Differential Regulation of the PanA and PanB Proteasome-Activating Nucleotidase and 20S Proteasomal Proteins of the Haloarchaeon *Haloferax volcanii*†

Christopher J. Reuter, Steven J. Kaczowka, and Julie A. Maupin-Furrow*

Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida

Received 25 May 2004/Accepted 16 August 2004

The halophilic archaeon *Haloferax volcanii* produces three different proteins ($\alpha 1$, $\alpha 2$, and β) that assemble into at least two 20S proteasome isoforms. This work reports the cloning and sequencing of two *H. volcanii* proteasome-activating nucleotidase (PAN) genes (*panA* and *panB*). The deduced PAN proteins were 60% identical with Walker A and B motifs and a second region of homology typical of AAA ATPases. The most significant region of divergence was the N terminus predicted to adopt a coiled-coil conformation involved in substrate recognition. Of the five proteasomal proteins, the $\alpha 1$, β , and PanA proteins were the most abundant. Differential regulation of all five genes was observed, with a four- to eightfold increase in mRNA levels as cells entered stationary phase. In parallel with this mRNA increase, the protein levels of PanB and $\alpha 2$ increased severalfold during the transition from exponential growth to stationary phase, suggesting that these protein levels are regulated at least in part by mechanisms that control transcript levels. In contrast, the β and PanA protein levels remained relatively constant, while the $\alpha 1$ protein levels exhibited only a modest increase. This lack of correlation between the mRNA and protein levels for $\alpha 1$, β , and PanA suggests posttranscriptional mechanisms are involved in regulating the levels of these major proteasomal proteins. Together these results support a model in which the cell regulates the ratio of the different 20S proteasome and PAN proteins to modulate the structure and ultimately the function of this central energy-dependent proteolytic system.

Proteasomes are large nanocompartments within the cell that catalyze the energy-dependent hydrolysis of proteins. The catalytic core particle (CP) responsible for peptide bond hydrolysis is a 20S cylinder of four stacked heptameric rings which is ubiquitous in archaea, eucaryotes, and actinomycetes (19, 22). The outer and inner rings of the CP are formed by proteins of related α and β superfamilies, respectively (6). A variety of components associate with the CP; most notable are the 19S cap regulatory particles (RP), which together with the CP form eucaryal 26S proteasomes. The RP of yeast can be separated into two multisubunit substructures: the lid and base domains. The base includes six Rpt (regulatory particle triple-A type I ATPase) subunits and not only exhibits chaperone activity (4) but also facilitates the energy-dependent degradation of globular proteins by 20S proteasomes (10). These Rpt subunits are closely related to archaeal proteasome-activating nucleotidase (PAN) proteins, of which only the *Methanocaldococcus jannaschii* Pan purified from recombinant *Escherichia coli* has been characterized (2, 38, 44). Substrate binding to this AAA ATPase activates the hydrolysis of nucleotides (e.g., ATP), which subsequently promotes the unfolding of substrates, opening of the 20S proteasome axial gate(s), and apparent translocation of substrate to the internal proteolytic chamber of 20S proteasomes.

Multicellular organisms synthesize CP and RP proteasomal subtypes by integrating distinct isoforms of one or more sub-

unit. A classic example of this is the immunoproteasome of vertebrates, in which the gamma interferon-inducible isoforms of $\beta 1$, $\beta 2$, and $\beta 5$ (designated as $\beta 1i$, $\beta 2i$, and $\beta 5i$) are incorporated into CP subtypes (16, 29). Although not as well defined, expression of insect (20, 42) and plant (24) proteasomal subunit isoforms has also been detected at specific developmental stages and in response to external stimuli. Furthermore, recent advances in proteomics have demonstrated that at least 15 *Arabidopsis* CP and RP isoforms are incorporated into 26S proteasomes (41). Thus, in multicellular eucarya there are a variety of proteasome subtypes available for decoration by differential posttranslational modification (26) to provide an array of flexibility and diversity of function.

In contrast to the various proteasomal subtypes synthesized by multicellular organisms, most microorganisms encode a set of proteins that assemble into single CP and AAA ATPase RP (i.e., Rpt, PAN, and ARC) complexes (22). Although yeast synthesize seven α -type and seven β -type proteins, these different subunits assemble into a single 20S proteasome (12). Likewise, the six Rpt subunits of yeast are proposed to assemble into a single hexameric ring which forms the base of the RP (4). The two α -type and two β -type proteins of the actinomycetes *Rhodococcus erythropolis* also assemble into a single 20S proteasome (43). Recent studies, however, demonstrate that the haloarchaeon *Haloferax volcanii* encodes three proteins ($\alpha 1$, $\alpha 2$, and β) (37) that assemble into at least two 20S proteasome (CP) subtypes (14). One of these is a symmetric complex of $\alpha 1$ and β subunits ($\alpha 1\beta$ CP), while the other ($\alpha 1\alpha 2\beta$ CP) appears asymmetric with homogenous rings of $\alpha 1$ and $\alpha 2$ at each end. In addition, genome sequences reveal that other archaea encode one α and two β proteins which may form multiple 20S proteasome subtypes (22).

* Corresponding author. Mailing address: Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611-0700. Phone: (352) 392-4095. Fax: (352) 392-5922. E-mail: jmaupin@ufl.edu.

† Florida Agricultural Experiment Station journal series no. R-10376.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Phenotype, genotype, or oligonucleotides for PCR amplification	Source
<i>E. coli</i> strains		
DH5 α	F ⁻ <i>recA1 endA1 hsdR17</i> (r _k ⁻ m _k ⁺) <i>supE44 thi-1 gyrA relA1</i>	Life Technologies
BL21(DE3)	F ⁻ <i>ompT</i> [<i>lon</i>] <i>hsd S_B</i> (r _B m _B) (an <i>E. coli</i> B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene	Novagen
ER1647	F ⁻ <i>fluA2Δ(lacZ)</i> <i>r1 supE44 trp31 mcrA1272::Tn10</i> (Tet ^r) <i>his-1 rpsL104</i> (Str ^r) <i>xyl-7 mtl-2 metB1 Δ(mcrC-mrr)102::Tn10</i> (Tet ^r) <i>recD1040</i>	Novagen
<i>H. volcanii</i> strains		
DS2	Isolate from the Dead Sea	23
DS70	DS2 cured of pHV2	36
WFD11	DS2 cured of pHV2	5
Plasmids		
pUC19	Ap ^r ; cloning vector	New England BioLabs
pCR-BluntII-TOPO	Km ^r ; cloning vector	InVitrogen
pET24b	Km ^r ; expression vector	Novagen
pJAM806	Ap ^r ; 0.36-kb PCR fragment of <i>H. volcanii</i> genomic DNA blunt-end ligated into HincII site of pUC19; carries <i>pan</i> -specific probe used in Southern blotting	This study
pJAM624	Ap ^r ; 2.7-kb <i>Rsr</i> II fragment of <i>H. volcanii</i> genomic DNA ligated into SmaI site of pUC19; carries <i>panB'</i>	This study
pJAM626	Ap ^r ; 6.2-kb SmaI fragment of <i>H. volcanii</i> genomic DNA ligated into HincII site of pUC19; carries <i>panA</i>	This study
pJAM631	Ap ^r ; 2.2-kb SacII genomic fragment from <i>H. volcanii</i> blunt-end ligated into HincII site of pUC19; carries <i>panA</i>	This study
pJAM638	Km ^r ; 1.2-kb fragment generated by PCR amplification from pJAM631 cloned into pCR-BluntII-TOPO; carries <i>panA</i> without a stop codon; 5'-CATATGATGACCG ATACTGTGGAC-3' and 5'- AGGCTTAGCAAACGCGCGGGAGAC-3' (NdeI and HindIII sites in bold)	This study
pJAM639	Km ^r ; 1.1-kb fragment generated by PCR amplification from pJAM631 cloned into pCR-BluntII-TOPO; carries <i>panAΔ120</i> without a stop codon; 5'-CATATGCGC GACAAGCTCCTCGAC-3' and 5'- AGGCTTAGCAAACGCGCGGGAGAC-3' (NdeI and HindIII sites in bold)	This study
pJAM642	Km ^r ; 1.2-kb NdeI-to-HindIII fragment of pJAM638 ligated into NdeI and HindIII sites of pET24b; PanA-His expressed in <i>E. coli</i>	This study
pJAM643	Km ^r ; 1.1-kb NdeI-to-HindIII fragment of pJAM639 ligated into NdeI and HindIII sites of pET24b; PanA(Δ1-40)-His expressed in <i>E. coli</i>	This study
pJAM1006	Km ^r ; 1.24-kb fragment PCR amplified from <i>H. volcanii</i> genomic DNA ligated into NdeI and XhoI sites of pET24b; PanB-His expressed in <i>E. coli</i> ; 5'-CATATGTCA CGCAGTCCATCTCTCC-3' and 5'- CTCGAGGTACTGGTAGTCCGTGAAG-3' (NdeI and XhoI sites indicated in bold)	This study

The demonstration that *H. volcanii* synthesizes more than one 20S proteasome subtype raises the possibility that select archaea may also synthesize more than one PAN regulatory protein. In this study, we demonstrate that *H. volcanii* synthesizes two PAN homologs (PanA and PanB), which are differentially regulated at the mRNA and protein levels in a manner coordinated with the CP isoforms. Although the transcripts of all five proteasomal components increased in parallel as cells entered stationary phase, at the protein level only PanB was coordinated with α 2, while the levels of PanA were relatively constant and more consistent with α 1 and β . These results suggest that differences in the levels of CP and RP proteins are involved in mediating the transition to stationary phase. In addition, these results suggest subunit isoforms form different proteasomal subtypes throughout growth to modulate energy-dependent proteolysis in *H. volcanii*.

MATERIALS AND METHODS

Materials. Biochemicals and amplification-grade DNase I were from Sigma-Aldrich (St. Louis, Mo.). Other organic and inorganic analytical-grade chemicals were from Fisher Scientific (Atlanta, Ga.). Hybond-P membranes were from Amersham Pharmacia Biotech (Piscataway, N.J.). Desalted oligonucleotides

were from Operon Technologies (Alameda, Calif.) and Integrated DNA Technologies (Coralville, Iowa). Digoxigenin-11-dUTP (2'-deoxyuridine-5'-triphosphate coupled by an 11-atom spacer to digoxigenin), alkaline phosphatase-conjugated antibody raised against digoxigenin, and nylon membranes for colony and plaque hybridizations were from Roche Molecular Biochemicals (Indianapolis, Ind.). Positively charged membranes for Southern hybridization were from Ambion (Austin, Tex.).

Strains, media, and plasmids. Strains, oligonucleotide primers used for cloning, and plasmids are summarized in Table 1 or were as described previously (14). *E. coli* DH5 α was used for routine recombinant DNA experiments. *E. coli* ER1647 was used to generate subgenomic libraries of *H. volcanii* chromosomal DNA. *E. coli* BL21(DE3) was used as a host for expression and purification of *H. volcanii* proteins. *E. coli* strains were grown at 37°C (200 rpm) in Luria-Bertani medium. *H. volcanii* strains were grown at 42°C (200 rpm) in complex medium ATCC 974. For analysis of mRNA and protein levels, *H. volcanii* WFD11 was selected to allow for future comparison to strains expressing genes from plasmids. WFD11 was grown to exponential phase (optical density at 600 nm [OD₆₀₀], <0.2) from an isolated colony, subcultured (1% [vol/vol]) to fresh medium, and grown to exponential phase (OD₆₀₀ of 0.080). This exponential-phase culture was used as a 1% (vol/vol) inoculum into fresh medium for the analysis.

DNA isolation and analysis. Genomic DNA was isolated from *H. volcanii* DS2 as described previously (37). Plasmid DNA was isolated from *E. coli* strains using the QIAprep Spin Miniprep kit (QIAGEN, Valencia, Calif.). DNA fragments were isolated from 0.8% (wt/vol) SeaKem GTG agarose (FMC Bioproducts, Rockland, Maine) gels in 1× TAE electrophoresis buffer (40 mM Tris acetate, 2

TABLE 2. Oligonucleotide primers used in quantitative RT-PCR

Primer	Direction	Sequence
α 1	Forward	5'-CGGCGACCAACAGCACC-3'
	Reverse	5'-GTCGTCTCTCGGTCGCCAACAG-3'
α 2	Forward	5'-CCCCGACGGACGCATCTATCA-3'
	Reverse	5'-GCGCGTCGTCGAGCTTGTGC-3'
β	Forward	5'-GCTCCGGCAGCCAGTACG-3'
	Reverse	5'-GTTCTGGTGGCGCTGGATG-3'
PanA	Forward	5'-GCAGGAGATCACC GCCCT-3'
	Reverse	5'-GGTGATTTCTGAACCGTCGCAA-3'
PanB	Forward	5'-ACCGCCACGCCGAGTTAGTC-3'
	Reverse	5'-TGCCGTGCTGCTTGATGATGAC-3'

mM EDTA; pH 8.5) using the QIAquick gel extraction kit (QIAGEN). Fidelity of PCR-amplified products was confirmed by DNA sequencing. Both strands of DNA were sequenced by the dideoxy chain termination method (27) with a LI-COR automated DNA sequencer (DNA Sequencing Facility, Department of Microbiology and Cell Science, University of Florida).

A DNA probe specific for *H. volcanii* genes encoding PAN homologs was generated using the degenerate oligonucleotides 5'-TAY GGN CCN CCN GGN CAN GGN AAR AC-3' and 5'-AA NGK NCC NGG NGK NAR NAD NGC NGG-3' (where R is A + G; Y is C + T; K is G + T; D is G, A + T; N is A + G + C + T). These oligonucleotides served as primers to amplify a 0.36-kb fragment from *H. volcanii* genomic DNA by PCR using Vent DNA polymerase. The PCR fragment was blunt-end ligated into the HincII site of pUC19 to generate plasmid pJAM806 (Table 1) and sequenced to confirm that the cloned DNA was *pan*-specific. The 0.36-kb XbaI-to-PstI fragment of pJAM806 was isolated and random prime labeled with digoxigenin-11-dUTP as previously described (37) to generate a *pan*-specific probe.

For Southern analysis, *H. volcanii* genomic DNA (2.5 μ g per lane) was cleaved by a variety of restriction enzymes, including SmaI, SacII and RsrII. DNA fragments were separated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized as previously described (37) with the following modifications. Membranes were equilibrated at 68°C for 4 h, after which the *pan*-specific probe was added to a final concentration of 25 ng per ml. After 14 h at 68°C, the membranes were washed twice with 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% (wt/vol) sodium dodecyl sulfate (SDS) (5 min per wash; 25°C) and twice with 0.5 $\times$ SSC and 0.1% (wt/vol) SDS (15 min per wash; 68°C). Based on these Southern blotting data, RsrII (2 to 4 kb), SmaI (5 to 7 kb), and SacII (1.5 to 3 kb) subgenomic libraries were generated in plasmid pUC19. Plasmids carrying the *panA* gene (pJAM626 and pJAM631) and partial *panB* gene (pJAM624) (Table 1) were isolated from the subgenomic libraries by hybridization to the 0.36-kb *pan*-specific probe.

PCR was used to introduce restriction enzyme sites for directional cloning of *panA*, *panA* Δ 120, and *panB* into plasmid pET24b for expression in *E. coli* (pJAM642, pJAM643, and pJAM1006) (Table 1). The start codons of these genes were positioned 8 bp downstream of the ribosome binding sequence of pET24b by using NdeI. For epitope tagging, the 3' end of the gene was modified by PCR to remove the stop codon and provide an in-frame C-terminal addition of poly-histidine residues.

RNA isolation and analysis. Total RNA was isolated from *H. volcanii* WFD11 with the RNeasy Mini kit for bacteria (QIAGEN) with the following modifications. Cells were centrifuged for 1 min at 18,000 $\times$ g and immediately resuspended in 100 μ l of RNase-free deionized water. RNA was treated with DNase I according to the supplier (Sigma), and removal of DNA was confirmed by PCR. Quality of RNA was determined by 0.8% (wt/vol) agarose gel electrophoresis in 1 $\times$ TAE buffer. One-step quantitative reverse transcription-PCR (RT-PCR) was performed using the Quantitect SYBR Green RT-PCR kit (QIAGEN) and iCycler MyiQ real-time PCR detection system (Bio-Rad). Quantitative RT-PCR was performed on a dilution series of total RNA using the oligonucleotide primers listed in Table 2. The total RNA concentration was determined by measuring absorbance at 260 nm. Reference standards used to determine the quantity of specific transcript included *psmC*-specific mRNA and plasmid DNA cleaved by HindIII which was specific for *psmA* (pJAM618), *psmB* (pJAM621), *panA* (pJAM642), and *panB* (pJAM1006) (14) (Table 1). For these latter genes, the PCR amplification efficiency for each dilution series of total RNA was equivalent to the amplification efficiency for the corresponding DNA standard. Thus, RT was not limiting and allowed the use of DNA standards for RT-PCR analysis. The *psmC*-specific mRNA standard was generated using the MAXI-

script in vitro transcription kit according to the supplier's protocol (Ambion). Plasmid DNA carrying the *psmC* gene (pJAM619) (14) was linearized with HindIII, extracted with Tris-saturated phenol (pH 8.0) and chloroform (1:1), and precipitated with ethanol to remove residual RNases prior to in vitro transcription. RT-PCR included an RT step (50°C, 30 min) and a reverse transcriptase inactivation and DNA polymerase activation step (95°C, 15 min). This was followed by 40 cycles of denaturation at 94°C (15 s), annealing at 57.5°C (30 s), and extension at 72°C (30 s). Data were analyzed using the MyiQ single-color real-time PCR detection system software, version 1.0 (Bio-Rad). All RT-PCR and PCR amplification efficiencies were 90 to 100%, and melt curve analysis was performed to verify the uniformity of product.

Protein purification and electrophoresis. *H. volcanii* proteins were expressed from plasmids pJAM643 (PanA Δ 1-40-His), pJAM642 (PanA-His), and pJAM1006 (PanB-His) in recombinant *E. coli* BL21(DE3) as previously described (21). Cells were lysed in six volumes (wet weight/vol) of 20 mM Tris buffer, pH 7.2, containing 150 mM NaCl and 10 mM imidazole by passage through a French pressure cell at 20,000 lb/in². Cell lysate was filtered (0.22 μ m) and applied to a Ni²⁺-Sephacel column (4.8 by 0.8 cm; Pharmacia) equilibrated in lysis buffer. The column was washed with 20 mM Tris buffer, pH 7.2, containing 2 M NaCl and 60 mM imidazole (10 ml). Protein was eluted in 20 mM Tris buffer, pH 7.2, containing 2 M NaCl and 500 mM imidazole.

Protein concentrations were determined by the Coomassie blue dye binding assay with bovine serum albumin as the standard (Bio-Rad) (3). Molecular masses of proteins were estimated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (17) using the standards phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa) (Bio-Rad).

Antibody preparation and immunoblotting. Prior to antibody preparation, the N-terminal sequences of PanA Δ 1-40-His and PanA-His were determined by Edman degradation, and the mass spectra of the tryptic fragments of PanB-His were determined by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Applied Biosystems QSTAR XL hybrid LC/MS/MS; University of Florida ICBP Protein Chemistry Core). PanA Δ 1-40-His and PanB-His proteins purified from recombinant *E. coli* were separated by SDS-7.5% PAGE, excised from the gel, and used as antigen to raise polyclonal antibodies in rabbit (Cocalico Biologicals, Reamstown, Pa.). For immunoblotting, cells were harvested by centrifugation (5,000 $\times$ g; 10 min at 4°C) and stored at -70°C. Cell pellets were resuspended in 1 ml of deionized water and passed through a French pressure cell at 10,000 lb/in². Proteins were separated by SDS-7.5% and 12% PAGE and electroblotted onto Hybond-P hydrophobic polyvinylidene difluoride membranes (Amersham Biosciences) (14.5 h; 20 V at 4°C). Antigens were detected using primary polyclonal antibodies (α 1, 1:20,000; α 2, 1:2,000; β , 1:10,000; PanA, 1:3,000; PanB, 1:10,000) (14) and secondary horseradish peroxidase-conjugated anti-rabbit antibody raised in goats (Southern Biotechnology Associates, Inc., Birmingham, Ala.) and chemiluminescence with ECL Plus according to the supplier's protocol (Amersham Biosciences). Dilutions of the secondary antibody used with each of the 20S proteasome and PAN proteins were as follows: α 1, 1:10,000; α 2, 1:1,000; β , 1:10,000; PanA, 1:3,000; PanB, 1:10,000. Reference standards included β Δ -His, α 1-His, and α 2-His purified from recombinant *E. coli* as previously described (14) and PanA-His and PanB-His purified from recombinant *E. coli* as described above. For quantitative immunoblotting, the concentrations of antibodies and cell lysate were optimized to ensure the chemiluminescence signal was within the linear range of the standard curve. Chemiluminescent signal was detected by using a charge-coupled device camera (Versa Doc; Bio-Rad) and X-ray film (Hyperfilm; Amersham Biosciences). The levels of proteasomal proteins were determined using the Quantity One software package (Bio-Rad).

DNA and protein sequence analyses. Clone Manager professional suite version 6.0 (Scientific & Educational Software, Durham, N.C.) and BioEdit sequence alignment editor version 5.0.9 (13) were used for DNA and protein sequence analyses. Deduced amino acid sequences were compared to sequences available in the GenBank, EMBL, and SWISS-PROT databases at the National Center for Biotechnology Information (Bethesda, Md.) by using the BLAST network server (1). For cluster analysis, protein sequences were aligned with Clustal W version 1.4 (30) using the neighbor-joining method set with a bootstrap value of 1,000. Amino acid extensions at N and C termini and insertions were removed. These core protein sequences were used to compute a distance matrix using the default settings of the Fitch-Margoliash and least-squares distance methods with PROTDIST version 3.5c. The TreeView program (25) was used to display these results as an unrooted dendrogram. The probability that a deduced protein sequence would adopt a coiled-coil conformation was predicted using COILS with weighted and unweighted MTK and MTKL scoring matrices set to scanning windows of 21 and 28 residues (18).

HvPanA	1	--MMTDTVDDVDLPYDKDSASOOEKITALOERLEVLETONEEEMRDKLLDT	48
HvPanB	1	MSRSPSLPERPHDLDPDPE-MSDAERLSALROHFERMVDVNRELDQRLQNA	49
HvPanA	49	NAENNKYQOKLERLTHENKKLKQSPLFVATVQEIITDEGVIIKQHGNNQEA	98
HvPanB	50	DDRHAELVDEVDOMKARNEALKTASYIATVEELTDDGVIIKQHGNNQEV	99
HvPanA	99	LTEVTDEMR-EELEPDARVAVNNSLSIVKRLDKETDVRARVMQVEHSPDV	147
HvPanB	100	LTEFAPSLNIDDIEPGDRVAINDSFVAVQTVLDDDETDAQAAMEVVESPTV	149
HvPanA	148	TYEDIGGLEEQMOEVRETVEMLDRPEMFAEVGIDPPSGVLLYGPPGTGK	197
HvPanB	150	TYDDIGGIDEQVREVREAVEQPLENPEMFAEVGIDPPSGVLLYGPPGTGK	199
		GXXGXGK	
HvPanA	198	TMLAKAVANQTNASFIKMGSELVHKFIGEGANVVRDLFEVARENEPAVI	247
HvPanB	200	TMLAKAVANETNASFIKMGSELVQKFIGEGARLVRLDFKLAAREPVPVV	249
		T	
HvPanA	248	FIDEIDATASKRTDSKTSGDAEVQRTMMQLLAEMDGFDERGNIRIIAATN	297
HvPanB	250	FIDEIDAVASKRTDSKTSGDAEVQRTMMQLLSEMDGFDDRGDIRIIAATN	299
		DEXD	TN
HvPanA	298	RFDMLDPAILRPGRFDRLEVPKPNEDGREIIFQIHTRKMNVSDDDVDFVE	347
HvPanB	300	RFDMLDEAILRPGRFDRLEVPKPAVEGRRHILDIHTRDMNVADDVDLDA	349
		XXXXDXAXXXRXD	
HvPanA	348	LAEMADNASGADIKAVCTEAGMFAIRDDRTEIFMQDFVDAWEKIQQEASD	397
HvPanB	350	LAELDDYSGADIASLTTEAGMFAIRDGRTEVTGADFDAHEKLSNVDES	399
HvPanA	398	ETEVSRAFA----	406
HvPanB	400	GTGPISGETDYQY	412

FIG. 1. Amino acid sequence alignment of *H. volcanii* PanA and PanB. Identical residues are shaded in black. Functionally conserved and semiconserved amino acid residues are shaded in grey. Dashes indicate gaps introduced in the protein sequence alignment. Boxed residues were predicted with a >90% probability to form a coiled-coil conformation (see Materials and Methods). Consensus sequences of the Walker A and B boxes of the P-loop nucleotidase core are indicated below the alignment as GX₂GXXGKT and DEXD, respectively (where X is any amino acid residue). The AAA ATPase second region of homology or SHR motif [(T/S)-(N/S)-X₅-DXA-X₂-R-X₂-RX-(D/E)] is also indicated. The N-terminal sequences of the PanA-His and PanA_{Δ1-40}-His antigens were identical to residues 2 to 14 and 41 to 51 of the deduced primary sequence, respectively. MALDI-TOF Q-STAR detected the mass spectra of 11 tryptic fragments of the PanB-His antigen, which encompassed 36% of the primary amino acid sequence. The masses (in daltons) and corresponding residue numbers of PanB-His were as follows: 716.3148, 30 to 34; 749.3711, 35 to 40; 1,004.5522, 330 to 337; 1,264.6408, 205 to 261; 1,307.7720, 317 to 328; 1,490.6807, 379 to 392; 1,548.7975, 301 to 313; 1,836.7691, 275 to 289; 2,180.0845, 118 to 137; 2,408.1564, 4 to 24; 2,739.3637, 138 to 162.

Nucleotide sequence accession number. The nucleotide sequences of the *panA* and *panB* genes of *H. volcanii* DS2 were assigned GenBank accession numbers AY627303 and AY627304.

RESULTS AND DISCUSSION

PAN genes (*panA* and *panB*) of *H. volcanii*. To date, studies on archaeal PAN proteins have been limited to the biochemistry of a single enzyme (*M. jannaschii* Pan) purified from recombinant *E. coli* (38, 44). To further understand the function of haloarchaeal PAN proteins, the *pan* gene was targeted for isolation from *H. volcanii*, one of the few archaea with an established genetic exchange system. Two degenerate oligonucleotides were synthesized based on the conserved regions YGPPGTG and FRGPR(F/L)(F/L/I)AP of archaeal PAN homologs and the codon bias of *H. volcanii* (34). Using these oligonucleotides, a *pan* probe was generated by PCR and used for DNA hybridization to *H. volcanii* genomic DNA. This enabled the isolation of two genes (*panA* and *panB*) encoding PAN homologs from subgenomic libraries of *H. volcanii*. The *panA* gene encoded a 406-amino-acid protein of 45,664.31 Da

and a calculated pI of 4.46 (Fig. 1). The deduced protein sequence was found to be identical to open reading frame RVO01394 and enabled closure of contigs 06262 and 06170 of the partial genome sequence of *H. volcanii* (<http://zdna2.umbi.umd.edu/>). In contrast to *panA*, the *panB* gene was not predicted by partial genome sequencing. The DNA sequence of this report enabled closure of contigs 06607 and 06375 of the partial genome sequence and completion of the entire *panB* gene. The *panB* gene was found to encode a protein of 412 amino acids (45,410.39 Da) and a calculated pI of 4.32. Based on these results combined with our group's previous work (37), all five proteasomal genes, including those encoding the α 1, α 2, and β subunits of the two 20S proteasome subtypes, are dispersed throughout the *H. volcanii* genome.

The deduced PanA and PanB protein sequences are closely related, with 60% identical and 78% similar amino acid residues (Fig. 1). The most significant region of identity was the central P-loop nucleotidase domain, which includes Walker A and B boxes, as well as the second region of homology typical of AAA ATPases (9). N-terminal residues of PanA and PanB

FIG. 2. Dendrogram summarizing the relationships between archaeal PAN and eucaryal Rpt proteins. Cluster analysis was performed as described in Materials and Methods. The scale bar represents 0.1 nucleotide substitutions per site. Organism abbreviations: Hs, *Homo sapiens*; Ce, *C. elegans*; Dm, *D. melanogaster*; At, *A. thaliana*; Sc, *S. cerevisiae*; Gl, *Giardia lamblia*; Af, *Archaeoglobus fulgidus*; Hm, *Haloarcula marismortui*; Hb, *Halobacterium* sp. strain NRC-1; Hv, *H. volcanii*; Mj, *M. jannaschii*; Mk, *Methanopyrus kandleri*; Ma, *Methanosarcina acetivorans*; Mb, *Methanosarcina barkeri*; Mm, *Methanosarcina mazei*; Mt, *Methanothermobacter thermoautotrophicus*; Pa, *Pyrococcus abyssi*; Pf, *Pyrococcus furiosus*; Ph, *Pyrococcus horikoshii*; Ap, *Aeropyrum pernix*; Ss, *Sulfolobus solfataricus*; Ne, *Nanoarchaeum equitans*. GenBank, EMBL, SwissProt, or locus tag accession numbers (in parentheses) were as follows: HsRpt1 (P35998), HsRpt2 (Q03527), HsRpt3 (P43686), HsRpt4 (Q92524), HsRpt5a (AAB24840), HsRpt5b (P17980), HsRpt6 (P47210), CeRpt1 (T20152), CeRpt2a (T31800), CeRpt2b (AAB42248), CeRpt3 (A88485), CeRpt4 (AAB70326), CeRpt5 (T33155), CeRpt6a (AAM48537), CeRpt6b (T27048), DmRpt1 (AAF59219), DmRpt2 (AAB34134), DmRpt3a (AAF48001), DmRpt3b (AAF54440), DmRpt4a (AAR46146), DmRpt4b (AAF08391), DmRpt4c (AAF49987), DmRpt5a (AAF56177), DmRpt5b (AAD24194), DmRpt6a (AAC63219), DmRpt6b (AAF57069), AtRpt1a (At1g53750), AtRpt1b (At1g53780), AtRpt2a (At4g29040), AtRpt2b (At2g20140), AtRpt3 (At5g58290), AtRpt4a (At1g45000), AtRpt4b (At5g43010), AtRpt5a (At3g05530), AtRpt5b (At1g09100), AtRpt6a (At5g19990), AtRpt6b (At5g20000), ScRpt1 (YKL145w), ScRpt2 (YDL007w), ScRpt3 (YDR394w), ScRpt4 (YOR259c), ScRpt5 (YOR117w), ScRpt6 (YGL048c), GlRpt1 (EAA40208), GlRpt2 (EAA39033), GlRpt3 (EAA42904), GlRpt4 (EAA41176), GlRpt5 (EAA42075), GlRpt6 (EAA42208), AfPan (AF1976), HmPanA (Contig93_11146_12363), HmPanB (Contig169_4249_3029), HbPanA (VNG0510g), HbPanB (VNG2000g), HvPanA (AY627303), HvPanB (AY627304), MjPan (MJ1176), MkPan (MK0878), MaPanA (MA4268), MaPanB (MA4123), MbPanA (Meth3002), MbPanB (Meth2182), MmPanA (MM1006), MmPanB (MM0798), MtPan (MTH728), PaPan (PAB2233), PfPan (PF0115), PhPan (PH0201), ApPan (APE2012), SsPan (SSO0271), NePan (NEQ186).

(position 17 to 72 and 39 to 73, respectively) are predicted to adopt a coiled-coil conformation. In contrast to the central domain, however, the N-terminal coiled-coil domains were not highly related (17% identity) and differed in length by 21

residues (three helical turns). Likewise, C-terminal residues 324 to 406 of PanA were only 49% identical to residues 322 to 412 of PanB. This divergence in the primary sequence of the N and C termini, combined with the highly conserved central

domain, suggests that PanA and PanB mediate similar yet distinct functions in *H. volcanii*. The N-terminal coiled-coil region of PAN/Rpt has been proposed to be involved in interactions with other RP subunits and/or substrate proteins (11). Furthermore, modification of the C terminus of Rpt1 (i.e., addition of a Flag-His₆ epitope tag in *Saccharomyces cerevisiae*) inhibits RP association with the CP in yeast (31). Thus, based on differences in the N and C termini, it is possible that the PanA and PanB proteins of *H. volcanii* recognize different substrate proteins and/or differ in their affinities for $\alpha 1\beta$ and $\alpha 1\alpha 2\beta$ 20S proteasome subtypes.

Cluster analysis was used to compare the *H. volcanii* PanA and PanB proteins to other archaeal PAN and eucaryal Rpt homologs (Fig. 2). The comparison revealed that PAN and Rpt proteins are related in primary sequence yet cluster into two major groups based on archaeal versus eucaryal origin. All of the eucaryal Rpt proteins were found to cluster into six subgroups (Rpt1 to -6), even when the genes were from multicellular organisms that encode more than six Rpt genes (i.e., humans, *Caenorhabditis elegans*, *Arabidopsis thaliana*, and *Drosophila melanogaster*). This subgrouping is consistent with biochemical evidence that suggests six different Rpt proteins assemble as a hexameric ring to form the RP interface which associates with the CP (35). The high level of sequence similarity among the PAN/Rpt proteins coupled with the species distribution of these genes has led to the suggestion that eucaryal Rpt genes originated by duplication and divergence from an ancestral proteasome-associated ATPase (32, 40). The few archaea that do not encode PAN proteins (i.e., *Thermoplasma acidophilum*, *Thermoplasma volcanium*, and *Pyrobaculum aerophilum*) likely encountered loss of this gene during evolution.

Of the archaea, only the haloarchaea and methanosarcina were found to encode more than one PAN homolog (indicated by group I [PanA] and group II [PanB]) (Fig. 2). Of these, the methanosarcina PanA proteins were more closely related to the single PAN homologs distributed among the *Crenarchaeota*, *Nanoarchaeota*, and *Euryarchaeota*. This close association suggests that the methanosarcina PanA are the most ancient of the PAN paralogs. In contrast, the haloarchaeal PanA and PanB proteins share a high level of identity, suggesting that they arose from a relatively recent gene duplication event which most likely occurred near the divergence of the haloarchaeal lineage. This is also based on the finding that the haloarchaeal PanA and PanB proteins are more closely related to their respective counterparts from other haloarchaea than to the homolog within the same species. Together these results suggest that the archaeal PAN paralogs, including those of *H. volcanii*, play nonredundant roles in cell physiology. In contrast, many of the eucaryal Rpt paralogs are nearly identical; for example, the Rpt2, Rpt4, Rpt5, and Rpt6 paralogs of *A. thaliana* share 94 to 99% identity.

PAN proteins (PanA and PanB) of *H. volcanii*. The synthesis of multiple archaeal PAN proteins has not yet been reported. In fact, most archaea encode only a single PAN homolog, and several do not encode any PAN (e.g., *Thermoplasma* spp., *Pyrobaculum*). To address whether *H. volcanii* synthesizes both the PanA and PanB proteins, an immunoblotting approach was employed. Polyclonal antibodies were raised in rabbits against PanA _{Δ 1-40}-His and PanB-His purified from recombinant *E.*

FIG. 3. PanA and PanB are produced in *H. volcanii*. Cell lysate of stationary-phase *H. volcanii* DS70 (10 μ g) (lanes 2 and 4) as well as PanA-His (45 ng) (lane 1) and PanB-His (20 ng) (lane 3) purified from recombinant *E. coli* were separated by SDS-7.5% PAGE. Protein was analyzed by Western blotting with anti-PanA (lanes 1 and 2) and anti-PanB (lanes 3 and 4) antibodies. Molecular mass standards are indicated to the right. Preimmune serum was included as a negative control (data not shown).

coli. Antigen fidelity was confirmed by N-terminal sequencing and Q-STAR MALDI-TOF (Fig. 1). Western blot analysis of cell lysate prepared from stationary-phase *H. volcanii* was performed with these antibodies (Fig. 3). Both PanA and PanB proteins were detected, and based on migration by SDS-PAGE their molecular masses were estimated to be 54 and 65 kDa, respectively. Under similar electrophoretic conditions, the PanA-His and PanB-His purified from recombinant *E. coli* migrated as slightly larger proteins (55 and 66 kDa) due to the addition of six C-terminal histidine residues. The similar migration of the PanA and PanB proteins purified from recombinant *E. coli* to those of *H. volcanii* supports the conclusion that the gene sequences isolated from the *H. volcanii* chromosome code for the PanA and PanB polypeptides detected in cell lysate. It remains to be determined why the molecular masses calculated from the deduced PanA and PanB protein sequences were at least 8 kDa less than those estimated by SDS-PAGE gels. Although the separation of halophilic proteins by SDS gel electrophoresis usually overestimates molecular weight (33), this alone is unlikely to account for the observed differences. It is possible that the N-terminal region of these PAN proteins adopts a coiled-coil conformation under these conditions which retards their electrophoretic migration.

Levels of 20S proteasome and PAN proteins in *H. volcanii*. Based on the results presented above coupled with our group's previous work (14), it is apparent that *H. volcanii* synthesizes five different proteasomal proteins, including three 20S ($\alpha 1$, $\alpha 2$, and β) and two PAN (PanA and PanB) proteins. This combination of proteasomal proteins is unique and suggests that the closely related α ($\alpha 1$ and $\alpha 2$) and PAN paralogs (PanA and PanB) may serve similar yet nonredundant functions in *H. volcanii* cell physiology. If the functions of these paralogs are distinct, it is quite possible that the levels of these proteins are differentially regulated based on growth and/or environmental conditions. To address this, the levels of the 20S proteasome and PAN proteins were estimated by quantitative immunoblot-

FIG. 4. Levels of 20S proteasome and PAN proteins of *H. volcanii* based on a quantitative immunoblot assay. Immunoblotting of $\alpha 1$ (●), PanA (○), β (■), $\alpha 2$ (□), and PanB (▲) and growth (OD₆₀₀ [Δ]) are shown. Time (in hours) refers to the amount of time elapsed during growth. Protein levels are the percentage of total protein of cell lysate from three independent experiments. The overall trend in protein levels (including the reduction in the levels of $\alpha 2$, PanB, PanA, and β proteins in log phase) was similar for all three experiments.

ting throughout the course of growth of *H. volcanii* (Fig. 4). Based on this analysis, significant differences were detected in the levels of the proteasomal proteins. In particular, the expression patterns of the paralogous $\alpha 1$ and $\alpha 2$ as well as PanA and PanB proteins differed. The PanA and β proteins remained relatively constant throughout growth, each accounting for $\sim 0.4\%$ of the total protein (Fig. 4). The $\alpha 1$ protein exhibited a modest but steady and linear ($r^2 = 0.9679$) increase over the same period, increasing by 0.0044% total protein per h from 0.16 to 0.39% of total protein as cells transitioned from exponential to stationary phase. In contrast, the PanB and $\alpha 2$ proteins increased rapidly as cells entered stationary phase.

Though dramatic increases were seen in the levels of the $\alpha 2$ and PanB proteins between exponential and stationary phase, these proteins were present at notably lower levels than the three other proteasomal proteins ($\alpha 1$, β , and PanA). In stationary phase, the $\alpha 2$ and PanB proteins each accounted for only about $\sim 0.12\%$ of the total soluble protein, one-third that of the $\alpha 1$, β , and PanA proteins.

Based on these results, 0.9 to 1.1% of the total protein of an exponential-phase culture of *H. volcanii* consists of 20S proteasomal and PAN proteins. During stationary phase, the overall levels of these proteins increase to $\sim 1.5\%$ of total protein. These estimates of the percentage of 20S proteasomal protein

to total protein are in good agreement with data based on specific activity of crude extract compared to 20S proteasomes purified from stationary-phase *H. volcanii* (data not shown) as well as that from other bacterial and eucaryotic cells (0.2 to 1.1% of the total soluble protein) (7, 15, 28). The estimates of *H. volcanii* PAN proteins are also consistent with the levels estimated for the *Rhodococcus erythropolis* ARC protein, a distantly related bacterial homolog. This ARC protein was determined to be 0.1% of total protein as analyzed by two-dimensional PAGE with polyclonal anti-ARC antibodies (39).

mRNA levels of 20S proteasome and PAN proteins in *H. volcanii*. The differential expression of the five proteasomal proteins throughout growth of *H. volcanii* raises the possibility that the levels of these proteins are coordinated at the transcriptional level. The availability of the *psmA*, *psmB*, *psmC*, *panA*, and *panB* genes encoding α 1, β , α 2, PanA, and PanB proteins, respectively, allowed the mRNA levels of these genes to be investigated by quantitative RT-PCR. Surprisingly, the transcript levels of all five genes were found to increase from four- to eightfold as cells transitioned from exponential to stationary phase (Fig. 5). The *psmC*- and *panB*-specific mRNA levels displayed the most pronounced increase (seven- to eightfold), while the increases in *panA*-, *psmB*-, and *psmA*-specific mRNA levels were somewhat less (four- to sixfold). Interestingly, throughout growth, the overall levels of the *psmC*- and *panA*-specific mRNA were more than 10-fold higher than those specific for *psmA*, *psmB*, and *panB*. The *psmC*- and *panA*-specific mRNA accounted for ~0.14 and 0.28%, respectively, of the total RNA from stationary-phase *H. volcanii* cells (Fig. 5). In contrast the *psmA*-, *psmB*-, and *panB*-specific mRNA accounted for only 0.01, 0.008, and 0.003%, respectively, of the total RNA in the same phase of growth (Fig. 5). The reason for these differences remains to be determined.

Since the genes coding for the 20S proteasome and PAN proteins are dispersed throughout the *H. volcanii* genome, a *trans*-activating factor(s) is most likely required to coordinate the regulated increase in transcript levels observed during the transition to stationary phase. The reason why all proteasomal gene transcript levels increased is not immediately evident, since only α 2 and PanB proteins displayed a substantial increase at the protein level during this same period of growth, while α 1, β , and PanA remained relatively constant. Previous analysis in eucaryotes, however, revealed that proteasome-specific mRNA levels do not necessarily correlate with protein (8). Thus, it appears that both transcriptional and posttranscriptional mechanisms are involved in regulating the levels of proteasomal proteins in *H. volcanii*.

Conclusion. Our group's previous work (14) demonstrated that *H. volcanii* synthesizes at least two 20S proteasomes (the symmetrical α 1 β CP and asymmetrical α 1 α 2 β CP). These proteasomal subtypes differ substantially in residues located at the ends of the 20S cylinder as well as residues in a loop restricting the channel opening (based on protein modeling). Thus, changes in the ratio of α 1 to α 2 were proposed to modulate distinct structural domains in 20S proteasomes. This in turn may alter the gating and/or the type of ATPase (e.g., PAN) that associates with 20S proteasomes and, subsequently, regulate the type of protein substrate recognized for destruction. In this study, we identified two *H. volcanii* PAN homologs (PanA and PanB) which, based on primary sequences, are predicted

FIG. 5. Levels of 20S proteasome and Pan-specific mRNA of *H. volcanii*. (A) mRNA levels were analyzed by quantitative RT-PCR from three independent experiments. Total RNA purified from exponential phase (gray) (OD_{600} of 1.1) and stationary phase (black) (OD_{600} of 3.2) is shown. (B) Total RNA concentration was determined by absorbance at 260 nm of a dilution series of the sample. rRNA separated by gel electrophoresis and estimated by ethidium bromide staining was used as an invariant control to ensure equal quality of sample. Lanes 1 and 2, total RNA prepared from exponential- and stationary-phase cells, respectively.

to differ in their affinities for substrate proteins and/or 20S proteasome subtypes. The high levels and relatively constant expression of the PanA protein throughout growth paralleled that of the 20S proteasomal α 1 and β proteins (Fig. 6). In contrast, the PanB and α 2 proteins were expressed at relatively low levels but were highly regulated as cells transitioned from exponential to stationary phase. This suggests that PanA may associate with the α 1 β 20S proteasome and play a central role in the housekeeping functions of the cell, while the PanB and α 1 α 2 β 20S proteasomes function in auxiliary tasks. The in-

FIG. 6. Working model for the modulation of 20S proteasome (CP) and Pan isoforms in *H. volcanii*. A transition is predicted to occur between a primarily $\alpha 1\beta$ CP and PanA complex in log phase to a mixture of $\alpha 1\beta$ and $\alpha 1\beta\alpha 2$ CP isoforms and PanA- and PanB-containing complexes in stationary phase. Whether PanA and PanB associate as PanA, PanB, and PanA/PanB complexes to allow for association with the 20S proteasome isoforms and recognize a greater diversity of protein substrates in this latter stage of growth remains to be determined.

crease in the protein levels of $\alpha 2$ and PanB from exponential to stationary phase raises the possibility that these subunits play a role during this transition period, perhaps acting to enhance proteolysis of specific proteins. Whether the PanA and PanB proteins associate as hetero- and/or homo-oligomers and whether other 20S proteasome subtypes exist in *H. volcanii* remain to be determined.

ACKNOWLEDGMENTS

We thank Francis Davis and Jack Shelton for DNA sequencing. Special thanks also to Julia Timofeev and Christopher Ainsley Davis for their assistance.

This work was supported in part by the National Institutes of Health (GM57498-03) and the Florida Agricultural Experiment Station.

REFERENCES

- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- Benaroudj, N., P. Zwickl, E. Seemuller, W. Baumeister, and A. L. Goldberg. 2003. ATP hydrolysis by the proteasome regulatory complex PAN serves multiple functions in protein degradation. *Mol. Cell* **11**:69–78.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Braun, B. C., M. Glickman, R. Kraft, B. Dahlmann, P. M. Kloetzel, D. Finley, and M. Schmidt. 1999. The base of the proteasome regulatory particle exhibits chaperone-like activity. *Nat. Cell Biol.* **1**:221–226.
- Charlebois, R. L., W. L. Lam, S. W. Cline, and W. F. Doolittle. 1987. Characterization of pHV2 from *Halobacterium volcanii* and its use in demonstrating transformation of an archaeobacterium. *Proc. Natl. Acad. Sci. USA* **84**:8530–8534.
- Coux, O., K. Tanaka, and A. L. Goldberg. 1996. Structure and functions of the 20S and 26S proteasomes. *Annu. Rev. Biochem.* **65**:801–847.
- Fernandez, M. P., M. J. Biscoglio, and S. Passeron. 2000. Purification and characterization of *Candida albicans* 20S proteasome: identification of four proteasomal subunits. *Arch. Biochem. Biophys.* **375**:211–219.
- Frentzel, S., I. Kuhn-Hartmann, M. Gernold, P. Gott, A. Seelig, and P.-M. Kloetzel. 1993. The major histocompatibility complex-encoded β -type proteasome subunits LMP2 and LMP7. Evidence that LMP2 and LMP7 are synthesized as proproteins and that cellular levels of both mRNA and LMP-containing 20S proteasomes are differentially regulated. *Eur. J. Biochem.* **216**:119–126.
- Frickey, T., and A. N. Lupas. 2004. Phylogenetic analysis of AAA proteins. *J. Struct. Biol.* **146**:2–10.
- Glickman, M. H., D. M. Rubin, O. Coux, I. Wefes, G. Pfeifer, Z. Cjeka, W. Baumeister, V. A. Fried, and D. Finley. 1998. A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell* **94**:615–623.
- Gorbea, C., D. Taillandier, and M. Rechsteiner. 2000. Mapping subunit contacts in the regulatory complex of the 26S proteasome. S2 and S5b form a tetramer with ATPase subunits S4 and S7. *J. Biol. Chem.* **275**:875–882.
- Groll, M., L. Ditzel, J. Löwe, D. Stock, M. Bochtler, H. D. Bartunik, and R. Huber. 1997. Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* **386**:463–471.
- Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**:95–98.
- Kaczowka, S. J., and J. A. Maupin-Furlow. 2003. Subunit topology of two 20S proteasomes from *Halobacterium volcanii*. *J. Bacteriol.* **185**:165–174.
- Klinkradt, S., R. J. Naude, K. Muramoto, and W. Oelofsen. 1997. Purification and characterization of proteasome from ostrich liver. *Int. J. Biochem. Cell Biol.* **29**:611–622.
- Kloetzel, P. M., and F. Ossendorp. 2004. Proteasome and peptidase function in MHC class I-mediated antigen presentation. *Curr. Opin. Immunol.* **16**:76–81.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Lupas, A. 1996. Prediction and analysis of coiled-coil structures. *Methods Enzymol.* **266**:513–525.
- Lupas, A., J. M. Flanagan, T. Tamura, and W. Baumeister. 1997. Self-compartmentalizing proteases. *Trends Biochem. Sci.* **22**:399–404.
- Ma, J., E. Katz, and J. M. Belote. 2002. Expression of proteasome subunit isoforms during spermatogenesis in *Drosophila melanogaster*. *Insect Mol. Biol.* **11**:627–639.
- Maupin-Furlow, J. A., H. C. Aldrich, and J. G. Ferry. 1998. Biochemical characterization of the 20S proteasome from the methanococcus *Methanospirillum thermophilum*. *J. Bacteriol.* **180**:1480–1487.
- Maupin-Furlow, J. A., M. A. Gil, I. M. Karadzic, P. A. Kirkland, and C. J. Reuter. 2004. Proteasomes: perspectives from the archaea (update 2004). *Front. Biosci.* **9**:1743–1758.
- Mullakhanbhai, M. S., and H. Larsen. 1975. *Halobacterium volcanii* sp. nov., a Dead Sea *Halobacterium* with a moderate salt requirement. *Arch. Microbiol.* **104**:207–214.
- Oguchi, S., H. Sassa, and H. Hirano. 2001. OsPAA2, a distinct $\alpha 1$ subunit gene for the 20S proteasome in rice (*Oryza sativa* L.). *Gene* **272**:19–23.
- Page, R. D. 1996. TreeView: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12**:357–358.
- Rivett, A. J., S. Bose, P. Brooks, and K. I. Broadfoot. 2001. Regulation of

- proteasome complexes by γ -interferon and phosphorylation. *Biochimie* **83**: 363–366.
27. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 28. Tamura, T., I. Nagy, A. Lupas, F. Lottspeich, Z. Cejka, G. Schoofs, K. Tanaka, R. De Mot, and W. Baumeister. 1995. The first characterization of a eubacterial proteasome: the 20S complex of *Rhodococcus*. *Curr. Biol.* **5**:766–774.
 29. Teoh, C. Y., and K. J. Davies. 2004. Potential roles of protein oxidation and the immunoproteasome in MHC class I antigen presentation: the “PrOxI” hypothesis. *Arch. Biochem. Biophys.* **423**:88–96.
 30. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673–4680.
 31. Verma, R., S. Chen, R. Feldman, D. Schieltz, J. Yates, J. Dohmen, and R. J. Deshaies. 2000. Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. *Mol. Biol. Cell* **11**:3425–3439.
 32. Volker, C., and A. N. Lupas. 2002. Molecular evolution of proteasomes. *Curr. Top. Microbiol. Immunol.* **268**:1–22.
 33. Vyazmensky, M., Z. Barak, D. M. Chipman, and J. Eichler. 2000. Characterization of acetoxy acid synthase activity in the archaeon *Haloferax volcanii*. *Comp. Biochem. Physiol. B* **125**:205–210.
 34. Wada, K., Y. Wada, F. Ishibashi, T. Gojobori, and T. Ikemura. 1992. Codon usage tabulated from the GenBank genetic sequence data. *Nucleic Acids Res.* **20**:2111–2118.
 35. Walz, J., A. Erdmann, M. Kania, D. Typke, A. J. Koster, and W. Baumeister. 1998. 26S proteasome structure revealed by three-dimensional electron microscopy. *J. Struct. Biol.* **121**:19–29.
 36. Wendoloski, D., C. Ferrer, and M. L. Dyll-Smith. 2001. A new simvastatin (mevinolin)-resistance marker from *Haloarcula hispanica* and a new *Haloferax volcanii* strain cured of plasmid pHV2. *Microbiology* **147**:959–964.
 37. Wilson, H. L., H. C. Aldrich, and J. A. Maupin-Furlow. 1999. Halophilic 20S proteasomes of the archaeon *Haloferax volcanii*: purification, characterization, and gene sequence analysis. *J. Bacteriol.* **181**:5814–5824.
 38. Wilson, H. L., M. S. Ou, H. C. Aldrich, and J. A. Maupin-Furlow. 2000. Biochemical and physical properties of the *Methanococcus jannaschii* 20S proteasome and PAN, a homolog of the ATPase (Rpt) subunits of the eucaryal 26S proteasome. *J. Bacteriol.* **182**:1680–1692.
 39. Wolf, S., I. Nagy, A. Lupas, G. Pfeifer, Z. Cejka, S. A. Müller, A. Engel, R. De Mot, and W. Baumeister. 1998. Characterization of ARC, a divergent member of the AAA ATPase family from *Rhodococcus erythropolis*. *J. Mol. Biol.* **277**:13–25.
 40. Wollenberg, K., and J. C. Swaffield. 2001. Evolution of proteasomal ATPases. *Mol. Biol. Evol.* **18**:962–974.
 41. Yang, P., H. Fu, J. Walker, C. M. Papa, J. Smalle, Y. M. Ju, and R. D. Vierstra. 2004. Purification of the *Arabidopsis* 26S proteasome: biochemical and molecular analyses revealed the presence of multiple isoforms. *J. Biol. Chem.* **279**:6401–6413.
 42. Yuan, X., M. Miller, and J. M. Belote. 1996. Duplicated proteasome subunit genes in *Drosophila melanogaster* encoding testes-specific isoforms. *Genetics* **144**:147–157.
 43. Zühl, F., T. Tamura, I. Dolenc, Z. Cejka, I. Nagy, R. De Mot, and W. Baumeister. 1997. Subunit topology of the *Rhodococcus* proteasome. *FEBS Lett.* **400**:83–90.
 44. Zwickl, P., D. Ng, K. M. Woo, H.-P. Klenk, and A. L. Goldberg. 1999. An archaeobacterial ATPase, homologous to ATPases in the eukaryotic 26S proteasome, activates protein breakdown by 20S proteasomes. *J. Biol. Chem.* **274**:26008–26014.