple polar flagella (12, 40). Their high optimal growth temperature in particular distinguishes them from other *Thermococcales* (12, 17, 125). *Pyrococcus* has been isolated almost exclusively from marine environments, including deep-sea vents (12, 39, 46, 56) and coastal hydrothermal environments (40, 149), although there has been one report of a *Pyrococcus* strain isolated from a terrestrial hot spring (71).

P. furiosus was the first species to be identified in the genus *Pyrococcus*, and was one of the first the hyperthermophile species isolated (3, 40). *P. furiosus* was isolated in 1986 from geothermally-heated marine sediments at Vulcano Island, Italy (40). It has a growth temperature range of 70 ^{o}C to 103 ^{o}C, with an optimal growth temperature of 100^{o}C (40). It can grow in a pH range of 5 - 9, and has a broad optimal pH range of 6 - 8 (40). Like all *Thermococcales*, *P. furiosus* is an obligate anaerobe (15, 40). It grows heterotrophically on peptides (40, 121), pyruvate (113), and select carbohydrates (40, 44, 74, 81). In the presence of elemental sulfur, *P. furiosus* will use sulfur as an electron acceptor and reduce it to hydrogen

sulfide (116). In the absence of elemental sulfur, it will reduce protons and evolve hydrogen gas (40, 109).

P. furiosus has many characteristics that make it a good model organism for hyperthermophilic archaea and for biological systems in general. It has a relatively short doubling time of 37 minutes under optimal conditions (40), it is relatively easy to culture (105, 131), and its genome sequence has been completed (101, 106). Prior studies have provided a considerable knowledge base for this species. This includes cDNA microarrays (115, 116, 120, 137, 143), enzymatic studies (25, 65, 74, 84, 110), and protein structures (34, 66, 134). In addition, the similarities between archaeal and eukaryotic systems have allowed studies of *P. furiosus* to provide insights into eukaryotic biology as well (119). All of these characteristics make *P. furiosus* an excellent model organism.

There are several existing and potential commercial and industrial applications for *P. furiosus*. For example, *P. furiosus* produces three distinct hydrogenase enzymes (25, 84, 110), which could potentially be used for industrial hydrogen production. The enzymes in hyperthermophiles can be very thermostable, with some enzymes exhibiting activity even at temperatures exceeding the growth temperature of the organisms from which they came (35). For example, α-glucosidase from *P. furiosus* exhibits optimal activity up to 115 ^{o}C, which is more than 10 ^{o}C higher than the maximum growth temperature of *P. furiosus* (33, 40). Due to their increased thermal stability, enzymes from hyperthermophilic archaea such as *P. furiosus* can have great economic value (129). In addition to being heat stable, some *P. furiosus* enzymes are resistant to denaturing agents such as SDS and urea (21). Such enzymes are potentially useful under harsh industrial conditions. One example of an enzyme from *P. furiosus* that is already sold commercially is the *Pfu* DNA polymerase, which is used in PCR reactions

(83). The error rate of *Pfu* DNA polymerase is 1.6 x 10^{-6}, which is approximately 10x lower than the commonly used *Taq* DNA polymerase (83). Another example is the enzyme *Pfu* dUTPase, which is sold in combination with *Pfu* DNA polymerase under the name *PfuTurbo* (57). *Pfu* dUTPase cleaves the PCR inhibitor dUTP, which is formed by dCTP deamination due to high temperatures (57). The inclusion of dUTPase to PCR reactions permits the successful amplification of longer PCR products (57).

Metabolism of *Pyrococcus furiosus*

P. furiosus is an obligate anaerobe that grows heterotrophically on peptides, pyruvate, and on select carbohydrates (40, 49, 74, 113, 121). It produces CO_2, alanine, and various organic acids such as acetic acid, propionic acid, isobutyric acid, and isovaleric acid as waste products (16, 40). In the presence of elemental sulfur, it produces H_2S; in the absence of sulfur, it produces H_2 instead (40). Several previous studies provide insights into the metabolism of this organism that are especially relevant to the work reported here (73, 109, 113, 116, 117).

Growth on proteinaceous substrates is a common trait in *Thermococcales* species, including *P. furiosus* (7, 40, 125). For *P. furiosus* to grow on peptides, it requires elemental sulfur as an electron acceptor (6). As might be expected, overall proteolytic activity increases in *P. furiosus* when it is grown on peptides only compared to when it is grown on both maltose and peptides (121). Proteolytic activity is also higher when *P. furiosus* is grown on a limiting concentration of peptides compared to growth on a high concentration of peptides (121). Several proteolytic enzymes from *P. furiosus* have been characterized, including prolidase (45), carboyxypeptidase (30), aminopeptidases (124, 127), and serine proteases (21, 132), although in many cases it is unclear whether the enzymes function primarily in peptide catabolism or in the degradation of damaged cellular proteins.

Once the proteins are degraded, the next step in protein catabolism is the transamination of the resulting free amino acids (7). The product of the transamination reaction is a 2-keto acid, which then serves as a substrate for 2-keto acid oxidoreductase in the next step of amino acid catabolism (7). Aminotransferases also catalyze the reverse reaction, producing an amino acid from a 2-keto acid, during amino acid biosynthesis (135, 136). Therefore, it is not certain if the aminotransferases characterized so far in *P. furiosus* function primarily in catabolism or anabolism (11, 135, 136). Four aminotransferases from *P. furiosus* have been purified and characterized: an alanine aminotransferase (AlaAT) (136), an aspartate aminotransferase (AspAT) (135), and two aromatic aminotransferases (AroAT I and AroAT II) (11, 135). AlaAT has the highest activity with alanine, although it does show some activity with aspartate and even has low levels of activity with the branched chain amino acids (136). AspAT has significant activity with aspartate and glutamate, and shows low levels of activity with alanine and the aromatic amino acids (135). AroAT I shows the highest activity with phenylalanine, but also shows significant activity with tyrosine and tryptophan (11). AroAT II shows equivalent activity towards all of the aromatic amino acids, and it also has low levels of activity with alanine and aspartate (135). There are several putative transaminases annotated in the *P. furiosus* genome that have not yet been experimentally characterized (106). These could potentially act on the remaining amino acids.

P. furiosus contains four ferredoxin-linked 2-keto acid oxidoreductases: pyruvate:ferredoxin oxidoreductase (POR), indolepyruvate ferredoxin oxidoreductase (IOR), 2-ketoisovalerate ferredoxin oxidoreductase (VOR), and 2-ketoglutarate ferredoxin oxidoreductase (KGOR), each specific for the 2-keto acid derivatives of different subsets of amino acids (18, 52, 77, 86, 87). POR oxidizes pyruvate to acetyl-CoA (18). POR is thought to function both in

carbohydrate metabolism using pyruvate derived from phosphoenolpyruvate (18), and in peptide metabolism using pyruvate derived from alanine (87, 88). KGOR oxidizes 2-ketoglutarate, which is derived from glutamate, to succinyl-CoA (86). IOR has the highest activity with aromatic 2-ketoacids, which it oxidizes to the corresponding aryl-CoA (87). IOR also shows some activity with 2-ketoisocaproate and 2-keto-γ-methylthiobutyrate, the transaminated derivatives of leucine and methionine respectively (87). VOR oxidizes the branched chain 2-keto acids as well as 2-keto-γ-methylthiobutyrate to the corresponding CoA derivatives (52).

The final step requires the ADP-dependent acetyl-CoA synthetase enzymes, ACS I and ACS II, which convert the various CoA derivatives to the corresponding acid concurrently with the production of ATP by substrate-level phosphorylation (88). ACS I and ACS II both use acetyl-CoA and the branched chain CoA derivatives, while only ACS II uses the aromatic CoA derivatives (88). Since neither ACS enzyme is active with succinyl-CoA, it is possible that the succinyl-CoA produced by KGOR is used for biosynthesis rather than in glutamate catabolism (86).

P. furiosus can grow directly on pyruvate by converting it first to acetyl-CoA using POR, and then to acetate (+ATP) using either ACS I or ACS II (18, 88, 113). There have been conflicting reports on the ability of *P. furiosus* to grow on individual amino acids (20, 40, 55, 105, 121, 135). The proposed pathway for protein metabolism contains the enzymes necessary for growth on some amino acids. However, factors such as the relative instability of some individual amino acids at the elevated temperatures required for *P. furiosus* may prevent some amino acids from being used directly to support growth (121).

P. furiosus can grow using select carbohydrates, including maltose, cellobiose, laminarin, lichenan, chitin, and starch (40, 44, 49, 74). It is unable to grow on glucose or other

monosaccharides directly (7, 73). This is thought to be due to the low stability of glucose at high temperatures (38). Unlike peptide metabolism, carbohydrate metabolism is not universally distributed in *Pyrococcus* species (12, 39, 40, 46, 50, 56, 71, 149). The Mal I operon that is responsible for maltose uptake and metabolism in *P. furiosus* is theorized to have been acquired by lateral gene transfer, which may explain why the ability to grow on maltose is absent in most *Pyrococcus* species (50). Sugar metabolism occurs via a modified Embden-Meyerhof (EM) pathway (73). In *P. furiosus*, two kinases, glucokinase and phosphofructokinase, are ADP-dependent, while in the traditional EM pathway these enzymes are ATP-dependent (73). Another key modification is that a single enzyme, the ferredoxin-dependent glyceraldehyde-3-phosphate:ferredoxin oxidoreductase (GAPOR), replaces two of the EM pathway enzymes, phosphoglycerate kinase and the NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase (97). POR, which is also ferredoxin-dependent, replaces the NAD-dependent pyruvate dehydrogenase (18).

Carbohydrate metabolism can occur in *P. furiosus* in the absence of elemental sulfur (6, 40, 44). When grown under these conditions, *P. furiosus* evolves hydrogen gas (40). *P. furiosus* contains three hydrogenase enzymes (25, 84, 110). The ferredoxin-linked membrane-bound hydrogenase (MBH) is the hydrogenase responsible for energy conservation during carbohydrate metabolism (109). As mentioned above, the GAPOR and POR enzymes in the modified EM pathway produce reduced ferredoxin (18, 97). The MBH complex uses the ferredoxin to reduce protons to hydrogen gas (109). In addition, the complex couples the production of hydrogen to the translocation of protons across the cell membrane, resulting in the production of additional ATP (109).

Both carbohydrate metabolism and peptide metabolism can be coupled to the reduction of elemental sulfur to hydrogen sulfide (114, 116). For carbohydrate metabolism, elemental sulfur is preferred over protons as an electron acceptor: The addition of sulfur causes a rapid down-regulation of MBH and an equally rapid initiation of H_2S production (116). Also, the cell yield doubles when grown on maltose with S^0 compared to growth on maltose alone (7, 114). Peptide metabolism is sulfur-dependent and only occurs when S^0 is present (6). The exact mechanism of sulfur reduction in the *Thermococcales* is not well understood, but it is believed to be distinct from known sulfur reduction pathways (6, 116, 118). Microarray analyses of *P. furiosus* have shown that a membrane-bound oxidoreductase complex (MBX) and a NAD(P)H sulfur oxidoreductase (NSR) are highly up-regulated in the presence of S^0, and are therefore thought to be involved in the sulfur reduction pathway (116). MBX has a high degree of homology to MBH, and is similarly believed to be involved in energy conservation during sulfur respiration (110). NSR was originally believed to be an NAD(P)H CoA disulfide reductase based on experiments performed on a close homologue in *P. horikoshii* (51). However, its high NAD(P)H and CoA dependent sulfur reductase activity, as well as its rapid up-regulation upon the addition of S^0 to the growth media, suggests that NSR's true physiological role is as a sulfur reductase during sulfur respiration (116).

Amino Acid Metabolism by *Pyrococcus furiosus*

The original characterization of the *P. furiosus* isolates described them as showing weak growth when using casamino acids as a carbon and energy source, and no growth when using DL-alanine or L-cysteine·HCl as the carbon and energy source (40). No attempt was made in that work to determine the amino acid auxotrophies of this organism. Since then, there have

been several attempts to elucidate amino acid utilization in *P. furiosus*. The results have been somewhat contradictory. These studies are summarized as follows:

One study by Blumentals et al. concerning various growth conditions for *P. furiosus* found that it could grow in an Artificial Seawater Medium supplemented with trace elements, elemental sulfur, 20 amino acids, and 0.01% tryptone (20). The tryptone was required for transferable growth in this media for undetermined reasons (20). *P. furiosus* reached a density of $1.5x10^7$ cells/mL in this medium within 9 hours, which was about 10-fold lower than the cell densities obtained when grown on 0.1% yeast extract and 0.5% tryptone (20). Under low-tryptone conditions, proline and cysteine were found to be essential amino acids (20). Tyrosine was not required (20).

A follow-up study by Snowden et al. on the proteolytic activity of *P. furiosus* confirmed the original description of *P. furiosus* as being able to use peptides as a sole carbon and energy source in the presence of elemental sulfur (40, 121). However, this study went further in concluding that peptides were required for growth (121). *P. furiosus* did not grow on maltose plus S^0 after 24 hours unless 0.01% whole casein was also present (121). Amino acids could not substitute for the peptide requirement: Media containing casamino acids supplemented with cysteine, tryptophan, and glutamine did not support growth even if another carbon and energy source, such as maltose, were present (121).

A third study by Hoaki et al. looked at the amino acid auxotrophies of *P. furiosus* as well as those of several other hyperthermophiles (55). It concluded that peptides were not required, since *P. furiosus* grew with individual amino acids after a long lag phase of 70+ hours (55). Furthermore, isoleucine and valine were the only amino acids that were strictly required for growth (55). *P. furiosus* reached low cell densities of 10^7 cells/mL in the absence of threonine,

leucine, or methionine (55). It reached a density of at least $5x10^7$ cells/ml in the absence of any one of the remaining 15 amino acids (55). The study also concluded that one or more of the following trace minerals was required for the growth of *P. furiosus*: Fe, Co, Zn, Cu, Al, Mo, Ni, and/or W (55). The proposed reason for the discrepancy in results between this study and the study by Snowden et al. was that it was a need for the trace mineral contaminants in the peptides, rather than a need for the peptides themselves, that prevented growth in peptide-free media (55).

A fourth study by Raven and Sharp used continuous culture to determine both the amino acid requirements and the vitamin requirements of *P. furiosus* in the absence of S^0 (105). It found that cysteine and proline were the only required amino acids, which is consistent with the amino acid requirements found the study by Blumentals et al. (20, 105). Several potential sulfur sources, including dithiothreitol, sodium sulfide, and sodium thiosulfate, were tested for their ability to substitute for cysteine (105). None could support growth in the absence of cysteine (105). D-biotin was the sole required vitamin (105). In the continuous culture, *P. furiosus* could grow to a density of $2.0x10^9$ cells/mL in a mineral salts medium containing only 7.2g/L maltose, 0.5g/L L-cysteine, 0.5g/L L-proline, and 0.001g/L D-biotin (105). However, when tested in batch culture, *P. furiosus* showed limited growth on this media, which was greatly improved when the maltose was replaced by 5g/L dextrin (105). *P. furiosus* could also be plated on a solid version of this media with a plating efficiency of 26% (105).

The results of these four studies are summarized in **Table 1.1**.

Project Goals

P. furiosus grows well on rich media, reaching densities as high as $4.5x10^8$ cells/mL in batch culture. Considerable work has been done to understand the dominant metabolic pathways when *P. furiosus* is grown on carbohydrates and protein. There is ongoing work by this lab and

Table 1.1 – Experimentally Determined Amino Acid Auxotrophies in *P. furiosus*

(-) Required by *P. furiosus*. (+/-) Poor growth in the absence of this amino acid. (+) Made by *P. furiosus*. (n.d.) Not determined. (**Y**) Peptides required for growth. (**N**) Peptides not required for growth.

Amino Acid	Blumentals et al.	Hoaki et al.	Raven and Sharp
Alanine	n.d.	+	+
Arginine	n.d.	+	+
Asparagine	n.d.	+	+
Aspartic Acid	n.d.	+	+
Cysteine	-	+	-
Glutamic Acid	n.d.	+	+
Glutamine	n.d.	+	+
Glycine	n.d.	+	+
Histidine	n.d.	+	+
Isoleucine	n.d.	-	+
Leucine	n.d.	(+/-)	+
Lysine	n.d.	+	+
Methionine	n.d.	(+/-)	+
Phenylalanine	n.d.	+	+
Proline	-	+	-
Serine	n.d.	+	+
Threonine	n.d.	(+/-)	+
Tryptophan	n.d.	+	+
Tyrosine	+	+	+
Valine	n.d.	-	+
Peptide Requirement?	**Y**	**N**	**N**

others to better understand the role of the three hydrogenase enzymes during fermentative growth, as well as to characterize the novel sulfur reduction pathway used for anaerobic respiration. However, less work has been done to characterize the growth of *P. furiosus* when grown on a defined medium.

The first goal of this project is to design a defined medium for *P. furiosus* that will permit reproducible growth to a high cell density. Some experiments require *P. furiosus* to grow under defined nutrient conditions. For example, the selection marker used to develop the targeted deletion protocol in *T. kodakarensis* required it to be grown in a uracil-free medium (111, 112). It would be useful to have more information about the growth of *P. furiosus* on a defined medium so that a similar targeted deletion system could be developed for this species.

The second goal of this project is to use the defined medium to determine the amino acid requirements of *P. furiosus* by selectively removing amino acids from the medium. Although there have been several previous studies that aimed to identify the required amino acids of *P. furiosus*, the results have not provided a definitive answer (20, 55, 105, 121). This study will attempt to definitively determine which amino acids can be produced by *P. furiosus*, and which must be acquired from the environment. This information will provide experimental evidence to support the existence or absence of the predicted amino acid biosynthetic pathways. This, in turn, will lead to a better overall understanding of the metabolic capabilities of *P. furiosus*.

CHAPTER 2

BIOINFORMATICS ANALYSIS OF AMINO ACID BIOSYNTHETIC PATHWAYS IN *PYROCOCCUS FURIOSUS*

Genomic Analysis and Predicted Amino Acid Biosynthetic Pathways

Genetic techniques to experimentally confirm predicted amino acid biosynthetic pathways in *Thermococcales* have only recently been developed (90, 111, 112). Therefore, there have been very few *in vivo* experiments that address amino acid biosynthetic pathways in any *Thermococcales* species (111, 112) and none so far in *P. furiosus*. Some enzymes from *P. furiosus* that are proposed to be involved in amino acid biosynthesis have been purified and characterized *in vitro* to confirm that their reaction rates and substrate specificities are consistent with their predicted roles (11, 85, 135, 136), but this has only been done for a limited number of enzymes. For the most part, the amino acid biosynthetic pathways currently annotated in *P. furiosus* are predictions based solely on homology to characterized enzymes and pathways in distantly-related organisms (101, 106).

According to bioinformatics analysis by the Kyoto Encyclopedia of Genes and Genomes (KEGG), there are complete biosynthetic pathways for 8 amino acids: asparagine, aspartic acid, glutamine, glutamic acid, phenylalanine, threonine, tryptophan, and tyrosine (67). It also shows nearly complete biosynthetic pathways for 9 additional amino acids: alanine, arginine, cysteine, glycine, histidine, leucine, proline, serine, and valine (67). Only the pathways for isoleucine, lysine and methionine biosynthesis are lacking homologues for multiple enzymes (67). Another pathway prediction site, MetaCyc, predicts that there are complete biosynthetic pathways present

for 8 amino acids: arginine, asparagine, aspartic acid, glutamic acid, leucine, lysine, threonine, and valine (28). It predicts that pathways are not present for another 8 amino acids: alanine, cysteine, glutamine, histidine, isoleucine, methionine, proline, and serine (28). In the cases of phenylalanine, tryptophan, and tyrosine, MetaCyc predicts complete pathways starting from chorismate and leading to the amino acids (28). However, it does not predict a biosynthetic pathway for D-erythrose-4-phosphate (D-E4P), which is required for chorismate synthesis (28). There is a predicted pathway to convert serine to glycine, but since the serine biosynthetic pathway is absent, an exogenous serine source would be required to produce glycine (28).

The predictions by KEGG and MetaCyc are summarized in **Table 2.1**. Four biosynthetic pathways are predicted by both databases to be complete in *P. furiosus*: asparagine, aspartic acid, glutamic acid, and threonine (28, 67). For the remaining 16 amino acids, the pathway is predicted to be incomplete by at least one of the databases (28, 67). The amino acid biosynthetic pathways are described in detail below. Enzymes in these pathways that are annotated in *P. furiosus* are mentioned, along with possible explanations and identities of the "missing" enzymes.

Alanine

Experimental evidence indicates that *P. furiosus* can produce alanine (75, 136). Under certain conditions, such as high hydrogen concentration, alanine is produced as an end product of fermentation (75). Alanine can be produced from pyruvate and glutamate by AlaAT (47). Cell extracts of *P. furiosus* contain considerable AlaAT activity (75). One AlaAT (PF1497) has been purified from *P. furiosus*, and since it functions well in both the forward and reverse reactions, it is proposed to function both in alanine biosynthesis and alanine degradation (136). In the BioCyc database, this protein is annotated as a putative transaminase, which is why MetaCyc

Table 2.1 – Predicted Amino Acid Biosynthetic Pathways in *P. furiosus*

(-) Required by *P. furiosus*. (+) Made by *P. furiosus*. (P) Partial pathway present.

Amino Acid	KEGG	MetaCyc
Alanine	P	-
Arginine	P	+
Asparagine	+	+
Aspartic Acid	+	+
Cysteine	P	-
Glutamic Acid	+	+
Glutamine	+	-
Glycine	P	P
Histidine	P	-
Isoleucine	-	-
Leucine	P	+
Lysine	-	+
Methionine	-	-
Phenylalanine	+	P
Proline	P	-
Serine	P	-
Threonine	+	+
Tryptophan	+	P
Tyrosine	+	P
Valine	P	+

does not predict that *P. furiosus* possesses an alanine biosynthetic pathway (28, 68). In KEGG, this protein is misassigned as an aspartate aminotransferase (67).

Lysine

There are two main pathways for lysine biosynthesis, the diaminopimelic acid (DAP) pathway and the α-aminoadipic (AAA) pathway (128). The DAP pathway produces lysine from aspartate with diaminopimelic acid as a key intermediate and is found in plants, some archaea, and nearly all bacteria that can produce lysine (41, 128, 130). *P. furiosus* does not appear to have a DAP pathway (67, 106). The AAA pathway produces lysine from α-ketoglutarate with α-aminoadipate as a key intermediate and was first identified in fungi (128). A modified version of the AAA pathway was recently characterized in the bacterium *Thermus thermophilus* (78, 99). An interesting feature of this pathway is that three out of five enzymes in the first half of the pathway (LysS, LysTU, HICDH) are homologous to enzymes in the leucine biosynthetic pathway (LeuA, LeuCD, LeuB) and to enzymes in the TCA cycle (GltA, Acn, Icd), and all four enzymes in the second half of the pathway (LysZ, LysY, LysJ, LysK) are homologous to enzymes in the arginine biosynthetic pathway (ArgB, ArgC, ArgD, ArgE) respectively (41, 99).

T. thermophilus is an obligately aerobic, thermophilic bacterium with a growth temperature range of 47°C to 85°C and an optimal growth temperature near 70°C (100). It is a member of the phylum *Deinococcus-Thermus*, and is not phylogenetically related to *P. furiosus* or to the other *Thermococcales* (4, 138, 144). Yet, the modified *T. thermophilus* AAA pathway is predicted to be present in *P. abyssi, P. furiosus*, and *P. horikoshii*, but its predicted distribution in the *Bacteria* is relatively limited compared to the distribution of the DAP pathway (28, 41, 99, 130). Homologues for nine out of eleven of the genes required for this pathway are arranged in *P. furiosus* as a single gene cluster (PF1678-PF1686), which is similar in terms of both gene

order and content to the gene clusters present described in *T. thermophilus* and *P. horikoshii* (9, 92, 94, 99, 106). A comparison of these three gene clusters is shown in **Figure 2.1**. Missing from the *P. furiosus* gene cluster is a clear homoisocitrate dehydrogenase (*hicdh*) homologue and an α-aminoadipate aminotransferase (*lysN*) homologue (9, 93, 96, 106).

HICDH is a β–decarboxylating dehydrogenase that converts homoisocitrate to 2-ketoadipate, and that has significant sequence homology with isocitrate dehydrogenase (ICDH) and 3-isopropylmalate dehydrogenase (IPMDH) in other organisms (93, 95). *P. furiosus* has two annotated β–decarboxylating dehydrogenases, ICDH (PF0202) and IPMDH (PF0940) (106, 123). Both genes are upregulated when *P. furiosus* is grown under protein-limited conditions (115). The sole β–decarboxylating dehydrogenase from *P. horikoshii* showed both isocitrate dehydrogenase activity and homoisocitrate dehydrogenase activity *in vitro*, as did the HICDH and ICDH from *T. thermophilus* (93, 95). It is possible that the putative ICDH in *P. furiosus* also possesses this dual activity, and can therefore function in the lysine biosynthetic pathway.

LysN is an aminotransferase that converts 2-ketoadipate to α-aminoadipate (96). The *T. thermophilus* LysN has a broad substrate specificity, showing activity with 2-ketoisocaproate, 2-ketoisovalerate, and 2-keto-3-methylvalerate (the precursors of leucine, valine, and isoleucine, respectively) in addition to 2-ketoadipate (96). The closest primary sequence homologue of the *T. thermophilus* LysK in *P.furiosus* is AroAT I (PF0121) (96). Although they have been purified, neither AroAT I nor the other three aminotransferases purified from *P. furiosus* have been tested for activity with 2-ketoadipate or α-aminoadipate (11, 135, 136). There are also several aminotransferases annotated in *P. furiosus* that have not been purified, so their activities have not been tested with any substrate (106). Therefore, it is unclear which of the aminotransferases, if any**,** is a functional LysN homologue in *P. furiosus*.

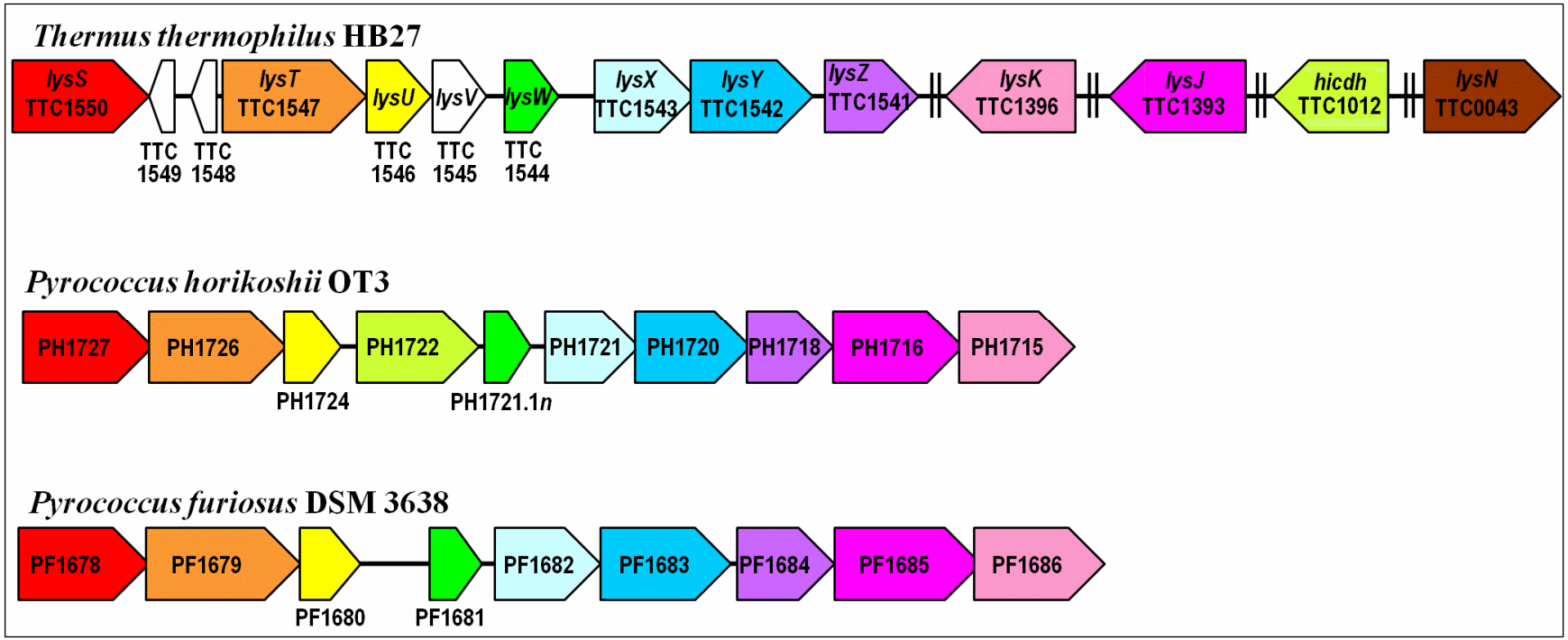

Figure 2.1 – Lysine Biosynthesis Genes in *T. thermophilus*, *P. horikoshii*, and *P. furiosus*

Colored block arrows ⌦⌦⌦⌦⌦⌦⌦⌦⌦⌦⌦ indicate ORFs determined experimentally (in the case of *T. thermophilus* HB27) or predicted by homology (in the case of *P. horikoshii* and *P. furiosus*) to be involved in lysine biosynthesis. Block arrows with identical colors are homologues. Clear block arrows ⌦ are intervening ORFs unrelated to lysine biosynthesis. Solid black lines — indicate intergenic regions. Broken lines —‖— indicate ORFs that are not adjacent.

Modified from: Nishida et al., 1999 (99)

Arginine

The first step in arginine biosynthesis is the acetylation of glutamate to *N*-acetylglutamate, which is typically performed by the enzyme *N*-acetylglutamate synthase (NAGS) (128, 147). *P. furiosus* does not contain a homologue for NAGS (67, 106). This is not surprising, as no homologue of NAGS has yet been indentified in archaea (54, 147). One proposed explanation is that it is a homologue to LysX that acetylates glutamate in some archaeal species, including *P. furiosus* (147). LysX is required for lysine biosynthesis in *T. thermophilus*, where it is believed to catalyze the acetylation of α-aminoadipate to N^2-acetyl-α-aminoadipate (99, 108). As mentioned previously, many of the proteins in the AAA pathway are phylogenically related to proteins in the arginine biosynthetic pathway (41, 130). However, this is NOT the case for LysX, which has no sequence homology and no phylogenic relationship with any known NAGS protein (41). Still, given the similarity between α-aminoadipate and glutamate, it was proposed that LysX may be able to catalyze the acetylation of glutamate as well (147). *P. furiosus* contains two homologues to the *T. thermophilus* LysX: PF0209 (43% identical and 61% similar to *T. thermophilus* LysX) and PF1682 (42% identical and 59% similar to *T. thermophilus* LysX) (9, 53, 106). Both PF0209 and PF1682 are currently annotated as ribosomal protein S6 modification proteins, but given that the S6 ribosomal protein has not been identified in *P. furiosus* or any other Archaeal species, this is very unlikely to be the true function (80, 106). Both genes are adjacent to other predicted arginine biosynthesis genes, and both are up-regulated in maltose media compared to peptide media (115).

There are annotated genes in *P. furiosus* for the rest of the typical arginine biosynthetic pathway (106, 128). The genes PF1683, PF1684, PF1685, PF1686 are annotated as *argC*, *argB*, *argD*, and *argE* respectively, which all belong to the section of the arginine biosynthetic pathway

that converts *N*-acetylglutamate to ornithine (106). However, these genes are also homologues of *lysY*, *lysZ*, *lysJ*, and *lysK* mentioned in the previous paragraph, which would be required for AAA lysine biosynthetic pathway (9, 99, 106). There is one other *argE* homologue, PF1185, annotated in *P. furiosus* (106). There are three other genes with homology to *argD*, PF1421, PF1232, and PF0513, that are currently annotated as 4-aminobutyrate aminotransferases (9, 106), but have not been biochemically characterized to determine their true function. On the other hand, PF1683 and PF1684 are the only recognizable *argC* and *argB* homologues, as well as the only *lysY* and *lysZ* homologues (9, 99, 106). So, which pathway do they belong to? The corresponding genes in *P. horikoshii* are believed to encode bifunctional proteins, acting in both arginine biosynthesis and lysine biosynthesis (41, 70, 99). If this is the case in *P. horikoshii*, it is possibly true for *P. furiosus* as well. PF0207, PF0208, and PF0594 are annotated as *argG*, *argH*, and *argF* respectively, which are the three genes required to convert ornithine to arginine (106). PF0207 and PF0208 are predicted to be in the same operon as the *lysX* homologue PF0209, and are up-regulated under protein-limited conditions (104, 115).

Leucine

Leucine is produced from 2-ketovalerate, an intermediate in the valine biosynthetic pathway (128). The pathway from 2-ketovalerate to leucine involves four enzymes: 2-isopropylmalate synthase (LeuA), isopropylmalate isomerase (LeuC and LeuD), 3-isopropylmalate dehydrogenase (LeuB), and an aminotransferase (27, 128). *P. furiosus* contains a set of five genes annotated as *leuACDBA* (PF0937-PF0941), which are predicted to be co-transcribed (104, 106). On a separate location on the chromosome, there is a set of three genes annotated as *leuACD* (PF1678-PF1680) that are also predicted to be co-transcribed (104, 106). Both of these putative operons are up-regulated at the mRNA level under protein-limited

conditions (115). The genes PF1678-PF1680 are probably part of the lysine biosynthetic pathway given their proximity to other potential lysine biosynthesis genes and the previously noted sequence homology between LeuA, LeuD, LeuC and LysS, LysT, LysU in other organisms (41, 99, 106). If this is indeed the case, then PF0937-PF0941 are likely to be the true leucine biosynthesis genes. It is unclear why there would be two *leuA* homologues in the same operon. Notably, *P. abyssi* has an identical gene arrangement (PAB0890-PAB0894) (31).

One common transaminase that can catalyze the final step in leucine biosynthesis is the branched chain aminotransferase IlvE (63, 107). IlvE participates in the biosynthesis of all three branched chain amino acids, and is a member of the Class IV aminotransferases (48, 63, 98). Although it has been identified in other archaea (54, 146), there is no *ilvE* gene annotated in *P. furiosus* (106). A search of the *P. furiosus* genome using the Class IV aminotransferase signature sequence E-X-[STAGCI]-X(2)-n-[LIVMFAC]-[FY]-X(6,12)-[LIVMF]-X-T-X(6,8)-[LIVM]-X-[GS]-[LIVM]-X-[KR] confirmed that there were no homologues of IlvE in *P. furiosus* (10, 62, 63, 106). *P. horikoshii* and *P. abyssi* also have nearly complete leucine biosynthetic pathways, but like *P. furiosus*, they are missing an annotated *ilvE* gene (31, 70, 130). Another aminotransferase that has been shown to function *in vivo* for leucine biosynthesis is TyrB, a broad-specificity aromatic aminotransferase that is a member of the Class I aminotransferases (62, 103). There are several putative Class I aminotransferases annotated in *P. furiosus* (106). One of these, the AlaAT from *P. furiosus*, does show aminotransferase activity *in vitro* with leucine, as well as with isoleucine and valine (136). However, its activity with the branched chain amino acids is only 4% of the activity with its preferred substrate, alanine (136). There are several other uncharacterized aminotransferases in *P. furiosus*, one of which could potentially function in leucine biosynthesis.

Valine

Valine is produced from pyruvate in a pathway involving four enzymes: acetohydroxyacid synthase (AHAS), ketol-acid reductoisomerase (IlvC), dihydroxy-acid dehydratase (IlvD), and an aminotransferase (28, 128). There are annotated genes in *P. furiosus* for the large subunit of acetohydroxyacid synthase (PF0935), *ilvC* (PF0936), and *ilvD* (PF0942) (106). These three genes are found adjacent to predicted leucine biosynthetic genes and are predicted to be transcribed together as part of an eight gene operon (104, 106). The biosynthetic pathways and regulation of leucine, isoleucine, and valine are interrelated (128), so it would not be surprising for these genes to be coregulated. In addition, the putative operon is up-regulated under protein-limited conditions, as would be expected if their true function were in amino acid biosynthesis (115).

All fully characterized AHAS enzymes consist of two subunits, a large catalytic subunit and a small regulatory subunit (91). There is a homologue of the AHAS catalytic subunit in *P. furiosus*, but there is no homologue of the regulatory subunit anywhere in the genome (9, 106). The regulatory subunit shows significant sequence conservation across the three domains of life (91). For example, the regulatory subunit from *Methanococcus aeolicus* is 62-67% similar and 37-43% identical to the *Escherichia coli* K12 regulatory subunits, 66% similar and 44% identical to the first domain of the *Arabidopsis thaliana* regulatory subunit, and 67% similar and 43% identical to the second domain of the *A. thaliana* regulatory subunit (9, 22, 82, 139). So, a *P. furiosus* regulatory subunit would probably be detected by homology if it were present. The AHAS large subunit from *P. furiosus* is approximately the same size as the ones characterized in other organisms (22, 106, 139); it does not contain the conserved domain found in the small subunit (89). Thus, it appears that the *P. furiosus* AHAS is not a fusion between the large and

small subunits. It is possible that the *P. furiosus* AHAS does not require the small subunit for activity. Although the AHAS from *M. aeolicus* is believed to be heterogeneous in its native state, the homogeneous form of the enzyme consisting solely of catalytic subunits still showed high specific activity and sensitivity to product inhibition (145). *P. abyssi* is also annotated as having an AHAS large subunit, but it contains no homologue for the small subunit (9, 31).

As in leucine biosynthesis, it is unclear which enzyme is performing the final transamination step to produce valine in *P. furiosus*. The general branched chain aminotransferase IlvE is the terminal aminotransferase in many organisms (67, 107), but it is absent in *P.furiosus*. Some species can use a valine-pyruvate aminotransferase (AvtA) to catalyze the final step of valine biosynthesis (14, 67, 140). Of the aminotransferases annotated in *P. furiosus*, AroAT (PF0121) has the closest primary sequence to AvtA, but its activity with valine has not been tested (9, 11). As mentioned previously, the *P. furiosus* AlaAT shows some activity with valine, although it is unclear if this activity is biologically relevant (136). Alternatively, it is possible that one of the many uncharacterized aminotransferases can catalyze the formation of valine.

Isoleucine

There are several known pathways for the production of isoleucine (28, 67, 128). Most of pathways differ only in their method of producing the precursor α-ketobutyrate; the steps from α-ketobutyrate to isoleucine are the same (28, 128). The only known exception is an isoleucine biosynthetic pathway identified in the bacteria *Bacteroides fragilis* and *Prevotella ruminicola* in which isoleucine is produced from α-methylbutyrate (8). *P. furiosus* does not contain an annotated gene for threonine deaminase, which is required for the first step of the standard isoleucine biosynthetic pathway that produces α-ketobutyrate from threonine (106, 128). In fact,

of the five different isoleucine biosynthetic pathways described in MetaCyc, none are predicted to exist in *P. furiosus* (28). From α-ketobutyrate, the enzymes for the remainder of the isoleucine biosynthetic pathway are the same as the ones in the valine biosynthetic pathway (128). As with the leucine and valine pathways, the isoleucine biosynthetic pathway in *P. furiosus* seems to be lacking the final aminotransferase (28, 67, 106).

Phenylalanine, Tryptophan, and Tyrosine

The biosynthetic pathways for the aromatic amino acids phenylalanine, tryptophan, and tyrosine are related in that they all require chorismate as a precursor (128). *P. furiosus* contains annotated homologues for the genes involved in the biosynthesis of all three aromatic amino acids from chorismate, and for the production of chorismate from D-E4P and phosphoenolpyruvate (106, 128). The vast majority of these genes are clustered together on the *P. furiosus* chromosome, where they are predicted to be organized into two operons: the first containing ORFs PF1687-PF1694 and the second containing ORFs PF1699-PF1711 (104, 106). The first operon contains one conserved hypothetical protein whose role in amino acid biosynthesis is unclear, but the remainder of the ORFs in the two operons have annotations that are consistent with a role in aromatic amino acid biosynthesis (106). Both of these predicted operons are upregulated when *P. furiosus* is grown on maltose compared to when it is grown on peptides (115). An exception is the prephenate dehydratase (PF0291) in the phenylalanine biosynthetic pathway, which is not upregulated during growth on maltose and not is predicted to be part of an operon (104, 106, 115). In addition to the tryptophan synthase subunit β (PF1706) located in the second putative operon, there is also a second annotated tryptophan synthase subunit β (PF1592) in a separate location on the chromosome (104, 106).

The final step in phenylalanine and tyrosine biosynthesis involves a transamination reaction (128). *P. furiosus* contains two experimentally characterized aromatic amino acid aminotransferases, AroAT I (PF0121) and AroAT II (PF1253) (11, 135). AroAT I shows its highest level of activity with phenylalanine, although it does also show significant activity with tyrosine (11). AroAT II shows equivalent activity with all three aromatic amino acids (135). Interestingly, it is the aspartate aminotransferase AspAT (PF1702) that is located in the second putative aromatic amino acid biosynthesis operon (104, 106). AspAT does show some activity with the aromatic amino acids, although it is only 1% of its relative activity with its preferred substrate, aspartate (135).

D-E4P is a precursor in the standard chorismate biosynthetic pathway (128). The MetaCyc database predicts that *P. furiosus* is missing the nonoxidative branch of the pentose phosphate pathway, which is used to produce D-E4P (28). The absence of a D-E4P biosynthesis pathway is has been noted in other species of *Archaea*, some of which are thought to have functional aromatic amino acid biosynthesic pathways (122). It was recently demonstrated that the Euryarchaeotes *Methanocaldococcus jannaschii* and *Methanococcus maripaludis* can produce chorismate using an alternative pathway that does not involve D-E4P (102, 142). It is possible that *P. furiosus* can also make chorismate using an alternate pathway. The KEGG database shows *P. furiosus* as having a partial nonoxidative branch of the pentose phosphate pathway, including a N-terminal subunit (PF1688) and C-terminal subunit (PF1689) of transketolase (67). Transketolase can catalyze the conversion of glyceraldehyde-3-phosphate and fructose-6-phosphate into D-xylulose-5-phosphate and D-E4P (67). Although transketolase is typically encoded by a single gene in *Bacteria*, it is encoded by two separate genes in some organisms, especially *Archaea* (126). Notably, the two transketolase subunits in *P. furiosus* are

located together in the first putative operon of aromatic amino acid biosynthetic genes (104, 106).

Bioinformatics Summary

A microarray analysis of *P. furiosus* concluded that the putative operons for the biosynthesis of 12 amino acids (glutamic acid, arginine, leucine, valine, isoleucine, serine, threonine, methionine, histidine, phenylalanine, tryptophan, and tyrosine) are up-regulated at least fivefold in a medium with maltose as the primary carbon source compared to a medium using peptides as the primary carbon source (115). This is consistent with the operons' predicted roles in amino acid biosynthesis. **Table 2.2** lists all of the *P. furiosus* open reading frames (ORFs) mentioned in the previous sections on amino acid biosynthetic pathways, with the ORFs that were up-regulated in the microarray experiment during growth on maltose indicated in bold. There was one ORF, AroAT II, that was down-regulated during growth on maltose (115).

In several amino acid biosynthetic pathways in *P. furiosus*, such as the ones for lysine, leucine, valine, and isoleucine, the missing enzyme is an aminotransferase (28, 67, 106). Many prokaryotic aminotransferases have broad substrate specificity (11, 63, 96, 135, 136, 146). Some have been shown to function in multiple biosynthetic pathways *in vivo*. *P. furiosus* is annotated as having 15 aminotransferases, most of which have not been experimentally characterized (106). As more of these aminotransferases are characterized, it is likely that more amino acid biosynthetic pathways will be shown to be complete.

Table 2.2 – *P. furiosus* ORFs Potentially Involved in Amino Acid Biosynthesis

ORFs in **bold** were up-regulated, and ORFs in *italics* were down-regulated, in *P. furiosus* during growth on maltose compared to growth on peptides, as shown in the microarray experiment by Schut et al. (115).

ORF	**Abbreviation**	**NCBI Annotation** (141)	**Experimentally Determined Function**
PF0121	AroAT I	putative aspartate aminotransferase	aromatic aminotransferase (11, 135)
PF0202	**ICDH**	**isocitrate dehydrogenase**	**isocitrate dehydrogenase (123)**
PF0207		**argininosuccinate synthase**	
PF0208		**argininosuccinate lyase**	
PF0209		**ribosomal protein s6 modification protein**	
PF0291	PheA	prephenate dehydratase	
PF0293		putative aminotransferase	
PF0362		glucosamine-fructose-6-phosphate aminotransferase	
PF0513		4-aminobutyrate aminotransferase	
PF0522		aspartate transaminase	
PF0594		ornithine carbamoyltransferase	
PF0935	**AHAS**	**acetolactate synthase**	
PF0936	**IlvC**	**ketol-acid reductoisomerase**	
PF0937	**LeuA**	**2-isopropylmalate synthase**	
PF0938	**LeuC**	**3-isopropylmalate dehydratase large subunit**	
PF0939	**LeuD**	**3-isopropylmalate dehydratase small subunit**	
PF0940	**IPMDH / LeuB**	**3-isopropylmalate dehydrogenase 2**	

Table 2.2 continued – *P. furiosus* ORFs Potentially Involved in Amino Acid Biosynthesis

ORFs in **bold** were up-regulated, and ORFs in *italics* were down-regulated, in *P. furiosus* during growth on maltose compared to growth on peptides, as shown in the microarray experiment by Schut et al. (115).

ORF	**Abbreviation**	**NCBI Annotation** (141)	**Experimentally Determined Function**
PF0941	**LeuA**	**putative α-isopropylmalate/homocitrate synthase family transferase**	
PF0942	**IlvD**	**dihydroxy-acid dehydratase**	
PF1066		putative aminotransferase	
PF1185		acetylornithine deacetylase	
PF1232		4-aminobutyrate aminotransferase	
PF1253	*AroAT II*	*aspartate aminotransferase*	*aromatic aminotransferase (135)*
PF1421		4-aminobutyrate aminotransferase	
PF1472		aspartate/serine transaminase	
PF1497	AlaAT	alanine aminotransferase	alanine aminotransferase (136)
PF1592	**TrpB**	**tryptophan synthase β subunit**	
PF1665		**histidinol-phosphate aminotransferase**	
PF1678	**LeuA**	**2-isopropylmalate synthase**	
PF1679	**LeuC**	**3-isopropylmalate dehydratase large subunit**	
PF1680	**LeuD**	**3-isopropylmalate dehydratase small subunit**	
PF1681		hypothetical protein	
PF1682		**ribosomal protein s6 modification protein**	
PF1683		**N-acetyl-γ-glutamyl-phosphate reductase**	

Table 2.2 continued – *P. furiosus* ORFs Potentially Involved in Amino Acid Biosynthesis

ORFs in **bold** were up-regulated, and ORFs in *italics* were down-regulated, in *P. furiosus* during growth on maltose compared to growth on peptides, as shown in the microarray experiment by Schut et al. (115).

ORF	**Abbreviation**	**NCBI Annotation** (141)	**Experimentally Determined Function**
PF1684		**acetylglutamate kinase**	
PF1685		**acetylornithine/acetyl-lysine aminotransferase**	
PF1686		**acetyl-lysine deacetylase**	
PF1687		**hypothetical protein**	
PF1688		**transketolase N-terminal section**	
PF1689		**transketolase C-terminal section**	
PF1690		**3-deoxy-7-phosphoheptulonate synthase**	
PF1691	AroB	3-dehydroquinate synthase	
PF1692	**AroD**	**3-dehydroquinate dehydratase**	
PF1693	**AroE**	**shikimate 5-dehydrogenase**	
PF1694		**shikimate kinase**	
PF1699	**AroA**	**3-phosphoshikimate 1-carboxyvinyltransferase**	
PF1700	**AroC**	**chorismate synthase**	
PF1701		**chorismate mutase**	
PF1702	**AspAT**	**aspartate aminotransferase**	**aspartate aminotransferase (135)**
PF1703	**TyrA**	**prephenate dehydrogenase**	
PF1704		hypothetical protein	
PF1705	TrpA	tryptophan synthase α subunit	

Table 2.2 continued – *P. furiosus* ORFs Potentially Involved in Amino Acid Biosynthesis

ORFs in **bold** were up-regulated, and ORFs in *italics* were down-regulated, in *P. furiosus* during growth on maltose compared to growth on peptides, as shown in the microarray experiment by Schut et al. (115).

ORF	**Abbreviation**	**NCBI Annotation** (141)	**Experimentally Determined Function**
PF1706	**TrpB**	**tryptophan synthase β subunit**	
PF1707		N-(5'-phosphoribosyl)anthranilate isomerase	
PF1708	**TrpG**	**anthranilate synthase component II**	
PF1709	**TrpE**	**anthranilate synthase component I**	
PF1710	**TrpD**	**anthranilate phosphoribosyltransferase**	
PF1711	**TrpC**	**indole-3-glycerol-phosphate synthase**	
PF1906		adenosylmethionine-8-amino-7-oxononanoate aminotransferase	

CHAPTER 3

MATERIALS AND METHODS

Strains

P. furiosus DSM 3638 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, Germany. The particular stock of *P. furiosus* DSM 3638 used in these experiments was obtained from DSMZ in 2002 and maintained in the Adams Lab by periodic transfers into rich media approximately every 6 months.

Media Preparation

The rich medium ("RM" medium) was prepared by adding 700 mL of diH_2O, 200 ml of 5xBase Salts (140 g/L NaCl [Fisher #BP358], 17.5 g/L $MgSO_4 \cdot 7H_2O$ [JT Baker #2550], 13.5 g/L $MgCl_2 \cdot 6H_20$ [JT Baker #2444], 1.65 g/L KCl [JT Baker #3040], 1.25 g/L NH_4Cl [JT Baker #0666], and 0.70 g/L $CaCl_2 \cdot 2H_20$ [Sigma #C3881] in diH_2O; sterile solution), 1.0 mL of Trace Minerals (1.00 ml/L 12.1M HCl [JT Baker #9535], 0.50 g/L Na_4EDTA [Sigma #E6511], 2.00 g/L $FeCl_3 \cdot 6H_2O$ [Sigma #F2877], 0.05 g/L H_3BO_3 [Sigma #B9645], 0.05 g/L $ZnCl_2$ [Sigma #Z4875], 0.03 g/L $CuCl_2 \cdot 2H_2O$ [JT Baker #1792], 0.05 g/L $MnCl_2 \cdot 4H_2O$ [JT Baker #2540], 0.05 g/L $(NH_4)_2MoO_4$ [Sigma #7302], 0.05 g/L $AlK(S0_4)_2 \cdot 12H_20$ [Sigma #A7167], 0.05 g/L $CoCl_2 \cdot 6H_2O$ [Sigma #C3169], 0.05 g/L $NiCl_2 \cdot 6H_2O$ [Aldrich #203866] in diH_2O; sterile solution), 0.1 mL of 100 mM $Na_2WO_4 \cdot 2H_20$ (33 g/L $Na_2WO_4 \cdot 2H_20$ [Sigma # S0765] in diH_2O; sterile solution), and 0.05 mL Resazurin (5 g/L Resazurin sodium salt [Sigma #R2127] in diH_2O). 0.5 g L-Cysteine • HCl and 0.5 g Na_2S were added as reductants, and 1.0 g $NaHCO_3$ [JT Baker #3506-05] was added as a CO_2 source. The pH was adjusted to 6.8 at room temperature, and the total volume was adjusted to 1 liter with diH_2O. The medium was sterilized by filtering

through a 0.22 μm Millipore Steritop vacuum filter [Fisher #SCGPT05RE]. The medium was then transferred as 40 mL aliquots into 100 mL serum bottles, and sealed with septum stoppers [Bellco Glass #204811800] held by 20 mm aluminum seals [Wheaton #224178-01]. The bottles were degassed with 99.998% Argon [National Welders #44841], and stored at room temperature until use. Immediately before use, 2.0 mL 10% Yeast Extract (100 g/L yeast extract [Difco #DF0886-08] in diH_2O; anaerobic and sterile solution), 2.0 mL 10% Casein (100g/L casein enzymatic hydrolysate [USB # 12855] in diH_2O; anaerobic and sterile solution), 0.4 mL of 50% Maltose (500 g/L ≥95% pure D-(+)-Maltose monohydrate [Sigma #M2250] in diH_2O); anaerobic and sterile solution), and 0.04 mL 1M Potassium Phosphate buffer, pH 6.8 (Monobasic potassium phosphate crystal [JT Baker #3246] and Dibasic potassium phosphate powder [JT Baker #3252] in diH_2O; anaerobic and sterile solution) were added aseptically to each serum bottle of media.

The defined medium containing all 20 amino acids ("20AA" medium) was prepared by adding 700 mL of diH_2O, 200 ml of 5x Base Salts, 1 mL of Trace Minerals, 5 mL of Vitamin Solution (10 mg/L Niacin [Sigma #N4126], 4 mg/L D-Biotin [USB #12115],10 mg/L DL-Pantothenic Acid hemicalcium salt [Sigma #P-9153], 10 mg/L Lipoic Acid [Sigma #T1395], 4 mg/L Folic Acid [Sigma #F8758], 10 mg/L *p*-Aminobenzoic Acid potassium salt [Sigma #A0250], 10 mg/L Thiamine • HCl [Sigma #T4625], 10 mg/L Riboflavin [Sigma #R9504], 10 mg/L Pyridoxine [Sigma #P5669], 10 mg/L Cobalamin [Sigma #C0884] in diH_2O; sterile solution), 0.1 mL of 100 mM $Na_2WO_4 \cdot 2H_20$, 0.05 mL of 5 g/L Resazurin, and amino acids (75 mg L-Alanine [Sigma #A7627], 125 mg L-Arginine • HCl [Fisher #BP372], 50 mg L-Aspartic acid [Fisher #BP374], 100 mg L-Asparagine • H_2O [Fisher #BP373], 200 mg L-Glutamic acid monosodium salt [Sigma #G-1626], 50 mg L-Glutamine [Sigma #G5763], 200 mg L-Glycine

[Sigma #G7403], 100 mg L-Histidine • HCl • H_2O [Sigma #H8000], 100 mg L-Isoleucine [Fisher #BP384], 100 mg L-Leucine [Fisher #BP385], 100 mg L-Lysine • HCl [Fisher #BP386], 75 mg L-Methionine [Fluka #64319], 75 mg L-Phenylalanine [Fisher BP391], 125 mg L-Proline [Fisher #BP392], 75 mg L-Serine [Fisher #BP393], 100 mg L-Threonine [Fisher #BP394], 75 mg L-Tryptophan [Fisher #BP395], 50 mg L-Tyrosine [Sigma #T3754], 50 mg L-Valine [Fisher #BP397]). 0.5 g L-Cysteine • HCl and 0.5 g Na_2S were added as reductants, and 1.0 g $NaHCO_3$ [JT Baker #3506-05] was added as a CO_2 source. The pH was adjusted to 6.8 at room temperature, and the total volume was adjusted to 1 liter with diH_2O. Serum bottles of media were prepared as with the rich medium, except that the solutions added after degassing were 0.4 mL of 50% ultrapure maltose (500 g/L ≥99% pure D-(+)-Maltose monohydrate [Sigma #M9171] in diH_2O; anaerobic and sterile solution) and 0.04 mL 1M potassium phosphate buffer, pH 6.8 (monobasic potassium phosphate crystal [JT Baker #3246] and dibasic potassium phosphate powder [JT Baker #3252] in diH_2O; anaerobic and sterile solution) These solutions were added aseptically to each serum bottle of media.

The defined medium containing 9 amino acids ("9AA" medium) was prepared as with the 20AA medium, except that the initial amino acids included only 75 mg L-Alanine, 125 mg L-Arginine, 50 mg L-Aspartic acid, 200 mg L-Glycine, 200 mg L-Glutamic acid monosodium salt, 100 mg L-Lysine • HCl, 125 mg L-Proline, and 75 mg L-Serine. (The ninth amino acid, 0.5 g L-Cysteine • HCl, was added later in the protocol as a reductant, as was done previously.)

The defined medium containing 6 amino acids ("6AA" medium) was prepared as with the 20AA medium, except that the initial amino acids included only 125 mg L-Arginine, 200 mg L-Glycine, 100 mg L-Lysine • HCl, 125 mg L-Proline, and 75 mg L-Serine. (The sixth amino

acid, 0.5 g L-Cysteine • HCl, was added later in the protocol as a reductant, as was done previously.)

The defined medium containing cysteine as the only amino acid ("Cys" medium) was prepared as with the 20AA medium, except that no amino acids were included other than the 0.5 g L-Cysteine • HCl.

Alterations to the standard media recipes are described in the text.

Growth Conditions

Unless otherwise specified, bottles were inoculated with 0.4 mL of *P. furiosus* overnight culture. *P. furiosus* was routinely grown in a 98 ^{o}C stationary incubator. Growth was monitored by direct cell counts using an Olympus BX41 light microscope under 400x magnification using a Petroff-Hausser Counting Chamber [Hausser Scientific #3900].

Storage Conditions

P. furiosus was routinely stored at room temperature as liquid cultures in sealed 100mL serum bottles.

For long term storage, overnight cultures were mixed 1:1 with a sterile solution of 40% glycerol in 1x Base Salts (28 g/L NaCl, 3.5 g/L $MgSO_4 \cdot 7H_2O$, 2.7 g/L $MgCl_2 \cdot 6H_20$, 0.33 g/L KCl, 0.25 g/L NH_4Cl, and 0.14 g/L $CaCl_2 \cdot 2H_20$) and frozen at -80 ^{o}C. For aerobic storage, 0.5 mL of overnight culture and 0.5 mL of 40% glycerol in 1x Base Salts were aliquoted into 1.5 mL screw-cap tubes [Phenix #SCS-015FS], mixed thoroughly by inverting, and stored at -80 ^{o}C immediately. For anaerobic storage, the 40% glycerol in 1x Base Salts solution was degassed in a sterile side-arm flask by stirring under vacuum for several hours, then replacing the head space with 99.998% Argon. 0.5 mL of overnight culture and 0.5 mL of degassed 40% glycerol in 1x Base Salts solution were transferred anaerobically to 2 mL glass vials [National Scientific

#B7999-1] sealed with 5x11mm sleeve stoppers [Wheaton #224100-020] containing 99.998% Argon. The cells were mixed thoroughly by inverting, and stored at -80 ^{o}C immediately.

Bioinformatics Analysis

Homology searches were performed using the Basic Local Alignment Search Tool (BLAST) algorithm from the National Center for Biotechnology Information (NCBI) web site (9): http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome General homology searches were performed with the blastp program using the default parameters (BLOSUM62 scoring matrix, Gap Existence Cost of 11 and Gap Extension Cost of 1, and conditional compositional score matrix adjustment) unless otherwise specified. Homology searches based on a known Signature Sequence were performed using the PHI-BLAST program using the default parameters (BLOSUM62 scoring matrix, Gap Existence Cost of 11 and Gap Extension Cost of 1, and a PSI-BLAST threshold of 0.005) unless otherwise specified.

CHAPTER 4

CHARACTERIZATION OF *PYROCOCCUS FURIOSUS* GROWTH IN DEFINED MEDIA

Introduction

The first step of this project was to design a defined medium for *P. furiosus*. The next step was to selectively remove amino acids from the defined medium to determine which amino acid supplements are absolutely required for growth. This information will either provide confirmation for the existence of the amino acid biosynthetic pathways predicted by bioinformatics analysis, or it will highlight contradictions with the bioinformatics analysis that can be used as a starting point for further experimentation.

Determining the minimal growth requirements of *P. furiosus* will provide valuable information about its metabolic capabilities. However, for some experiments, it is better to have a defined medium that supports fast, high density growth rather than one that contains the absolute minimal requirements for growth. To that end, the growth of *P. furiosus* on the various defined media is compared to its growth on a standard rich medium ("RM" medium) containing yeast extract, casein, and maltose. The defined media that showed high density growth comparable to the rich medium, along with the defined medium that contains the absolute minimum number of amino acids, are characterized in terms of their doubling times and maximum cell densities. In addition, storage conditions are tested to determine a good method to preserve *P. furiosus* cultures adapted to defined media.

Defined Medium with 20 Amino Acids

The first step in characterizing the growth of *P. furiosus* under defined nutrient conditions was testing its growth in a defined medium that contained all twenty amino acids ("20AA" medium). The 20AA medium was based on the standard growth medium used for *P. furiosus* and a defined medium developed for *T. kodakaraensis* (112). Maltose was used as the primary carbon and energy source. All of the 20AA cultures were grown for 24 hours at 98 ^{o}C. A stock culture of *P. furiosus* in rich medium ("RM" medium) was used as the inoculum for the first transfer into the 20AA medium. After 24 hours of growth, the first transfer was used to inoculate the second transfer. Similarly, all subsequent transfers were inoculated from the previous transfer. A schematic diagram of this procedure is shown in **Figure 4.1**. Each transfer was performed in triplicate. The growth was followed for nine transfers and was measured by direct cell counts. The results of the 20AA endpoint growth experiment are shown in **Figure 4.2**.

P. furiosus grew to a cell density of $1.5x10^{8}$- $4x10^{8}$ cells/mL after 24 hours of growth in the 20AA medium at 98 ^{o}C. For comparison, *P. furiosus* typically reaches a final cell density of $4.5x10^{8}$ cells/mL after 12-15 hours in RM at 98^{o}C. Growth persisted for all nine transfers in the 20AA media. This suggested that the 20AA medium included all of the required components for growth of *P. furiosus*, and that the growth was not due to carryover of nutrients from the RM inoculum.

The growth parameters of *P. furiosus* in 20AA medium were further analyzed by performing growth curves. The inoculums for the growth curves was a culture of *P. furiosus* that had been stored in RM medium and was subsequently transferred 3x in 20AA medium. Triplicate cultures of 20AA media were inoculated at hour 0. Cell counts were performed on

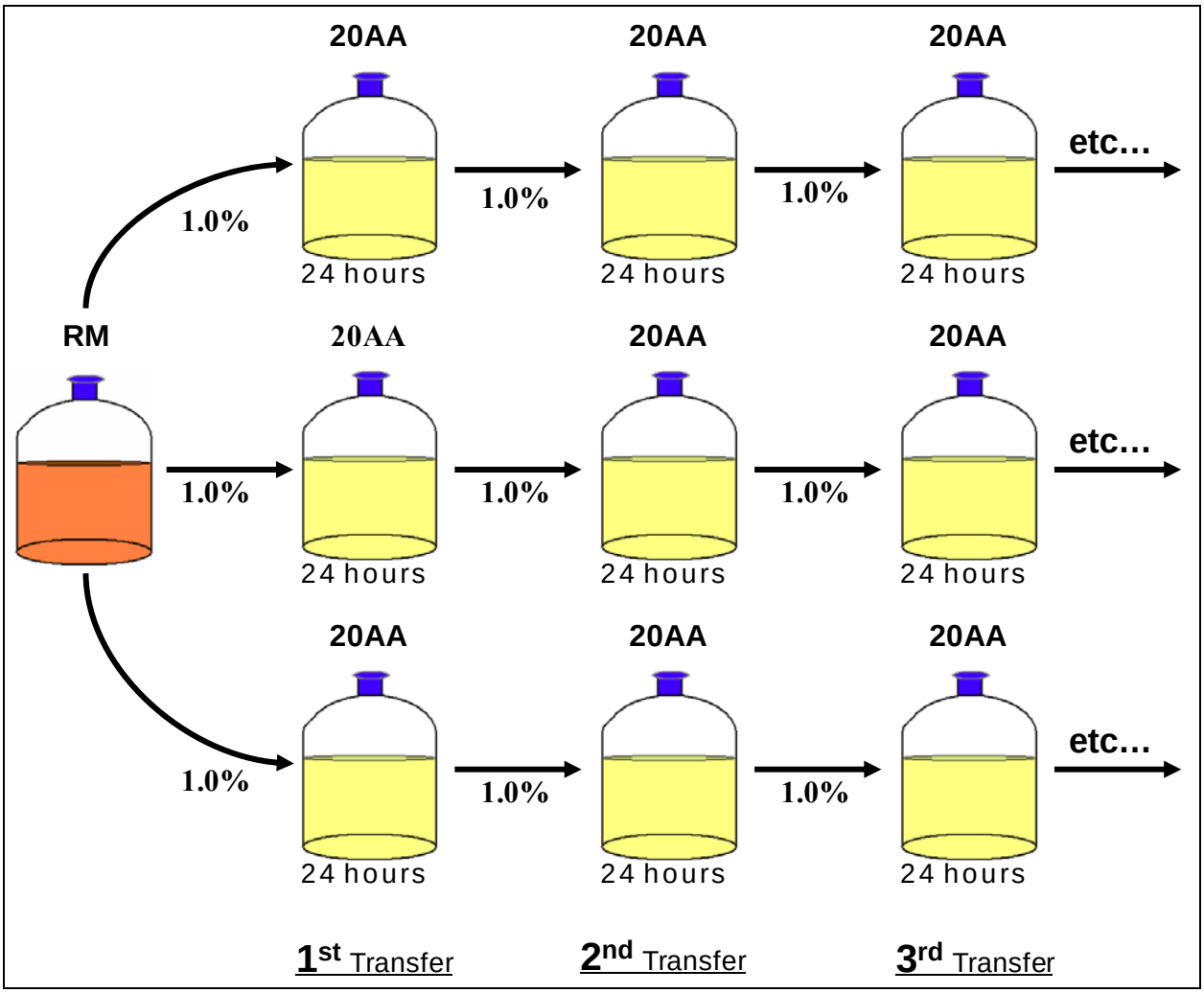

Figure 4.1 – Inoculation Procedure for Endpoint Growth Experiments

This diagram illustrates the procedure used for the 20AA defined medium. A similar procedure was used for testing the other defined media.

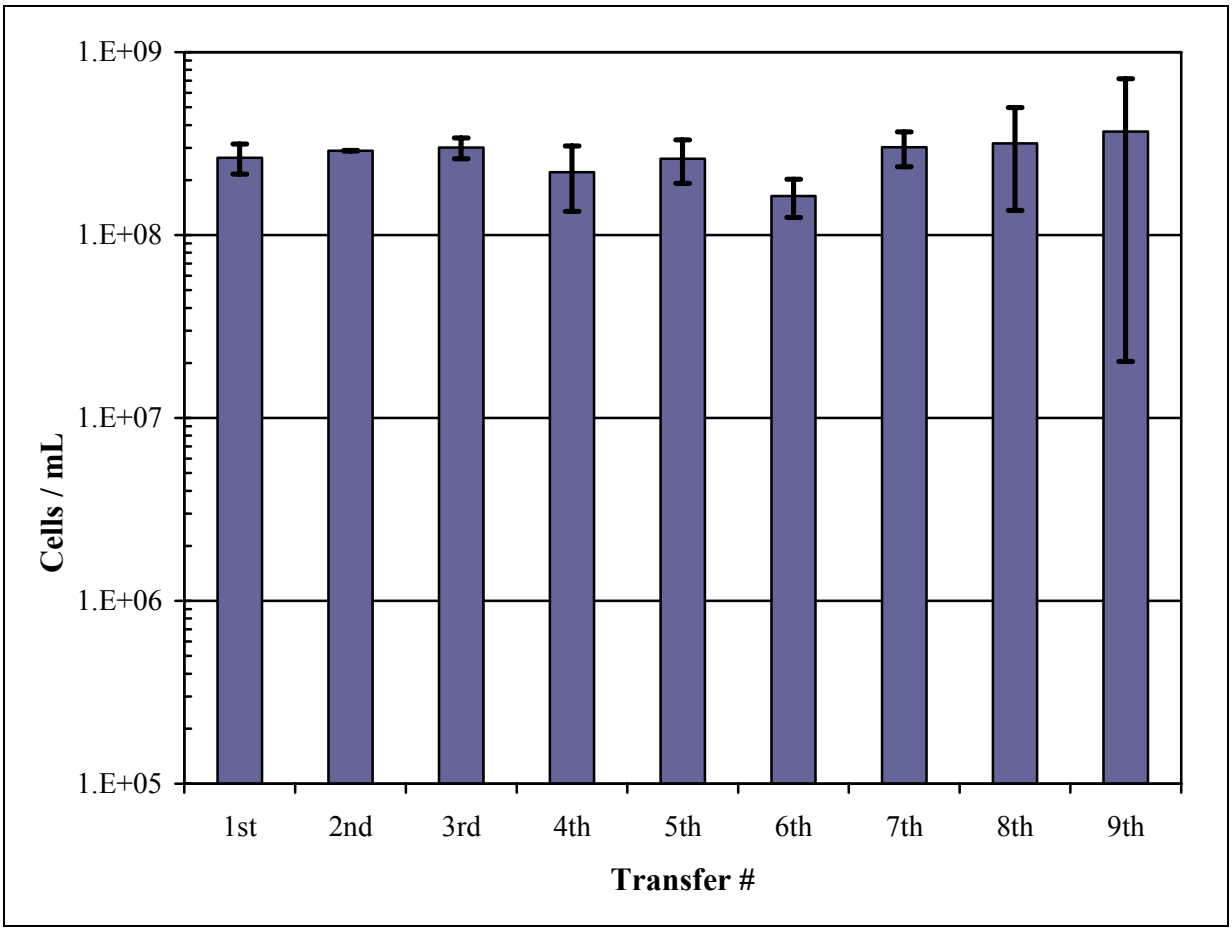

Figure 4.2 – Endpoint Growth Measurements of *P. furiosus* in 20AA Medium

Each bar represents the average of three independent cultures. Cell densities were determined by direct cell counts at the end of a 24 hour growth period at 98 ^{o}C. Error bars represent ±1 standard deviation.

these cultures every 3 hours for 24 hours. A schematic diagram of the set-up for the growth curves is shown in **Figure 4.3**. Growth curves were repeated on three separate days. The results of the three growth curves of *P. furiosus* in 20AA medium are shown in **Figure 4.4**.

The maximum cell density obtained by *P. furiosus* in 20AA medium ranged from $1.75x10^8$ to $4x10^8$ cells/mL. The cultures required 15-18 hours of growth at 98 ^oC to reach their maximum density. The doubling time was 100-130 minutes. In RM, the doubling time is typically 75-90 minutes. The observed lag phase in 20AA medium was approximately 3 hours, although this is only a rough estimate given the time intervals between measurements.

Defined Medium with 9 Amino Acids

The second medium tested was a defined medium that contained only 9 of the amino acids ("9AA" medium): arginine, cysteine, glycine, lysine, proline, and serine. Maltose was used as the carbon and energy source. For comparison, cultures in 20AA medium were grown alongside the culture in 9AA medium as a positive control. The first transfer was inoculated by a stock culture of *P. furiosus* in RM. After growing for 24 hours at 98 ^oC, the first transfer was used to inoculate the second transfer. The second was used to inoculate the third transfer, and so on, as was done for the original 20AA tests. Each transfer was performed in triplicate. All of the cultures were grown for 24 hours at 98 ^oC. The growth was followed for four transfers. Growth was measured by direct cell counts. The results of the 9AA endpoint growth experiment are shown in **Figure 4.5**.

In the 20AA medium, *P. furiosus* grew to a cell density of $1.5x10^8$- $4x10^8$ cells/mL after 24 hours at 98 ^oC. The maximum cell density showed little variation from one transfer to the next. This is in contrast to the growth pattern of *P. furiosus* in the 9AA medium. For the first transfer, the cultures in 9AA medium reached a cell density of $1.0x10^8$ cells/mL after 24 hours at

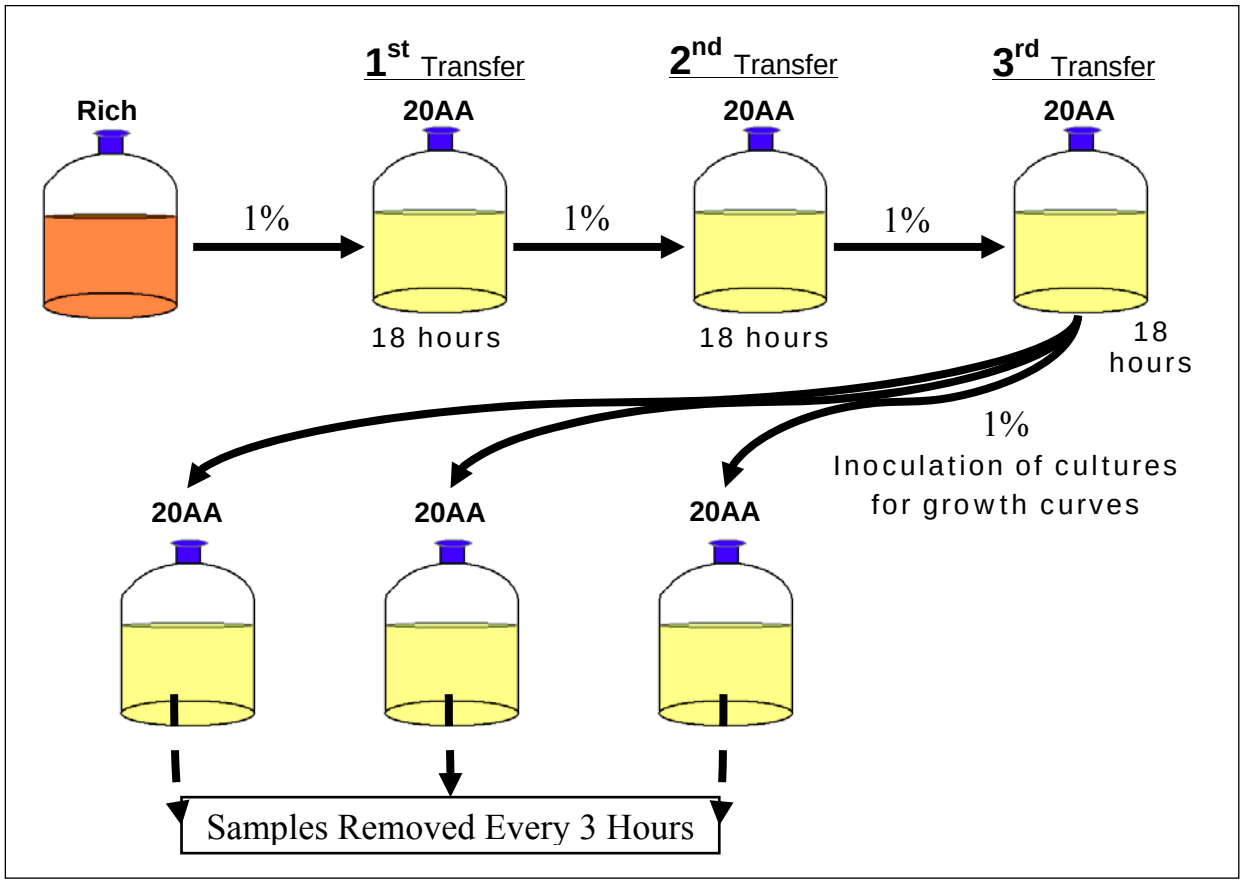

Figure 4.3 – Inoculation Procedure for Growth Curves

This diagram illustrates the procedure used for the 20AA defined medium. A similar procedure was used for testing the other defined media.

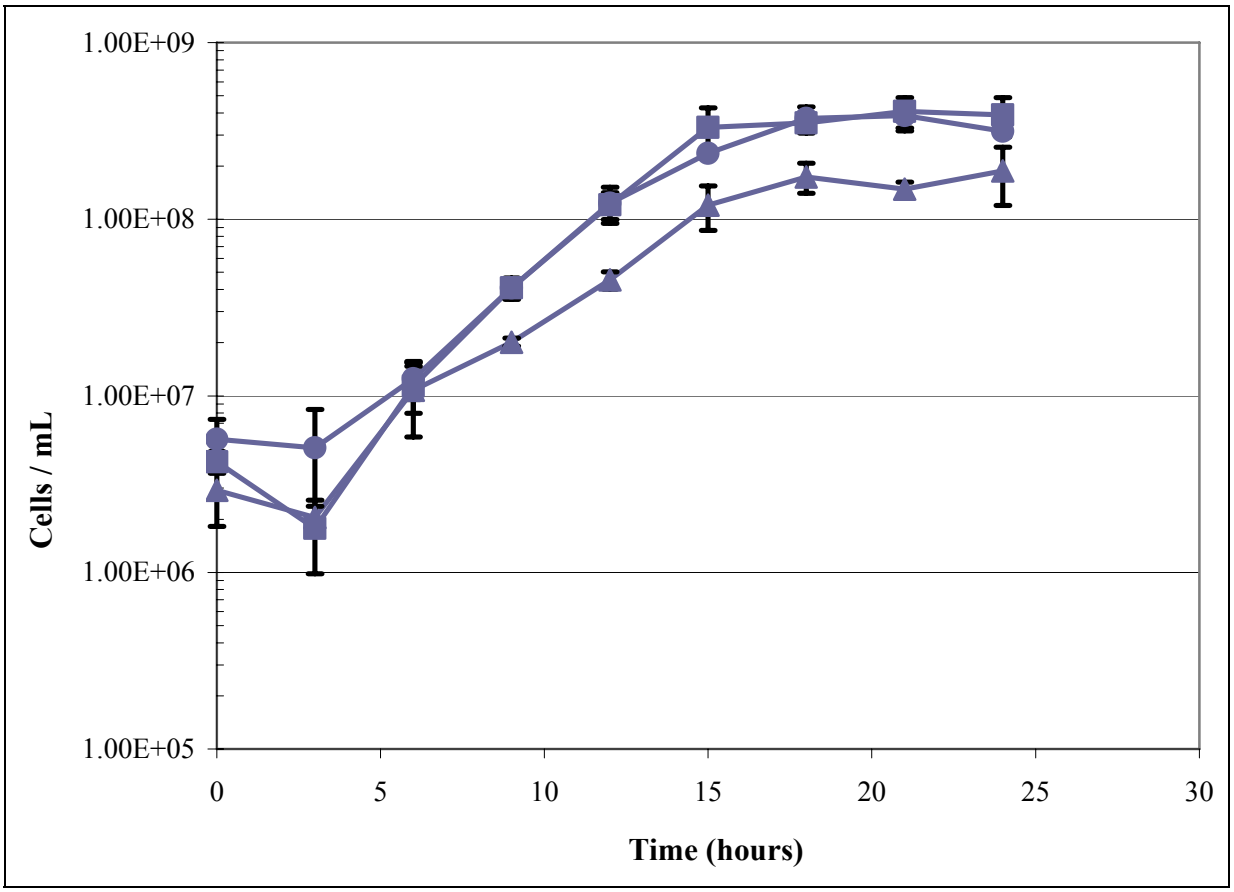

Figure 4.4 – Growth Curves of *P. furiosus* in 20AA Medium

Each series represents the average of independent three cultures. Cell densities were determined by direct cell counts at 3 hour intervals during growth at 98 ^{o}C. Error bars represent ±1 standard deviation. (●) growth curve performed on May 11th, 2006. (▲) growth curve performed on August 1st, 2006. (■) growth curve performed September 20th, 2006. The three experiments were otherwise identical.

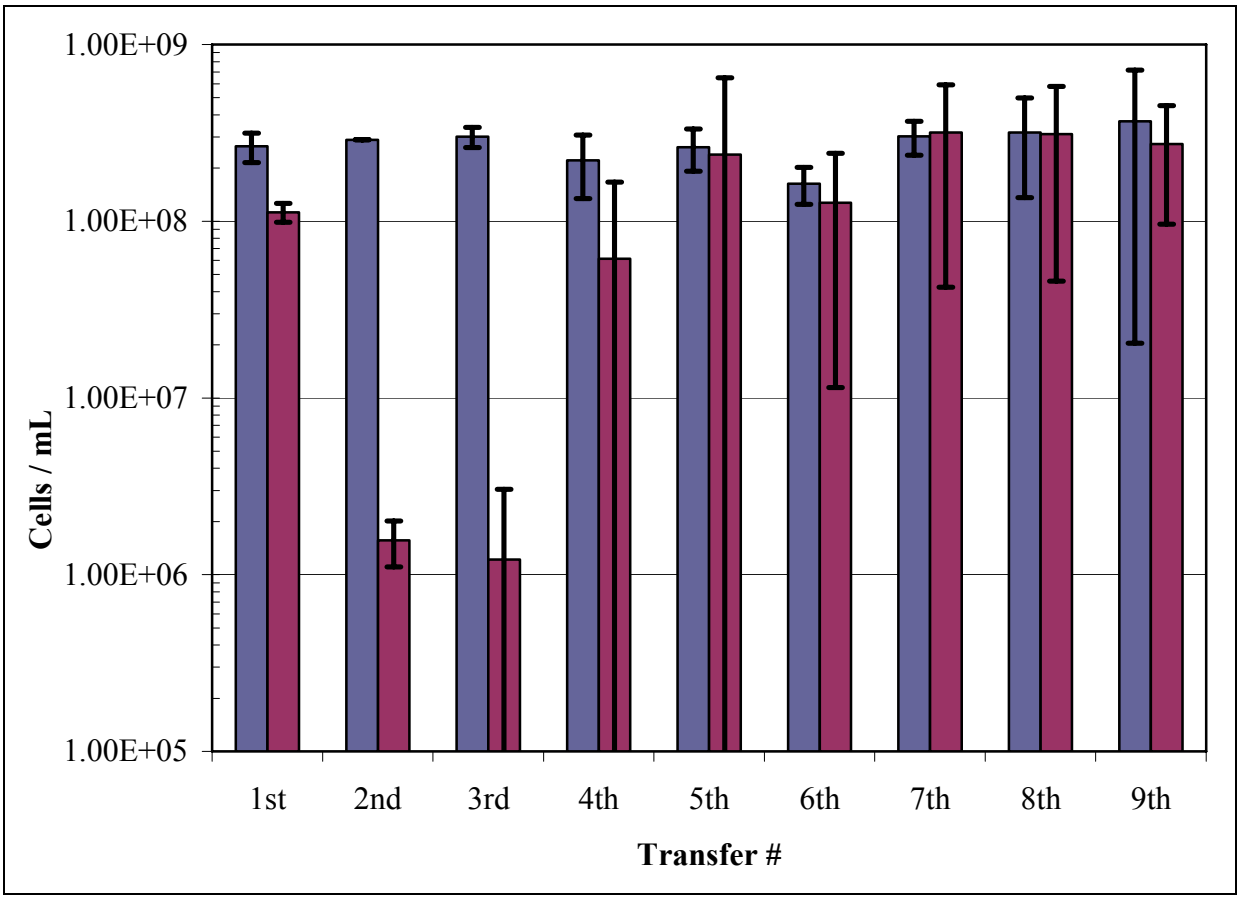

Figure 4.5 – Endpoint Growth Measurements of *P. furiosus* in 20AA and 9AA Medium

Each bar represents the average of three independent cultures. Cell density determined by direct cell counts at the end of a 24 hour growth period at 98 ^{o}C. Error bars represent ±1 standard deviation. Blue bars ( ) grown in 20AA medium. Purple bars ( ) grown in 9AA medium.

98 ^{o}C. However, for the second transfer, the final cell density was 1.5x10^{6} cells/mL, which corresponds to less than one doubling in 24 hours. For the third transfer, the final cell density remained low - only 1.25x10^{6} cells/mL. Remarkably, the growth of the 9AA cultures starts to improve between the fourth and sixth transfers. The large error bars for the fourth and fifth transfers are due to the fact that the growth of the three 9AA cultures do not recover at the same time. The cell density eventually stabilized at 2.75x10^{8} cells/mL. The growth pattern observed for the 9AA was reproducible, as the same pattern appeared when the experiment was repeated.

Defined Medium with 6 Amino Acids

The next medium tested was a defined medium that contained only 6 amino acids ("6AA" medium): arginine, cysteine, glycine, lysine, proline, and serine. Maltose was used as the carbon and energy source. For comparison, cultures in 20AA medium were grown alongside the 6AA medium. The first transfer for one set of cultures was inoculated by a stock culture of *P. furiosus* in RM. The first transfer for another set was inoculated from a culture of *P. furiosus* that had been grown for 6 transfers in the 9AA medium. Subsequent transfers were performed as in the previous experiments. Each transfer was performed in triplicate. All of the cultures were grown for 24 hours at 98 ^{o}C. The growth was followed for four transfers. Growth was measured by direct cell counts. The results of the 6AA endpoint growth experiment are shown in **Figure 4.6**.

In the 20AA medium, *P. furiosus* grew to a cell density of 2x10^{8}- 3x10^{8} cells/mL after 24 hours of growth, which is similar to what was observed in previous experiments. There was not a large difference between the 20AA cultures inoculated from RM and the ones inoculated from 9AA medium. An interesting pattern emerged with the cultures grown in 6AA medium. The

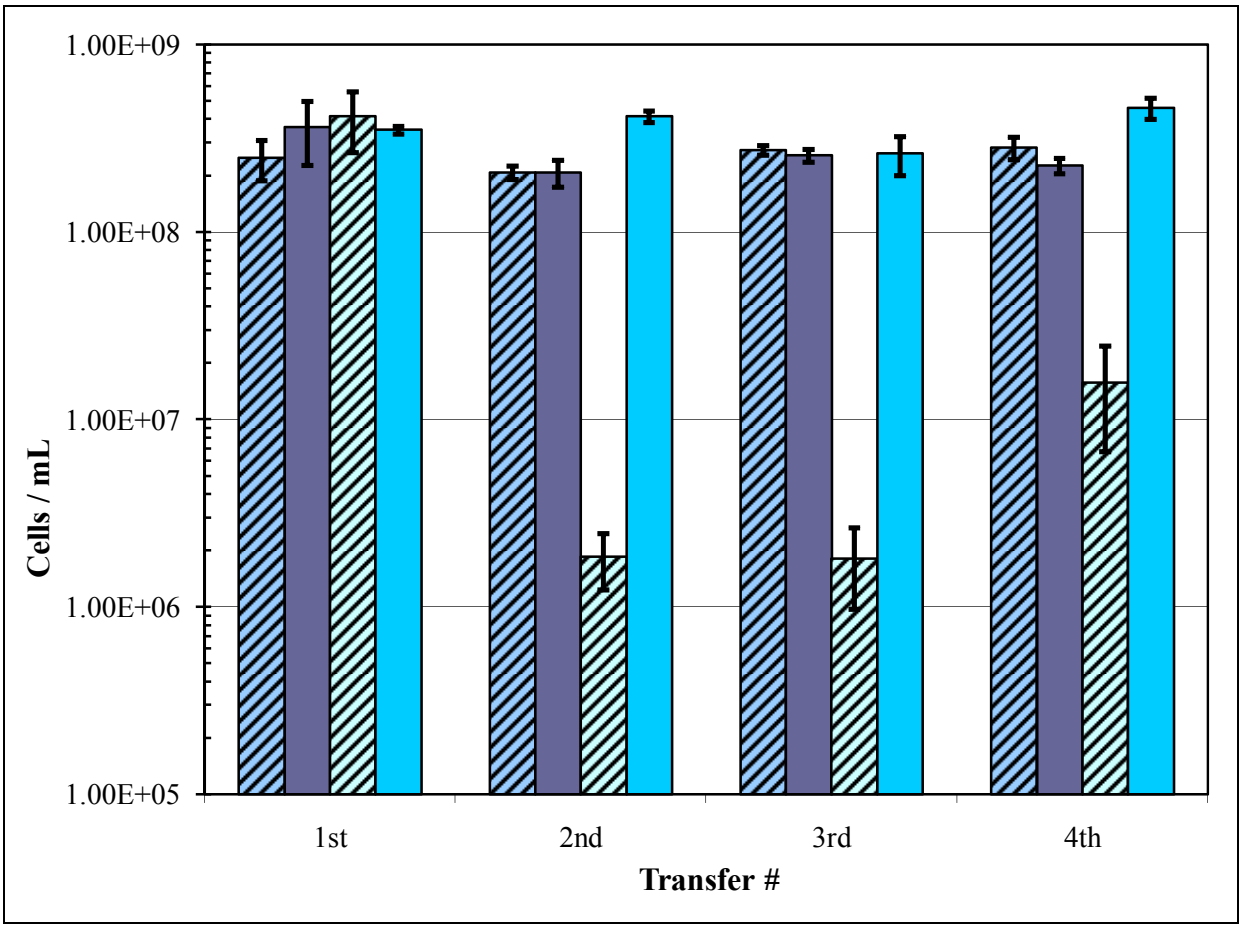

Figure 4.6 – Endpoint Growth Measurements of *P. furiosus* in 20AA and 6AA Medium

Each bar represents the average of three independent cultures. Cell density determined by direct cell counts at the end of a 24 hour growth period at 98 °C. Error bars represent ±1 standard deviation. Dark blue bars (,) grown in 20AA medium. Light blue bars (,) grown in 6AA medium. Diagonal striped bars inoculated from RM. Solid bars inoculated from 9AA medium.

6AA cultures that were inoculated from RM grew well in the first transfer, but grew poorly in the second transfer. The growth did improve somewhat by the fourth transfer, reaching a density of $1.5x10^7$ cells/mL. This is similar to the growth pattern observed with cultures in 9AA medium that were inoculated from RM. However, the 6AA cultures inoculated from 9AA medium grew well for all four transfers, reaching a maximum density of $2.5x10^8$- $4.5x10^8$ cells/mL after 24 hours. The type of media used for the inoculum culture affects how *P. furiosus* behaves when transferred to the 6AA medium.

Defined Medium with 1 Amino Acid

The final medium was a defined medium that contained only 1 amino acid, cysteine ("1AA" medium). Maltose was used as the carbon and energy source. For comparison, cultures in 20AA medium were grown at the same time. One set of cultures was inoculated by a stock culture of *P. furiosus* in RM, and another set was inoculated by a culture of *P. furiosus* grown in 6AA medium. Subsequent transfers were performed as in the previous experiments. Each transfer was performed in triplicate. All of the cultures were grown for 24 hours at 98 ^oC. Growth of all of the cultures was followed for nine transfers. Growth was measured by direct cell counts. The results of the 1AA endpoint growth experiment are shown in **Figure 4.7**.

The *P. furiosus* cultures in 20AA medium grew to a density of $1.5x10^8$- $4x10^8$ cells/mL, with little difference between the cultures started from RM and 6AA medium. The growth of *P. furiosus* in the 1AA did depend on the source of the initial inoculum. Cultures that were started from the RM stock showed little or no growth in the 1AA medium. The cultures started from the 6AA medium did grow in the 1AA medium, although the densities reached were quite variable. The growth of the 1AA cultures were followed for five additional transfers, for a total of fourteen transfers to see if the growth would stabilize. These results are shown in **Figure 4.8**.

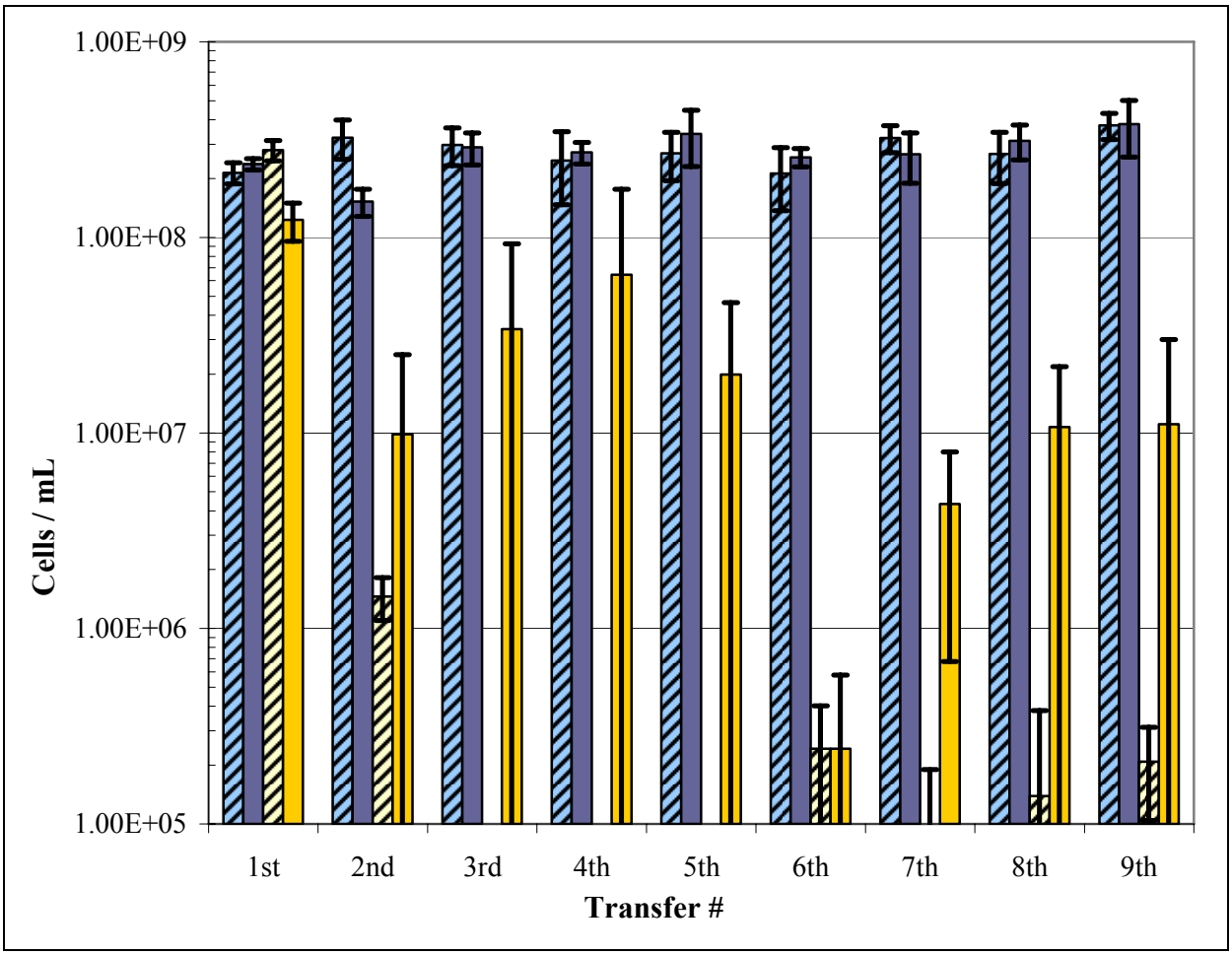

Figure 4.7 – Endpoint Growth Measurements of *P. furiosus* in 20AA and 1AA Medium

Each bar represents the average of three independent cultures. Cell density determined by direct cell counts at the end of a 24 hour growth period at 98 ^{o}C. Error bars represent ±1 standard deviation. Dark blue bars (,) grown in 20AA medium. Yellow bars (,) grown in 1AA medium. Diagonal striped bars inoculated from RM. Solid bars inoculated from 6AA medium.

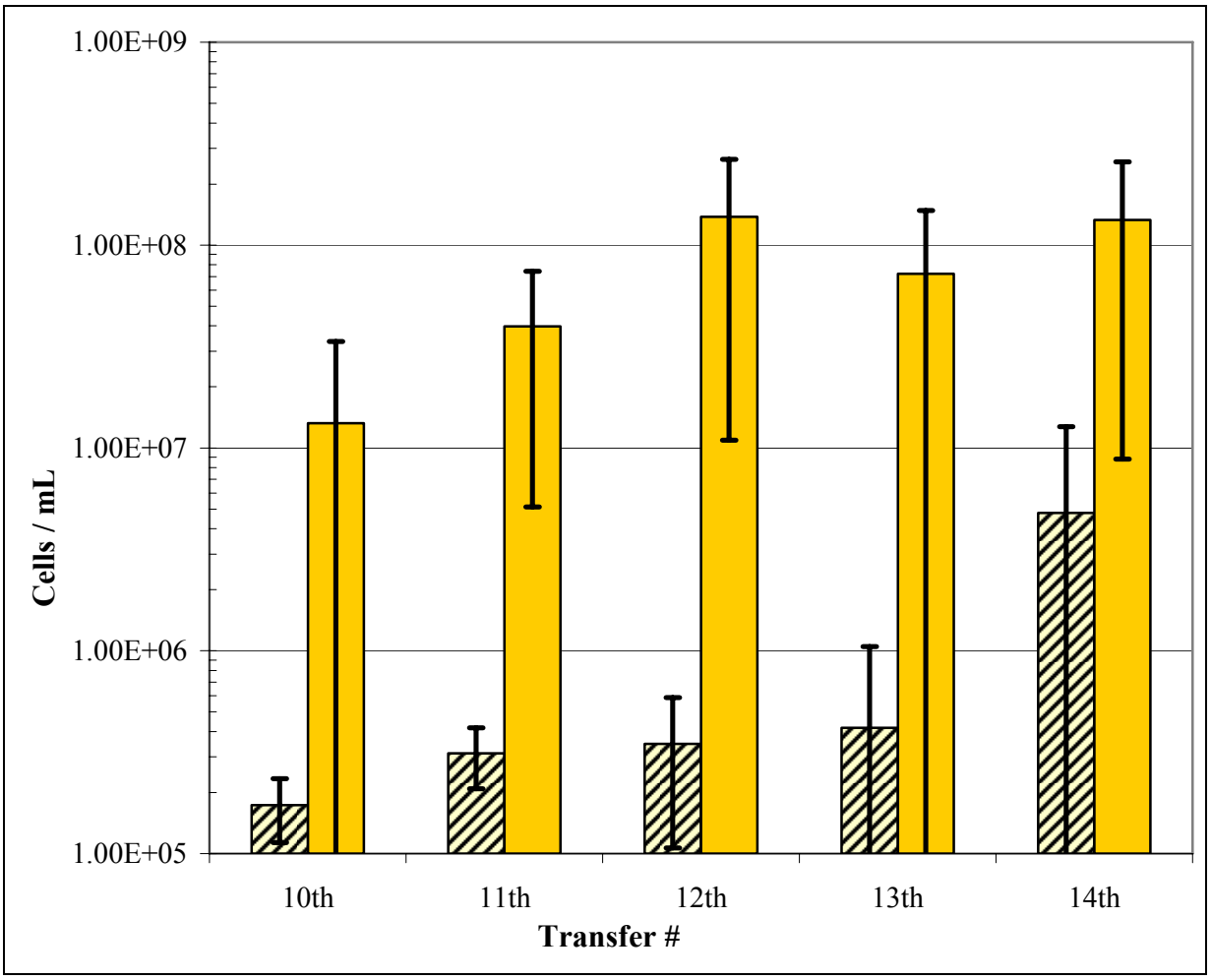

Figure 4.8 – Endpoint Growth Measurements of *P. furiosus* in 1AA Medium; Transfers 10-14.

Each bar represents the average of three independent cultures. Cell density determined by direct cell counts at the end of a 24 hour growth period at 98 °C. Error bars represent ±1 standard deviation. Yellow bars (,) grown in 1AA medium. Diagonal striped bars inoculated from RM. Solid bars inoculated from 6AA medium.

For the 1AA cultures that were initially inoculated from the 6AA medium, the growth did seem to stabilize at $1x10^8$ cells/mL. The 1AA cultures that were initially inoculated from RM continued to show poor growth.

All of the growth media used for *P. furiosus* contains 0.5 g/L (approximately 4.7 mM) of NH_4Cl as part of the salt solution. In the 1AA medium, the only other nitrogen source is cysteine, which is present at a concentration of 0.5 g/L (approximately 4.1 mM). The following experiment was designed to test if *P. furiosus* is using NH_4Cl or cysteine as a nitrogen source during growth on the 1AA medium: Growth of cultures in the standard 1AA medium were compared to the grown of cultures in a modified 1AA medium that lacks NH_4Cl. The inoculum culture was a 1AA culture that was transferred 13 times in the standard 1AA medium. Five transfers were performed as in previous experiments. The growth was measured by direct cell counts. The results of the endpoint growth experiments comparing growth in 1AA medium with and without NH_4Cl are shown in **Figure 4.9**.

If NH_4Cl were the only nitrogen source being used *P. furiosus* by in the 1AA medium, then the cultures would show little or no growth in the modified 1AA medium lacking NH_4Cl. This experiment showed that the *P. furiosus* cultures consistently reached a moderate final cell density of $5.5x10^7$- $8.25x10^7$ cells/mL in the 1AA medium without NH_4Cl. The cultures in the standard 1AA medium reached a final density of $5x10^7$- $2x10^8$ cells/mL. (The large error bars for the 5^{th} transfer was due to the fact that two out of the three cultures failed to grow.) Thus, the nitrogen provided by cysteine in the 1AA medium is sufficient to maintain growth in the absence of NH_4Cl, although perhaps not to the same cell density as the equivalent 1AA medium containing 4.7 mM NH_4Cl. Further experiments are needed to determine if the difference in

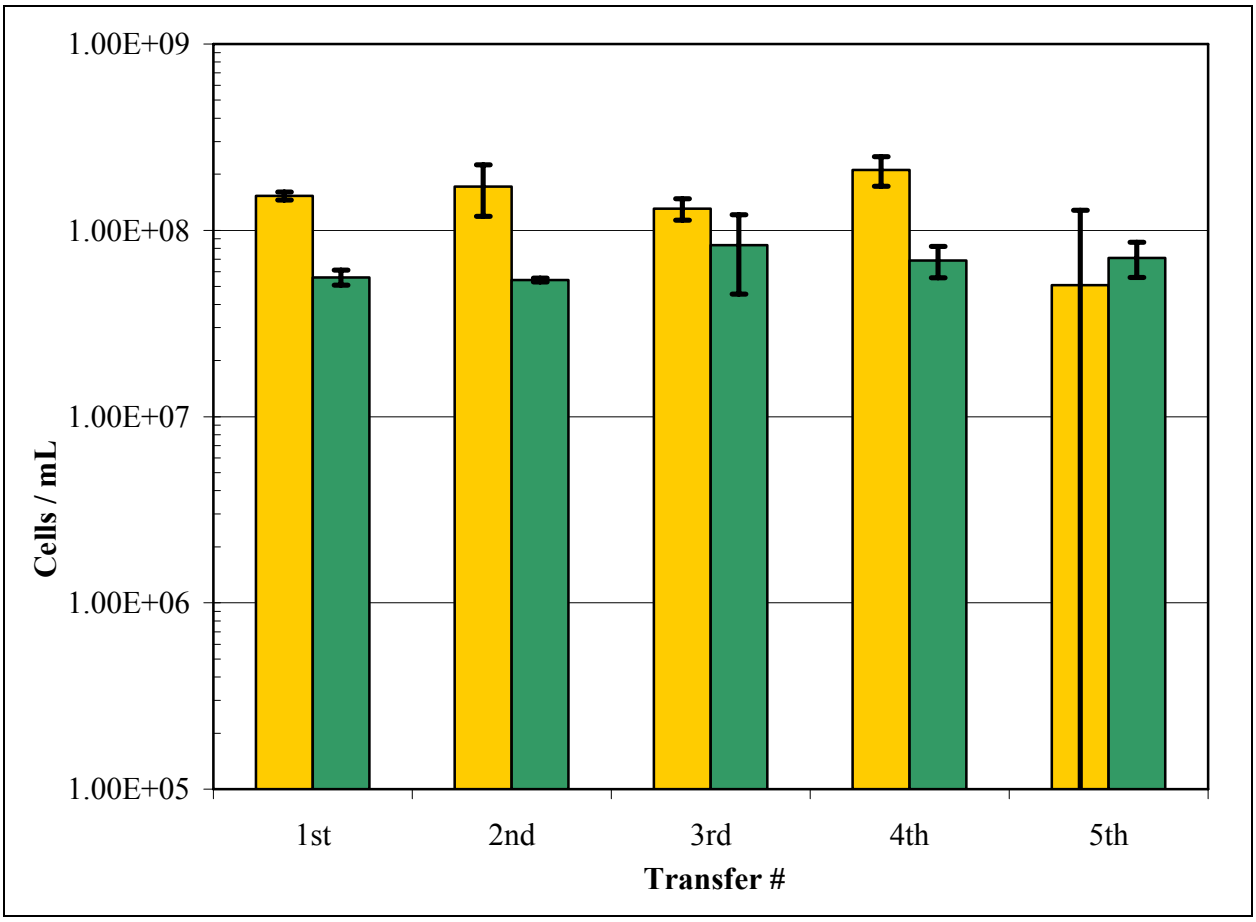

Figure 4.9 – Endpoint Growth Measurements of *P. furiosus* in 1AA Medium ±NH_4Cl

Each bar represents the average of three independent cultures. Cell density determined by direct cell counts at the end of a 24 hour growth period at 98 °C. Error bars represent ±1 standard deviation. Yellow bars ( ) grown in 1AA medium with the usual amount of NH_4Cl (4.7 mM). Green bars ( ) grown in 1AA medium without NH_4Cl (0 mM).

final cell density between the standard 1AA medium and the modified 1AA medium lacking NH_4Cl is reproducible.

After obtaining the results from the 1AA medium experiments, the ability of *P. furiosus* to grow in media containing no amino acids ("0AA" medium) was tested. A culture that had undergone 13 transfers in the 1AA medium was used as the inoculum in this experiment. *P. furiosus* failed to grow in the 0AA medium. *P. furiosus* also failed to grow if the 0AA medium was supplemented with equimolar amounts of other potential sulfur sources, namely methionine, thioglycolate, or elemental sulfur. No conditions were identified under which *P. furiosus* could grow in the absence of cysteine. However, it was difficult to interpret some of the experiments because the standard 1AA cultures sometimes failed to grow, as was seen in the 5th transfer of the $\pm NH_4Cl$ experiment.

Comparison of Media Types

Growth curves were performed for four media types: RM, 20AA, 6AA, and 1AA. The inoculum culture for the RM growth curves was from a stock culture of *P. furiosus* in RM that was subsequently transferred 3x in RM to obtain fresh cells. The inoculum for the 20AA growth curves was derived from the same RM stock culture that had been transferred 3x in 20AA medium. Similarly, the inoculum for the 6AA growth curves came from the RM stock culture that had been transferred 3x in 6AA medium. Because *P. furiosus* takes numerous transfers before it can grow well in the 1AA medium, the inoculum for the 1AA growth curves came from a 13th transfer culture from the previous 1AA experiment that had been transferred 3 additional times in 1AA medium. Triplicate cultures for each media type were inoculated at hour 0. Cell counts were performed on these cultures every 3 hours for 30 hours. These growth curves are shown in **Figure 4.10**

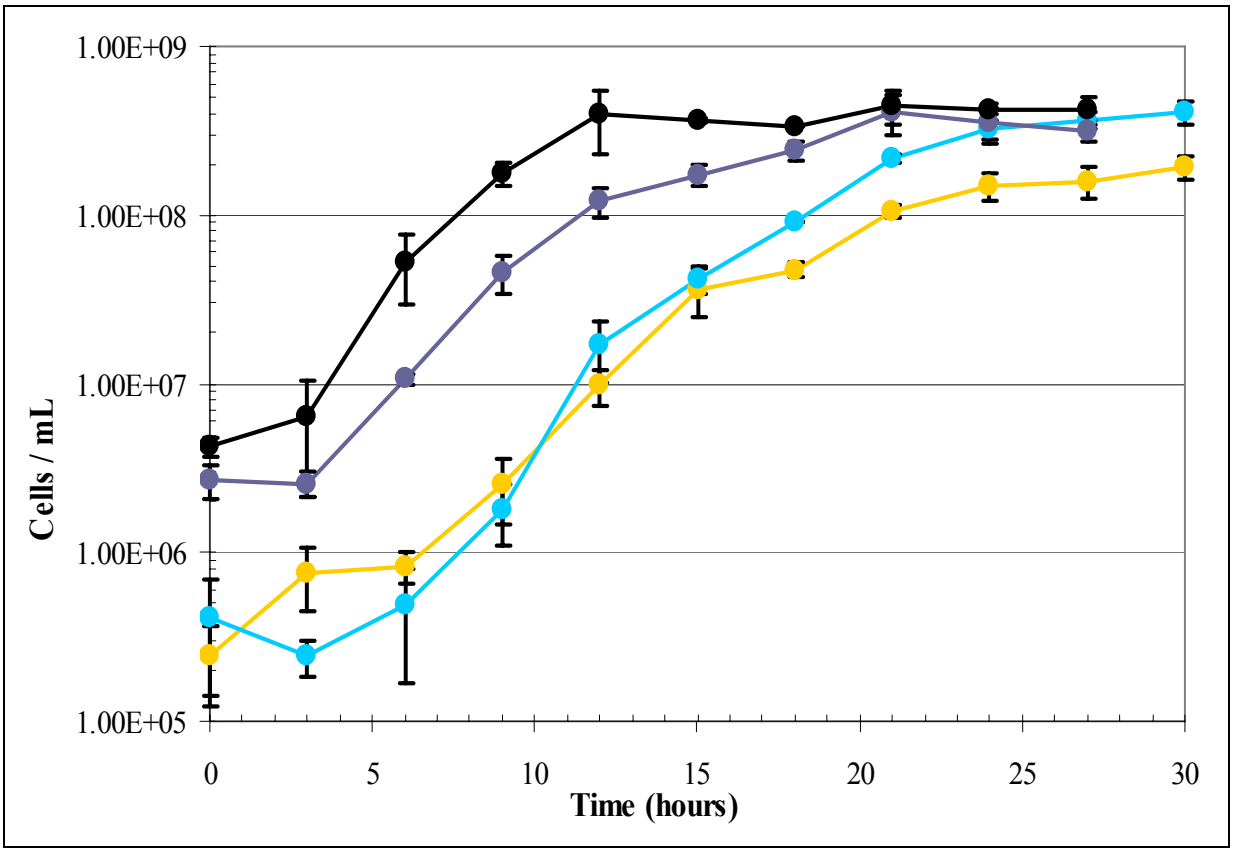

Figure 4.10 – Growth Curves of *P. furiosus* in Various Media

Each growth curve represents an average of three independent cultures. Cell densities were determined by direct cell counts at 3 hour intervals during growth at 98°C. Error bars represent ±1 standard deviation. (●) 1AA Medium, (●) 6AA Medium, (●) 20AA Medium, and (●) RM.

In RM, *P. furiosus* reached a maximum cell density of $4x10^8$ cells/mL after 12 hours. The doubling time was 75 minutes. In 20AA medium, *P. furiosus* reached a maximum cell density of $4x10^8$ cells/mL after 21 hours. The doubling time was 95 minutes during the initial exponential phase. In 6AA medium, *P. furiosus* reached a maximum cell density of $3.5x10^8$ cells/mL after 24 hours. The doubling time was 75 minutes during the initial exponential phase. The 6AA cultures continued to grow at a slower doubling time of 150 minutes for 12 hours after the end of the initial growth phase until the cultures reached their maximum cell density. In 1AA medium, *P. furiosus* reached a maximum cell density of $1.5x10^8$ cells/mL after 24 hours. The doubling time was 100 minutes during the initial exponential phase. The 1AA cultures continued to grow at a slower doubling time of 230 minutes for 9 hours after the end of the initial growth phase until the cultures reached their maximum cell density. The growth rates and maximum cell densities in all four media types were high enough for the media to be practical to use for routine experiments.

Storage Conditions

Given the observed variability in the growth *P. furiosus* when adapted to different growth media, it became necessary to find a way to preserve adapted cultures. A previous study by Connaris et al. tested several methods for storing *P. furiosus* (DSM 3638) that had been grown in rich media (32). They found that *P. furiosus* remained viable for at least 1 year in a sealed glass capillary tube when stored at -80 ^oC or in liquid nitrogen vapor phase (32). Viability of the cultures improved when either 5% DMSO or 10% glycerol were added as a cryprotectant prior to freezing in liquid nitrogen vapor phase, or when 5% DMSO was added prior to freezing at -80 ^oC (32).

A modified storage protocol was tested on *P. furiosus* cultures that were adapted to growth on 6AA medium. Anaerobic stocks were prepared in glass vials at -80 ^{o}C in 20% glycerol using the procedure described in the Materials and Methods section. Although it is an obligate anaerobe, *P. furiosus* is reported to be insensitive to oxygen at low temperatures (64). Stocks were prepared under both aerobic and anaerobic conditions to determine if it made a difference to viability. Aerobic stocks were prepared in screw-cap vials at -80 ^{o}C in 20% glycerol using the procedure described in the Materials and Methods section.

Samples from the frozen stocks were thawed and tested for viability in 6AA medium after 1 day. Their growth was compared to a culture adapted to 6AA medium that had been stored in liquid culture at room temperature for 1 day, as well as to a fresh culture that was adapted to 6AA medium. Glycerol was added to some of the cultures inoculated from the non-frozen cultures to control for the effects of glycerol on the growth of *P. furiosus*. All of the cultures inoculated from the frozen stocks contained glycerol from the storage buffer. The growth curves for these cultures are shown in **Figure 4.11.**

Samples from frozen stocks were again thawed and tested for viability in 6AA medium after 14 days. As in the previous experiment, their growth was compared to a culture stored in liquid culture at room temperature for 14 days, as well as to a fresh culture. Glycerol was again added to some of the cultures as a control. The growth curves for these cultures are shown in **Figure 4.12.**

Both the aerobically and the anaerobically stored cultures of *P. furiosus* remained viable for 14 days when frozen at -80^{o}C in 20% glycerol. Further tests will be needed to determine the maximum length of time that *P. furiosus* will remain viable when stored under these conditions.

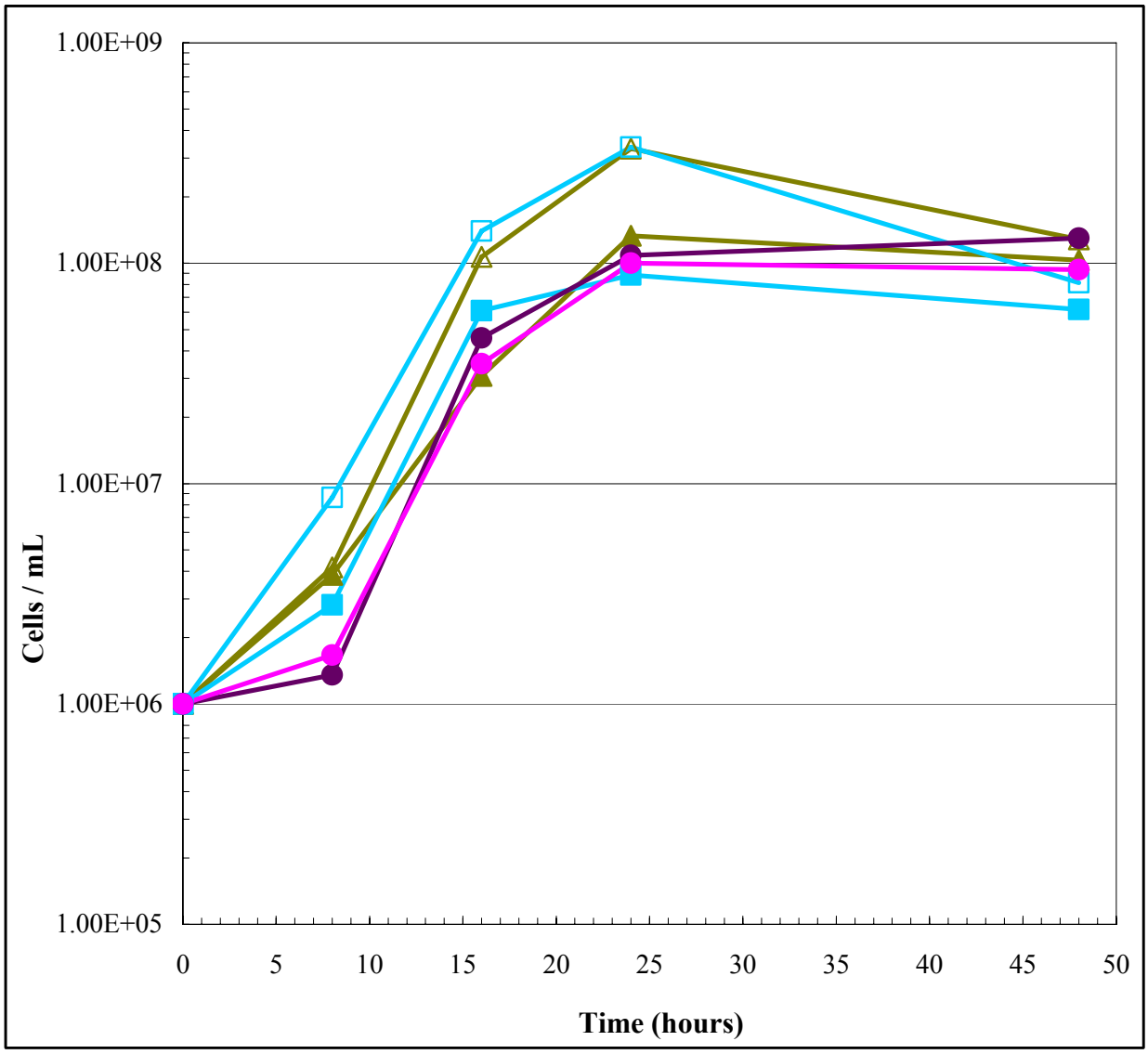

Figure 4.11 – Viability of *P. furiosus* 6AA Frozen Stocks after 1 Day at -80°C

Each growth curve represents a single culture in 6AA medium. Growth monitored by cell counts at 8, 16, 24, and 48 hours. For each growth curve, the inoculum came from the following sources: (△,▲) liquid culture stored at room temperature for 24 hours, (□,■) fresh liquid culture in stationary phase, (•) anaerobic frozen stock stored at -80 °C for 24 hours, and (•) aerobic frozen stock stored at -80 °C for 24 hours. Closed symbols (▲,■,•,•) indicate cultures that contain glycerol. Note the change in X-axis scale from previous growth curves.

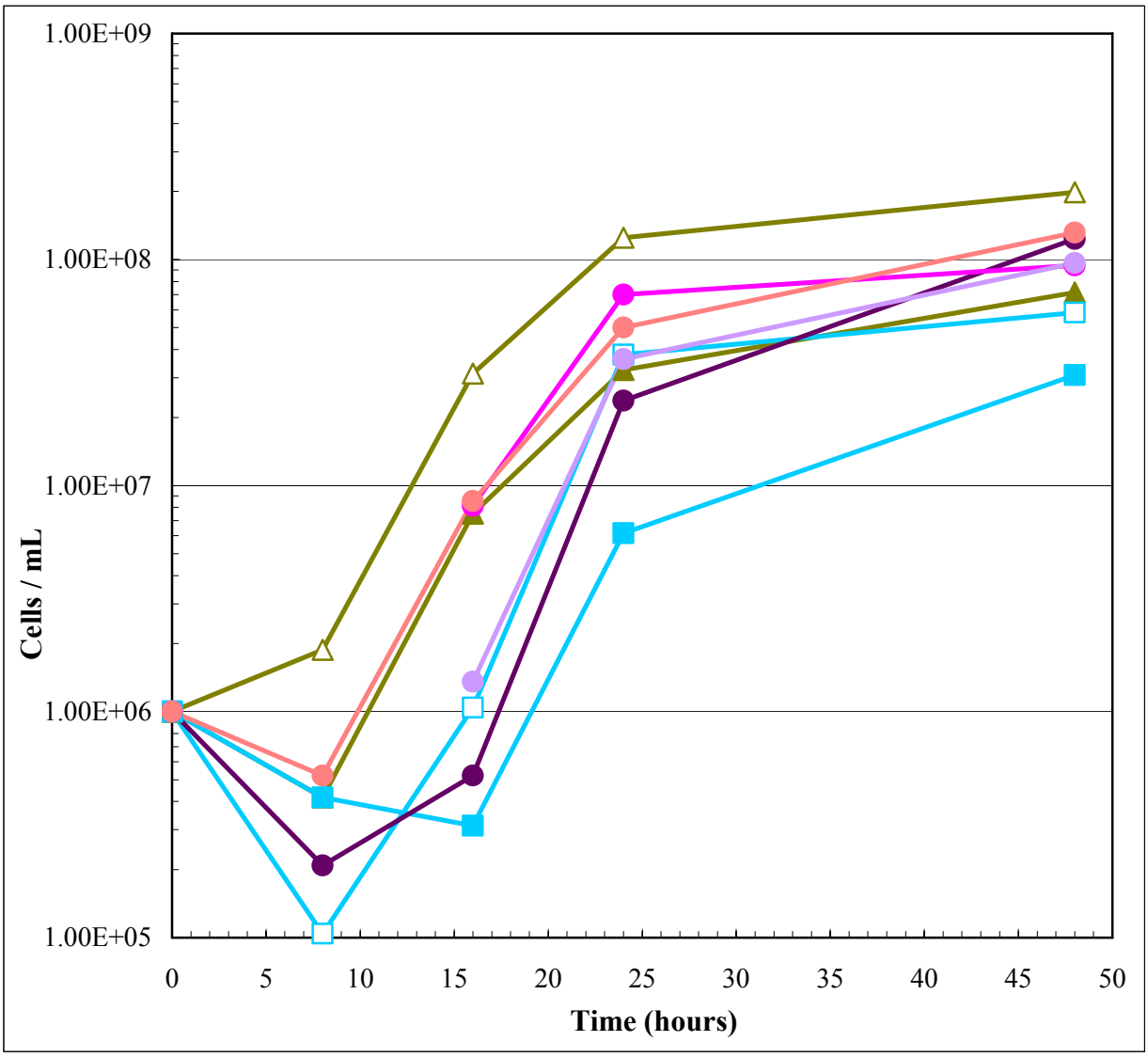

Figure 4.12 –Viability of *P. furiosus* 6AA Frozen Stocks after 14 Days at -80°C

Each growth curve represents a single culture in 6AA medium. Growth monitored by cell counts at 8, 16, 24, and 48 hours. For each growth curve, the inoculum came from the following sources: (△,▲) liquid culture stored at room temperature for 14 days, (□,■) fresh liquid culture in stationary phase, (●) anaerobic frozen stock stored at -80 °C for 14 days, (●) aerobic frozen stock stored at -80 °C for 14 days, (●) anaerobic frozen stock that was thawed after 24 hours and then refrozen for 13 days, (●) aerobic frozen stock that was thawed after 24 hours and then refrozen for 13 days. Closed symbols (▲,■, ●, ●, ●, ●) indicate cultures that contain glycerol. The scale of the X-axis is the same as in Figure 4.9.

CHAPTER 5

DISCUSSION

The results of this study are compared to the results of previous experiments and to the bioinformatics predictions in **Table 5.1**. The current study shows that *P. furiosus* can grow in media containing individual amino acids instead of peptides, in contrast to the results reported previously by Blumentals et al. (20) and Snowden et al. (121). The growth rate and cell densities reached in the 20AA medium are high enough for this medium to be convenient to use for standard growth and enzyme purification experiments. The following 14 amino acids were not required for growth: alanine, asparagine, aspartic acid, glutamine, glutamic acid, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine. These results contradict those of Hoaki et al. (55), who concluded that *P. furiosus* has a strict requirement for isoleucine and valine. The following 5 amino acids were not absolutely required, but the inclusion of at least one of the five improved growth yield and reproducibility: arginine, glycine, lysine, proline, and serine. There was not a clear difference between these 5 amino acids, i.e. it did not seem to matter which one of the 5 were added. It is as of yet unclear why the growth of *P. furiosus* in the 1AA medium was variable – sometimes reaching a cell density of up to 1.5×10^8 cells/mL**,** but at other times failing to grow at all. It does seem fairly certain that cysteine is required for growth of *P. furiosus* under the conditions tested. This is in partial agreement with the conclusions of Blumentals et al. (20) and Raven and Sharp (105), who

Table 5.1 – Comparison of Results with Previous Experiments and Bioinformatics Analyses

(-) Required by *P. furiosus*. (+/-) Poor growth in the absence of this amino acid. (+) Made by *P. furiosus*. (P) Partial pathway present. (n.d.) Not determined. (**Y**) Peptides required for growth. (**N**) Peptides not required for growth.

Amino Acid	Blumentals et al.	Hoaki et al.	Raven and Sharp	KEGG	MetaCyc	This Study
Alanine	n.d.	+	+	P	-	+
Arginine	n.d.	+	+	P	+	(+/-)
Asparagine	n.d.	+	+	+	+	+
Aspartic Acid	n.d.	+	+	+	+	+
Cysteine	-	+	-	P	-	-
Glutamic Acid	n.d.	+	+	+	+	+
Glutamine	n.d.	+	+	+	-	+
Glycine	n.d.	+	+	P	P	(+/-)
Histidine	n.d.	+	+	P	-	+
Isoleucine	n.d.	-	+	-	-	+
Leucine	n.d.	(+/-)	+	P	+	+
Lysine	n.d.	+	+	-	+	(+/-)
Methionine	n.d.	(+/-)	+	-	-	+
Phenylalanine	n.d.	+	+	+	P	+
Proline	-	+	-	P	-	(+/-)
Serine	n.d.	+	+	P	-	(+/-)
Threonine	n.d.	(+/-)	+	+	+	+
Tryptophan	n.d.	+	+	+	P	+
Tyrosine	+	+	+	+	P	+
Valine	n.d.	-	+	P	+	+
Peptide Requirement?	**Y**	**N**	**N**	n.d.	n.d.	**N**

also concluded that cysteine was required. However, both of the prior studies concluded that proline was required as well (20, 105), while the results of this experiment suggest that growth can occur in the absence of proline. In terms of the cysteine requirement, it was unclear if it was the amino acid itself that was required, or if the lack of growth in the absence of cysteine was merely a reflection of the requirement for an organic nitrogen and/or organic sulfur source. Further experimentation with alternative sulfur and nitrogen sources should provide further insight into the exact nature of the cysteine requirement.

In this study, *P. furiosus* remained viable for 14 days in 20% glycerol at -80^{o}C when stored anaerobically in glass vials or aerobically in screw-cap tubes. The study of *P. furiosus* storage conditions by Connaris et al. (32) concluded that the cells were not viable when stored in plastic cryotubes after 1 month. They detected oxygen in these culture tubes and theorized that the loss of viability was due to oxygen diffusing through the plastic (32). Therefore, it is possible that a difference between aerobic and anaerobic storage conditions would become apparent if the cultures were tested after freezing for a longer time period.

The recent progress in the development of genetic techniques for *T. kodakaraensis* has raised the exciting possibility of exploring the possible amino acid biosynthetic pathways in *Thermococcales* species *in vivo* (90, 111, 112). Two possible deletion targets in *P. furiosus* would be the *lysX* homologues. It would be interesting to confirm whether one of these genes is required for lysine biosysthesis, as was shown in *T. thermophilus* (99), and/or is required for arginine biosynthesis, as was proposed by Xu et al. (147). *P. furiosus* differs from *T. thermophilus* and *P. horikoshii* in that *P. furiosus* has two *lysX* homologues that are widely-spaced on the chromosome instead of just a single *lysX* gene (53, 70, 99, 106). It would be interesting to compare growth of the *P. furiosus* two *lysX* deletion mutants to determine if each

LysX enzyme was specific for either lysine or arginine biosynthesis, or if they had overlapping specificities and could function in either pathway.

Another potentially interesting area of future study would be the investigation of the isoleucine and methionine biosynthetic pathways in *P. furiosus*. The data from this study show that *P. furiosus* does not require an exogenous source of isoleucine and methionine. However, neither KEGG nor MetaCyc predict the existence of one of the known biosynthetic pathways for either of these amino acids in *P. furiosus* (28, 67). This raises the possibility that there are novel enzymes, or even novel pathways, for the biosynthesis of isoleucine and methionine in *P. furiosus*.

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APPENDIX A

ABBREVIATIONS

1AA	1 Amino Acid
6AA	6 Amino Acids
9AA	9 Amino Acids
20AA	20 Amino Acids
AAA	α-Aminoadipic Acid
ACS I	Acetyl-CoA Synthetase I
ACS II	Acetyl-CoA Synthetase II
ADP	Adenosine 5'-Diphosphate
AHAS	Acetohydroxyacid Synthase
AlaAT	Alanine Aminotransferase
AroAT I	Aromatic Aminotransferase I
AroAT II	aromatic aminotransferase
AspAT	Aspartate Aminotransferase
ATP	Adenosine 5'-Triphosphate
BLAST	Basic Local Alignment Search Tool
BLOSUM	Blocks Substitution Matrix
cDNA	Complementary DNA
CoA	Coenzyme A

DAP	Diaminopimelic Acid
dCTP	2’-Deoxycytidine 5’-Triphosphate
D-E4P	D-Erythrose-4-Phosphate
diH2O	Deionized Water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
dUTP	2'-Deoxyuridine 5'-Triphosphate
EM	Embden-Meyerhof
GAPOR	Glyceraldehyde-3-Phosphate:Ferredoxin Oxidoreductase
HICDH	Homoisocitrate Dehydrogenase
ICDH	Isocitrate Dehydrogenase
IOR	Indolepyruvate Ferredoxin Oxidoreductase
IPMDH	3-Isopropylmalate Dehydrogenase
KEGG	Kyoto Encyclopedia of Genes and Genomes
KGOR	2-Ketoglutarate Ferredoxin Oxidoreductase
Mal I	Maltose Metabolism Operon I
MBH	Membrane-Bound Hydrogenase
MBX	Membrane-Bound Oxidoreductase
mRNA	Messenger Ribonucleic Acid
NAD+	Nicotinamide Adenine Dinucleotide (oxidized form)
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
NADP+	Nicotinamide Adenine Dinucleotide Phosphate (oxidized form)

NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NAD(P)H	either NADH or NADPH
NAGS	*N*-Acetylglutamate Synthase
NCBI	National Center for Biotechnology Information
NSR	NAD(P)H Sulfur Oxidoreductase
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PHI-BLAST	Pattern Hit Initiated Basic Local Alignment Search Tool
POR	Pyruvate Ferredoxin Oxidoreductase
RM	Rich Medium
SDS	Sodium Dodecyl Sulfate
TCA	Tricarboxylic Acid
VOR	2-Ketoisovalerate Ferredoxin Oxidoreductase

APPENDIX B

P. FURIOSUS ORFS

Table B.1 – *P. furiosus* ORFs Relevant to This Study

ORF	Abbreviation	NCBI Annotation (141)	Experimentally Determined Function
PF0121	AroAT I	putative aspartate aminotransferase	aromatic aminotransferase (11, 135)
PF0202	ICDH	isocitrate dehydrogenase	isocitrate dehydrogenase (123)
PF0207		argininosuccinate synthase	
PF0208		argininosuccinate lyase	
PF0209		ribosomal protein s6 modification protein	
PF0291	PheA	prephenate dehydratase	
PF0293		putative aminotransferase	
PF0362		glucosamine-fructose-6-phosphate aminotransferase	
PF0464	GAPOR	glyceraldehyde-3-phosphate:ferredoxin oxidoreductase	glyceraldehyde-3-phosphate:ferredoxin oxidoreductase (97)
PF0513		4-aminobutyrate aminotransferase	
PF0522		aspartate transaminase	
PF0532	ACS II	hypothetical protein	ADP-forming acetyl-CoA synthetase II subunit α (88)
PF0533	IOR	indolepyruvate ferredoxin oxidoreductase subunit α	indolepyruvate:ferredoxin oxidoreductase subunit α (87)

Table B.1 continued – *P. furiosus* ORFs Relevant to This Study

ORF	Abbreviation	NCBI Annotation (141)	Experimentally Determined Function
PF0534	IOR	indolepyruvate oxidoreductase subunit β	Indolepyruvate:ferredoxin oxidoreductase subunit β (87)
PF0594		ornithine carbamoyltransferase	
PF0935	AHAS	acetolactate synthase	
PF0936	IlvC	ketol-acid reductoisomerase	
PF0937	LeuA	2-isopropylmalate synthase	
PF0938	LeuC	3-isopropylmalate dehydratase large subunit	
PF0939	LeuD	3-isopropylmalate dehydratase small subunit	
PF0940	IPMDH / LeuB	3-isopropylmalate dehydrogenase 2	
PF0941	LeuA	putative α-isopropylmalate/homocitrate synthase family transferase	
PF0942	IlvD	dihydroxy-acid dehydratase	
PF0965	POR	pyruvate ferredoxin oxidoreductase subunit β	pyruvate:ferredoxin oxidoreductase subunit β (18, 77)
PF0966	POR	pyruvate ferredoxin oxidoreductase subunit α	pyruvate:ferredoxin oxidoreductase subunit α (18, 77)
PF0967	POR	pyruvate ferredoxin oxidoreductase subunit δ	pyruvate:ferredoxin oxidoreductase subunit δ (18, 77)
PF0968	VOR	2-ketoisovalerate ferredoxin oxidoreductase subunit β	2-ketoisovalerate:ferredoxin oxidoreductase subunit β (52)
PF0969	VOR	2-ketoisovalerate ferredoxin oxidoreductase subunit α	2-ketoisovalerate:ferredoxin oxidoreductase α (52)
PF0970	VOR	2-ketoisovalerate ferredoxin oxidoreductase subunit δ	2-ketoisovalerate:ferredoxin oxidoreductase subunit δ (52)

Table B.1 continued – *P. furiosus* ORFs Relevant to This Study

ORF	Abbreviation	NCBI Annotation (141)	Experimentally Determined Function
PF0971	POR / VOR	pyruvate ferredoxin oxidoreductase subunit γ	pyruvate:ferredoxin oxidoreductase subunit γ (18, 77) 2-ketovalerate:ferredoxin oxidoreductase subunit γ (52, 77)
PF1066		putative aminotransferase	
PF1185		acetylornithine deacetylase	
PF1186	NSR	NADH oxidase	NAD(P)H sulfur oxidoreductase (116)
PF1232		4-aminobutyrate aminotransferase	
PF1253	AroAT II	aspartate aminotransferase	aromatic aminotransferase (135)
PF1421		4-aminobutyrate aminotransferase	
PF1423	MBH1	putative monovalent cation/H+ antiporter subunit E	membrane bound hydrogenase complex protein (110)
PF1424	MBH2	putative monovalent cation/H+ antiporter subunit F	membrane bound hydrogenase complex protein (110)
PF1425	MBH3	putative monovalent cation/H+ antiporter subunit G	membrane bound hydrogenase complex protein (110)
PF1426	MBH4	hypothetical protein	membrane bound hydrogenase complex protein (110)
PF1427	MBH5	hypothetical protein	membrane bound hydrogenase complex protein (110)

Table B.1 continued – *P. furiosus* ORFs Relevant to This Study

ORF	Abbreviation	NCBI Annotation (141)	Experimentally Determined Function
PF1428	MBH6	putative monovalent cation/H+ antiporter subunit B	membrane bound hydrogenase complex protein (110)
PF1429	MBH7	putative monovalent cation/H+ antiporter subunit C	membrane bound hydrogenase complex protein (110)
PF1430	MBH8	putative monovalent cation/H+ antiporter subunit D	membrane anchor of the membrane bound hydrogenase complex (110)
PF1431	MBH9	hypothetical protein	membrane bound hydrogenase complex protein (110)
PF1432	MBH10	NADH dehydrogenase subunit	membrane bound hydrogenase complex protein (110)
PF1433	MBH11	membrane bound hydrogenase beta (NADH dehydrogenase)	membrane bound hydrogenase complex protein (110)
PF1434	MBH12	membrane bound hydrogenase alpha (NADH dehydrogenase)	membrane bound hydrogenase catalytic NiFe subunit (110)
PF1435	MBH13	NADH dehydrogenase subunit	membrane bound hydrogenase complex protein (110)
PF1436	MBH14	NADH-plastoquinone oxidoreductase subunit	membrane bound hydrogeanse complex protein (110)
PF1472		aspartate/serine transaminase	
PF1497	AlaAT	alanine aminotransferase	alanine aminotransferase (136)
PF1540	ACS I	ADP forming acetyl coenzyme A synthetase	ADP-forming acetyl-CoA synthetase I subunit α (88)
PF1592	TrpB	tryptophan synthase β subunit	

Table B.1 continued – *P. furiosus* ORFs Relevant to This Study

ORF	Abbreviation	NCBI Annotation (141)	Experimentally Determined Function
PF1665		histidinol-phosphate aminotransferase	
PF1678	LeuA	2-isopropylmalate synthase	
PF1679	LeuC	3-isopropylmalate dehydratase large subunit	
PF1680	LeuD	3-isopropylmalate dehydratase small subunit	
PF1681		hypothetical protein	
PF1682		ribosomal protein s6 modification protein	
PF1683		N-acetyl-γ-glutamyl-phosphate reductase	
PF1684		acetylglutamate kinase	
PF1685		acetylornithine/acetyl-lysine aminotransferase	
PF1686		acetyl-lysine deacetylase	
PF1687		hypothetical protein	
PF1688		transketolase N-terminal section	
PF1689		transketolase C-terminal section	
PF1690		3-deoxy-7-phosphoheptulonate synthase	
PF1691	AroB	3-dehydroquinate synthase	
PF1692	AroD	3-dehydroquinate dehydratase	
PF1693	AroE	shikimate 5-dehydrogenase	
PF1694		shikimate kinase	
PF1699	AroA	3-phosphoshikimate 1-carboxyvinyltransferase	
PF1700	AroC	chorismate synthase	

Table B.1 continued – *P. furiosus* ORFs Relevant to This Study

ORF	Abbreviation	NCBI Annotation (141)	Experimentally Determined Function
PF1701		chorismate mutase	
PF1702	AspAT	aspartate aminotransferase	aspartate aminotransferase (135)
PF1703	TyrA	prephenate dehydrogenase	
PF1704		hypothetical protein	
PF1705	TrpA	tryptophan synthase α subunit	
PF1706	TrpB	tryptophan synthase β subunit	
PF1707		N-(5'-phosphoribosyl)anthranilate isomerase	
PF1708	TrpG	anthranilate synthase component II	
PF1709	TrpE	anthranilate synthase component I	
PF1710	TrpD	anthranilate phosphoribosyltransferase	
PF1711	TrpC	indole-3-glycerol-phosphate synthase	
PF1767	KGOR	2-keto acid:ferredoxin oxidoreductase subunit δ	2-ketoglutarate:ferredoxin oxidoreductase subunit δ (86)
PF1768	KGOR	2-oxoglutarate ferredoxin oxidoreductase subunit α	2-ketoglutarate:ferredoxin oxidoreductase subunit α (86)
PF1769	KGOR	2-oxoglutarate ferredoxin oxidoreductase subunit β	2-ketoglutarate:ferredoxin oxidoreductase subunit β (86)
PF1770	KGOR	2-oxoglutarate ferredoxin oxidoreductase subunit γ	2-ketoglutarate:ferredoxin oxidoreductase subunit γ (86)
PF1837	ACS II	hypothetical protein	ADP-forming acetyl-CoA synthetase II subunit β (88)
PF1906		adenosylmethionine-8-amino-7-oxononanoate aminotransferase	