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Sequence, Expression in *Escherichia coli*, and Analysis of the Gene Encoding a Novel Intracellular Protease (PfpI) from the Hyperthermophilic Archaeon *Pyrococcus furiosus*

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A previously identified intracellular proteolytic activity in the hyperthermophilic archaeon *Pyrococcus furiosus* (I. I. Blumentals, A. S. Robinson, and R. M. Kelly, Appl. Environ. Microbiol. 56:1992–1998, 1990) was found to be a homomultimer consisting of 18.8-kDa subunits. Dissociation of this native *P. furiosus* protease I (PfpI) into a single subunit was seen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) but only after trichloroacetic acid precipitation; heating to 95°C in the presence of 2% SDS and 80 mM dithiothreitol did not dissociate the protein. The gene (*pfpI*) coding for this protease was located in genomic digests by Southern blotting with probes derived from the N-terminal amino acid sequence. *pfpI* was cloned, sequenced, and expressed in active form in *Escherichia coli* as a fusion protein with a histidine tag. The recombinant protease from *E. coli* showed maximum proteolytic activity at 95°C, and its half-life was 19 min at this temperature. This level of stability was significantly below that previously reported for the enzyme purified by electroelution of a 66-kDa band from SDS-PAGE after extended incubation of cell extracts at 98°C in 1% SDS (>30 h). The *pfpI* gene codes for a polypeptide of 166 amino acid residues lacking any conserved protease motifs; no protease activity was detected for the 18.8-kDa PfpI subunit (native or recombinant) by substrate gel assay. Although an immunological relationship of this protease to the eukaryotic proteasome has been seen previously, searches of the available databases identified only two similar amino acid sequences: an open reading frame of unknown function from *Staphylococcus aureus* NCTC 8325 (171 amino acid residues, 18.6 kDa, 41% identity) and an open reading frame also of unknown function in *E. coli* (172 amino acid residues, 18.8 kDa, 47% identity). Primer extension experiments with *P. furiosus* total RNA defined the 5′ end of the transcript. There are only 10 nucleotides upstream of the start of translation; therefore, it is unlikely that there are any pre- or pro-regions associated with PfpI which could have been used for targeting or assembly of this protease. Although PfpI activity appears to be the dominant proteolytic activity in *P. furiosus* cell extracts, the physiological function of PfpI is unclear.

Hyperthermophilic microorganisms (those that grow above 90°C with an optimum temperature of at least 80°C), which are mostly archaea (53), have been investigated for clues to evolutionary processes as well as to uncover biological strategies underlying life at elevated temperatures. Most of the focus has been on characterization of new isolates (3, 45) and on the mechanisms responsible for heightened levels of biomolecular thermostability (1). Although just as important, the physiology of this novel group of organisms has been less studied (20). This is not surprising since hyperthermophiles are generally difficult to culture, in addition to the fact that no genetic systems are available for directed analysis of cellular phenomena. Thus, the nature of metabolic pathways and regulatory strategies within this group of microorganisms and the relationship, physiological in addition to phylogenetic, to more thoroughly studied organisms and cells merit further examination.

From a physiological perspective, the most studied hyperthermophilic archaeon to date is *Pyrococcus furiosus*, which was originally isolated from hot springs near Vulcano Island, Italy (13). This heterotrophic anaerobe grows optimally between 98 and 100°C on a variety of complex carbon sources, including peptides and starch, and will reduce sulfur for additional energetic benefit. Several proteins from this organism have been purified and characterized biochemically, and their corresponding genes have been sequenced (11, 19, 23, 49, 54). Some physiological characteristics of *P. furiosus*, including glycolytic pathways (21, 30, 31, 43), gluconeogenesis (42), and the bioenergetics of sulfur reduction (26, 44), have been investigated.

A nutritional characteristic of *P. furiosus*, and other hyperthermophilic heterotrophs, is the utilization of peptides for growth; as such, proteolytic activity has been studied in *P. furiosus* (7, 12, 46). Specific intracellular proteolytic activity in *P. furiosus* is also heightened under peptide-limited conditions and reduced by the addition of maltose to the media (46). Blumentals et al. (4) showed that *P. furiosus* produces several intracellular proteolytic species, as determined by clearing zones on gelatin overlays by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell extracts. Of these, a protease with an electrophoretic mobility of 66 kDa by SDS-PAGE was previously purified, characterized, and shown to be highly thermostable (a half-life of over 30 h at 98°C) and resistant to several denaturants (4). In fact, the purification process for this protease involved the incubation of *P. furiosus*

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cell extracts in 1% SDS at 98°C for periods up to 24 h or longer, after which SDS-PAGE revealed only a few protein species and active enzyme. This protease apparently hydrolyzed completely most other *P. furiosus* proteins under these conditions, forming very small (<10-kDa) proteolysis products.

To date, only a few other proteases from thermophilic (6, 14, 18, 24) and hyperthermophilic (8, 22, 29, 33) archaea have been purified and characterized, and the genes for a putative prolyl oligopeptidase (37) and a putative subtilisin-like protease (50) have been sequenced. To further examine the significance of the intracellular protease identified by Blumentals et al. (4) in *P. furiosus* and compare it with other multisubunit intracellular proteases such as La (51), ClpP (27), the archaeal proteasome (55, 56), and the eukaryotic proteasome (16, 32), efforts to isolate and clone the gene encoding the enzyme were undertaken. Here, we report the nucleotide sequence encoding the subunit of *P. furiosus* protease I (PfpI). In addition, we show that this subunit, when expressed as a fusion protein in *Escherichia coli*, assembles into a multimeric conformation which has optimal proteolytic activity at 95°C.

MATERIALS AND METHODS

Culture of microorganisms. *P. furiosus* (DSM 3638) was obtained from Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany. Cultures were grown on an artificial seawater-based medium as described previously (46). One gram of yeast extract (Difco, Detroit, Mich.) per liter, 5 g of tryptone (Difco) per liter, and 10 g of elemental sulfur (Fisher Scientific, Atlanta, Ga.) per liter were added to the artificial seawater-based medium. Anaerobic conditions were obtained by adding 2.5 to 5 ml of a 200-g/liter $Na_2S \cdot 9H_2O$ stock solution to each liter of 98°C medium while sparging with nitrogen. Cells were grown in batch mode in a 98°C oil bath; typical yields were 0.3 to 0.5 g (wet weight) per liter.

Preparation of cell extracts and SDS-resistant proteases. Cell extracts were prepared as described previously (4), except in 50 mM Tris buffer (pH 7.5). This cell extract was incubated at 98°C for 4 h and then concentrated by using an Amicon stirred cell or a Centricon gravitational concentrator with a molecular mass cutoff of 10 kDa. The concentrate was electrophoresed and stained, and the band corresponding to 66 kDa was electroeluted as described previously (4). Purity of the electroeluted protein was verified by silver-stained SDS-PAGE.

Analytical protein chemistry. A sample of electroeluted PfpI was precipitated with trichloroacetic acid and dissolved in 70% formic acid. PfpI was cleaved with CNBr, dissolved in 8 M GuHCl–50 mM Tris (pH 8.5), reduced, and alkylated with iodoacetamide (28). Peptides generated from the CNBr cleavage protease were separated on a Waters high-performance liquid chromatography system as described previously (28), with a Waters Deltapak C_{18} column (3.9 by 150 mm) at a flow rate of 0.5 ml/min. Selected fractions were analyzed by laser desorption-mass spectrometry on a Finnigan Lasermat instrument. PfpI species corresponding to molecular masses of approximately 20, 45, 66, and 86 kDa were blotted onto pro-Blott polyvinylidene difluoride membranes for sequence analysis. The 45- and 66-kDa species were microsequenced on an Applied Biosystems model 477A amino acid sequencer that was connected on-line to a model 120A amino acid analyzer. Data were collected and analyzed by using ABI model 610A software. The 20- and 86-kDa species were N-terminally microsequenced in similar fashion at the Microsequencing Facility at the University of Georgia.

Electrophoresis and gelatin overlay assays. Gelatin (0.1%) was polymerized in a 10% acrylamide gel in order to visualize bands containing proteolytic activity which were represented by cleared zones in the stained gelatin. Protein samples containing 1 to 50 μg of protein were electrophoresed, washed, and overlaid for 6 to 12 h at 75°C in 50 mM sodium phosphate buffer (pH 7.5) and stained as described previously (4).

TCA precipitation. Protein samples (>1 mg/ml) were trichloroacetic acid (TCA) precipitated by the addition of a 0.5 volume of cold 15% TCA. After incubation on ice for at least 20 min, precipitated proteins were removed by microcentrifugation at 4°C for 20 min and washed with 50 μl of ice-cold acetone. The precipitate was dried in a centrifuge vacuum dryer for several minutes and resuspended in standard buffer at room temperature.

Molecular biology reagents, bacterial strains, and plasmids. The restriction enzymes *Eco*RI and *Hin*dIII, T4 polynucleotide kinase and T4 DNA ligase, and pUC19 and M13mp19 vectors were obtained from New England Biolabs (Beverly, Mass.). Shrimp alkaline phosphatase and Sequenase 2.0 were purchased from United States Biochemicals (Cleveland, Ohio). Electrocompetent *E. coli* DH10B and XL1-Blue were obtained from Gibco BRL (Gaithersburg, Md.). Synthetic oligonucleotides were synthesized with an ABI model 381A DNA synthesizer, obtained from the North Carolina State University Molecular Biology Center or IDT, Inc. (Coralville, Iowa). Radio-labeled nucleotides were purchased from NEN Research Products (Boston, Mass.) or Amersham Life Sciences (Arlington Heights, Ill.). Plasmid pET-15b; *E. coli* BL21, BL21(DE3), and BL21(DE3)pLysS; and bacteriophage CE6 were obtained from Novagen (Madison, Wis.).

Genomic DNA purification. *P. furiosus* DSM 3638 was grown to stationary phase, and the cells were pelleted by centrifugation and then washed twice in isotonic buffer. After the cells were disrupted by freeze-thawing, they were resuspended in buffer containing 1% SDS. After several phenol (Tris-EDTA equilibrated) and chloroform-isoamyl alcohol (24:1) (Fisher, Atlanta, Ga.) extractions, the DNA was ethanol precipitated, dissolved in Tris-EDTA buffer, and treated with RNase (Gibco BRL) and proteinase K (Gibco BRL). Finally, the solution was extracted with phenol and chloroform-isoamyl alcohol several times and ethanol precipitated.

Recombinant DNA procedures. *P. furiosus* genomic DNA was digested with a series of restriction endonucleases and separated by electrophoresis through a 1% agarose gel in standard Tris-acetate-EDTA buffer. For hybridization studies, the DNA was transferred to 0.2-μm-pore-size MSI NitroBind filters by using a Stratagene Posiblotter with 10× standard SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as the transfer buffer and cross-linked by using a Stratagene model 1800 UV cross-linker. Prehybridization of the filters was carried out at 37°C in standard hybridization solutions (40) for 6 to 24 h. After prehybridization, the filters were washed twice, immersed in 10 ml of hybridization solution, and incubated at 37°C for approximately 14 h in a Stratagene roller hybridizer. Degenerate oligonucleotide probes, based on the PfpI N-terminal amino acid sequence, were labeled by incubation with T4 polynucleotide kinase and [γ-^{32}P]ATP. The filters were washed three times for 15 min each in solutions containing 6× SSC and 1% sodium PP_i. The wash temperature ranged from 41 to 61°C, depending on the nature of the DNA sample and oligonucleotide probe. *P. furiosus* DNA hybridized to the ^{32}P-labeled probe was detected by using a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, Calif.). Hybridizations of plasmid DNA with the same oligonucleotides were carried out as mentioned previously. Bacterial colonies were transferred to Schleicher & Schuell BA85 0.45-μm-pore-size nitrocellulose filters, the cells were lysed, and the filters were freed of cell debris by standard procedures (40). The sequences of the degenerate probes used in the hybridization studies were ATGAARATWCTHTTY CTHWSHGCHAAYGARTTYGARGAYGTWGARCTHATWTAYCCATA YCAYAG, corresponding to amino acids M-K-I-L-F-L-S-A-N-E-F-E-D-V-E-L-I-Y-P-Y-H-R, and GCHAAYGARTTYGARGAYGTWGARCTHATWTAYC CATAYCAYAG, corresponding to amino acids A-N-E-F-E-D-V-E-L-I-Y-P-Y-H-R, where H is A, C, or T; R is A or G; S is C or G; Y is C or T; and W is A or T.

Nucleotide sequencing of *P. furiosus* DNA cloned into M13mp19 employed the dideoxynucleotide chain termination method (41). The sequences for both strands of the *P. furiosus* DNA were determined by using a set of oligonucleotide primers complementary to each DNA strand.

RNA isolation and primer extension. Total RNA was extracted from *P. furiosus* DSM 3638, grown to stationary phase, by a method based on that of DiRuggiero and Robb (10), with additional phenol-chloroform extractions. Primer extension to locate the start of transcription was carried out as described by Sambrook et al. (40). Twenty micrograms of total RNA and approximately 5 pmol of γ-^{32}P-labeled primer were used. Annealing took place at 30°C overnight, and reverse transcription at 37°C for 1 h was followed by an incubation at 40°C for 1 h.

Gene expression and recombinant protein purification. The *pfpI* gene was amplified during PCR (25 cycles) of a 100-μl reaction mixture containing 700 ng of *P. furiosus* DNA, 1 μM primers complementary to each end of the gene, 1× Vent reaction buffer, and 2 U of Vent DNA polymerase. The upstream primer contained an *Nde*I restriction site at the N-terminal methionine of PfpI and the downstream primer contained a *Bam*HI restriction site, immediately following the *pfpI* stop codon. PCR-induced changes in the *pfpI* sequence were minimized by amplification of a relatively short (550-bp) segment with a polymerase with exonuclease proof-reading ability and no additional Mg^{2+} in the buffer. The ~550-nucleotide PCR product was phenol extracted, ethanol precipitated, restriction digested, and gel purified. The double-digested, gel-purified pET-15b plasmid was ligated to the PCR product overnight; resulting vectors were electrotransformed into *E. coli* BL21 (Novagen). Constructs pSH3-1H, pSH3-2H, pSH3-3H, and pSH3-4H were verified to contain one copy of the *pfpI* gene by restriction mapping and electrophoresis.

The clone containing pSH3-4H was grown in liquid culture to an optical density at 600 nm of 0.6 at 30°C and then induced by the addition of CE6 λ phage to a final concentration of 2.3×10^9 PFU/ml and 200 mM $MgSO_4$ to a final concentration of 10 mM. After 3 h at 30°C, cells were harvested by centrifugation at 5,000 × *g* for 5 min and resuspended in ice-cold 1× binding buffer (Novagen). *E. coli* was disrupted by sonication for 1 min on ice, and debris was removed by centrifugation at 12,000 × *g* for 20 min and filtration. The recombinant protein was separated from other soluble proteins by using the HisBind Resin and Buffer Kit (Novagen) according to the protocol provided. After purification, the expression product was dialyzed in 20 mM Tris-HCl (pH 8.4)–150 mM NaCl–2.5 mM $CaCl_2$ and concentrated by using a Centricon gravitational concentrator with a molecular mass cutoff of 10 kDa. The histidine tag was removed by adding thrombin protease as directed (Novagen).

Computer analysis. Database searches were done using the TFASTA and FASTA programs of the University of Wisconsin Genetics Computer Group

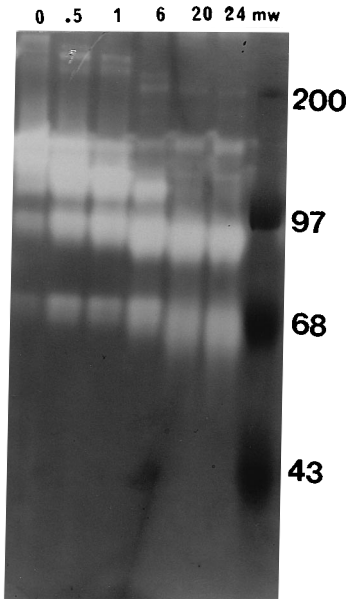

FIG. 1. Changes in proteolytic activity in *P. furiosus* cell extracts upon incubation at 98°C in 1% SDS. Gelatin overlay of a 15% SDS slab gel containing extracts that were incubated for the length of time (in hours) indicated above each lane. Two micrograms of total protein was loaded per lane. Lane mw, molecular mass markers (top to bottom) myosin (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), and ovalbumin (43 kDa).

Gene Bank (9). Gene manipulation, translation, and alignments were also done using the same package.

RESULTS

Identification of proteolytic species in *P. furiosus* cell extracts. *P. furiosus* cell extracts were incubated at 98°C in 1% SDS for various amounts of time and then assayed by gelatin overlay (Fig. 1). Two bands, corresponding to molecular masses of 66 and 86 kDa, retained high levels of proteolytic activity after 24 h. Both of these proteolytic bands had the same nonambiguous N termini as determined by protein microsequencing following electroelution from a gelatin substrate polyacrylamide gel (Fig. 2). Two weaker activities at higher molecular masses were also evident after incubation and may be related to PfpI on the basis of immunological cross-reactivity with anti-PfpI antibodies (data not shown). Active species smaller than 66 kDa were not detected by this type of gelatin overlay.

PfpI and formation of larger, stable oligomers. The proteolytic activity corresponding to 66 kDa was purified by electroelution, concentrated to 1.0 mg/ml, and stored at 4°C. When this concentrated solution was later applied to SDS-PAGE, an 86-kDa band and one considerably larger (>200 kDa) withstood both heating in dissociation buffer and the denaturing conditions of SDS-PAGE (Fig. 3, lanes 2 and 3). Bands at 47 and 20 kDa were also present, representing the dimer and subunit, respectively. In the less-concentrated solution of 0.1 mg/ml, only the 66-kDa species and a faint band at 20 kDa were noted (Fig. 3, lane 4) either before or after storage at 4°C. In vivo, PfpI may exist as a larger assembly as antibodies raised against the electroeluted 66-kDa species also recognized higher-molecular-mass species in *P. furiosus* extracts (4).

Peptide	Amino Acid Sequence
N-term. - 86 kDa band	M-K-I-L-F-L-S-A-N-E-F-E-D-V-E-L-I-Y-P-Y
N-term. - 66 kDa band	M-K-I-L-F-L-S-A-N-E-F-E-D-V-E-L-I-Y-P-Y-H-R
N-term. - 47 kDa band	M-K-I-L-F-L-S-A-N-E-F-E-D-V-E-L-I-Y-P-Y
N-term. - 20 kDa band	M-K-I-L-F-L-S-A-N-E-F-X-D-V-E-L-I-Y-P-Y-H-R-D-K-E-X-G or A- or -V- or -D- or -I-
CNBr-28 fragment	R-E-F-V-K-L-L-K
CNBr-61 fragment	I-N-A-G-V-E-W-I-D-R-E-V-V-V-D-G-N-W-V-S-S-R-H-P-G-D-L-Y-A

FIG. 2. Amino acid sequences of PfpI species and fragments.

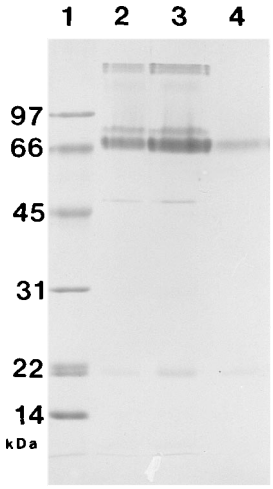

FIG. 3. Self-assembly of PfpI into larger oligomers. Proteins were separated by SDS-PAGE (15%) and stained with Coomassie blue R-250. Lanes: 1, molecular mass markers (top to bottom) were phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme with the molecular masses indicated on the left; 2 and 3, concentrated PfpI that had been purified by electroelution and stored at 4°C for 2 weeks; 4, dilute PfpI that had been purified by electroelution and stored at 4°C for 2 months.

PfpI consists of 19-kDa subunits. PfpI appears to be a stable oligomer with more than one functional form that retains structure and activity under various denaturing conditions. Figure 4, lane 1, shows that typical SDS-PAGE conditions, including buffers containing β-mercaptoethanol and dithiothreitol and heating to 95°C for several minutes, were not sufficient to denature PfpI. However, when PfpI, purified by high-temper-

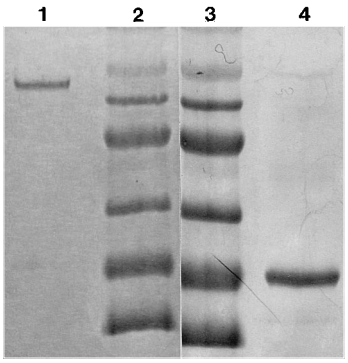

FIG. 4. Disassembly of PfpI into its subunits. Proteins were separated by SDS-PAGE (15%) and stained with Coomassie Blue R-250 and amido black. Lanes: 1, PfpI purified by electroelution; 2 and 3, molecular mass markers as described in the legend to Fig. 1 with the addition of carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa); 4, PfpI purified by electroelution from SDS-PAGE and then TCA precipitated.

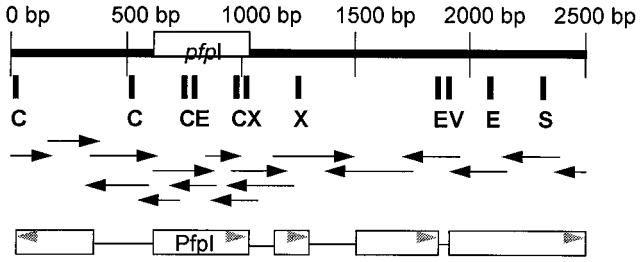

FIG. 5. Sequencing strategy for *pfpI*. A restriction map of the 2.5-kb *P. furiosus* DNA fragment containing the *pfpI* gene for the subunit of PfpI is shown. Restriction sites are *Hin*cII (C), *Eco*RI (E), *Sac*I (S), *Eco*RV (V), and *Xba*I (X). The black arrows indicate nucleotide sequences obtained with internal oligonucleotide primers. The open boxes and arrowheads represent putative open reading frames and the directions of transcription, respectively.

ature incubation and electroelution, was precipitated with TCA, washed with cold acetone, and resuspended in denaturation buffer, it dissociated into approximately 20-kDa subunits (Fig. 4). This subunit was not active as determined by gelatin and fluorescent substrate gels (data not shown). An apparent dimer of two 20-kDa subunits was also stable and was often seen by SDS-PAGE of electroeluted PfpI at approximately 47 kDa (Fig. 3, lanes 2 and 3). These 20- and 47-kDa bands both have 20 N-terminal amino acids that are identical to the nonambiguous 20 N-terminal amino acids of the 66- and 86-

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                                                                  #
  1  AACAATTAGCTATGTGTTCAGGCATGTTTGGATAGCTTGAACAGTAATTTTTCCAGGCTT   60

     ####
 61  TATATCCATTCTCACTCACCTCCAGATTAGGCTCGCCTAACTTAAATACCAGAATAATCA  120

     ######
121  TTTAAAAAGTTATTTACCTTCATTTAACCAACAACATTTTTTGAAAACTAATTCATTAAA  180

                                                    *
181  CAGTATTGTTAACCCAAAATGCCTTAAAGAAAAGCCACGAATAAAGTCTTTGGTGATAGG  240

      $$$
241  AATGAAGATACTGTTCTTGAGTGCAAACGAATTTGAAGATGTAGAGCTAATCTACCCCTA  300
181   M  K  I  L  F  L  S  A  N  E  F  E  D  V  E  L  I  Y  P  Y   200

301  CCACAGGCTCAAGGAAGAAGGTCACGAAGTTTACATAGCGAGCTTCGAGAAGGGTGTAAT  360
201   H  R  L  K  E  E  G  H  E  V  Y  I  A  S  F  E  K  G  V  I   220

361  TACAGGAAAGCACGGATATTCAGTCAAGGTGGACTTAACCTTTGACGAGGTTAACCCCGA  420
221   T  G  K  H  G  Y  S  V  K  V  D  L  T  F  D  E  V  N  P  D   240

421  CGAATTCGACGCTTTAGTTCTCCCAGGAGGAAGGGCCCCAGAGAGAGTAAGGCTTAACGA  480
241   E  F  D  A  L  V  L  P  G  G  R  A  P  E  R  V  R  L  N  E   260

481  GAAAGCAGTTGAGATAGCAAGAAAAATGTTCACCGAAGGAAAGCCAGTGGCAACTATCTG  540
261   K  A  V  E  I  A  R  K  M  F  T  E  G  K  P  V  A  T  I  C   280

541  TCATGGTCCTCAGATATTGATTTCCGCTGGAGTGCTAAAGGGAAGAAAGGGAACAAGCTA  600
281   H  G  P  Q  I  L  I  S  A  G  V  L  K  G  R  K  G  T  S  Y   300

601  CATAGGAATAAGAGACGACATGATTAATGCCGGAGTAGAGTGGATAGACAGAGAGGTTGT  660
301   I  G  I  R  D  D  M  I  N  A  G  V  E  W  I  D  R  E  V  V    32

661  TGTTGACGGAAACTGGGTTAGTTCTAGACACCCAGGAGACCTTTACGCTTGGATGAGAGA  720
321   V  D  G  N  W  V  S  S  R  H  P  G  D  L  Y  A  W  M  R  E   340

                                                        ^^^^^^^^^^
721  ATTTGTTAAATTACTTAAGTGATCATTGTAAGTATTCATAATTCATAAATTTTTTATATT  780
341   F  V  K  L  L  K                                            360

     ^^^^^^^^^^^^^^^^
781  TTTGTTAGTCCTTTTTCATTGGTGAATACTAGTGAGAAGGGGGCTATCATTACTTGGTTT  840

841  TATTATTGGTGCAGTTATTGGGATAATAATAGGAGCACTAATTTTAGCAGGAATATTCCT  900
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FIG. 6. Nucleotide sequence and translation of the gene (*pfpI*) for 18.8-kDa subunit of PfpI. The following features of the sequence are indicated: conserved archaeal promoters (#), start of transcription (*), start of translation ($), putative termination region (^), and agreement with amino acid microsequencing (_).

```
           1                                                    50
  1        MtKKVAIilA NEFEDIEYsS PkEaLenAGf ntVvIgdtAn seVvGKHGE.
  2        MsKKIAVLit dEFEDsEFtS PaDeFrkAGH EvItIekqAg ktVkGKkGEa
  3        ..mKIlfLsA NEFEDVELiy PyhrLkeeGH E.VyIasfek gvItGKHG.y
Consensus  M-KKIA-L-A NEFED-E--S P---L--AGH E-V-I---A- --V-GKHGE-

           51                                                   100
  1        kVTVDvgIaE akPEDYDALL iPGGfSPDhL RGDtegRYgT FAkyFtkndv
  2        SVTIDksIDE VtPaEFDALL LPGGhSPDyL RGD..nRFVT FtRdFvnsGK
  3        SVkVDltfDE VnPDEFDALv LPGGraPErv R..lnekaVe iARkmfteGK
Consensus  SVTVD--IDE V-P-EFDALL LPGG-SPD-L RGD---R-VT FAR-F---GK

           101                                                  150
  1        PtFAICHGPQ ILIdtDdLKG RtLTAVlnVR kDLsNAGAhv VDesVVVD.n
  2        PVFAICHGPQ lLISADVirG RKLTAVkpIi iDvkNAGAEF yDqEVVVDkd
  3        PVatICHGPQ ILISAgVLKG RKgTsyigIR dDMiNAGvEW IDrEVVVD.g
Consensus  PVFAICHGPQ ILISADVLKG RKLTAV--IR -D--NAGAE- -D-EVVVD--

           151                  174
  1        NiVTSRvPDD LddFNREiVK qLq.
  2        qlVTSRtPDD LpAFNREalr LLga
  3        NwVsSRhPgD LyAWmREfVK LLk.
Consensus  N-VTSR-PDD L-AFNRE-VK LL--
```

FIG. 7. Alignment of PfpI subunit sequence with *E. coli* and *S. aureus* homologs. Alignment was generated by using the University of Wisconsin Genetics Computer Group program PileUp (with a gap weight of 3.0 and a gap length weight of 0.1) followed by the program Pretty (implementing the case and consensus parameters). Capital letters indicate agreement of the amino acid in each sequence with that determined for the consensus strand. 1, *S. aureus* PfpI homolog; 2, *E. coli* PfpI homolog; 3, *P. furiosus* PfpI.

kDa active species (Fig. 2), as determined by amino acid sequencing of the individually electroeluted or blotted proteins.

Cloning and sequencing of gene encoding PfpI subunit. On the basis of the N-terminal amino acid sequence determined for the 66-kDa protease and its 20-kDa subunit, two degenerate and overlapping oligodeoxynucleotides (a 65-mer and a 44-mer) were synthesized and used to probe restriction digests of *P. furiosus* genomic DNA. This Southern blot identified a single, ~2.5-kb *Hin*dIII DNA fragment that strongly hybridized to both ^{32}P-labeled oligonucleotide probes. These size-selected *Hin*dIII fragments were electroeluted and ligated into pUC19. Colonies resulting from transformation of these constructs were screened with the degenerate 44-base probe. Several independent colonies were selected for further analysis. One (containing plasmid pSH26-1) had a single 2.5-kb insert that remained hybridized to the 44-mer probe after the blots were washed for 45 min at 61°C. This unique *P. furiosus* *Hin*dIII DNA was subcloned into M13mp19 for nucleotide sequence determination.

The sequenced portions of pSH26-1, its open reading frames, and a restriction map are presented in Fig. 5. The sequence of the structural and promoter regions of the *pfpI* gene (coding for PfpI) and its deduced amino acid sequence are shown in Fig. 6. The *pfpI* gene codes for a protein with a size of 18.8 kDa, which corresponds to the molecular mass of the PfpI protease subunit identified by SDS-PAGE (Fig. 4, lane 4) and laser desorption-mass spectrometry. The exact agreement of the 86-, 66-, 47-, and 20-kDa N-terminal sequences and the predicted amino acid sequence is underlined in Fig. 6 and shown in Fig. 2. In addition, two internal CNBr-derived peptides of electroeluted PfpI correspond to the expected subunit sequence and are also underlined in Fig. 6 and included in Fig. 2. The molecular mass of one of these peptides (determined by laser desorption-mass spectrometry), R-E-F-V-K-L-L-K, indicates a C-terminal lysyl residue and is not derived by cleavage after methionine. This observation suggests that the C terminus of the subunit composing PfpI matches that of the *pfpI* gene open reading frame.

Analysis of *pfpI* and promoter. When the amino acid sequence deduced from the *pfpI* gene was compared with translated sequences in the University of Wisconsin Genetics Com-

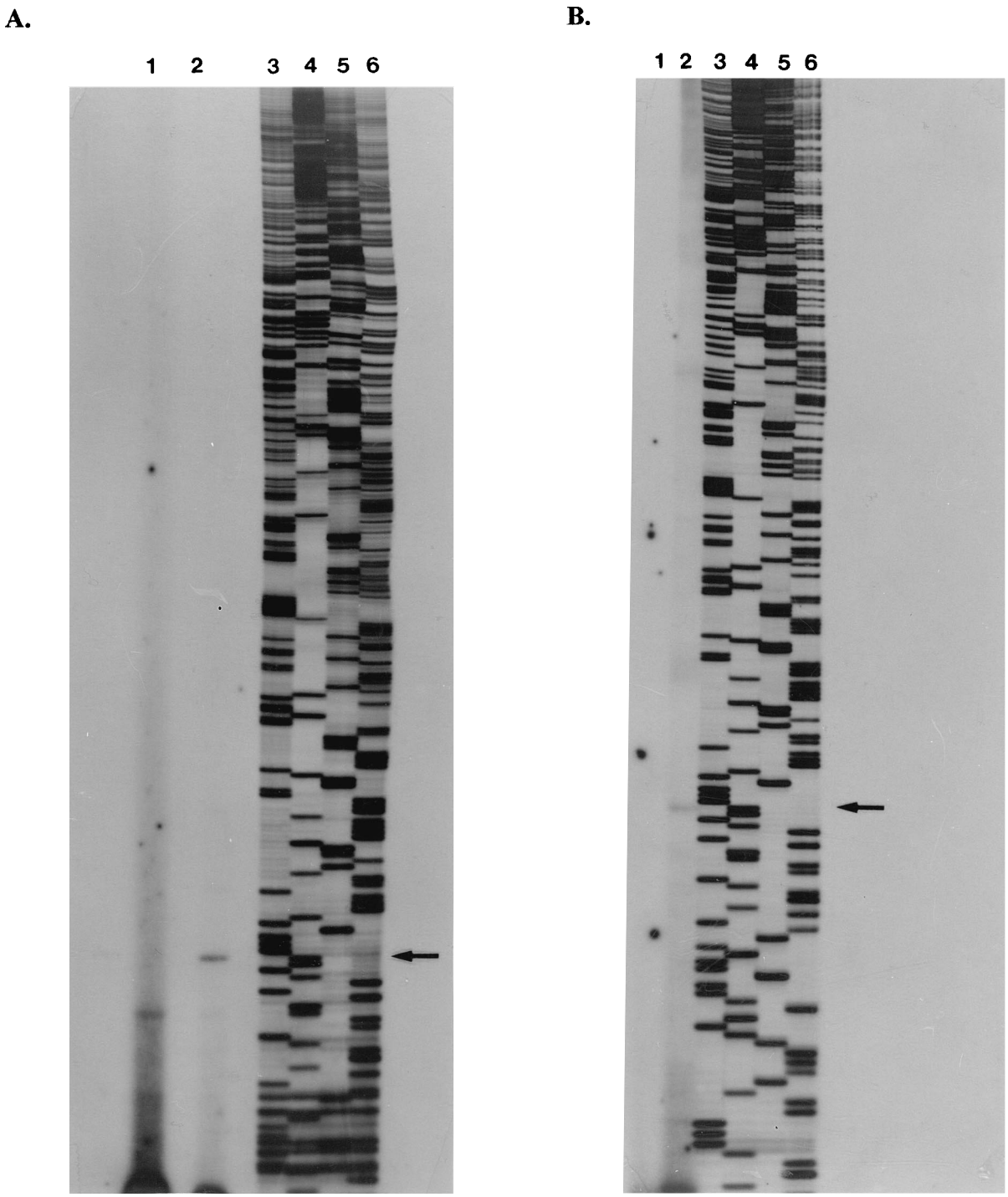

FIG. 8. Primer extension by reverse transcription of *P. furiosus* total RNA. The start of transcription is indicated by the arrows and occurs 10 nucleotides upstream of the start of translation. Lanes: 1, labeled oligonucleotide only; 2, half of the primer extension reaction mixture; 3, A; 4, C; 5, G; 6, T. The primer corresponds to the complement of either bases 58 through 74 (A) or 76 through 92 (B) in the coding region of the *pfpI* gene.

puter Group database, significant similarity was found in only two cases. The subunit was 41% identical to an entire open reading frame coded for by a gene located downstream of the peptidoglycan hydrolase gene in *Staphylococcus aureus* NCTC 8325 (5) (Fig. 6). The *S. aureus* gene codes for a 171-amino-acid polypeptide with a size of 18.6 kDa. Borchardt et al. (5) were unable to detect expression of this gene in *E. coli* maxicell or in vitro transcription-translation experiments. The PfpI subunit that was 47% identical to an open reading frame in *E. coli* was also detected (Fig. 7) and coded for a 172-amino-acid polypeptide with a size of 18.8 kDa (34); no known function of this putative protein has been proposed. In addition, PfpI does not have a conserved active site or structure as determined by structure, motif, and molecular simulation programs (9, 52) and appears to be a novel proteolytic species.

Analysis of the DNA sequence flanking the *pfpI* gene iden-

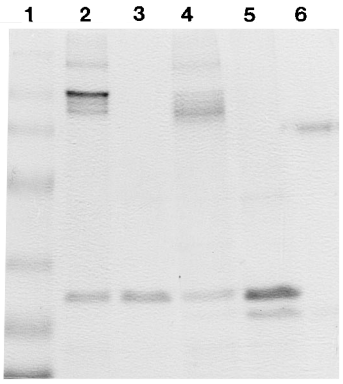

FIG. 9. Assembly and disassembly of PfpI expression product from *E. coli*. Proteins were separated by SDS-PAGE (15%) and stained with Coomassie blue R-250 and amido black. Lanes: 1, molecular mass markers as described in the legend to Fig. 3; 2, purified histidine-PfpI fusion protein; 3, same as lane 2 but TCA precipitated; 4, purified histidine-PfpI fusion protein with histidine tag removed by addition of thrombin protease; 5, same as lane 4 but TCA precipitated; 6, PfpI purified by electroelution.

tified strongly conserved box A (36) archaeal promoter sequences TTATA at −180 and TTTAAA at −116 and another possible promoter sequence TTAAA at −36 and −63 nucleotides 5′ to the initiation codon (Fig. 6). The *pfpI* transcriptional start site was located by primer extension with three different oligonucleotides complementary to bases 1 through 17, 58 through 74, and 76 through 92 of the *pfpI* gene on *P. furiosus* RNA. Results with all three primers indicated that the start of transcription was 10 nucleotides upstream of the start of translation (Fig. 8), occurring at a pyrimidine followed by a purine of the weakly conserved archaeal box B consensus sequence (17). Thus, the ribosome binding site must lie within the sequence GGTGATAGGA, immediately preceding the start of translation. This primer extension experiment also suggests that there is no pre- or pro-sequence involvement in PfpI targeting or assembly. Finally, the sequence downstream of the TGA stop codon is thymidine rich and could function in transcription termination.

Expression of *pfpI* in *E. coli*. Preliminary attempts to express

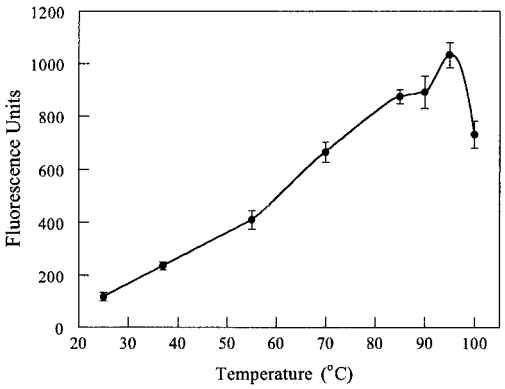

FIG. 10. Effect of temperature on proteolytic activity of histidine-PfpI fusion protein expression product. Proteolytic activity was determined by the release of MCA by the AAF-MCA peptide substrate after 5 min at the indicated temperature. Four measurements at each time point were taken by adding 5 micrograms of purified histidine-PfpI (at each temperature) to 100 μl of 100 mM sodium phosphate buffer (pH 7) containing 50 μM AAF-MCA (at indicated temperature). The background fluorescence at each temperature was subtracted; it was determined by adding 100 mM sodium phosphate buffer (pH 7) instead of the enzyme.

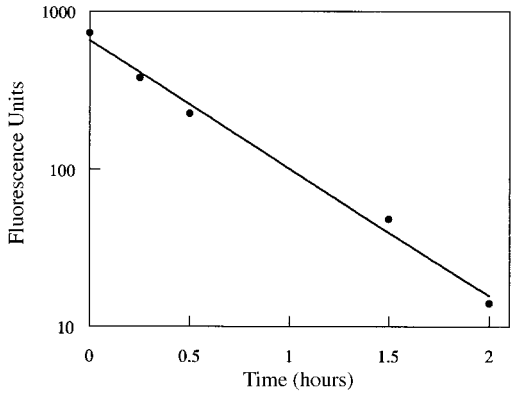

FIG. 11. Stability of histidine-PfpI fusion protein at 95°C. Purified histidine-PfpI fusion protein (6 μg) in 20 mM Tris (pH 7.9)–150 mM NaCl–2 mM $CaCl_2$ was incubated at 95°C for the indicated lengths of time. The remaining proteolytic activity was determined by the release of MCA by the AAF-MCA peptide substrate at 95°C. Six micrograms of purified histidine-PfpI (at 95°C) was added to 100 ml of 100 mM sodium phosphate buffer (pH 7) containing 50 μM AAF-MCA (at 95°C). The background fluorescence at 95°C was subtracted; it was determined by adding 100 mM sodium phosphate buffer (pH 7) instead of the enzyme.

PfpI in *E. coli* under the *lac* promoter yielded unstable vector constructs in stationary phase or induced cells; this is usually an indication of product toxicity. To improve expression levels and to obtain proteolytically active product, PfpI was expressed as a fusion protein in *E. coli* with a T7 promoter as described above. T7 RNA polymerase was added at mid-log growth phase by infection with λ phage CE6. The expressed fusion protein consisted of a histidine 6 tag followed by S-S-G-L-V-P-R-G-S-H, which is a thrombin protease recognition site used for the removal of the histidine tag, followed by the PfpI sequence. The soluble expression product was separated from the *E. coli* proteins on a histidine affinity column as described above. With this system, yields of up to 1.0 mg of purified protein from 100 ml of starting culture were obtained. The expressed subunit, with and without the histidine tag, and its stable oligomers are shown in Fig. 9.

High-temperature fluorometric protease assays were used to determine the temperature optimum (Fig. 10) and the stability at 95°C (Fig. 11) of the histidine fusion protein expression product. The highest level of activity of the recombinant protease with the histidine tag occurred at 95°C, with a half-life of 19 min at 95°C. These measurements are much lower than those observed for native PfpI obtained by electroelution, which showed a temperature optimum above 100°C and a half-life of 33 h at 98°C (4).

DISCUSSION

In this communication, we describe the cloning, sequence, and expression in *E. coli* of the gene encoding the 18.8-kDa subunit for the homooligomeric PfpI protein from the hyperthermophile *P. furiosus*. To date, the sequence from only one other hyperthermophilic protease gene, also from *P. furiosus*, has been reported (37), although the corresponding protein has yet to be isolated and characterized. Previous studies found that PfpI is a predominant intracellular protease, responsive to growth conditions (4, 46). Furthermore, the 66-kDa species shares the same N terminus as the 86-kDa species, both of which remain active after 24 h of incubation at 98°C in 1% SDS. Although these two forms are apparently responsible for the degradation of most other proteins during the incubation

(4), they do not appear to be degradation products of larger proteins. However, these two species may result from the disassociation of a larger complex containing the PfpI subunit or associated polypeptides. The 86-kDa version has higher specific activity than does the 66-kDa species as judged from a comparison of Coomassie-stained gels and visualization of proteolytic activity by fluorescence or SDS-PAGE (4). Both of these proteases appear to consist of 18.8-kDa subunits, which contain no known conserved proteolytic motifs and do not disassociate under normally denaturing conditions. As noted earlier, few gene sequences are available for hyperthermophilic archaea (11, 19, 23, 37, 38, 48, 49, 50, 54), and amino acid sequence comparisons between hyperthermophilic and mesophilic proteins have shed little light on the basis for thermostability (2). As such, the three-dimensional structures of the subunit and proteolytically active, assembled PfpI will be required to address questions of extreme thermostability.

The recombinant PfpI was found to be less active and less stable than the native enzyme. There are many possible reasons for this, including the fact that it was expressed and assayed as a fusion protein. When thrombin was added to remove the His tag, only a fraction of the fusion proteins was cleaved (Fig. 9, lanes 4 and 5). The subunits that were properly folded may not have been susceptible to cleavage by thrombin. In addition, three amino acids of the fusion protein remained after cleavage by thrombin and it is not clear how the additional amino acid residues impact protein function. A second fusion protein (PfpI preceded by the periplasmic locator leader sequence pelB) showed proteolytic activity approximately equal to that of the histidine fusion protein when the periplasmic fraction was assayed at 87°C with the Ala-Ala-Phe–7-amido-4-methylcoumarin (MCA) substrate. However, this expression system did not provide sufficient material or a means by which to purify it. When PfpI was expressed as a nonfusion protein under the *lac* promoter, expression levels were too low to be visualized by SDS-PAGE, and the product was not sufficiently active to be purified by conventional means. These low expression levels were attributed to toxicity of the protease (the native form shows low, but detectable, levels of activity at 30°C) which resulted in selection of plasmids having deletions encompassing the *pfpI* gene and vector DNA flanking this gene.

Whether PfpI is related to other multimeric, intracellular proteases of types already reported (16, 27, 35, 51) remains to be determined. We have not detected ATPase activity in electroeluted PfpI, but the possibility of an energetic effector or regulator cannot be ruled out, especially if the in vivo form of the protease is significantly larger than 66 kDa, as suggested by Western blots (immunoblots) of cell extracts (4). We have shown previously that antibodies directed against the bovine pituitary proteasome recognized PfpI (46), although the recognition pattern on a partially denaturing gel was different from that seen for purified bovine pituitary proteasome or for extracts of *Thermoplasma acidophilum* (data not shown), which is known to contain a proteasome (55). Pühler et al. (35) have concluded that *T. acidophilum* is the only member of the archaea that contains a proteasome which is closely related in sequence and structure to the eukaryotic proteasome (25). According to 16S phylogenetic trees (53), *P. furiosus* is one of the more primitive organisms yet studied. As such, in light of recent studies linking archaea to eukaryotes (39), the physiological relationship of PfpI to other proteolytic complexes is an intriguing, but still open, question. The role of PfpI in the metabolism of *P. furiosus* is at present unknown, but the availability of the gene for at least one of the subunits of a presumably primitive proteolytic complex will be useful in examining the role of intracellular proteases in archaea and how this relates to the other domains of life.

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REFERENCES

1. **Adams, M. W. W.** 1993. Enzymes and proteins from organisms that grow near and above 100°C. Annu. Rev. Microbiol. **47:**627–658.
2. **Adams, M. W. W., and R. M. Kelly.** 1994. Thermostability and thermoactivity of enzymes from hyperthermophilic microorganisms. Bioorg. Med. Chem. **2:**659–667.
3. **Baross, J. A., and J. W. Deming.** 1995. Growth at high temperatures: isolation, taxonomy, physiology and ecology, p. 169–217. *In* D. Karl (ed.), Microbiology of deep sea hydrothermal vent environments. CRC Press, Telford, N.J.
4. **Blumentals, I. I., A. S. Robinson, and R. M. Kelly.** 1990. Characterization of sodium dodecyl sulfate-resistant proteolytic activity in the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Appl. Environ. Microbiol. **56:**1992–1998.
5. **Borchardt, S. A., A. V. Babwah, and R. K. Jayaswal.** 1993. Sequence analysis of the region downstream from a peptidoglycan hydrolase-encoding gene from *Staphylococcus aureus* NCTC8325. Gene **137:**253–258.
6. **Burlini, N., P. Magnani, A. Villa, F. Macchi, P. Tortora, and A. Guerritore.** 1992. A heat-stable serine proteinase from the extreme thermophilic archaebacterium *Solfolobus solfataricus*. Biochim. Biophys. Acta **1122:**283–292.
7. **Connaris, H., D. Cowan, and R. Sharp.** 1991. Heterogeneity of proteinases from the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. J. Gen. Microbiol. **137:**1193–1199.
8. **Cowan, D. A., K. A. Smolenski, R. M. Daniel, and H. W. Morgan.** 1987. An extremely thermostable extracellular proteinase from a strain of the archaebacterium *Desulfurococcus* growing at 88°C. Biochem. J. **247:**121–133.
9. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
10. **DiRuggiero, J., and F. T. Robb.** RNA extraction from sulfur-utilizing thermophilic archaebacteria. *In* F. T. Robb (ed.), Protocols for archaeal research, in press. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
11. **Eggen, R., A. Geerling, K. Waldkotter, G. Antranikian, and W. de Vos.** 1993. The glutamate dehydrogenase-encoding gene of the hyperthermophilic archaeon *Pyrococcus furiosus*: sequence, transcription, and analysis of the deduced amino acid sequence. Gene **132:**143–148.
12. **Eggen, R., A. Geerling, J. Watts, and W. de Vos.** 1990. Characterization of pyrolysin, a hyperthermoactive serine protease from the archaebacterium *Pyrococcus furiosus*. FEMS Microbiol. Lett. **71:**17–20.
13. **Fiala, G., and K. O. Stetter.** 1986. *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotropic archaebacteria growing optimally at 100°C. Arch. Microbiol. **145:**56–60.
14. **Fusek, M., X.-L. Lin, and J. Tang.** 1990. Enzymic properties of thermopsin. J. Biol. Chem. **265:**1496–1501.
15. **Fusi, P., M. Villa, N. Burlini, P. Tortora, and A. Guerritore.** 1991. Intracellular proteases from the extremely thermophilic archaebacterium *Sulfolobus solfataricus*. Experientia **47:**1057–1060.
16. **Goldberg, A., and K. Rock.** 1992. Proteolysis, proteasomes, and antigen presentation. Nature (London) **357:**357–379.
17. **Hain, J., W. D. Reiter, U. Hudepohl, and W. Zillig.** 1992. Elements of an archaeal promoter defined by mutational analysis. Nucleic Acids Res. **20:**5423–5428.
18. **Hanner, M., B. Redl, and G. Stoeffler.** 1990. Isolation and characterization of an intracellular aminopeptidase from the extreme thermophilic archaebacterium *Sulfolobus solfataricus*. Biochim. Biophys. Acta **1033:**148–153.
19. **Heltzel, A., E. T. Smithe, and M. W. W. Adams.** 1994. Cloning, expression and molecular characterization of the gene encoding an extremely thermostable (4Fe-4S) ferredoxin from the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Bacteriol. **176:**4790–4793.
20. **Kelly, R. M., and M. W. W. Adams.** 1994. Metabolism in hyperthermophilic microorganisms. Antonie van Leeuwenhoek J. Microbiol. **66:**247–270.
21. **Kengen, S. W. M., F. A. M. de Bok, N.-D. van Loo, C. Dijkema, A. J. M. Stams, and W. M. de Vos.** 1994. Evidence for the operation of a novel

Embden-Meyerhof pathway that involves ADP-dependent kinases during sugar fermentation by *Pyrococcus furiosus*. J. Biol. Chem. **269:**17537–17541.
22. **Klingeberg, M., B. Galunsky, C. Sjoholm, V. Kasche, and G. Antranikian.** 1995. Purification and properties of a highly thermostable, sodium dodecyl sulfate-resistant and stereospecific proteinase from the extremely thermophilic archaeon *Thermococcus stetteri*. Appl. Environ. Microbiol. **61:**3098–3104.
23. **Laderman, K., K. Asada, and T. Uemori.** 1993. Alpha-amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Cloning and sequencing of the gene and expression in *Escherichia coli*. J. Biol. Chem. **268:**24402–24407.
24. **Lin, X.-L., and J. Tang.** 1990. Purification, characterization, and gene cloning of thermopsin, a thermostable acid protease from *Sulfolobus acidocaldarius*. J. Biol. Chem. **265:**1490–1495.
25. **Löwe, J., D. Stock, B. Jap, P. Zwickl, W. Baumeister, and R. Huber.** 1995. Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4 Å resolution. Science **268:**533–539.
26. **Ma, K., R. N. Schicho, R. M. Kelly, and M. W. W. Adams.** 1993. Hydrogenase of the hyperthermophile *Pyrococcus furiosus* is an elemental sulfur reductase or sulfhydrogenase: evidence for a sulfur-reducing hydrogenase ancestor. Proc. Natl. Acad. Sci. USA **90:**5341–5344.
27. **Maurizi, M., W. Clark, Y. Katayama, S. Rudikoff, J. Pumphrey, B. Bowers, and S. Gottesman.** 1990. Sequence and structure of ClpP, the proteolytic component of the ATP-dependent Clp protease of *E. coli*. J. Biol. Chem. **265:**12536–12545.
28. **McDonald, O. B., B. M. Merrill, M. M. Bland, L. C. E. Taylor, and N. Sahyoun.** 1993. Site and consequences of the autophosphorylation of Ca^2/calmodulin-dependent protein kinase type "Gr." J. Biol. Chem. **268:**10054–10059.
29. **Morikawa, M., Y. Izawa, N. Rashid, T. Hoaki, and T. Imanaka.** 1994. Purification and characterization of a thermostable thiol protease from a newly isolated hyperthermophilic *Pyrococcus* sp. Appl. Environ. Microbiol. **60:**4559–4566.
30. **Mukund, S., and M. W. W. Adams.** 1991. The novel tungsten-iron-sulfur protein of the hyperthermophilic archaebacterium *Pyrococcus furiosus* is an aldehyde ferredoxin oxidoreductase: evidence for its participation in a unique glycolytic pathway. J. Biol. Chem. **266:**14208–14216.
31. **Mukund, S., and M. W. W. Adams.** 1995. Glyceraldehyde-3-phosphate ferredoxin oxidoreductase, a novel tungsten-containing enzyme with a potential glycolytic role in the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Biol. Chem. **270:**8389–8396.
32. **Orlowski, M.** 1993. The multicatalytic proteinase complex (proteasome) and intracellular protein degradation: diverse functions of an intracellular particle. J. Lab. Clin. Med. **121:**187–189.
33. **Peters, J., M. Nitsch, B. Kühlmorgen, R. Golbik, A. Lupas, J. Kellermann, H. Engelhardt, J.-P. Pfander, S. Müller, K. Goldie, A. Engel, K.-O. Stetter, and W. Baumeister.** 1995. Tetrabrachion: a filametous archaebacterial surface protein assembly of unusual structure and extreme stability. J. Mol. Biol. **245:**385–401.
34. **Plunkett, G.** 1994. University of Wisconsin Genetics Computer Group direct submission from the *Escherichia coli* genome project, no. U18997. University of Wisconsin, Madison.
35. **Pühler, G., F. Pitzer, and P. Zwickl.** 1994. Proteasomes: multisubunit proteinases common to *Thermoplasma* and eukaryotes. Syst. Appl. Microbiol. **16:**734–741.
36. **Reiter, W., U. Hudepohl, and W. Zillig.** 1990. Mutational analysis of an archaebacterial promoter: essential role of a TATA box for transcriptional efficiency and start-site selection *in vitro*. Proc. Natl. Acad. Sci. USA **87:**9509–9513.
37. **Robinson, K. A., D. A. Bartley, F. T. Robb, and H. J. Scheier.** 1995. A gene from the hyperthermophile *Pyrococcus furiosus* whose deduced product is homologous to members of the prolyl oligopeptidase family of protease genes. Gene **152:**103–106.
38. **Robinson, K. A., and H. J. Schreier.** 1994. Isolation, sequence and characterization of the maltose-regulated *mlrA* gene from the hyperthermophioic archaeum *Pyrococcus furiosus*. Gene **151:**1173–1176.
39. **Rowlands, T., P. Baumann, and S. P. Jackson.** 1994. The TATA-binding protein: a general transcription factor in eukaryotes and archaebacteria. Science **264:**1326–1329.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
42. **Schafer, T., and P. Schönheit.** 1993. Gluconeogenesis from pyruvate in the hyperthermophilic archaeon *Pyrococcus furiosus*: involvement of reactions of the Embden-Meyerhof pathway. Arch. Microbiol. **159:**354–363.
43. **Schäfer, T., K. B. Xavier, H. Santos, and P. Schönheit.** 1994. Glucose fermentation to acetate and alanine in resting cell suspensions of *Pyrococcus furiosus*: proposal of a novel glycolytic pathway based on ^{13}C labelling data and enzyme activities. FEMS Microbiol. Lett. **121:**107–114.
44. **Schicho, R. N., K. Ma, M. W. W. Adams, and R. M. Kelly.** 1993. Bioenergetics of sulfur reduction in the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Bacteriol. **175:**1823–1830.
45. **Segerer, A., S. Burggraf, G. Fiala, G. Huber, R. Huber, U. Pley, and K. O. Stetter.** 1993. Life in hot springs and hydrothermal vents. Origins Life Evol. Biosphere **23:**77–90.
46. **Snowden, L. J., I. I. Blumentals, and R. M. Kelly.** 1992. Regulation of intracellular proteolysis in the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Appl. Environ. Microbiol. **58:**1134–1139.
47. **Thompson, M. W., and M. R. Maurizi.** 1994. Processive degradation of proteins by the ATP-dependent Clp protease from *Escherichia coli*. Requirement for the multiple array of active sites in ClpP but not ATP hydrolysis. J. Biol. Chem. **269:**18201–18208.
48. **Tiboni, O., P. Cammarano, and A. M. Sanangelantoni.** 1993. Cloning and sequencing of the gene encoding glutamine synthetase I from the archaeum *Pyrococcus woesei*: anomalous phylogenies inferred from the archaeal and bacterial glutamine synthetase I sequences. J. Bacteriol. **175:**2961–2969.
49. **Uemori, T., Y. Ishino, H. Toh, K. Asada, and I. Kato.** 1993. Organization and nucleotide sequence of the DNA polymerase gene from the archaeon *Pyrococcus furiosus*. Nucleic Acids Res. **21:**259–265.
50. **Volkl, P., P. Markiewicz, K. O. Stetter, and J. H. Miller.** 1994. The sequence of a subtilisin-type protease (aerolysin) from the hyperthermophilic archaeon *Pyrobaculum aerophilum* reveals sites important to thermostability. Protein Sci. **3:**1329–1340.
51. **Waxman, L., and A. Goldberg.** 1985. Protease La, the *lon* gene product, cleaves specific fluorogenic peptides in an ATP-dependent reaction. J. Biol. Chem. **260:**12022–12028.
52. **Wessen, L., and D. Eisenberg.** 1992. Atomic solvation parameters applied to molecular dynamics of proteins in solution. Protein Sci. **1:**227–235.
53. **Woese, C., O. Kandler, and M. Wheelis.** 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc. Natl. Acad. Sci. USA **87:**4576–4579.
54. **Zwickl, P., S. Fabry, C. A. Bogedain, and R. Hensel.** 1990. Glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic archaebacterium *Pyrococcus woesei*: characterization of the enzyme, cloning and sequencing of the gene, and expression in *Escherichia coli*. J. Bacteriol. **172:**4329–4338.
55. **Zwickl, P., A. Grziwa, G. Puhler, B. Dahlmann, F. Lottspeich, and W. Baumeister.** 1992. Primary structure of the *Thermoplasma* proteasome and its implications to the structure, function, and evolution of the multicatalytic proteinase. Biochemistry **31:**964–972.
56. **Zwickl, P., F. Lottspeich, and W. Baumeister.** 1992. Expression of functional *Thermoplasma acidophilum* proteasomes in *Escherichia coli*. FEBS Microbiol. Lett. **312:**157–160.