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**Bioenergetic and Physiological Studies of
Hyperthermophilic Archaea**

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FINAL REPORT

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Summary

This project focuses on physiological and bioenergetic characteristics of two representative hyperthermophilic archaea: *Thermococcus litoralis* (T_{opt} 88°C) and *Pyrococcus furiosus* (T_{opt} 98°C). Both are obligately anaerobic heterotrophs which grow in the presence or absence of reducible sulfur compounds. *T. litoralis* was studied in relation to information previously developed for *P. furiosus*: effect of sulfur reduction on bioenergetics, preferred fermentation patterns, tungsten requirement, etc. A defined medium was developed for *T. litoralis* consisting of amino acids, vitamins and nucleotides. This serves as the basis for continuous culture studies probing metabolic response to media changes. *P. furiosus* and *T. litoralis* have also been found to produce a polysaccharide in the presence of maltose and yeast extract. The composition and chemical structure of this polysaccharide was investigated as well as the metabolic motivation for its production. A novel and, perhaps, primitive intracellular proteolytic complex (previously designated as protease S66) in *P. furiosus* was isolated and the gene encoding the subunit of the complex was cloned, sequenced and the protease expressed in active form in *Escherichia coli*. Among other issues, the role of this complex in protein turnover and stress response was examined in the context of this organism in addition to comparing it to other complexes in eubacterial and eukaryotic cells. Biochemical characteristics of the protease have been measured in addition to examining other proteolytic species in *P. furiosus*.

Significant accomplishments during this project period

- o The gene encoding the 18.8 kDa subunit for S66, the predominant intracellular protease in the hyperthermophilic archaeon *Pyrococcus furiosus*, has been isolated, cloned, sequenced and expressed in active form in *Escherichia coli*. This gene was found not to be homologous to any other gene yet identified, except for two of unknown function in *Staphylococcus aureus* and *E. coli*.
- o Protease S66 was purified from cell extracts of *P. furiosus* and characterized biochemically. By gel filtration, two proteolytic species corresponding to molecular masses of 59 kDa and 115-130 kDa were isolated and found to be composed of only the 18.8 kDa subunit previously identified through cloning efforts.

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- o Protease S66, when expressed in *E. coli*, was found to have a T_{opt} of 95°C and a half-life of 19 minutes at this temperature.
- o Western blots using antibodies for the rat liver proteasome show strong cross reaction to S19, the subunit of S66, and some cross reaction with cell extract and purified S66. The relationship between S66 and intracellular protease complexes from eukaryotic, eubacterial and archaeal sources is being investigated.
- o Another intracellular protease in *P. furiosus*, of approximate molecular mass 500 kDa, was purified to homogeneity and characterized. This protease is composed of two subunits with molecular masses of 20 and 28 kDa. It appears to be a methionyl protease but additional characterization is underway.
- o Using a probe derived from the N-terminus of S66, the gene for a protease of molecular mass >40 kDa was identified. The sequencing of this gene is underway but it appears to be related to a metalloprotease with a consensus Zn binding site, based on sequence homology.
- o A defined medium was developed for *Thermococcus litoralis* which provides cell densities equivalent to complex media. *T. litoralis* was found require 16 amino acids, vitamins and nucleotide bases. Growth rates and cell densities similar to those obtained with complex media were obtained.
- o Significant amounts of an extracellular polysaccharide were produced by *T. litoralis* when grown in continuous culture at 88°C on complex media containing yeast extract. This polysaccharide is currently being characterized chemically.
- o The physiological motivation for polysaccharide production by *T. litoralis* is being investigated with the possible relationship to sulfur reduction as a focus.

Intracellular proteases in *Pyrococcus furiosus*

S19 gene sequence: *Pyrococcus furiosus* protease S66, which runs at 66 kDa on both SDS and native PAGE, has been shown to disassemble into a 19 kDa subunit under extreme conditions (heating to 125°C in 6 M urea) or after acid precipitation (5% TCA). The gene that corresponds to the N-terminus of S66 and the S19 species has been located by Southern blot on *P. furiosus* genomic DNA using a set of degenerate oligos. The gene sequence codes for an 18.8 kDa protein which shows no sequence homology to other known proteins or proteases in available databases. However, the amino acid sequence is approximately 40% identical to genes of unknown function found in *Eschericia coli* and *Staphylococcus aureus*. This *S. aureus* gene was not expressed in *in vitro* transcription/translation experiments in *E. coli*, so it remains to be determined whether this homologous protein is utilized in *S. aureus*. The *S aureus* gene is directly downstream of a phage insertion site, but codon usage patterns lead us to believe that the gene is in fact functional in *S. aureus*. This putative mesophilic protease in *S. aureus* and *E. coli* may provide us with a mesophilic counterpart to our thermophilic protease and allow for comparative stability and physiology studies in the future. The promoter region of the S19 gene has also been analyzed. Primer extension experiments on *P. furiosus* RNA show that the start of transcription is only 10 nucleotides upstream of the start of translation, ruling out possible pre- or pro- sequence involvement in targeting or assembly of the protease. The promoter region also contains conserved archaeal Box A and Box B sequences, but the Box A promoter is over 100 bp from the initiation codon.

Expression of the S19 gene product in *E. coli*: The gene for the S19 subunit of the S66 protease was amplified by PCR and inserted, out of frame, within the *lacZ* gene of pUC19 under a *lac* promoter. Two *E. coli* ribosome binding sites were added upstream of the S19 gene in order to help overcome translation failure due to rare codon usage in the *P. furiosus* gene. Using this system, we were able to obtain low-level expression of the protease subunit which consistently ran at 18.8 kDa on SDS-PAGE. Occasionally, the 18.8 kDa subunit ran as a larger, distinct species as detected by SDS-PAGE and S66 polyclonal antibodies; these species probably represented assembled S19 subunits. To increase expression levels and plasmid stability, we placed the S19 gene under the T7 RNA polymerase promoter. Because the gene proved to be extremely toxic, T7 RNA polymerase was added by lambda phage infection at mid-log phase. We also created a fusion protein, containing a histidine leader sequence, which allowed for one-step affinity purification of the S19 subunit expression product. The purified protein ran at both 21 kDa (19 kDa protein plus 2 kDa leader) and at 72 kDa (possibly the assembled S66 protease). The expression product has a $t_{1/2}$ of 20 minutes at 95°C, its temperature optimum, but has low specific activity compared to the native enzyme. Currently, further analysis and characterization of this expression product are underway. Furthermore, a fusion protein to a periplasmic locator signal (PelB) or an *in vitro* transcription-translation system are being considered as methods to recover more of the proteolytic activity or increase expression levels.

Characterization of the S66 protease in relation to other known proteases:

Gene sequence comparisons show no homology of the S66 protease to other known proteases from archaea, eukarya, or eubacteria. This conclusion was reached using computer generated % similarity and % identity calculations based on both evolutionary and structural criteria. However, we have seen immunological cross-reaction of S66 with yeast, bovine, frog, and rat proteasome polyclonal antibodies in Western blots. We have recently confirmed this result using fresh anti-rat liver proteasome antibodies and purified material. The rat liver proteasome antibodies also recognize the expression product. No immunological cross-reactivity to S66 was seen with ClpP (from *E. coli*) or the archaeal proteasome (from *T. acidophilum*) polyclonal antibodies.

***P. furiosus* metalloprotease gene sequence:** As mentioned above, the S66 protease gene was located by screening *P. furiosus* genomic DNA with a set of degenerate oligos. These oligos helped to locate a second *P. furiosus* protease gene that contains 6 consecutive, identical amino acids that are present in the S66 gene. This portion of the genome has been partially sequenced as well and codes for an open reading frame of at least 1.1 kb. When translated, the sequence contains a conserved Zn^{2+} metalloprotease binding region, and has a predicted structure of very tight alpha-helix, such as that found in structural proteins. Aside from the 6 amino acids, this metalloprotease has no protein sequence relationship to S66--there are no matches between internal peptide sequence obtained by CNBr cleavage of S66 and the amino acid sequence predicted by this metalloprotease gene. Sequencing and analysis of this gene continue, and expression of the gene in a mesophile is planned.

Screening of *P. furiosus* extracts for proteases: We have screened *P. furiosus* cell extracts for the presence of proteases that will cleave a variety of 7-amido-4-methylcoumarin-linked peptides at high temperature. Extracts were separated by both SDS and native PAGE and incubated with the fluorometric substrates at 80°C for 5 minutes. The sizes of the proteases that cleaved the given substrates are summarized in the chart below:

**Table 1. Proteolytic Activities in *Pyrococcus furiosus* Cell Extract
by Substrate Gel Assay**

Peptide Substrate	Molecular Mass (kDa) SDS - PAGE			Molecular Mass (kDa) NATIVE - PAGE							
	>200	120	66	250	200	100	84	66	56	35	25
N-Cbz-GGR-MCA		+	++		++	++		++++			+
N-Cbz-RR-MCA			+		+	+		+++		+	
N-Succ-GPLGP-MCA			++	+			+	++++	++++	+	+
N-Succ-AAF-MCA	+	+	+++		+	++		+++		++	
N-Succ-RPFHLLVY-MCA			+					++			
N-Cbz-VKM-MCA	+++				++++	+		+			
N-Succ-LY-MCA		+	++		++			+++			
N-Succ-AFK-MCA		+	+		++			+++			

Purification of S66 by column chromatography: In the past, S66 has been purified by incubating *P. furiosus* extract in 1% SDS at high temperature followed by electroelution of one of the few remaining bands that showed proteolytic activity. To increase our final yield of active protease and confirm the purity of our product, we have been developed a purification protocol for S66 using column chromatography. Results this far are summarized in the chart below. We have determined that S66 occurs in at least two distinct active forms (Halio et al., in preparation). On native PAGE, these forms conform to molecular masses of 66 and 86 kDa, with the latter species being predominant. On SDS-PAGE, only the 66 kDa species is noted, along with two inactive bands at 45 and 19 kDa, both of which have the same N-terminus as S66 and which are recognized by anti-S66 antibodies. Gel filtration analysis has shown that S66 corresponds to an approximately 59 kDa molecular mass while S86 corresponds to a molecular mass 115-130 kDa. This suggests that S66 is a homotrimer and S86 consists of 6 or 7 of the 18.8 kDa subunits. Unexpectedly, S86 purified by column chromatography has a lower T_{opt} and thermostability than S66 obtained by incubating *P. furiosus* cell extracts for 24 hours in 1% SDS (85°C vs. 95-100°C; $t_{1/2}$ of a few minutes at 98°C vs. more than 24 hours). We have shown that protease activity (as measured by hydrolysis of AAF-MCA) of *P. furiosus* cell extracts rapidly decreases to less than 30% of initial activity after 12 hours at 98°C, but does not further decrease to any extent for at least the next 12 hours. Experiments are underway to determine the reason for these differences and to follow the fate of S86 over the incubation time. From previous efforts, it appears that S66 represents the most stable form of this proteolytic activity and confirms observations made in our earlier work (Blumentals et al., 1990).

**Table 2. Purification of Protease S66 from *P. furiosus*
(based on activity to *N*-succinyl-AAF-MCA)**

SAMPLE	Total protein (mg)	Specific activity (units/mg) $\times 10^{-6}$	Activity tot units $\times 10^{-9}$	FOLD	RECOVERY (%)
Cell extract	25,350	0.5	6.2	1.0	100.0
DEAE	1,740	2.2	3.8	4.5	61.3
Hydroxyapatite	1,600	1.4	2.2	3.2	34.9
Phenyl Sepharose	45	21.9	1.0	44.5	16.0
Mono Q	7.3	43.5	0.3	88.5	5.0
Gel filtration	1.2	99.3	0.1	202.0	2.0

Physiological/Bioenergetic Studies of *Thermococcus litoralis*

We are doing comparative physiological and bioenergetic studies of *P. furiosus* and *T. litoralis*. Since these species appear to be representative of the two major genera found in marine hydrothermal sites, their metabolic characteristics merit close examination. In previous work, we have studied *P. furiosus* in terms of sulfur reduction and growth on maltose-based media. *T. litoralis* is being examined from this perspective. We expect to take into account the findings of our protease studies here, also.

***Thermococcus litoralis* media development:** In order to make future physiological experiments more meaningful, efforts have been directed at developing a defined media. *T. litoralis*, and other heterotrophic hyperthermophiles, usually require a complex media. Initial efforts focused on the elimination of yeast extract and tryptone. Tryptone did not have a significant effect on the growth of *T. litoralis*, but yeast extract proved difficult to remove. The lowest yeast extract concentrations that still promoted growth were used to investigate amino acid dependence. We were trying to establish a medium to use for continuous culture studies, and thus disregarded conditions where *T. litoralis* did not grow sufficiently well. Four amino acids, alanine, asparagine, glutamine, and glutamate, were eliminated with no effect on growth rate or maximum cell density. In addition, we found yeast extract could be removed if four nucleotides, adenine, cytosine, thymine, and uracil, were supplemented in the media. Only a small decline in growth rate and maximum cell density resulted from this modification. The addition of maltose seemed to boost growth and prolong the stationary phase, but no growth occurred in media lacking a significant source of peptides or amino acids. Table 3 summarizes the defined medium formulation developed for *T. litoralis*. This medium was used as the basis to investigate the effect of medium composition of growth as seen in Figure 1. In brief, the addition of maltose to the sulfur-free defined medium reduces doubling times from about 150 minutes to 80 minutes. The addition of sulfur to maltose-containing defined media then brings doubling times back to that obtained in maltose-containing media. When yeast extract is added to maltose-containing, sulfur-free media, doubling times on order of 50 minutes are obtained. The

implications of these results are being examined relative to similar experiments with *P. furiosus*.

Table 3. Composition of the Defined Medium for *Thermococcus litoralis*

Classi-fication	Component	Conc. (mg/l)	Classification	Component	Conc. (mg/l)
Base Medium	MgCl ₂ ·6H ₂ O	1000	Vitamins	folic acid	2
	Na ₂ SO ₄	1000		pyridoxine-HCl	10
	NaCl	15,000		thiamine-HCl	5
	CaCl ₂ ·2H ₂ O	75		riboflavin	4
	KCl	350		nicotinic acid	5
	NaBr	50		biotin	2
	H ₃ BO ₃	20		DL-Ca-pantothenate	5
	KI	20		Vitamin B-12	0.1
	SrCl ₂ ·6H ₂ O	10		p-aminobenzoic acid	5
	Na ₂ WO ₄ ·2H ₂ O	3		DL-6,8-thioctic acid (lipoic acid)	5
	K ₂ HPO ₄	140			
	Rezasurin	1			
Trace Elements	Nitrilotriacetate	150	Amino Acids	All (except Ala, Asn, Gln, Glu)	200
	MnSO ₄ ·7H ₂ O	50			
	FeSO ₄ ·7H ₂ O	140	Nucleotides	Adenine	10
	NiCl ₂ ·6H ₂ O	20		Uracil	10
	CoSO ₄ ·7H ₂ O	36.2			
	Na ₂ MoO ₄ ·2H ₂ O	1			
	CuSO ₄ ·5H ₂ O	1			

Continuous culture development: Extensive modifications have been made to the previous chemostat design to decrease time spent maintaining the system and improve sterility and sampling (see Figure 2). Long-term cultures of *P. furiosus* and *T. litoralis* have been successfully run in the updated system.

Production of polysaccharide by *T. litoralis* and *P. furiosus*: A polysaccharide-like material has been found in *T. litoralis* and *P. furiosus* under certain growth conditions. Preliminary investigations have revealed a composition of 2.1% mannose, 0.8% glucose, and 0.3% xylose for the substance in *P. furiosus*. A similar substance has been observed in *T. litoralis*. Preliminary experiments were performed to assess the possibility of the use of the substance to aid in attachment to surfaces. Figure 3 shows preliminary evidence that *T. litoralis* forms what we propose is a biofilm in complex media with both yeast extract and maltose but lacking sulfur; *P. furiosus* does not respond in a similar fashion on comparable media. Further experiments are underway to characterize this phenomenon.

Publications (6/93-5/96) acknowledging DOE-EBS support:

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