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Correlating the Transcriptome, Proteome, and Metabolome in the Environmental Adaptation of a Hyperthermophile

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We have performed a comprehensive characterization of global molecular changes for a model organism *Pyrococcus furiosus* using transcriptomic (DNA microarray), proteomic, and metabolomic analysis as it undergoes a cold adaptation response from its optimal 95 to 72 °C. Metabolic profiling on the same set of samples shows the down-regulation of many metabolites. However, some metabolites are found to be strongly up-regulated. An approach using accurate mass, isotopic pattern, database searching, and retention time is used to putatively identify several metabolites of interest. Many of the up-regulated metabolites are part of an alternative polyamine biosynthesis pathway previously established in a thermophilic bacterium *Thermus thermophilus*. Arginine, agmatine, spermidine, and branched polyamines N^4-aminopropylspermidine and N^4-(*N*-acetylaminopropyl)spermidine were unambiguously identified based on their accurate mass, isotopic pattern, and matching of MS/MS data acquired under identical conditions for the natural metabolite and a high purity standard. Both DNA microarray and semiquantitative proteomic analysis using a label-free spectral counting approach indicate the down-regulation of a large majority of genes with diverse predicted functions related to growth such as transcription, amino acid biosynthesis, and translation. Some genes are, however, found to be up-regulated through the measurement of their relative mRNA and protein levels. The complimentary information obtained by the various "omics" techniques is used to catalogue and correlate the overall molecular changes.

Keywords: metabolomics • hyperthermophile • polyamine • spermidine • mass spectrometry • DNA microarray • tandem mass spectrometry • *Pyrococcus furiosus*

Introduction

Correlating the interrelation of the genome, proteome, and metabolome to gain an understanding of the microbial response to external stressors is fundamental to characterizing the underlying changes in biochemistry. The completion of genomes for many organisms has facilitated the monitoring of the global molecular changes at the transcriptomic and proteomic level and the identification of protein biomarkers for cells undergoing a stress response. However, the metabolite level changes have not been as widely investigated. Unlike techniques used to sequence genomes, measure mRNA, and identify proteins which are fairly mature, the analysis of metabolites offers significant challenges in comparative chromatographic analysis since normal fluctuations in chromatographic retention time makes comparative LC/MS analyses of thousands of metabolites difficult. A second more significant challenge is in their structural characterization since unlike proteins and genes there are no common building blocks that can simplify identification. Metabolites can have unique structures and mass spectral profiles that cannot be easily interpreted. To address both of these issues, we have developed a platform that provides a nonlinear correction solution to retention time, XCMS,[1] which is also linked to METLIN (http://metlin.scripps.edu),[2] a freely available metabolite database, that provides initial putative identification information on previously characterized metabolites. This publicly available repository now contains thousands of metabolite structures that can be a starting point in identifying a metabolite.

For the determination of protein level changes, a diverse set of techniques are now available. Relative protein quantitation with mass spectrometry can involve the use of a coupled technique with stable isotope labeling such as isotope coded affinity tag (ICAT),[3] ^{18}O proteolytic labeling,[4] and metabolic labeling of amino acids (SILAC)[5] or a decoupled technique such as label-free proteomics.[6–8] Recently, the label-free semiquantitative proteomic profiling methods have been gaining popularity due to their simplicity and cost. They have the advantage of avoiding typical losses due to multiple steps of processing and chromatography often seen in techniques like ICAT. However, the label-free methods tend to be more subject to

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variability. Spectral counting is a label-free approach that relies on a comparison of the relative number of identified spectra between separate analyses. For our analysis of the system-wide molecular changes in *Pyrococcus furiosus*, we chose a spectral counting approach due to its simplicity. In addition, the low cysteine content of the *P. furiosus* and other hyperthermophiles made the ICAT technique less suitable.[9]

P. furiosus is a so-called hyperthermophile and grows optimally near 100 °C, the normal boiling point of water. It represents a biochemically and evolutionarily distinct organism that has adapted to the unique challenges of molecular and structural stability of volcanic marine vents. While DNA, RNA, and proteins can undergo deleterious changes at higher temperatures for most organisms,[10] these effects are largely overcome by hyperthermophiles suggesting adaptations to their geothermal habitats through novel mechanisms of DNA and protein stabilization, many of which are still poorly understood. In addition, *P. furiosus* has only 2065 open reading frames (ORFs) in its annotated genome.[11] Many of the predicted proteins remain conserved hypothetical without any predicted or observed function. The analysis of its cold adaptation response at the molecular level provides an excellent opportunity for gaining greater understanding of fundamental biochemistry of this fascinating and understudied microorganism.

Here we present a comprehensive platform for the transcriptomic, proteomic, and metabolite profiling using gene array measurements and mass spectrometry that allows the characterization of transcript, protein, and metabolite level changes. The combination of these technologies have been used to monitor global molecular changes in response to environmental stress and for mapping networks that define connectivity in function between gene expression, proteins, and metabolites for the model organism, *P. furiosus*.

Materials and Methods

Growth and DNA Microarray Analysis of *P. furiosus*. The organism was routinely grown with maltose as the primary carbon source in a 20 L custom fermentor as previously reported at 95 and 72 °C.[12] To prepare cell extracts from adapted cultures, cells were harvested in late log phase (~2×10^8 cells/mL) and fractionated. The construction of DNA microarrays containing all of the 2065 ORFs in the annotated genome of *P. furiosus* (http://comb5-156.umbi.umd.edu/genemate/), preparation of cDNA from the RNA samples, and hybridization experiments were all performed as previously described. For the adapted experiments, each $\log_2$ value was an average of four hybridization experiments performed in duplicate using cDNA derived from four different cultures of *P. furiosus* (two grown at 95 °C, two grown at 72 °C). Individual *t* test procedures were conducted to identify the significantly expressed ORFs, and Holm's step-down *p*-value adjustment procedure was performed to give modified *p*-values.[13]

Metabolite Characterization Using ESI-TOF. A 50 uL sample was taken for TCA precipitation for the removal of proteins. 100 μL of 20% TCA in cold acetone was added to the sample before vortexing for 20 s, followed by sonication for 3 min. The sample was left at −20 °C for 30 min after which it was pelleted at 13000 rpm in a high speed centrifuge (Eppendorf, model 4500). The pellet was washed twice with ice cold acetone, and the supernatant was combined and transferred to a new tube and dried down in a speed-vac. The sample was reconstituted in 20 μL of water prior to analysis with ESI-TOF in triplicate.

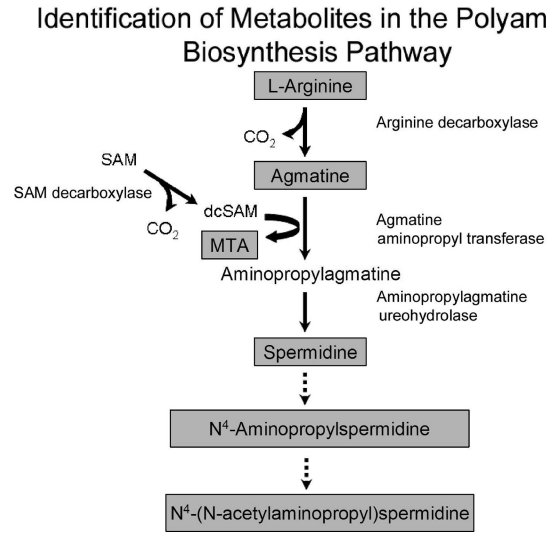

Figure 1. Many of the metabolites involved in a new biosynthesis pathway of polyamines using L-arginine as a starting material, proposed by Oshima and co-workers for another hyperthermophile *T. thermophilus*,[16] were identified in our experiments with *P. furiosus* (highlighted in gray). Oshima also identified the novel long chain polyamine N^4-aminopropylspermidine in archaea[19] which is most likely the precursor to the novel metabolite N^4-(*N*-acetylaminopropyl)spermidine. The dotted line indicates that the enzymatic pathways for these molecular conversions are still unknown (Figure was adapted from reference 16).

The comparative analysis of the two metabolite extracts of *P. furiosus* cells grown at 95 and 72 °C was performed using an Agilent MSD-TOF mass spectrometer coupled to an Agilent 1100 capillary LC system. A Zorbax 300 SB C18 column (0.5 mm ID, 15 cm, 5 μm particle size) was used to perform a reverse phase separation at 6 μL/min. A reverse phase gradient was used from 5% acetonitrile (0.1% formic acid) to 95% acetonitrile (0.1% formic acid) over 65 min and maintained for 5 min. The LC-MS profiling data for soluble fractions of cells grown at both temperatures were analyzed for distinguishing features with XCMS software developed in the Siuzdak laboratory.[1] This program performs a nonlinear alignment of the two data sets, normalization, and outputs a list of significant differences (m/z values, corrected retention times and integrated intensities), along with *p*-values from their Student's *t* test results. Approximately 6000 distinct ions were detected by XCMS in each sample. The top 1500 most significant ions by their calculated *p*-values with absolute intensity exceeding 10000 (arbitrary units), corresponding to a S/N of >10, were targeted for identification. For these 123 ions, accurate mass information was used to calculate probable elemental composition. For a small group of interesting metabolites, this information, along with collision induced dissociation (CID) data, was used for identification. A ratio between the areas of the integrated selected ion chromatogram peaks provided by XCMS (A^{72}/A^{95}) was used as a measure of the fold change for each metabolite.

CID of metabolites of interest was performed on Q-TOF micro (Waters Corp., Waltham, MA) and an Agilent Q-TOF mass spectrometer. Previously collected fractions were concentrated and electrosprayed at 3500 V in positive ion mode. Ions were isolated with a 4 m/z isolation window, and the collision energy was optimized to induce optimum fragmentation with <10% precursor ion remaining.

Protein Digestion. Protein pellets reserved from the metabolite analysis were redissolved in an acid cleavable detergent

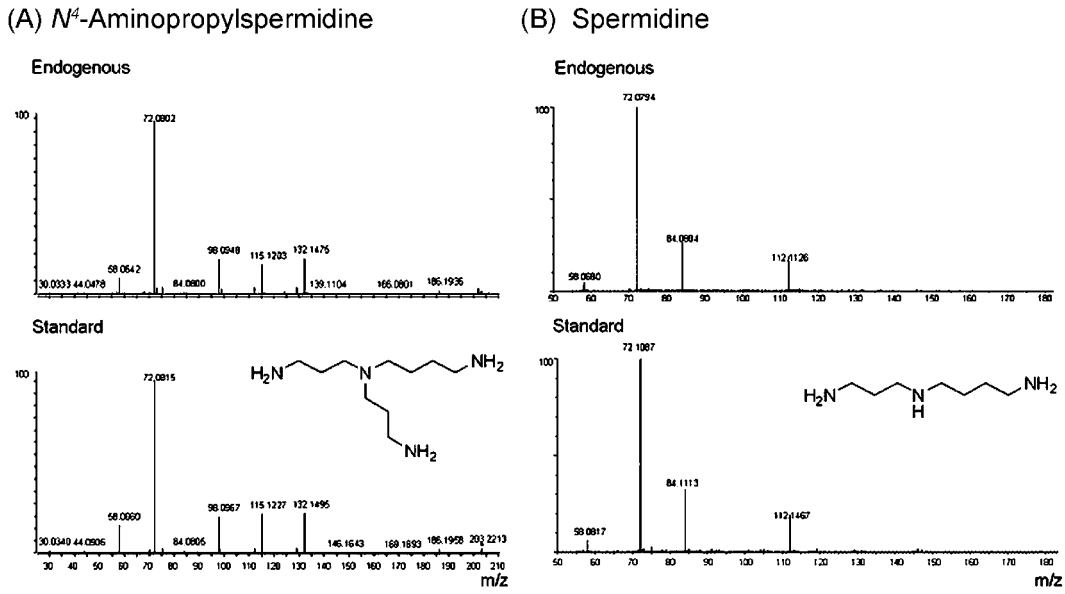

Figure 2. Identification of endogenous polyamine metabolites involved the use of accurate mass to ascertain possible molecular formulas and a comparison of the metabolite's MS/MS pattern to that of a high-purity standard for structural confirmation using an ESI-q-TOF mass spectrometer. Here this methodology is demonstrated for two of the molecules that are part of the polyamine biosynthetic pathway: (A) MS/MS pattern for N^4-aminopropylspermidine with a measured accurate mass of 202.2151 (theoretical 202.2157) and (B) MS/MS pattern for spermidine with a measured accurate mass of 145.1571 (theoretical 145.1579).

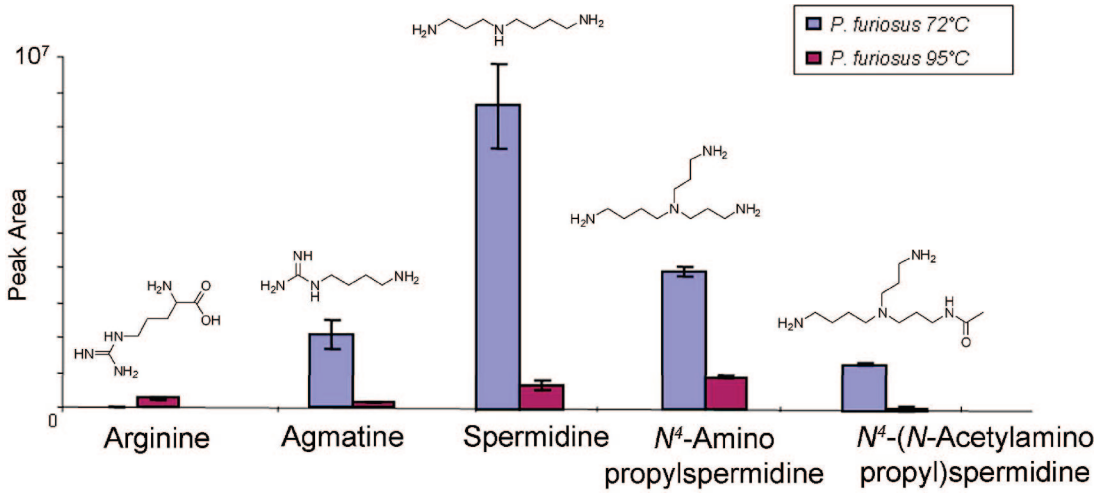

Figure 3. Relative quantitation of metabolomic changes for *P. furiosus* grown at 95 and 72 °C for five polyamine related compounds along the polyamine biosynthesis pathway is plotted as a bar graph of their normalized integrated peak areas.

(0.1% Rapigest, Waters Corp.). The protein concentrations for the two sample types were measured using a Bradford assay and normalized to 1 mg/mL. The disulfide bonds contained in the proteins were reduced in 10 mM D,L-dithiothrietol (Sigma) for 1 h and alkylated with 55 mM iodoacetamide (Sigma) for 30 min in the dark prior to an 18 h trypsin digestion at 37 °C using a 1:30 (w/w) enzyme to substrate ratio. The digestion was halted by lowering the pH to 2.0 for the acid hydrolysis and removal of Rapigest by first incubating for 1 h at 37 °C pelleting at 13000 rpm using a high-speed centrifuge.

Separation of Peptides and Mass Spectrometry. The peptides contained in the two sample types were analyzed by reverse phase chromatography prior to mass spectrometry analysis using the following method. Nanoelectrospray capillary coumns tips were made in house using a P-100 laser puller (Sutter Instruments). The columns were packed with Zorbax SB-C18 stationary phase (Agilent) purchased in bulk (5 μm particles, 15 cm length, 75 μm inner diameter). The reverse phase gradient separation was performed using water and acetonitrile (0.1% formic acid) as the mobile phases. The gradient starts at 5% acetonitrile and is ramped to 8% acetonitrile over 10 min. The acetonitrile is ramped to 35% acetonitrile over 20 min, then increased to 90% acetonitrile for another 20 min and maintained for another 10 min prior to re-equilibration to 5% acetonitrile.

The data-dependent MS/MS data were obtained on the LTQ linear ion trap mass spectrometer using a home-built nanoelectrospray source at 2 kV at the tip. The instrument was used in data-dependent MS/MS mode. One MS spectrum was followed by 4 MS/MS scans on the most abundant ions after the application of the dynamic exclusion list. Protein identification was performed using Mascot (Matrix Science, London, UK, version 2.1.04) at the 95% confidence level. A minimum of two peptides were used for the identification of each protein

Table 1. Proteins Found to be Significantly Up-Regulated in *P. furiosus* Adapted to 72 °C when Compared to 95 °C

accession	ORF	annotation	$\log_2$(ratio)
gi|18976415	PF0043	phosphoenolpyruvate synthase	0.68
gi|18976466	PF0094	glutaredoxin-like protein	6.82
gi|18976605	PF0233	hypothetical protein PF0233	0.71
gi|18977396	PF1024	cysteinyl-tRNA synthetase	2.06
gi|18977448	PF1076	hypothetical TLDD protein	2.34
gi|18977476	PF1104	homoserine dehydrogenase	1.18
gi|18977655	PF1283	Rubrerythrin	0.48
gi|18978019	PF1647	DNA-directed RNA polymerase subunit d	1.80
gi|18978215	PF1843	chromosome segregation protein smc	1.20
gi|18978304	PF1932	hypothetical protein PF1932	2.75
gi|18978338	PF1966	hypothetical protein PF1966	1.12

determined to be differentially regulated. Five replicates were performed for the cells grown at each temperature, and a Student's *t* test was used to differentiate and identify the significant differences using a *p*-value of 0.05.

Tandem mass spectra were extracted by the Xcalibur software. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.1.04). Mascot was set up to search *P. furiosus* (strain DSM 3638) proteins contained in the NCBInr protein database assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 2.0 Da. Iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Oxidation of methionine was specified in Mascot as a variable modification.

Scaffold (version Scaffold-01_06_03, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm.[14] Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.[15] Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

The determination of change in protein levels was determined from five replicate nano-LC-MS/MS analyses performed at each temperature. Using the relative number of peptides identified, a Student's *t* test was performed to differentiate the significantly changed proteins using a *p*-value of 0.05. A ratio was determined between the average number of peptides at each temperature. The $\log_2$(ratio) determined for each of these significantly changing proteins provides a convenient measure of the relative change. Negative values indicated a downward regulation, while positive values indicated an upward regulation for the cold adaptation response.

Results and Discussion

Metabolomic Profiling and Identification. Metabolite profiling of the two sets of samples revealed vast differences in the LC-MS features. Tentative assignments of metabolite identities were made based on accurate mass (±5 ppm) and a search of known metabolites found in the METLIN and KEGG databases. Most metabolites were down-regulated in samples grown at 72 °C compared to 95 °C. Some of these down-regulated metabolites could be putatively identified as indolacetic acid, naphthoic acid/vitamin K3, pantothenic acid, indolpropionic acid, valine, lysine, arginine, methionine, histidine, tyrosine, adenine, and 5′-methylthioadenosine (MTA) through their accurate mass (<5 ppm) and retention time. There were some putatively assigned metabolites that underwent a significant up-regulation including agmatine, spermidine, and higher-order polyamines such as N^4-aminopropylspermidine aminopropylspermidine and thermine. Higher-order polyamines like N^4-aminopropylspermidine and thermine have been found in thermophilic bacteria. Oshima and co-workers provided evidence that the higher-order and branched polyamines like N^4-aminopropylspermidine in some thermophiles are produced by an alternative pathway to polyamine synthesis than found in other prokaryotes.[16] This alternative pathway which was demonstrated in *T. thermophilus* using reverse genetics involves the biosynthesis of polyamines from L-arginine, as shown in Figure 1, and does not use putrescine as an intermediate for spermidine synthesis like in other prokaryotes. They speculated that a similar alternative pathway may be operative in other thermophilic organisms for the stabilization of biomolecules at the higher temperatures. We observed many ions that correspond to metabolites involved in this pathway including arginine, agmatine, spermidine, 5′-methylthioadenosine (MTA), aminopropylspermidine, and acetylaminopropylspermidine. The homologous enzymes involved with the biosynthesis of polyamines in *P. furiosus* for these processes correspond to arginine decarboxylase SpeA (PF1623), agmantine ureohydrolase SpeB (PF1957), and SAM decarboxylase SpeD (PF1930). A significant change in the expression of these genes was not observed in the DNA microarray analysis, and the corresponding proteins were not identified in the differential proteomic analysis possibly due to low abundance. In addition, the fourth enzyme, which catalyzes the aminopropyl transfer step, was recently characterized from *P. furiosus* and has unusual properties.[17] It is encoded by PF0127 and was termed agmantine/cadaverine aminopropyl transferase (ACAPT) as it uses a range of amine acceptors including cadaverine and agmantine. From microarray analysis, all four genes (PF127, PF1623, PF1930, and PF1957) are expressed at significant levels at 95 °C, but none were significantly regulated by a change in growth temperature. It is interesting that the amount of arginine in the two cell types is inversely related to the amount of spermidine (higher in 72 °C-grown cells), although how the pathway is regulated remains unknown.

A selected group of ions determined to undergo significant increase upon cold adaptation were targeted for identification through a combination of accurate mass and collision induced dissociation (CID). The elemental composition could be narrowed down through accurate mass measurements (<5 ppm) and isotopic pattern at higher resolution (6000) on an ESI-TOF mass spectrometer. The identity of each molecule was confirmed through the simultaneous MS/MS characterization of the natural product from *P. furiosus* and a high purity standard. The rigorous approach involving a comparison of the MS/MS pattern obtained from high purity standards for the identification of metabolites is illustrated for the case of N^4-aminopropylspermidine and spermidine in Figure 2. This methodology

Table 2. Proteins Found to be Significantly Down-Regulated in *P. furiosus* Adapted to 72 °C when Compared to 95 °C

accession	ORF	annotation	$\log_2$(ratio)
gil18976380	PF0008	hit family protein	−6.65
gil18976382	PF0010	1-aminocyclopropane-1-carboxylate deaminase	−7.73
gil18976394	PF0022	putative RNase E	−4.30
gil18976465	PF0093	replication factor C, small subunit	−1.61
gil18976474	PF0102	hypothetical protein PF0102	−8.67
gil18976485	PF0113	hypothetical protein PF0113	−4.94
gil18976493	PF0121	putative aspartate aminotransferase	−7.86
gil18976500	PF0128	hypothetical protein PF0128	−7.49
gil33359454	PF0132	hypothetical protein PF0132	−7.15
gil18976510	PF0138	hypothetical protein PF0138	−8.34
gil18976574	PF0202	isocitrate dehydrogenase	−7.57
gil18976579	PF0207	argininosuccinate synthase	−8.03
gil18976580	PF0208	argininosuccinate lyase	−6.14
gil18976608	PF0236	ribose-phosphate pyrophosphokinase	−5.93
gil33359463	PF0239	hypothetical protein PF0239	−3.47
gil18976637	PF0265	2-amino-3-ketobutyrate CoA ligase	−7.13
gil18976661	PF0289	gtp - hyd. phosphoenolpyruvate carboxykinase	−6.50
gil18976675	PF0303	hypothetical protein PF0303	−2.67
gil18976718	PF0346	aldehyde:ferredoxin oxidoreductase	−0.88
gil18976755	PF0383	hypothetical protein PF0383	−7.13
gil18976777	PF0405	methanol dehydrogenase regulatory protein	−7.03
gil18976822	PF0450	glutamine synthetase i	−9.99
gil18976857	PF0485	cell division inhibitor mind-like protein	−6.33
gil18976867	PF0495	reverse gyrase	−4.12
gil18976894	PF0522	aspartate transaminase	−8.15
gil18976905	PF0533	indolepyruvate ferredoxin oxidoreductase sub. a	−1.53
gil18976906	PF0534	indolepyruvate ferredoxin oxidoreductase sub. b	−7.15
gil18976918	PF0546	hypothetical protein PF0546	−6.64
gil18976919	PF0547	hypothetical protein PF0547	−0.68
gil18976931	PF0559	hydrogenase expression regulatory protein	−2.26
gil18976932	PF0560	hypothetical protein PF0560	−7.41
gil18976969	PF0597	hypothetical iaa-amino acid hydrolase 1 precursor	−0.76
gil18976970	PF0598	aspartate carbamoyltransferase, regulatory subunit	−6.65
gil18976973	PF0601	hypothetical phosphomethylpyrimidine kinase	−6.91
gil18976979	PF0607	hypothetical protein PF0607	−7.98
gil18976980	PF0608	alcohol dehydrogenase	−3.24
gil18976987	PF0615	hydrogenase expression/ formation protein	−7.79
gil18976988	PF0616	nucleotide-binding protein, mrp/nbp35 family	−6.78
gil18977015	PF0643	hypothetical protein PF0643	−2.48
gil18977038	PF0666	nol1-nop2-sun family putative nucleolar prot. IV	−0.91
gil18977114	PF0742	putative ferritin	−1.90
gil18977123	PF0751	flavoprotein	−2.31
gil18977124	PF0752	thioredoxin peroxidase	−7.13
gil18977126	PF0754	2-keto acid:ferredoxin oxidoreductase subunit α	−1.20
gil18977140	PF0768	acetyl/acyl transferase related protein	−2.70
gil18977217	PF0845	2-keto acid:ferredoxin oxidoreductase subunit α	−8.00

Table 2. continued

accession	ORF	annotation	$\log_2$(ratio)
gil18977236	PF0864	transcriptional regulatory protein, asnC family	−4.74
gil18977263	PF0891	sulfhydrogenase beta subunit	−7.13
gil18977266	PF0894	sulfhydrogenase alpha subunit	−7.32
gil18977335	PF0963	cell division control protein 48, aaa family	−5.16
gil18977337	PF0965	pyruvate ferredoxin oxidoreductase sub. beta-2	−0.68
gil18977338	PF0966	pyruvate ferredoxin oxidoreductase sub. alpha-2	−2.37
gil18977341	PF0969	2-ketovalerate ferredoxin oxidored. alpha-2	−6.78
gil18977343	PF0971	pyruvate ferredoxin oxidoreductase/gamma	−3.13
gil18977355	PF0983	pcna sliding clamp (proliferating-cell nuc. antigen)	−4.13
gil18977361	PF0989	phenylalanyl-tRNA synthetase, alpha-subunit	−3.94
gil18977363	PF0991	possible threonine 3-dehydrogenase	−8.88
gil18977393	PF1021	hypothetical protein PF1021	−0.93
gil18977402	PF1030	methionyl-tRNA synthetase	−1.40
gil18977485	PF1113	hypothetical protein PF1113	−7.03
gil33359545	PF1127	hypothetical protein PF1127	−1.12
gil18977512	PF1140	translation initiation factor eIF-2, subunit alpha	−3.58
gil18977569	PF1197	NADH oxidase /nitrite reductase	−3.07
gil18977576	PF1204	seryl-tRNA synthetase	−1.08
gil18977586	PF1214	hypothetical protein PF1214	−8.20
gil18977654	PF1282	Rubredoxin	−3.06
gil18977659	PF1287	putative ABC transporter	−3.50
gil18977739	PF1367	LSU ribosomal protein L7AE	−2.20
gil18977766	PF1394	phosphoglycerate dehydrogenase	−1.61
gil18977806	PF1434	mbh12 membrane bound hydrogenase alpha	−6.79
gil18977831	PF1459	hypothetical protein PF1459	−1.98
gil18977833	PF1461	l-asparaginase (l-asparagine amidohydrolase)	−2.67
gil18977843	PF1471	hypothetical protein PF1471	−3.15
gil18977848	PF1476	hypothetical protein PF1476	−8.04
gil18977869	PF1497	putative transaminase	−0.88
gil18977888	PF1516	GMP synthase	−7.66
gil18977908	PF1536	hypothetical protein PF1536	−3.52
gil18977912	PF1540	ADP forming acetyl coenzyme A synthetase	−1.53
gil18977974	PF1602	glutamate dehydrogenase	−2.23
gil18977983	PF1611	cell division inhibitor mind	−2.74
gil18978013	PF1641	enolase (2-phosphoglycerate dehydratase)	−2.50
gil18978046	PF1674	hypothetical protein PF1674	−7.02
gil18978054	PF1682	ribosomal protein s6 modification –protein	−6.91
gil18978097	PF1725	hypothetical protein PF1725	−3.53
gil18978117	PF1745	hypothetical protein PF1745	−4.35
gil18978136	PF1764	hypothetical protein PF1764	−0.85
gil18978159	PF1787	acetyl-CoA synthetase	−2.47
gil18978166	PF1794	inosine-5′-monophosphate dehydr. rel. protein I	−7.66
gil18978189	PF1817	hypothetical protein translation factor sui1	−2.05
gil18978199	PF1827	hypothetical protein PF1827	−2.29
gil33359575	PF1828	hypothetical protein PF1828	−7.93
gil18978201	PF1829	nucleotidyltransferase	−7.43

Table 2. continued

accession	ORF	annotation	$\log_2$(ratio)
gil18978203	PF1831	archaeal histone a1	−0.88
gil18978209	PF1837	hypothetical protein PF1837	−4.18
gil18978234	PF1862	DNA-binding protein	−7.32
gil18978238	PF1866	*S*-adenosylmethionine synthetase	−0.51
gil18978298	PF1926	recombinase, radA	−7.87
gil18978318	PF1946	methylmalonyl-CoAmutase, subunit alpha	−6.65
gil18978320	PF1948	hypothetical protein PF1948	−1.49
gil18978325	PF1953	inosine-5′-monophosphate dehydr. rel. protein II	−4.75
gil18978346	PF1974	thermosome, single subunit	−0.57
gil18978359	PF1987	hypothetical protein PF1987	−1.69
gil18978360	PF1988	hypothetical cell division protein ftsZ	−4.25
gil18978365	PF1993	LSU ribosomal protein L10E	−3.86
gil18978366	PF1994	LSU ribosomal protein L12A	−1.45
gil18978372	PF2000	glycine dehydrogenase (decarboxylating) sub. 2	−2.51

was applied to the identification of spermidine, arginine, agmatine, and a previously unidentified and the novel metabolite N^4-(*N*-acetylaminopropyl)spermidine. This novel metabolite was identified through an iterative process of chemical synthesis and comparative MS/MS analysis (Jaroslaw Kalisiak, unpublished results). Interestingly, these molecules can be traced to the alternative polyamine biosynthetic pathway shown in Figure 1. N^4-(*N*-Acetylaminopropyl)spermidines, along with other higher-order polyamines, are probably synthesized from spermidine, similarly to the alternative pathway outlined in Figure 1. The relative quantitation for the different metabolites identified along the biosynthesis pathway is shown in Figure 3. For the adaptation response of *P. furiosus* to 72 °C, arginine was down-regulated upon cold adaptation ($A^{72}/A^{95} = 0.10 \pm 0.0031$), while agmatine ($A^{72}/A^{95} = 8.01 \pm 1.99$), spermidine ($A^{72}/A^{95} = 12.48 \pm 0.61$), N^4-aminopropylspermidine ($A^{72}/A^{95} = 4.28 \pm 0.19$), and N^4-(*N*-acetylaminopropyl)-spermidine ($A^{72}/A^{95} = 15.38 \pm 10.79$) were significantly up-regulated. These results indicate a consistent up-regulation of the polyamine biosynthetic pathway. Although the presence of spermidine and N^4-aminopropylspermidine has been reported in *P. furiosus*,[18,19] to our knowledge, it is the first report of the up-regulation of spermidine and other polyamines upon adaptation to 72 °C. In *T. thermophilus*, a moderately thermophilic bacterium, spermidine synthesis has been reported to increase at relatively higher temperatures of 75 °C, along with the higher-order and branched polyamines.[20] These molecules are believed to play an important role in the survivability of thermophilic organisms at higher temperatures. However, it is important to note that 75 °C used in the original study with *T. thermophilus* is very close to our cold adaptation temperature of 72 °C for the hyperthermophile *P. furiosus*, for which we see a consistent up-regulation of this part of the polyamine pathway. It is possible that there are multiple mechanisms of achieving viability at higher temperatures. Specifically, there are certain pathways which are more active at ∼70 °C than at the significantly higher temperature of 95 °C for the stability of the biomolecules and the survival of the organism. The same polyamine pathway may be used at ∼70 °C for both *T. thermophilus* and *P. furiosus* for an increased synthesis of branched polyamines, while at the significantly higher temperatures approaching 100 °C, other unique mechanisms may by utilized.

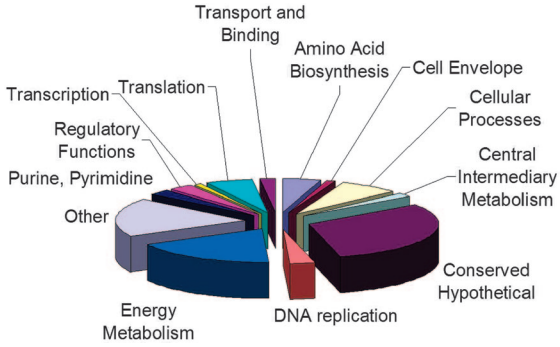

Figure 4. Distribution of 107 proteins based on predicted functions (NCBInr) which were found to be significantly down-regulated using our semiquantitative proteomics analysis of the cold adaptation response in *P. furiosus*.

Spermidine is most likely a building block for higher-order polyamines such as N^4-aminopropylspermidine, by an, as of yet, uncharacterized enzymatic pathway. While agmantine/cadaverine aminopropyl transferase (ACAPT) has been shown to use several polyamines as acceptors including spermidine, it does not seem to be the natural substrate for its conversion to N^4-(*N*-acetylaminopropyl)spermidine due to its low relative activity toward it when compared to cadaverine (0.8%).[18] In addition, it appears that N^4-aminopropylspermidine is being acetylated in the organism to form N^4-(*N*-acetylaminopropyl-)spermidine, a previously uncharacterized product, by still an unknown pathway.

Correlating Microarray Analysis with Protein Expression. Tandem mass spectrometry based experiments were performed to examine proteomic changes in response to cold adaptation. One hundred ninety five proteins were identified with at least two peptides with >95% confidence level using the Mascot search engine. A negative value of the $\log_2$(ratio) indicated a down-regulation, while a positive value indicated a relative up-regulation. While many proteins were found to be in common under these two conditions, there were also significant differences observed (p-value < 0.05). The proteins

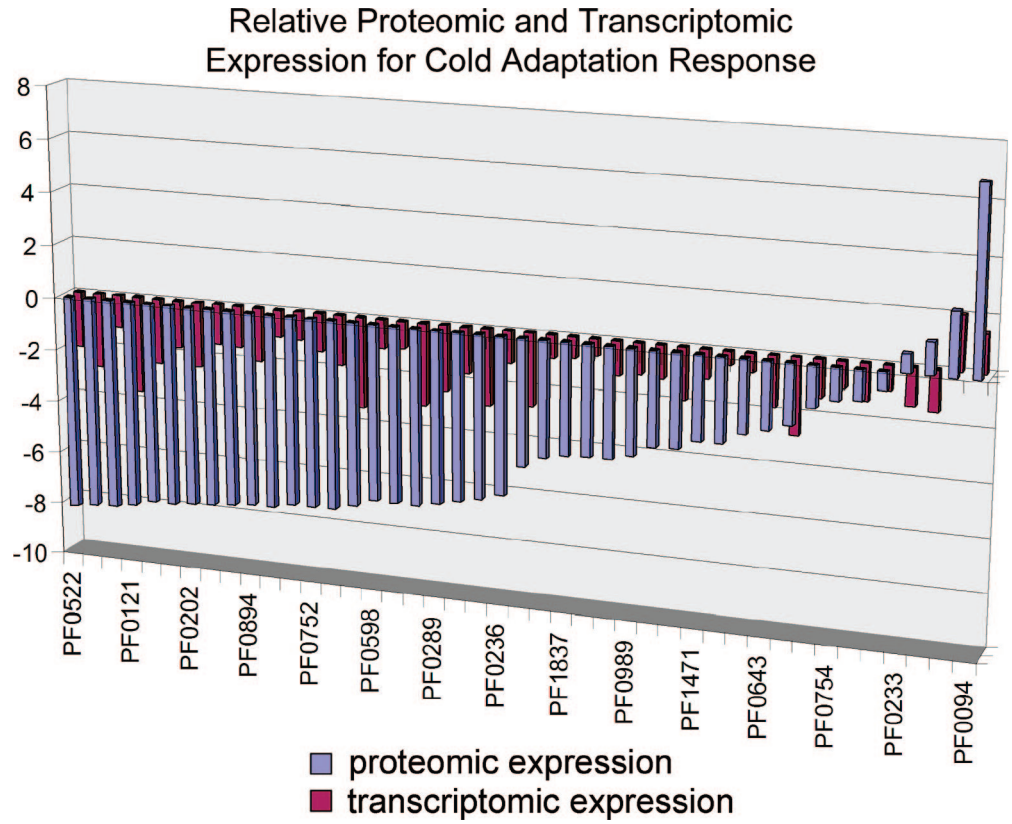

Figure 5. Comparison between relative transcriptomic expression levels (log_2(ratio), p-values < 0.05) and proteomic expression (log_2(ratio) of percentage of total spectra identified for each protein at the two temperatures) plotted for the proteins identified for which reliable gene array values are available (>2 peptide identification and a minimum of 1 peptide identified at each temperature). An excellent directional correspondence is found for these cases where most proteins found to be significantly altered using the defined criteria are also found to have a corresponding change in gene expression.

found to be up- and down-regulated using these criteria are given in Tables 1 and 2, respectively. In many cases, a significant change in mRNA was not detected for proteins found to be significantly regulated through the proteomics experiment. In cases where a significant change was identified, there was good directional correspondence between the two results. There were two proteins PF1843 (chromosome segregation protein smc, gil18978215) and PF0233 (hypothetical protein PF0233) that showed a down-regulation, while the mRNA levels recorded a significant up-regulation. In these cases, the increase in mRNA level may not directly result in an increased level of the protein since it may be regulated at the post-translational level. All proteins that showed significant down-regulation through our proteomic measurements also showed a significant down-regulation with DNA microarray measurements. Eleven proteins were found to be up-regulated (Table 1) during cold adaptation. Two of these are conserved hypothetical proteins, and therefore no information is known about their possible function. One of the most strongly up-regulated proteins is the glutaredoxin-like protein (PF0094). Glutaredoxin is known to be involved in removal of oxygen reactive species (ROS) and the reduction of oxidative stress in archaea.[21] Independently performed microarray experiments also indicate the up-regulation of this gene. Hypothetical TLDD protein, believed to be involved in regulatory functions, is also determined to be up-regulated by both gene expression and mass spectrometry based proteomics measurements.

The expression of most proteins was down-regulated. One hundred seven proteins were found to be significantly down-regulated for cold adapted *P. furiosus* using our proteomic analysis method (Table 2). These included 19 proteins with predicted involvement in energy metabolism and in central intermediary metabolism (PF1602, PF1946), proteins active in translation (PF0597, PF1030, PF1140, PF1367, PF1682, PF1817), amino acid biosynthesis (PF0121, PF157, PF0207, PF0450), those necessary for binding and transport (PF0472, PF1287), and transcription (PF1647, PF0060, and PF1564). Figure 4 summarizes the distribution of protein by predicted and known function for down-regulated proteins identified by the proteomics experiments. Figure 5 illustrates the correlation between the DNA microarray and the proteomic data by plotting the log_2(ratio) for protein expression taken as a ratio of total identified spectra at the two temperatures and the log_2(ratio) from the microarray cold adaptation analysis. There is good directional correspondence for the eight genes for cases where both reliable microarray and proteomics data are available.

Conclusions

We have performed the comprehensive analysis of transcriptomic, proteomic, and metabolite level changes in a model organism *P. furiosus* by combining gene array measurements and mass spectrometry. Mass spectrometry is used to identify several polyamine related metabolites that change upon adaptation to 72 °C. A significant decrease in the level of arginine and an increase in levels of agmatine, spermidine, N^4-aminopropylspermidine, and N^4-(*N*-acetylaminopropyl)spermidine indicate the up-regulation of the pathway at 72 °C when

compared to 95 °C. To further investigate metabolite changes revealed in this work, future experiments using LC-MS based metabolite profiling experiments could be designed involving knockout of genes for the predicted arginine decarboxylase (PF1623), agmantine ureohydrolase (PF1957), and SAM decarboxylase (PF1930, PF1866) in *P. furiosus* to measure their effect on the relative amounts of the metabolites such as spermidine and to confirm its regulatory role in the biosynthetic pathway of other polyamines in *P. furiosus*. The discovery of a novel metabolite N^4-(*N*-acetylaminopropyl)spermidine will ultimately allow the uncovering of new enzymatic pathways and the annotation of new enzymes involved in these molecular transformations. We believe this system-wide approach can be used generally to map the networks that define the interdependence of molecular changes as a microbe undergoes a stress response.

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References

(1) Smith, C. A.; Want, E. J.; O'Maille, G.; Abagyan, R.; Siuzdak, G. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem.* **2006**, *78* (3), 779–87.

(2) Smith, C. A.; O'Maille, G.; Want, E. J.; Qin, C.; Trauger, S. A.; Brandon, T. R.; Custodio, D. E.; Abagyan, R.; Siuzdak, G. METLIN: a metabolite mass spectral database. *Ther. Drug Monit.* **2005**, *27* (6), 747–51.

(3) Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **1999**, *17* (10), 994–9.

(4) Yao, X.; Freas, A.; Ramirez, J.; Demirev, P. A.; Fenselau, C. Proteolytic 18O labeling for comparative proteomics: model studies with two serotypes of adenovirus. *Anal. Chem.* **2001**, *73* (13), 2836–42.

(5) Ong, S. E.; Blagoev, B.; Kratchmarova, I.; Kristensen, D. B.; Steen, H.; Pandey, A.; Mann, M. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* **2002**, *1* (5), 376–86.

(6) Wiener, M. C.; Sachs, J. R.; Deyanova, E. G.; Yates, N. A. Differential mass spectrometry: a label-free LC-MS method for finding significant differences in complex peptide and protein mixtures. *Anal. Chem.* **2004**, *76* (20), 6085–96.

(7) Old, W. M.; Meyer-Arendt, K.; Aveline-Wolf, L.; Pierce, K. G.; Mendoza, A.; Sevinsky, J. R.; Resing, K. A.; Ahn, N. G. Comparison of label-free methods for quantifying human proteins by shotgun proteomics. *Mol. Cell. Proteomics* **2005**, *4* (10), 1487–502.

(8) Smith, R. D.; Shen, Y.; Tang, K. Ultrasensitive and quantitative analyses from combined separations-mass spectrometry for the characterization of proteomes. *Acc. Chem. Res.* **2004**, *37* (4), 269–78.

(9) Bragg, J. G.; Thomas, D.; Baudouin-Cornu, P. Variation among species in proteomic sulphur content is related to environmental conditions. *Proc. Biol. Sci.* **2006**, *273* (1591), 1293–300.

(10) Marmur, J.; Doty, P. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. Mol. Biol.* **1962**, *5*, 109–18.

(11) Robb, F. T.; Maeder, D. L.; Brown, J. R.; DiRuggiero, J.; Stump, M. D.; Yeh, R. K.; Weiss, R. B.; Dunn, D. M. Genomic sequence of hyperthermophile, Pyrococcus furiosus: implications for physiology and enzymology. *Methods Enzymol.* **2001**, *330*, 134–57.

(12) Weinberg, M. V.; Schut, G. J.; Brehm, S.; Datta, S.; Adams, M. W. Cold shock of a hyperthermophilic archaeon: Pyrococcus furiosus exhibits multiple responses to a suboptimal growth temperature with a key role for membrane-bound glycoproteins. *J. Bacteriol.* **2005**, *187* (1), 336–48.

(13) Holm, S. A simple sequentially rejective multiple test procedure to give modified p values. *Scand. J. Stat* **1979**, *6*, 65–70.

(14) Keller, A.; Nesvizhskii, A. I.; Kolker, E.; Aebersold, R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* **2002**, *74* (20), 5383–92.

(15) Nesvizhskii, A. I.; Keller, A.; Kolker, E.; Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **2003**, *75* (17), 4646–58.

(16) Ohnuma, M.; Terui, Y.; Tamakoshi, M.; Mitome, H.; Niitsu, M.; Samejima, K.; Kawashima, E.; Oshima, T. N1-aminopropylagmatine, a new polyamine produced as a key intermediate in polyamine biosynthesis of an extreme thermophile, Thermus thermophilus. *J. Biol. Chem.* **2005**, *280* (34), 30073–82.

(17) Cacciapuotil, G.; Porcelli, M.; Moretti, M. A.; Zappia, V.; Liu, Z.-J.; Tempel, W.; Shubot, F.; Rose, J. P.; Wang, B.-C.; Brereton, P. S.; Jenney, F. E.; Adams, M. W. W. The first agmantine/cadaverine aminopropyl transferase: biochemical and structural characterization of the enzyme involved in polyamine biosynthesis in the hyperthermophilic archaeon Pyrococcus furiosus. *J. Bacteriol.* **2007**,in press.

(18) Cacciapuoti, G.; Porcelli, M.; Moretti, M. A.; Sorrentino, F.; Concilio, L.; Zappia, V.; Liu, Z. J.; Tempel, W.; Schubot, F.; Rose, J. P.; Wang, B. C.; Brereton, P. S.; Jenney, F. E.; Adams, M. W. The first agmatine/cadaverine aminopropyl transferase: biochemical and structural characterization of an enzyme involved in polyamine biosynthesis in the hyperthermophilic archaeon Pyrococcus furiosus. *J. Bacteriol.* **2007**, *189* (16), 6057–67.

(19) Hamana, K.; Hamana, H.; Niitsu, M.; Samejima, K.; Sakane, T.; Yokota, A. Occurrence of tertiary and quaternary branched polyamines in thermophilic archaebacteria. *Microbios* **1994**, *79* (319), 109–19.

(20) Oshima, T., Unique polyamines produced by an extreme thermophile, Thermus thermophilus. *Amino Acids* **2007**.

(21) Pedone, E.; Bartolucci, S.; Fiorentino, G. Sensing and adapting to environmental stress: the archaeal tactic. *Front Biosci.* **2004**, *9*, 2909–26.

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