Effect of proteasome inhibitor *clasto*-lactacystin- β -lactone on the proteome of the haloarchaeon *Haloferax volcanii*

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Proteasomes play key roles in a variety of eukaryotic cell functions, including translation, transcription, metabolism, DNA repair and cell-cycle control. The biological functions of these multicatalytic proteases in archaea, however, are poorly understood. In this study, *Haloferax volcanii* was used as a model to determine the influence the proteasome-specific inhibitor *clasto*-lactacystin- β -lactone (cL β L) has on archaeal proteome composition. Addition of 20–30 μ M cL β L had a widespread effect on the proteome, with a 38–42% increase in the number of 2-D gel electrophoresis (2-DE) protein spots, from an average of 627 to 1036 spots. Protein identities for 17 of the spots that were easily separated by 2-DE and unique and/or increased 2- to 14-fold in the cL β L-treated cells were determined by tandem mass spectrometry (MS/MS). These included protein homologues of the DJ-1/ThiJ family, mobilization of sulfur system, translation elongation factor EF-1 A, ribosomal proteins, tubulin-like FtsZ, divalent metal ABC transporter, dihydroxyacetone kinase DhaL, aldehyde dehydrogenase and 2-oxoacid decarboxylase E1 β . Based on these results, inhibition of *H. volcanii* proteasomes had a global influence on proteome composition, including proteins involved in central functions of the cell.

INTRODUCTION

Proteasomes are large, barrel-shaped proteases found in all three domains of life (Maupin-Furlow *et al.*, 2004). The 20S proteolytic core consists of four stacked heptameric rings with 6 to 14 N-terminal nucleophile (Ntn) hydrolase-active sites sequestered within the complex interior. Protein degradation by 20S proteasomes requires protein unfolding, which is mediated by ATPases associated with various cellular activities (AAA+) such as the proteasome-activating nucleotidases (PANs) of archaea and homologous regulatory particle ATPases of eukaryal 26S proteasomes (Smith *et al.*, 2006).

Eukaryal 26S proteasomes, in association with the ubiquitin conjugation system, are essential for regulating a myriad of cellular functions such as antigen processing for major histocompatibility complex presentation

(Kloetzel & Ossendorp, 2004), circadian rhythmicity (Casal & Yanovsky, 2005), cell division (Devoy *et al.*, 2005), metabolism (Asher *et al.*, 2006), transcription (Lipford *et al.*, 2005) and translation (Baugh & Pilipenko, 2004; Arora *et al.*, 2005; Jiang & Wek, 2005). Unlike eukaryotes, archaea do not encode a conserved ubiquitin conjugation system for tagging proteins for proteasome-mediated destruction. However, many fundamental aspects of physiology and biochemistry are conserved between these two domains, including highly related proteasomes.

Recently, *clasto*-lactacystin- β -lactone (cL β L) was shown to inhibit 20S proteasomes of the halophilic archaeon *Haloferax volcanii* (*in vivo* and *in vitro*) (Reuter & Maupin-Furlow, 2004). The β -lactone component of cL β L irreversibly and specifically inhibits 20S proteasome activity via modification of the Ntn-threonine residue of the β -type subunits (Fenteany & Schreiber, 1998). Thus, proteins that change in abundance after cL β L treatment of *H. volcanii* are likely to provide insight into the types of proteins that are regulated by archaeal proteasomes and expand our understanding of the role these multicatalytic proteases play in archaeal cell physiology.

In this communication, protein identities for 17 spots that increased in *H. volcanii* cells treated with the proteasome inhibitor cL β L were determined by MS/MS. These included homologues of proteins known to be essential in a variety of functions, including cell division, translation and

Abbreviations: 2-DE, 2-D gel electrophoresis; AAA+, ATPases associated with various cellular activities; Ac, acetyl; ALDH, aldehyde dehydrogenase; AMC, 7-amido-4-methylcoumarin; Boc, *tert*-butoxy-carbonyl; cL β L, *clasto*-lactacystin- β -lactone; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; EF-1 A, elongation factor-1 A; ISC, iron-sulfur (Fe-S) cluster; OADH, 2-oxoacid dehydrogenase; PTS, phosphoenolpyruvate:sugar phosphotransferase system; SMC, structural maintenance of chromosomes; Suc, succinyl; SUF, mobilization of sulfur.

A supplementary table and two supplementary figures are available with the online version of this paper.

metabolism. Evidence suggests that a subset of these proteins may be modified post-translationally.

METHODS

Materials. Biochemicals were purchased from Sigma-Aldrich. Other organic and inorganic analytical-grade chemicals were from Fisher Scientific and Bio-Rad.

Strains, media and culture conditions. All experiments were performed in triplicate. *H. volcanii* DS70 was grown in rich medium (ATCC 974 at 42 °C, 200 r.p.m.) supplemented with *c*LβL and DMSO as indicated below. Growth was monitored as OD₆₀₀ using a 50–2000 µl, 220–1600 nm UVette (Eppendorf) and a SmartSpec 3000 spectrophotometer (Bio-Rad). For growth curves, 6 ml cultures (125 ml Erlenmeyer flasks) were inoculated at 0.33 % (v/v) from 1 ml cultures (OD₆₀₀ 0.40–0.55, 13 × 100 mm tubes) of freshly isolated colonies. Proteasome inhibitor *c*LβL (0, 20 and 30 µM) and 0.5 % (v/v) DMSO were added to cultures at an OD₆₀₀ of 0.15–0.20 (15 h growth). For preparation of proteins for 2-D gel electrophoresis (2-DE) and/or peptidase assay, 25 ml cultures were grown similar to above, with group 1 (with or without 20 µM *c*LβL) grown to an OD₆₀₀ of 1.7–1.9 (18–24 h growth) and group 2 (with or without 30 µM *c*LβL) grown to an OD₆₀₀ of 1.0. Cultures were chilled on an ice water bath for 15–30 min and cells were harvested at 12000 g (5 min, 4 °C).

Peptide-hydrolysing assay. To assess the influence of *c*LβL on peptidase activity, cell pellets of group 2 cultures were resuspended in 1 ml buffer A (2 M NaCl, 20 mM Tris/HCl at pH 7.2) and lysed by sonication (10 s followed by 1 min on ice, repeated three times) using a Sonifier Cell Disruptor on power level 20 and micro tip limit 7 (Heat Systems Ultrasonics). The cell lysate was clarified by centrifugation at 12000 g (15 min, 4 °C). Protein concentration was determined by Bio-Rad Protein Assay using BSA as the standard. Triplicate peptidase assays (300 µl) were performed at 60 °C in buffer A with 200 µg cell lysate protein ml⁻¹, 0.4 % (v/v) DMSO and 20 µM 7-amido-4-methylcoumarin (AMC)-linked peptide substrate (Sigma-Aldrich). Substrates incubated in buffer alone were subtracted from each assay. Release of AMC was monitored over a 30 min period by fluorescence using excitation wavelengths of 340–380 nm and an emission wavelength of 460 nm with an Aminco Fluoro-Colorimeter (American Instrument).

Preparation and separation of proteins by 2-DE. Protein was purified from cell pellets of group 1 and group 2 cultures (0.4 mg wet weight) using a Trizol-based method and separated by 2-DE, as previously described (Kirkland *et al.*, 2006). Three biological replicate 2-DE gels were generated per culture and harvest condition to ensure accuracy and to determine the statistical significance of each variable spot. 2-DE was performed using immobilized pH gradient strips (11 cm, pI 3.9–5.1) for the first dimension and Criterion pre-cast SDS-polyacrylamide gels (12.5 %) for the second dimension (Bio-Rad). Proteins were stained in-gel with SYPRO Ruby and imaged with a Molecular Imager FX Scanner using a 532 nm excitation laser and a 555 nm LP emissions filter according to the supplier (Bio-Rad). Acquired images were statistically analysed with PDQuest 7.0.1 (Bio-Rad) as previously described (Karadzic & Maupin-Furlow, 2005). Protein spots of interest were excised using the Bio-Rad ProteomeWorks spot cutter with fluorescent enclosure.

In-gel tryptic digestion and MS/MS protein analysis. 2-DE excised gel spots were reduced, alkylated, and digested with trypsin (Promega) in-gel, using an automated platform for protein digestion (ProGest; Genomics Solutions). Capillary reversed phase HPLC

separation of protein digests (desalted with a PepMap C18 cartridge) was performed using a PepMap C18 column (15 cm × 75 µm i.d.) and an UltiMate Capillary HPLC System (LC Packings). A linear gradient of 5–40 % (v/v) acetonitrile for 25 min at 200 nl min⁻¹ was used for separation. Tandem mass spectrometric analysis was performed online using a hybrid quadrupole time-of-flight instrument (TOF, QSTAR XL hybrid LC/MS/MS) equipped with a nanoelectrospray source (Applied Biosystems) and operated with the Analyst QS 1.1 data acquisition software.

Information-dependent acquisition was employed, in which each cycle consisted of a full scan from *m/z* 400 to 1500 (1 s) followed by MS/MS (3 s) of the two ions that exhibited the highest signal intensity. In the full-scan acquisition mode, ions were focused through the first quadrupole by focusing and declustering potentials of 275 V and 55 V, respectively, and guided to the TOF region via two quadrupole filters operated in rf-only mode. Ions were orthogonally extracted, accelerated through the flight tube (plate, grid, and offset voltages were 340, 380 and –15 V, respectively), and refocused to a four-anode microchannel plate detector via an ion mirror held at 990 V. The same parameters were utilized with MS/MS mode of operation; however, the second quadrupole was employed to filter a specific ion of interest while the third quadrupole operated as a collision cell. Nitrogen was used as the collision gas and collision energy values were optimized automatically using the rolling collision energy function based on *m/z* and the charge state of the peptide ion.

Mass spectrometric data were searched against the deduced proteome of *H. volcanii* DS2 (4208 total ORFs) (<http://archaea.ucsc.edu/>; 05/26/06 annotation) and GenBank, EMBL and SWISS-PROT databases at the National Center for Biotechnology Information using the Mascot (Matrix Science) search algorithm. Carbamidomethylation of cysteines was allowed as a fixed modification, and variable modifications of methionine oxidation, pyroglutamate, acetylation and phosphorylation of serine, threonine and tyrosine residues were also included in the search parameters. Precursor and fragment ion mass tolerances were set to 0.3 Da. Probability-based Mowse scores above the calculated threshold value (*P* < 0.05) were considered for protein identification. The pI and molecular mass values for deduced proteins were calculated according to Gasteiger *et al.* (2005). The probability that a deduced protein will adopt a coiled-coil conformation was predicted using COILS with weighted and unweighted MTK and MTIKL scoring matrices set to scanning windows of 21 and 28 residues (Lupas, 1996). Orthologue neighbourhood analysis was performed according to JGI/IMG (<http://img.jgi.doe.gov/cgi-bin/pub/main.cgi>).

RESULTS

Little is known regarding the types of proteins targeted for proteasome-mediated degradation or the role these multicatalytic proteases play in archaeal cells. To provide insight, this communication focused on establishing a set of proteins that increased in abundance and/or changed in isoform migration when *H. volcanii* cells were treated with the proteasome inhibitor *c*LβL. Previously, we demonstrated that *c*LβL-treatment (15 µM) of recombinant *H. volcanii* cells expressing the β-His subunit of 20S proteasomes results in the purification of 20S proteasomes with decreased (~15 %) peptide-hydrolysing activity (Reuter & Maupin-Furlow, 2004). Although insolubility in the high-salt growth medium of *H. volcanii* is responsible at least in part for incomplete inhibition, *c*LβL treatment does provide a mechanism to analyse partial inhibition of

proteasome function in the context of an archaeal cell. Another advance was our recent development of a Trizol-based method to enhance 2-DE separation of halophilic proteins isolated from as little as 2 ml cultures of *H. volcanii* (Kirkland *et al.*, 2006). Prior to this advance, detailed proteomic analysis of halophilic cells treated with cL β L was prevented by the restrictive expense of this type of proteasome-specific inhibitor, coupled with the relatively large volumes of culture needed to obtain protein samples amenable to 2-DE separation (e.g. 100 ml cultures) (Karadzic & Maupin-Furlow, 2005).

Growth of *H. volcanii* in the presence of proteasome inhibitor

To facilitate downstream proteomic analysis, the growth of *H. volcanii* was monitored in the presence of cL β L (0, 20 and 30 μ M) supplemented with DMSO (0.5 %) to enhance inhibitor solubility (Fig. 1a). Cultures devoid of proteasome inhibitor (negative controls) grew the fastest and reached the highest cell yield, with doubling times of 2.1 h and final OD₆₀₀ values of 3.8. In comparison, cells treated with 20 μ M and 30 μ M cL β L grew more slowly with doubling times of 3.1, and reached lower cell yields with maximal OD₆₀₀ values of 2.9 and 2.8, respectively. Thus, addition of the proteasome inhibitor reduced overall cell yield and growth rate. However, increasing the concentration of cL β L from 20 to 30 μ M resulted in little change in these growth characteristics. No gross differences in cell morphology were detected in the presence of proteasome inhibitor as determined by phase-contrast microscopy (data not shown).

Analysis of cell lysate revealed that growth in the presence of cL β L (30 μ M) inhibited the major peptidase activities of purified *H. volcanii* 20S proteasomes (Wilson *et al.*, 1999). This included a 68 % decrease in AE-AMC hydrolysis as well as a 28–61 % decrease in chymotrypsin-like activity as measured by the hydrolysis of succinyl (Suc)-AAF-AMC, Suc-LLVY-AMC and Suc-IIW-AMC (Fig. 1b). Little to no change was observed in the hydrolysis of *tert*-butyloxycarbonyl (Boc)-FSR-AMC or acetyl (Ac)-YVAD-AMC, both of which are not hydrolysed by purified *H. volcanii* 20S proteasomes (Wilson *et al.*, 1999). Thus, other cellular proteases are not sufficient to compensate for the lack of proteasome efficacy during growth in the presence of cL β L.

Global differences in *H. volcanii* proteome in the presence of cL β L

Proteins were extracted from *H. volcanii* cells grown in the presence and absence of cL β L (20 and 30 μ M) and were analysed for differences in proteome composition by 2-DE as described in Methods. Based on this analysis, the total number of 2-DE-separated protein spots from cells grown in the presence of proteasome inhibitor was dramatically higher than that from untreated cells (Fig. 2). Cells grown in the presence of 20 and 30 μ M cL β L had respective

Fig. 1. Cellular response of *H. volcanii* to proteasomal inhibition. (a) Growth of *H. volcanii* in the presence and absence of cL β L. ■, 0 μ M, ▲, 20 μ M and ▽, 30 μ M cL β L was added to cells at an OD₆₀₀ of 0.20 (15 h growth). DMSO (0.5 %, v/v) was added to all cultures at this time. (b) Select peptide-hydrolysing activities of cell lysate prepared from *H. volcanii* grown in the presence or absence of cL β L. Peptide substrates and type of peptide-hydrolysing activity are listed on the x-axis. PGPH, peptidyl-glutamyl peptide hydrolysis; CL, chymotrypsin-like activity; TL, trypsin-like activity.

averages of 1072 and 1000 total spots. In contrast, the average spot number of the uninhibited controls for the 20 and 30 μ M cL β L-treated cells was 669 and 584, respectively. Thus, there was a 38–42 % increase in the total number of spots detected when cells were grown in the presence vs absence of cL β L. The respective spot differences were 403 ± 48 and 436 ± 33 for the 20 and 30 μ M cL β L-treated vs untreated controls. This contrasts with the modest and inconsistent spot number differences observed between the cL β L-treated groups (72 ± 51) and the uninhibited controls (105 ± 74). These results also contrast with 2-DE proteome maps of *H. volcanii* aminopeptidase (ORF01128), S2P metalloprotease (ORF00083) and proteasome-activating nucleotidase A (ORF01103) knockout mutants vs their parent strains (Zhou, Kirkland, Gil, Humbard and J. A. M.-F., unpublished results). Using methods similar to those of this study, the 2-DE maps

Fig. 2. Modified Gaussian 2-DE images of *H. volcanii* proteomes isolated from cells grown in the presence (a, c) and absence (b, d) of cL β L. Cultures were divided into two groups based on the phase of growth at the time of harvest: (a, b) group 1, harvested at an OD₆₀₀ of 1.0 and 1.3, respectively; (c, d) group 2, harvested at an OD₆₀₀ of 1.7 and 1.9, respectively.

display only a 0.1% (ORF01128 and 00083) to 7.5% (ORF01103) difference in total spot number.

To compare the relative abundance of individual proteins within the 2-DE sets for this study, the statistical significance for the relative intensity of each protein spot was set to a threshold value of 4. The cultures supplemented with 20 and 30 μ M of the proteasome inhibitor (group 1 and 2, respectively) yielded a total of 89 spots that were at or above this threshold. Of these spots, 60 were common to both groups, two were restricted to group 2, and 27 were restricted to group 1. In contrast, the number of spots with relative intensities at least fourfold below that of the uninhibited controls was only 14, with two spots common to both groups, two restricted to group 1 and 12 restricted to group 2. Based on these results, a number of consistent and significant differences within the proteome could be detected by 2-DE when *H. volcanii* cells were treated with cL β L. Thus, in addition to a notable reduction in the growth of proteasome-inhibited cells, changes in 2-DE migration and/or abundance of a large group of proteins were observed.

Identification of *H. volcanii* protein isoforms which increase in the presence of proteasome inhibitor

A total of 24 spots 'unique' and/or increased 2- to 14-fold in cells cultivated in the presence of cL β L were selected, excised and pooled from triplicate 2-DE gels to provide enough protein for in-gel tryptic digestion and MS/MS identification (see Fig. 3 for examples). Criteria for spot selection included (i) reproducible and significant differences between the 2-DE gels of cL β L-treated and non-treated cells, (ii) sufficient protein quantity as determined by SYPRO Ruby fluorescent staining and (iii) adequate separation from neighbouring protein spots by 2-DE. Protein identities for 17 of these spots were determined via HPLC-electrospray ionization (ESI) MS/MS using a

QSTAR XL hybrid LC/MS/MS and are listed in Table 1 along with their corresponding probability-based Mascot ion scores, peptide coverage and fold increase in the presence vs absence of proteasome inhibitor. These protein identities are well within the significant range ($P < 0.05$), with Mascot ion scores from 53 to 839 (average of 268) and peptide coverage of 6.9–60.3%, with an average of 5.5 tryptic peptide fragment ions detected per protein. In two cases, more than one protein was identified per spot (a3 and b2) that cannot be contributed by protein carryover from one sample to another (Table 1; see Supplementary Figure S1, available with the online version of the paper, for all spot images). Spot a3 appears as a protein chain that is not well separated, yet all of the proteins within that chain appear to increase in the presence of cL β L. The reason for the identification of three proteins within spot b2 is less clear, since this spot appears well separated from surrounding proteins. However, only one protein identified within this spot migrated similarly in molecular mass to that calculated from the deduced polypeptide. The remaining seven protein spots out of the 24 total had overall Mascot ion scores below 53 and were excluded from the list.

The majority of proteins identified migrated within 9 kDa of the molecular mass and 0.4 pI units of that calculated for the deduced polypeptide (Table 1). Exceptions included the ORF 01073 protein and 30S ribosomal protein S4, which migrated more acidic (by 0.5 units); the actin-like protein (ORF02969), which migrated more basic (by 0.5 units); and a number of 'outlier' proteins which migrated at least 10 kDa greater or less than calculated. Whether these differences are due to post-translational modification, incomplete denaturation (in 7 M urea and 2 M thiourea) or other factors remains to be determined. In particular, ORF01073 is predicted to adopt a coiled-coil conformation (residues 60–289) which may be somewhat resistant to unfolding. A number of the proteins identified appear to undergo N-terminal methionine excision, based on the identification of tryptic peptide ions with cleaved

Fig. 3. Magnified regions of 2-DE proteome maps of *H. volcanii* cells grown in the absence (right) and presence (left) of cLβL. Landmark spots common to both gels are indicated by lower-case letters. Protein spots whose intensity increased in the presence of cLβL are circled, and those identified by MS/MS are labelled with arrows. (a) ThiJ/Pfp1 cellular protease homologue was 5-fold higher than the negative control. (b) FtsZ cell division protein homologue with a relative intensity value 4-fold that of the uninhibited control. (c) S3Ae ribosomal protein homologue exhibiting one of the highest differences in relative intensity in this study at almost 14-fold over the uninhibited control.

N-termini (individual Mascot ion scores of 49–91) (see Supplementary Table S1, available with the online version of this paper). These include the 30S ribosomal protein S4, the 2-oxoacid decarboxylase E1β, ORF01073 and the elongation factor (EF)-1A. In addition, the detection of an STHDVDPATVEVIR tryptic fragment with an N-acetyl group (Mascot ion score of 62; *E* value 4.4×10^{-5}) suggests that the hydantoinase/oxoprolinase homologue is cleaved by the methionine aminopeptidase and acetylated on the resulting N-terminal serine. These results are consistent with what has been observed for other haloarchaeal proteins (Falb *et al.*, 2006; Humbard *et al.*, 2006), but do not account for the aberrant migration of the subset of proteins described above. Ions of FtsZ and ORF01073 tryptic fragments were correlated with methylated and phosphorylated residues; however, their identity was only tentative (individual Mascot ion scores below 32) (see Supplementary Table S1).

The protein spots that increased in the presence of proteasome inhibitor and were linked to protein sequence by MS/MS fell into three major categories: (i) protein quality control, translation and degradation; (ii) metabolism/transport; and (iii) cell division/conserved proteins of unknown function. Those which fell into the category of protein quality control and translation were the most extensive, with 9 proteins identified by a total of 33 tryptic peptide fragment ions. The majority of proteins in this group were homologues of the 30S and 50S ribosomal subunits (S3Ae, S17, S13, S4 and L7). In addition, members of the DJ-1/ThiJ/PfpI, sulfur mobilization system (SUF) iron–sulfur (Fe–S) cluster (ISC) assembly and EF-1 A families were found. The second category of spots identified were 4 proteins known and/or proposed to be

involved in metabolism and transport, and were identified via a total of 22 peptide fragment ions with the highest Mascot score average of 437. The group included orthologues of the divalent metal-binding lipoproteins of ABC-type transporters, 2-oxoacid decarboxylase E1β (EC 1.2.4.–), dihydroxyacetone kinase (EC 2.7.1.29) and aldehyde dehydrogenase (ALDH) (EC 1.2.1.–). The final category included a homologue of the cell division protein FtsZ and two conserved proteins, ORF02998 and ORF01703, which respectively cluster to COG1077 and COG1340. Although these latter two COGs encompass proteins of unknown function, ORF02998 and ORF01703 have low (20%) identity to actin-like (e.g. *Magneto-spirillum magneticum* amb0965) and structural maintenance of chromosomes (SMC)-like proteins (e.g. *Haloarcula marismortui* rrnAC1639), respectively. In further support of the potential role of the coiled-coil ORF01703 protein in cell division, some of its orthologues appear to be cotranscribed with *ftsZ*-like genes, based on neighbourhood analysis of archaeal genomes.

DISCUSSION

Ribosomal proteins

Many of the protein spots which increased in the presence of cLβL were homologues of ribosomal proteins, including S4p (S9e), S17p (RpsQ, S11e), S13p (S18e), S3Ae and L30p (L7e). All of these are functionally versatile and/or key to the initiation of ribosome biogenesis. In mammalian cells, ribosomal proteins are ubiquitinated and degraded by proteasomes (Kim *et al.*, 2006b). Although this type of proteasome specificity has yet to be established in archaea,

Table 1. Proteins unique and/or increased in *H. volcanii* cells cultivated in the presence vs absence of cLβL

Grp/spot*	ORF no.†	Homologue description	Increase (± SD)‡	pI(cal./gel)§	Mass, kDa (cal./gel)§	Coverage (%/pep no.)	Mascot¶
Protein quality control, degradation and translation:							
2/a3	00875	DJ-1/ThiJ family [EC 3.4.-.-]	5.1 ± 0.97	4.0/4.2	24/33	27.2/4	220
2/a3	01094	Fe-S assembly protein SufC	5.1 ± 0.97	4.2/4.2	33/33	11/3	145
1,2/b1	01093	Fe-S assembly protein SufB	3.0 ± 1.0, 6.6 ± 0.40	4.6/4.8	53/54	13.9/5	287
1,2/a6	01599	Translation elongation factor EF-1 A	4.4 ± 0.25	4.6/4.7	46/50	25.4/5	224
1/b7	00801	30S ribosomal protein S3Ae	13.8 ± 1.4	4.8/4.5	25/34	38.6/6	359
1,2/a10	02196	30S ribosomal protein S13p/S18e	2.4 ± 0.6, U	5.1/4.8	19/16	22.8/3	154
1/b8	02197	30S ribosomal protein S4	U	5.2/4.7	20/14	18.3/3	176
1/c5	02443	50S ribosomal protein L30p	6.5 ± 2.1	4.6/4.8	17/22	23.4/3	104
2/a4	02431#	30S ribosomal protein S17	2.1 ± 0.53	4.7/4.4	13/15	35.5/1	53
Metabolism/transport:							
1/b11	02598	Lipoprotein of divalent metal ABC transporter	U	4.4/4.6	40/32	18.2/5	265
2/a9	00393	Dihydroxyacetone kinase DhaL [EC 2.7.1.29]	5.3 ± 0.98	4.4/4.6	25/27	41.8/10	328
1/c8	02023	2-Oxoacid decarboxylase E1β chain [EC 1.2.4.-]	2.7 ± 1.1	4.6/4.8	36/27	38.2/12	494
2/b2	B00047	Aldehyde dehydrogenase [EC 1.2.1.-]	4.0 ± 2.4	4.3/4.1	53/48	38.9/11	661
Cell division/conserved proteins of unknown function:							
1,2/b5	02794	FtsZ	U	4.6/4.7	42/35	12.4/3	67
2/b6	01073	Coiled-coil protein of COG1340	5.0 ± 1.4	4.7/4.2	36/42	24.1/4	179
2/a8	02998	Conserved protein related to actin-like proteins of COG1077	3.0 ± 0.66	4.1/4.6	38/27	11/2	84
Outlier data:							
2/a7	02574	Hydroxymethylglutaryl CoA synthetase	2.9 ± 0.47	4.5/4.8	50/15	9.4/3	206
2/a7	01093	Fe-S assembly protein SufB	2.9 ± 0.47	4.6/4.8	53/15	6.9/2	60
2/a12	01599	Translation elongation factor EF-1 A	3.3 ± 0.38	4.6/4.9	46/20	60.3/14	839
2/a12	02915	4-Aminobutyrate aminotransferase	3.3 ± 0.38	4.6/4.9	48/20	8.4/2	103
1,2/a6	01073	Coiled-coil protein of COG1340	4.4 ± 0.25	4.7/4.7	36/50	31.9/8	212
2/b2	A00018	Hydantoinase B /oxoprolinase	4.0 ± 2.4	4.3/4.1	64/48	34.2/13	636
2/b2	01503	Thermosome subunit 2	4.0 ± 2.4	4.2/4.1	59/48	15.2/6	355

*Group and spot numbers of protein samples analysed by mass spectrometry, where group 1 and 2 correspond to exposure to 20 and 30 μM cLβL, respectively.

†ORF number based on the *H. volcanii* DS2 genome sequence released by TIGR (05/26/06 assembly available at <http://archaea.ucsc.edu/>). Polypeptide sequences deduced from genome and identified by MS are included with this ORF number in the Supplementary Figure S2, available with the online version of this paper, for comparison of polypeptide to future annotations.

‡Increase in intensity of protein spot of cells grown in the presence/absence of cLβL ± SD. U, Unique protein spot detected only in cells grown in the presence of cLβL.

§pI and molecular mass (Mass) estimated (gel) by 2-DE and calculated (cal.) based on deduced protein sequence.

||Coverage (per cent) of the deduced protein sequence and number of peptide ions detected.

¶Overall Mascot ion score of protein detected by mass spectrometry. Protein sequences of detected peptide ions with individual Mascot ion scores and *E* values are included in Supplementary Table S1, available with the online version of this paper.

#ORF number for which the polypeptide sequence was extended from the annotation.

the changes observed in the isoform abundance of ribosomal proteins do suggest a widespread influence of proteasome inhibition on archaeal cell function. Similar alterations in the levels of ribosomal protein 2-DE spots have been observed for other archaea after global challenges, including changes in energy source (Dopson *et al.*, 2005), cold

adaptation (Goodchild *et al.*, 2004), heat shock (Shukla, 2006) and osmotic stress (Shukla, 2006). Of these ribosomal proteins which are altered, the L30p homologue commonly increases after cLβL-treatment of *H. volcanii* as well as after the addition of Fe²⁺ (an electron donor) to *Ferroplasma acidarmanus* Fer1 (Dopson *et al.*, 2005).

Elongation factor 1 A

EF-1 A is a major translational factor, which catalyses the first step of the elongation cycle. In eukaryotes, EF-1 A binds proteasomes (e.g. the Rpt1 subunit) (Verma *et al.*, 2000; Coux, 2003) and ubiquitinated proteins after ATP depletion (Chuang *et al.*, 2005), and is essential for ubiquitin-proteasome dependent degradation of *N*^ε-acetylated proteins (Gonen *et al.*, 1994). EF-1 A also has isopeptidase (Gonen *et al.*, 1996) and chaperone activities (Caldas *et al.*, 2000), and binds polypeptides unable to fold after their release from the ribosome (Hotokezaka *et al.*, 2002). Based on these multifunctional properties of EF-1 A, a number of biological factors may be responsible for the increased levels of the EF-1 A protein spot in κ L β L-treated *H. volcanii* cells. In eukaryotes, changes in EF-1 A abundance and post-translational modification are correlated with growth rate, cell proliferation and differentiation, and intracellular pH (Krieg *et al.*, 1989; Grant *et al.*, 1992; Zobel-Thropp *et al.*, 2000; Ransom-Hodgkins *et al.*, 2000; Lopez-Valenzuela *et al.*, 2003). In archaea, the intensities of EF-1 A 2-DE spots are altered by heat shock (Shukla, 2006), cold adaptation (Goodchild *et al.*, 2004) and energy source (Dopson *et al.*, 2005). In addition, multiple archaeal EF-1 A isoforms have been detected (Giometti *et al.*, 2002), and post-translational modification sites are predicted based on similarity to known eukaryal sites (Whiteheart *et al.*, 1989; Wang & Poovaiah, 1999; Lopez-Valenzuela *et al.*, 2003).

DJ-1/ThiJ/PfpI superfamily

The DJ-1/ThiJ/PfpI superfamily (Bandyopadhyay & Cookson, 2004) protein, which accumulated as a 2-DE spot in the presence of κ L β L, has the conserved Cys, His and Asp residues proposed to function as a catalytic triad in peptide-bond hydrolysis (Malki *et al.*, 2005). Members of this peptide-hydrolysing clan are often induced by stressful conditions, such as heat shock (Sastry *et al.*, 2002) and peptide starvation (Snowden *et al.*, 1992), and may hydrolyse short peptides generated by energy-dependent proteases such as proteasomes (Maupin-Furlow *et al.*, 2006). The *Escherichia coli* Hsp31 member of this superfamily interacts with EF-1 A and ClpA (Malki *et al.*, 2005), a ClpP-associated AAA+ ATPase related to the proteasome-activating nucleotidases (PANs) of archaea.

Cell division

FtsZ, actin and SMC are important in the division of prokaryotic cells. FtsZ forms a cytokinetic ring early in cell division (Margolin, 2005), actin-like proteins (e.g. MreB) are partners with RNA polymerase in providing the force needed for chromosome segregation (Kruse *et al.*, 2006), and coiled-coil SMC proteins are key subunits of complexes that perform essential tasks in chromosome dynamics (Nasmyth & Haering, 2005). In bacteria, the levels and/or activities of many of these cell division

proteins are controlled by proteolysis. In *E. coli*, the FtsZ inhibitor SulA is targeted for regulated proteolysis by HslVU (Kanemori *et al.*, 1999) and Lon proteases (Mizusawa & Gottesman, 1983). In *Streptomyces coelicolor*, the half-life of FtsZ is controlled (Del Sol *et al.*, 2006), and in *Bacillus subtilis*, an SMC protein is degraded by Lon and Clp proteases as cells enter stationary phase (Mascarenhas *et al.*, 2005). In addition, the AAA+ ATPase ClpX associates with and inhibits FtsZ assembly in *E. coli* (Flynn *et al.*, 2003) and *B. subtilis* (Weart *et al.*, 2005), respectively. In archaea, it is not clear whether the proteasome and/or proteolysis control events in cell division. However, the κ L β L-dependent accumulation of FtsZ-, actin- and SMC-like protein spots correlates well with the reduced growth rate of *H. volcanii* cells under these conditions.

2-Oxoacid dehydrogenase (OADH) (EC 1.2.4.-)

All archaea use ferredoxin oxidoreductases to oxidize 2-oxoacids to their CoA derivatives. This has led to the question whether archaea synthesize and/or need functional OADH complexes of E1 α β , E2, and E3 (Jolley *et al.*, 2000). Although E1 and E3 enzyme activities have been detected (Danson *et al.*, 1984; Heath *et al.*, 2004), a functional OADH has yet to be demonstrated in archaea. The E1 β -like protein spot that increased in the presence of κ L β L is encoded within a four-gene operon with coding capacity for the E1 α , E1 β , E2 and E3 components of an OADH (Jolley *et al.*, 2000), and is separate from the genomic region coding only for an E1 α β required for nitrate-respirative growth (Wanner & Soppa, 2002). This, combined with the finding that an E1 α -type OADH protein of *F. acidarmanus* Fer1 increases several-fold during chemo-organotrophic vs chemomixotrophic growth (Dopson *et al.*, 2005), suggests that archaea may modulate the E1 α β 2-oxoacid decarboxylase component of OADH complexes as nutrients shift and/or become limiting. Whether proteasomes are involved in this process remains to be determined.

Dihydroxyacetone kinase (EC 2.7.1.29)

Dihydroxyacetone kinases phosphorylate dihydroxyacetone (DHA), D-glyceraldehyde or other short-chain ketoses and aldoses (Erni *et al.*, 2006). The source of the high-energy phosphate is either ATP or a phosphoprotein of the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The *E. coli* DHA kinase is composed of the three subunits DhaL, DhaK and DhaM (Erni *et al.*, 2006), the latter of which is phosphorylated by the PTS. This phosphoryl group is displaced from DhaM by a tightly bound ADP coenzyme of DhaL and transferred to the substrate, which is covalently bound to DhaK. Besides catalysis, DhaL and DhaK serve antagonistic roles in binding the sensing domain of an AAA+ ATPase transcriptional regulator, DhaR (Bachler *et al.*, 2005). Whether a DHA kinase- and PTS-related global regulatory system functions in *H. volcanii* and is controlled by

proteasomes is unknown. However, the DhaL-like protein spot which accumulated in ϵ L β L-treated *H. volcanii* cells is encoded within a region of the genome with coding capacity for DhaK and the PTS EIIA- and HPr-type domains of DhaM.

Aldehyde dehydrogenase (ALDH) (EC 1.2.1.-)

The ALDH-like protein (pfam00171; *E* value 7×10^{-100}) that accumulated as a 2-DE spot in the presence of ϵ L β L includes conserved catalytic and NAD(P)-binding residues. Thus, it is likely to function in the oxidation of aldehydes to their corresponding carboxylic acids and, ultimately, detoxify a wide variety of reactive organic compounds. Interestingly, the levels of a highly related ALDH isoform (GI no. 10581906; *E* value 6×10^{-147}) are reduced when *Halobacterium salinarum* cells are cultured in high vs optimal salt conditions (6 vs 4.3 M NaCl) (Kim *et al.*, 2006a), suggesting that the levels of these types of proteins are modulated.

ISC assembly

Unlike most archaea (Lill & Muhlenhoff, 2005), haloarchaea such as *H. volcanii* have the coding capacity for both ISC/Nif- and SUF-like ISC assembly systems. This is analogous to *E. coli*, in which the ISC system plays a 'housekeeping' role and SUF is required during iron starvation (Outten *et al.*, 2004) and is induced after oxidative stress (Lee *et al.*, 2004). The reason for the increased abundance of the SufB and SufC protein homologues in ϵ L β L-treated *H. volcanii* cells remains to be determined; however, both proteins are encoded in an apparent operon and are thus likely to be co-ordinately regulated. It is possible that inhibition of proteasome activity increased the levels of oxidatively damaged and/or improperly folded proteins and triggered an overall increase in the SUF system to facilitate ISC assembly under these conditions.

Divalent metal transport

The ORF02598 protein that accumulated as a 2-DE spot in the presence of ϵ L β L is related to 'cluster 9' lipoproteins. These proteins are proposed to regulate the high-affinity uptake of divalent metals by ABC transporters for repair of metalloenzymes, resistance to oxidative stress and/or maintenance of intracellular redox homeostasis (Claverys, 2001; Johnston *et al.*, 2004; Hantke, 2005). Interestingly, heat shock of *Halobacterium* NRC-1 results in the elevation of a 2-DE protein spot related to an ABC transporter lipoprotein which functions in the import of nutrients and/or release of toxic products (Shukla, 2006). Thus, the accumulation of unfolded and damaged proteins after cellular stresses such as proteasomal inhibition or heat shock may be a general signal that modulates the levels (or isoform status) of various ABC transporter-associated lipoproteins in the halophilic archaea.

Conclusions

A number of protein spots, which represent a variety of cellular functions, differentially accumulated in *H. volcanii* cells treated with the 20S proteasomal inhibitor ϵ L β L. The general classes of proteins identified through the proteomic analysis of chemically inhibited cells provide insight into specific cellular functions that may be regulated by archaeal proteasomes. Many of the proteins identified represent key components of processes vital to cell function, thereby making them prime candidates for theoretical proteasomal control points. To further understand the role proteasomes play in these changes observed, future studies are aimed at determining the influence ϵ L β L has on transcript levels, protein half-life and/or covalent modification of the protein spots identified to increase in the presence of proteasome inhibitor.

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