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Heat Shock Response of *Archaeoglobus fulgidus*†

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The heat shock response of the hyperthermophilic archaeon *Archaeoglobus fulgidus* strain VC-16 was studied using whole-genome microarrays. On the basis of the resulting expression profiles, approximately 350 of the 2,410 open reading frames (ORFs) (ca. 14%) exhibited increased or decreased transcript abundance. These span a range of cell functions, including energy production, amino acid metabolism, and signal transduction, where the majority are uncharacterized. One ORF called AF1298 was identified that contains a putative helix-turn-helix DNA binding motif. The gene product, HSR1, was expressed and purified from *Escherichia coli* and was used to characterize specific DNA recognition regions upstream of two *A. fulgidus* genes, AF1298 and AF1971. The results indicate that AF1298 is autoregulated and is part of an operon with two downstream genes that encode a small heat shock protein, Hsp20, and cdc48, an AAA$^+$ ATPase. The DNase I footprints using HSR1 suggest the presence of a *cis*-binding motif upstream of AF1298 consisting of CTAAC-N5-GTTAG. Since AF1298 is negatively regulated in response to heat shock and encodes a protein only distantly related to the N-terminal DNA binding domain of Phr of *Pyrococcus furiosus*, these results suggest that HSR1 and Phr may belong to an evolutionarily diverse protein family involved in heat shock regulation in hyperthermophilic and mesophilic *Archaea* organisms.

Heat shock is a well-studied physiological response in both the *Eucarya* and *Bacteria*, and for many years it has been used as a basis for exploring gene regulation (24, 25). It is well established that heat and other cell stresses modulate the expression of genes encoding the so-called heat shock proteins (HSPs), which are involved in a variety of cellular processes, including membrane transport and stability, protein folding, and cell signaling (11, 12, 29, 42). The predominant HSPs are classified by their molecular masses as Hsp104, Hsp90, Hsp70, Hsp60, Hsp40, Hsp20, and Hsp10 or as heat-inducible proteases, such as Clp and Lon (21). With the development of gene arrays, it has become clear that a great many genes in addition to HSPs are influenced by heat (14, 30, 40). In *Escherichia coli*, for example, microarray analyses indicate that 119 genes are significantly induced by heat (30). More generally, in *E. coli* as well as *Campylobacter jejuni* and *Bacillus subtilis*, whole-genome microarrays indicate that about 20% of all genes are differentially regulated at various levels by heat shock. Similarly, microarray data for *Saccharomyces cerevisiae* indicate that over 25% of the genome is differentially expressed 10 min after a temperature shock from 25 to 37°C (9). In *Caenorhabditis elegans* 28 of 11,917 open reading frames (ORF) showed consistent induction during heat shock (13).

In contrast, relatively little is known about heat shock in the *Archaea*. Genome sequence information indicates that some archaea lack the HSPs that are common in *Eucarya* and *Bacteria*, which were previously thought to be universal (22). For example, Hsp70 (DnaK), Hsp40 (DnaJ), and Hsp60 (GroEL) are absent in members of the *Crenarchaeota*, although they are found in some members of the *Euryarchaeota*. The *Crenarchaeota* have a separate class of Hsp60s that is related to a eucaryotic protein known as TCP1 and only distantly related to the highly conserved bacterial Hsp60s (42, 43). Some crenarchaeal Hsp60s are heat inducible (16, 19), but others are regulated by low temperatures (17) as has also been shown for the related TCP1 proteins in yeast (39). Sequence analyses indicate that some archaea have an ORF for a protein related to the bacterial heat-inducible protease known as Lon, but the archaeal ORF is missing the ATP binding domain and the protein has not yet been biochemically characterized (8, 47).

While it has been proposed that archaeal transcription factors are similar to those in the *Eucarya*, with TATA binding proteins and transcription factor II B (TFIIB) (4), no heat shock factor homologues have been identified and the few known regulatory proteins in the *Archaea* have similarities to transcription regulators in the *Bacteria*. The heat shock promoters, studied by mutagenesis in the halophilic archaeon *Haloferax volcanii*, revealed a conserved region (5′-CGAA-3′) upstream of the TATA box important for both basal and heat shock gene expression and two regions downstream of the TATA box important for heat shock expression (41). In other archaea, the region upstream of the TATA box is conserved but not unique to heat shock-regulated genes, and the downstream regions are not conserved. In the hyperthermophilic archaeon *Pyrococcus furiosus*, a heat shock regulator (Phr) binds to promoter regions upstream of Hsp20, an ATPase, and its own gene (46), increasing the synthesis of Hsp20 and ATPase but decreasing the synthesis of its own gene product.

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† Supplemental material for this article may be found at http://jb.asm.org/.

Here we analyze the whole-genome heat shock response of the hyperthermophilic archaeon *Archaeoglobus fulgidus*. A microarray was designed and constructed to minimize cross-hybridization between homologous ORFs. Its application revealed heat-induced changes in the expression of 350 genes out of the 2,410 genes, where 189 exhibited increased mRNA abundance and 161 had reduced abundance over the 60-min time period examined. One differentially expressed ORF, AF1298, that encodes a potential DNA binding protein, was cloned and expressed in *E. coli* and purified to homogeneity. Electrophoresis mobility shift assays (EMSA) and DNase I footprinting assays were used to document the DNA binding properties of the protein at two heat shock-induced promoters. A role for the protein in *A. fulgidus* gene regulation and heat shock response is discussed.

MATERIALS AND METHODS

Strain and culture conditions. *Archaeoglobus fulgidus* strain VC-16 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) (DSM 4304) and grown in 500-ml cultures anaerobically without shaking at 78°C. The growth medium was prepared according to the methods of Zellner et al. (49), but without yeast extract and peptone. It contained, per liter, 3.45 g $MgSO_4 \cdot 7H_2O$, 4.26 g Na_2SO_4, 0.34 g KCl, 2.75 g $MgCl_2 \cdot 6H_2O$, 0.25 g NH_4Cl, 18.0 g NaCl, 1 mg resazurin, 0.18 g $K_2HPO_4 \cdot 3H_2O$, 0.14 g $CaCl_2 \cdot 2H_2O$, 2.0 mg $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 2.4 mg $Ni(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.38 mg $NaWO_4 \cdot 2H_2O$, 20 μg biotin, 20 μg folic acid, 100 μg pyridoxine hydrochloride, 50 μg riboflavin, 50 μg thiamine, 50 μg nicotinic acid, 50 μg pantothenic acid, 1 μg vitamin B_{12}, 50 μg *p*-aminobenzoic acid, 50 μg thiotic acid, 7.6 g piperazine-*N*,*N′*-bis(2-ethanesulfonic acid), and 4.5 g 70% lactic acid.

Microarray probe selection and PCR primer design. Microarray DNA probes were designed using the software MyPROBES available at the website www.seas.ucla.edu/~liaoj/MyPROBES. This program designs optimal microarray probes of all genomic ORFs along with appropriate pairs of PCR primers for amplification from chromosomal DNA. The parameters set in the program included a probe length range of 500 to 2,000 bp, PCR primer melting temperature range of 59 to 63°C, primer length of 20 to 25 bases, GC content range of 50 to 60%, and a BLAST identity score of 100. For the reverse transcription-PCR (RT-PCR) primers, MyPROBES was used with a melting temperature of 59 to 63°C, primer length of 20 to 25 bases, GC content of 50 to 60%, product length of 90 to 120 bp, and BLAST identity score of 25.

PCR amplification of microarray probes. Duplicate 50-μl-volume PCRs were performed in 96-well plates using custom-synthesized (Illumina, San Diego, CA) primers selected by MyPROBES. Chromosomal DNA (1.5 ng) from *A. fulgidus* VC-16 and the Eppendorf MasterTaq kit (Westbury, NY) were used in the PCRs. The quality of all PCR products was examined by electrophoresis using 1.4% (wt/vol) agarose gels. The products were purified using 96-well filter plates (Millipore Corp., Bedford, MA), resuspended in 50 μl of water, dried to completion in a vacuum concentrator (CentriVap; Labconco, Kansas City, MO), and then resuspended in 10 μl of 3× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Microarray spotting. PCR products were transferred to 384-well plates and spotted onto GAPS II slides (Corning, Corning, New York) using a robotic arrayer Virtek ChipWriter Pro (Bio-Rad, Hercules, CA). The diameter of each spot was approximately 150 μm, and the distance between the centers of each spot was 200 μm. Slides were hydrated with steam for 2 to 3 s and snap dried on a 100°C heating block. The probes were cross-linked to the surface of the slide by UV light (Stratalinker; Stratagene, La Jolla, CA) at 250 mJ, and the slides were then baked at 80°C for 3 h. To minimize background, the slides were blocked by soaking for 15 min in 250 ml of 1-methyl-2-pyrrolidone with 4 g of succinic anhydride and 28 ml of 0.2 M sodium borate solution (pH 8.0). After blocking, the slides were washed with 95°C water for 2 min and transferred to 95% ethanol at room temperature for 1 min and then dried by centrifugation.

Microarray experimental design. The 500-ml cultures were incubated at 78°C without shaking for 12 to 16 h to an optical density at 600 nm (OD_{600}) of 0.2 before transferring to six argon-purged, prewarmed (78°C) culture flasks (60 ml/flask) to ensure a homogenous temperature shock for all cells. After 2 to 3 h, cultures reached an OD_{600} of 0.3 and 30 ml from each flask was quick cooled in an ethanol-dry ice bath. Cells were harvested by centrifugation (7,000 rpm at 4°C) and resuspended in RNAlater (Ambion). Non-heat-shocked cells were pooled and used as controls. The remaining cultures were transferred to an 89°C water bath and sampled after 5, 10, 15, 30, and 60 min (Fig. 1). At each time point two 15-ml samples were taken from two different flasks. Samples were immediately transferred to an ethanol-dry ice bath before harvesting cells by centrifugation and resuspending in 1 ml RNAlater buffer for RNA isolation. The experiment was repeated three times. For microarray calibration, a zero time point sample was divided in half, labeled with Cy3 and Cy5, and hybridized to each of two slides. This calibration experiment was performed three times to obtain a reference distribution in the data analysis (15, 44).

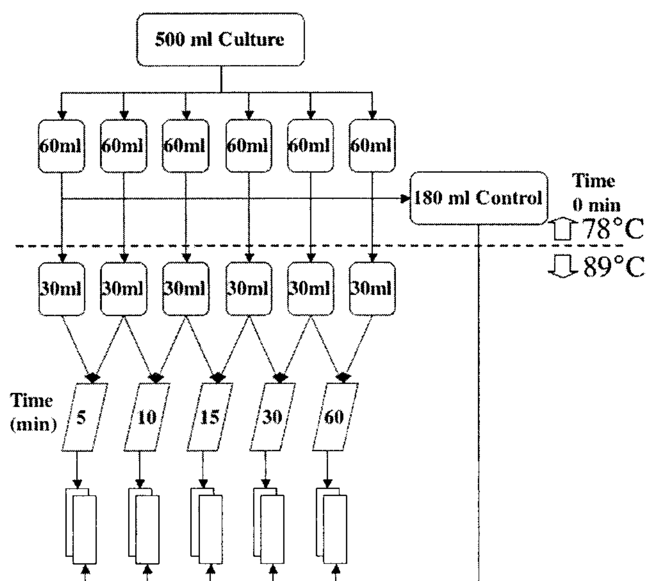

FIG. 1. Schematic plan of heat shock experiments. An initial *A. fulgidus* 500-ml culture was divided into six smaller flasks and incubated at 78°C. At an OD_{600} of 0.3, 30 ml of medium was removed from each flask and used as the reference and control. Each flask was then transferred from 78 to 89°C, and samples were removed at 5, 10, 15, 30, and 60 min after the temperature shift. Samples were taken so that each time point shared a common flask with the previous time point. RNA was purified from each sample and used for hybridization with two microarray slides. The reference samples were pooled together prior to slide hybridization.

RNA purification and labeling. For microarray experiments, total RNA was purified from 30 ml of cells using the RNAwiz (Ambion, Austin, TX) following the manufacturer's instructions. The purified RNA was treated with DNase I as described in reference 26. Total RNA (20 μg) was labeled using indirect labeling with amino allyl-dUTP with either Cy3 or Cy5 monofunctional NHS-ester (Amersham Bioscience, Piscataway, NJ). The reverse transcription mixture included 400 units of Superscript II RNase H reverse transcriptase (Invitrogen), random hexamers, 0.5 mM dATP, dCTP, and dGTP, 0.2 mM dTTP, and 0.3 mM amino allyl-dUTP (Sigma, St. Louis, MO) for 2 h. The Superscript was added in two steps, 200 U at time zero and 200 U after 1 h. After reverse transcription, the RNA was hydrolyzed by incubating at 65°C for 40 min after adding 10 μl of 0.5 M EDTA (pH 8.0) and 10 μl of 1 N NaOH. The cDNAs were neutralized with 25 μl 1 M HEPES and purified with Microcon-30 and dried. The dried cDNA was resuspended in 5 μl water plus 5 μl of 0.1 M sodium bicarbonate and transferred to a dry aliquot of the dye for the coupling reaction. The unheated sample (i.e., control) was labeled with Cy3, and the heated sample was labeled with Cy5 for 1 hour. The coupling reaction was quenched by adding 4.5 μl 4 M hydroxylamine. The two dyes were combined, cleaned up with a QiaQuick column (QIAGEN, Valencia, CA), and concentrated to 1 to 2 μl by using Microcon-30 (Millipore).

Hybridization, scanning, and data analysis. The concentrated Cy3 and Cy5 cDNA was hybridized and washed as described in reference 26. The dried slides were analyzed using a Virtek scanner at 5 μm. The scanner creates a TIFF file for each channel, Cy3 and Cy5, where two images were simultaneously analyzed in an image analysis program Imagene5 (Biodiscovery, Marina Del Ray, CA) to find spot intensities. Microarray spot intensity data were normalized, and the

95% confidence level was calculated using the software lcDNA (http://receptor.seas.ucla.edu/lcDNA) (15, 44).

Real time RT-PCR. The real time RT was performed using Superscript II (Invitrogen) according to the manufacturer's recommended protocol with gene-specific primers and 50 ng of total RNA. The reaction was heat inactivated at 70°C for 10 min. cDNA from the RT reaction (2 μl) was subsequently used in a 25-μl-volume QuantiTect SYBR Green PCR (QIAGEN) according to the manufacturer's recommendations. The real-time PCRs were conducted on a Cephid Smart Cycler unit (Cephid, Sunnyvale, CA) using a four-step program consisting of denaturing, annealing, extension, and acquisition steps. Each primer pair was calibrated using genomic DNA. AF0700, a gene that showed no significant up- or down-regulation in the microarray experiments, was used as the control to which all gene data were normalized.

Cloning of AF1298. The gene encoding AF1298 was PCR amplified using Turbo *Pfu* (Stratagene, San Diego, CA) using oligonucleotide primers AF1298for (5′-CATATGAAGGGATTAGTGCCCCGAG-3′) and AF1298rev (5′-GCGGCCGCCTTTATCATCCAAACAACTTC-3′) containing restriction sites NdeI and NotI, respectively. After the amplification, the fragment was incubated at 72°C with MasterTaq (Eppendorf) for 10 min before ligation into plasmid vector pTOPO4 (Invitrogen). The sequence of the insert was verified by DNA sequencing. The vector containing the insert and pET29b was cut with NdeI and NotI. PET29b was dephosphorylated using calf intestinal alkaline phosphatase (Promega, Madison, WI). The linearized vector and the cut insert were gel purified using a QIAGEN gel purification kit (QIAGEN, Valencia, CA). The two products were ligated using a rapid ligation kit (Roche, Indianapolis, IN). The resulting plasmid was transformed into BL21codonplus(DE3)-RIL for protein expression.

Expression and purification of AF1298. *E. coli* BL21codonplus cells were grown in LB medium containing 200 μl/ml ampicillin and 35 μl/ml chloramphenicol at 37°C with shaking. At an OD_{600} of 1.0, the culture was induced with isopropyl-β-D-thiogalactopyranoside with a final concentration of 0.6 mM. After 4 h of incubation, the cells were harvested by centrifugation and resuspended in buffer (20 mM phosphate buffer, pH 7.4, 0.5 M NaCl, 20 mM imidazole). The resuspended cells were broken with a French press at 100 mPa, and the cell suspension was centrifuged at 8,000 rpm for 10 min at 4°C. The supernatant was then filtered with a 22-μm filter and applied to a 1-ml HisTrap HP (Amersham, Piscataway, NJ) column, and the column was washed with 5 ml of the phosphate buffer. The recombinant protein was eluted with a step gradient in imidazole, 2 ml for each step, starting at 100 mM imidazole and increasing with 100 mM steps to 400 mM imidazole. Fractions (1 ml) were collected where the recombinant protein eluted in the second fraction of 400 mM imidazole. The protein was concentrated, and the buffer was exchanged to 20 mM Tris, pH 7.6, 150 mM KCl, 0.5 mM $MgCl_2$, 10% glycerol using a YM-30 column (Millipore). Purity was evaluated by polyacrylamide gel electrophoresis, and the protein concentration was measured using the DC protein assay (Bio-Rad).

EMSA. Promoter fragments extending between −175 to +50 relative to the ORF start codon were PCR amplified from genomic DNA using primers containing EcoRI sites on the forward primers and HindIII or XbaI sites on the reverse primers. The PCR products were purified using QiaQuick columns, digested using EcoRI and HindIII or XbaI, and repurified using QiaQuick columns (QIAGEN). The fragments were cloned into pUC18 and transformed into XL1-Blue, and the intended DNA sequences were confirmed.

For electrophoretic mobility shift assays, promoter fragments were generated by PCR using the above plasmids, gel purified on a 6% Tris-borate-EDTA-polyacrylamide gel electrophoresis gel and then extracted overnight in 800 μl of Crush and Soak buffer with shaking at 37°C. The resulting solution was diluted in five volumes of BP buffer, and DNA was purified using a QiaQuick column (QIAGEN). The DNA binding assay was carried out in 17.5 μl of binding buffer (10 mM Tris, pH 7.5, 50 mM KCl, 7 mM $CaCl_2$, 8.6% glycerol, 0.0036% bovine serum albumin) containing 20 ng of DNA and protein at the indicated concentrations. The AF1298 protein stock solution (0.05 M Tricine, pH 8.0, 0.2 M KCl, 20 mM $CaCl_2$, 0.5 mM dithiothreitol) concentration was 400 nM. The reaction mixtures were preincubated for 12 min at room temperature before separation on a 6% DNA retardation gel (Invitrogen, Carlsbad, CA) at 100 V for 45 min. The gels were soaked for 20 min in 50 ml 1× TBE containing SybrGreen, washed twice in MilliQ water for 10 seconds, and analyzed using a Kodak EDA290 camera system. Protein analysis was performed with a Bio-Rad dye kit (Bio-Rad Laboratories).

DNase I footprinting. The PCR product was digested with EcoRI, HindIII, or XbaI, end labeled with [α-^{32}P]dATP or [α-^{32}P]dCTP (ICN, Inc.) using the Klenow fragment of DNA polymerase I, and purified using a Quick spin column clean-up kit (Roche). DNase I footprinting assays were carried out in 30 μl of binding buffer, as for the EMSA, with a final DNA concentration of 2 nM. The AF1298 protein was diluted to the indicated concentrations and incubated at room temperature for 10 min. DNase I (Sigma) was added (2 μl of a 1:4,000 dilution of 10-mg/ml stock in water), and incubation continued for 8 min at room temperature. Reactions were stopped by the addition of 7 μl of stop buffer (0.1 M ETDA, pH 8, 1.7 M sodium acetate, pH 5.2). Following precipitation, the samples were resuspended in loading dye, subjected to electrophoresis on an 8% polyacrylamide gel containing 6 M urea, and detected by a Molecular Dynamics PhosphorImager.

Identifying potential binding sites. A Perl script was written to identify potential DNA binding sites with either a perfect match or a 1-bp mismatch for the palindromic CAATC-5N-GATTG sequence, where N is either A, C, G, or T.

RESULTS

Construction of whole-genome DNA array for *A. fulgidus*. To generate a whole-genome microarray, primer pairs were custom designed for 2,436 ORFs in the *A. fulgidus* genome using the MyPROBES software program (see Materials and Methods). Following PCR of the ORFs from chromosomal DNA, the products were purified, quantified, and spotted on Corning GAPS II glass slides in duplicate. In total, 2,410 ORFs are represented on the slides. The slide arrays were then blocked, washed, and dried prior to usage in the microarray experiments. Calibration and application of the arrays are described in Materials and Methods.

Heat shock response in *A. fulgidus*. *A. fulgidus* grows within the range of 60°C to 95°C, and optimal cell growth occurs at 83°C (18). A limited heat shock response study was previously documented using cultures shifted from 78°C to 89°C (7). To examine the whole-genome transcription profile in *A. fulgidus* in response to heat shock, total cellular RNA was prepared from cells at six intervals over a 60-min time period (Fig. 1). As shown in the experimental approach, cDNA prepared for each time point was hybridized to two slides to monitor changes in transcript abundance with increasing time following heat shock (i.e., at 5, 10, 15, 30, and 60 min). The experiment was replicated two additional times to give a total of 12 data sets. Calibration experiments with no heat shock (i.e., 78°C versus 78°C at time zero) were also conducted to determine the statistical confidence level of each transcript measurement. By comparing the average intensities of each ORF using the Cy3 and Cy5 channels, the hybridization efficiencies were determined to have no bias (data not shown). The 95% confidence interval for the expression ratio of each ORF was calculated using the software package lcDNA (see Materials and Methods).

Within the above confidence intervals, 118 of the 2,410 *A. fulgidus* ORFs exhibited increased mRNA levels, while 120 ORFs exhibited decreased mRNA levels by 5 min after the temperature shift (Tables 1 and 2). This represents a change in approximately 10% of the genome. In general, the expression of the genes remained elevated (or reduced) during the next 55 min. By 60 min, 189 ORFs showed increased expression, while 161 ORFs exhibited decreased expression, totaling 14% of the genes in the *A. fulgidus* genome. When categorized by function, the differentially expressed genes were broadly dispersed across a variety of predicted cellular roles (Tables 1 and 2). The most frequently affected categories, with increased or decreased abundance of mRNA, were "energy production and conservation," and "not categorized" functions. A complete summary of the results for these genes is available as Table S1 in the supplemental material.

TABLE 1. Genes significantly up-regulated categorized by cluster of orthogonal genes

Function	Total no. of genes	No. of genes significantly up-regulated at time (min)				
		5	10	15	30	60
Not categorized	540	17	18	26	34	24
General function prediction only	315	17	19	18	30	27
Amino acid transport and metabolism	150	11	11	14	18	18
Signal transduction mechanisms	69	10	10	13	13	10
Lipid metabolism	107	9	12	13	15	9
Energy production and conversion	214	6	13	17	17	15
Transcription	94	6	10	8	14	12
Posttranslational modification, protein turnover, chaperones	58	6	7	6	12	7
Inorganic ion transport and metabolism	77	6	8	7	12	10
Carbohydrate transport and metabolism	53	5	5	8	11	10
Translation, ribosomal structure, and biogenesis	152	5	7	10	3	6
Function unknown	260	5	6	9	16	12
Nucleotide transport and metabolism	48	4	5	4	7	3
DNA replication, recombination, and repair	84	4	3	5	11	9
Cell envelope biogenesis, outer membrane	44	2	2	1	2	4
Secondary metabolite biosynthesis, transport and catabolism (poorly characterized)	27	2	4	4	4	3
Cell division and chromosome partitioning	14	1	2	2	3	1
Coenzyme metabolism	98	1	9	16	13	9
Cell motility and secretion	33	1	3	4	3	0
Total genes	2,437	118	154	185	238	189

Thirteen genes in the *A. fulgidus* genome have been previously either annotated (18) or predicted (10) to be heat shock related. Six of these were observed to be induced within the first 5 minutes (AF1296, AF1297, AF1298, AF1451, AF2238, and AF1971). Five other genes were not differentially regulated over the 60-min heat shock period and included AF0337 (archaeal histone A1, *hpyA1-1*), AF1493 (archaeal histone A1, *hpyA1-2*), AF2098 (cell division control protein 48, *cdc48-2*), AF1285 (putative cell division control protein), and AF0949 (glutamine synthetase, *glnA*).

The time course of expression for 11 of the most significantly induced genes is shown in Fig. 2. Three of the genes form an apparent operon consisting of AF1298 (a hypothetical protein), AF1297 (cell division control protein 48, *cdc48-1*), and AF1296 (small heat shock protein, *hsp20-1*). Maximum expression of each gene occurred at 5 min, followed by a slight reduction over the next 55 min. AF1971, which encodes a putative small heat shock protein (*hsp20-2*), exhibited the greatest elevation in mRNA levels (ca. 12-fold) (Fig. 2). Other genes that showed at least a threefold change by 5 minutes included AF1813 (TATA binding protein-interacting protein TIP49, *tip49*), AF1323 (group II carboxylase), AF1835 (con-

TABLE 2. Genes significantly down-regulated, categorized by cluster of orthogonal genes

Function	Total no. of genes	No. of genes significant down-regulated at time (min)				
		5	10	15	30	60
Not categorized	540	30	51	33	51	42
General function prediction only	315	19	25	20	23	18
Energy production and conversion	214	17	23	20	27	39
Function unknown	260	10	13	13	19	10
Amino acid transport and metabolism	150	9	9	12	6	8
Coenzyme metabolism	98	4	6	4	7	6
Lipid metabolism	107	4	3	4	4	7
Translation, ribosomal structure, and biogenesis	152	4	7	5	17	7
Posttranslational modification, protein turnover, chaperones	58	4	4	4	6	4
Signal transduction	69	4	7	4	6	3
Cell envelope biogenesis, outer membrane	44	3	3	2	2	3
Nucleotide transport and metabolism	48	2	1	2	2	3
Carbohydrate transport and metabolism	53	2	2	1	1	1
Transcription	94	2	4	1	5	2
Inorganic ion transport and metabolism	77	2	7	7	6	6
Secondary metabolite biosynthesis, transport, and catabolism (poorly characterized)	27	2	5	1	2	2
DNA replication, recombination, and repair	84	1	2	1	1	0
Cell motility and secretion	33	1	1	0	2	0
Cell division and chromosome partitioning	14	0	0	0	0	0
Total genes	2,437	120	173	134	187	161

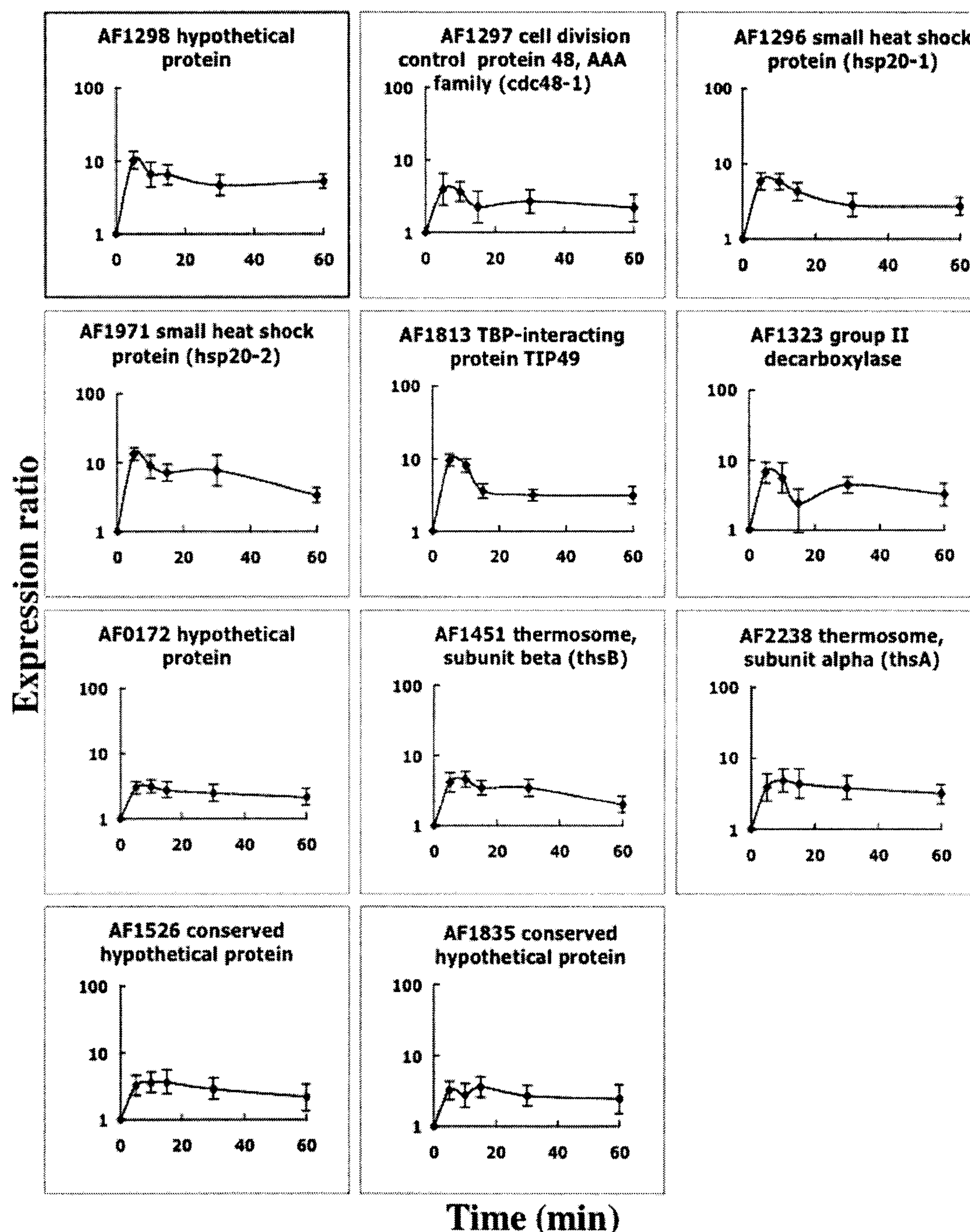

FIG. 2. Temporal pattern of gene expression in response to heat shock. Data for each gene were plotted if a threefold or greater abundance of mRNA was observed at 5 min post-temperature shift. The *x* axis is expressed in minutes, while the *y* axis is the expression change in fold mRNA abundance. The error bars represent one standard deviation.

served hypothetical protein), AF1526 (conserved hypothetical protein), AF0172 (hypothetical protein), AF1451 (thermosome beta subunit, *thsB*), and AF2238 (thermosome alpha subunit, *thsA*).

Verification of microarray data. To verify the quality of the *A. fulgidus* heat shock microarray data, real-time RT-PCR was performed to determine the level of mRNA for 12 selected ORFs at 0, 5, and 10 min following heat shock (Fig. 3). These included four that had increased levels, four that had decreased levels, and four that had no change. The gene expression values were determined in triplicate and compared to the non-heat-shocked condition at 0 min. This value was then plotted against the mean log ratio value for the corresponding gene from the microarray experiment. For each of the 11 genes for which data were obtained, the real-time RT-PCR measurement correlated well ($R = 0.944$) to that observed from the

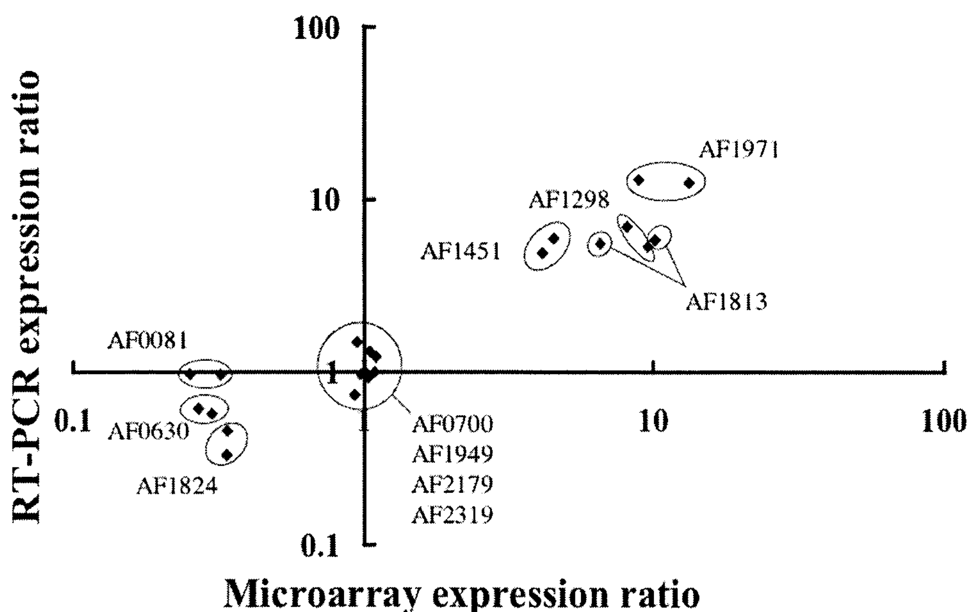

FIG. 3. Comparison of real-time RT-PCR data versus the corresponding DNA microarray data. The mRNA change in response to heat shock was measured as described in Materials and Methods using microarray hybridization and real-time RT-PCR. Data for the 5- and the 10-min changes relative to the 0-min time are indicated, where the correlation coefficient was 0.944.

microarray data regardless of whether the mRNA abundance was increased, decreased, or remained unchanged (Fig. 3).

AF1298 encodes a putative *A. fulgidus* heat shock regulator called HSR1. One ORF identified in the above experiments, AF1298, was among the most highly induced genes at 5 min. It was previously annotated as a hypothetical protein of 27,085 kDa in size (18). An amino acid BLAST search using AF1298 against the nonredundant database (NCBI) returned 10 genes with E-values less than 1e-10. All genes were from the Archaea, where AF1298 has the highest similarity to five genes of unknown function. These include *cmi2* of *Haloferax volcanii*, VNG1843C of *Halobacterium* sp. strain NRC-1, MA4576 of *Methanosarcina acetivorans*, MM1257 of *Methanosarcina mazei*, and METH0903 of *Methanosarcina barkeri*. More distantly related genes include PF1790 (*phr*) in *Pyrococcus furiosus*, PAB0208 in *Pyrococcus abyssi*, PH1744 in *Pyrococcus horikoshii*, and MTH1288 in *Methanothermobacter thermautotrophicus* strain Delta H. Alignment of the corresponding amino acid sequences revealed a high similarity among all proteins within their N-terminal domains, while the C-terminal regions were of lower similarity (Fig. 4). The N-terminal region of AF1298 contains a putative helix-turn-helix (HTH) motif positioned from position 28 to 52.

One ORF in *P. furiosus*, designated PF1790, was recently shown to be a transcription regulator with a proposed role in heat shock induction (46). To test if the *A. fulgidus* ORF AF1298 product called HSR1 protein serves a related role, the gene was cloned into an *E. coli* expression vector and the gene product was purified to homogeneity (see Materials and Methods). The AF1298 protein was then tested for its ability to bind to the promoter elements of 6 of the 11 heat-shocked ORFs shown in Fig. 2. By an EMSA, the upstream promoter regions of two of these (AF1298 and AF1971) were bound as indicated by their altered gel mobility shifts (Fig. 5). For AF1298, a DNA fragment extending from −175 to +50 relative to the start codon was shifted by 125 nM protein, where 250 nM gave a second apparent DNA protein complex (Fig. 5). The apparent K_d was approximately 200 nM (data not shown), where nonspecific binding was seen at 1,000 to 2,000 nM protein as indicated by control DNA fragments.

Of the five other promoter elements of the remaining heat shock-induced genes, AF1451, AF1323, AF1813, AF1971, and AF2238, only the DNA fragment for AF1971 was gel shifted by 125 nM protein (Fig. 5). All other DNA fragments gave no mobility shifts with up to 1,000 nM protein (Fig. 5 and data not shown). Two heat shock genes, AF1297 and AF1296, are positioned immediately downstream of AF1298 and appear to comprise an operon with AF1298. Since the three genes have 15-bp intergenic spacers, the two downstream gene regions were not tested in the EMSA. The AF1297 and AF1296 genes also lack apparent TATA or BRE boxes, indicative of archaeal promoters (discussed below).

Identification of *cis*-acting sequences of HSR1. To further analyze the DNA binding region recognized by the AF1298 gene product, DNase I footprinting was performed on the promoter elements for AF1298 and AF1971. The DNA binding protein protected a 70-bp region on the noncoding strand of the AF1971 promoter element (Fig. 6A) and a 45-bp region on the coding strand (Fig. 6B). At the AF1298 promoter, HSR1 protected a 60-bp region on the noncoding strand (Fig. 7A) and a 30-bp region on the coding strand (Fig. 7B). Since the AF1298 protein binds to its own promoter element, it appears to constitute an autoregulatory heat shock-inducible regulator.

DISCUSSION

Using a whole-genome microarray to study the heat shock response of *A. fulgidus*, we observed changes in mRNA levels

```
                                                               :   ::*::* :.** *: :* ..*
    cmi2 ------------------------------------------------------MDSAVLLDLLGNENRRRILRLLSHKP   26
VNG1843C ------------------------------------------------------MDSGALLDLLGNENRRRILQLLARKP   26
  MA4576 ------------------------------------------------------MDPAKLLDILGNENRRKIIQLLANRP   26
  MM1257 ------------------------------------------------------MDPAKLLDILGNENRRKIIQLLANRP   26
METH0903 -------------------------------HNHNSLALFARHVTNLDYYQRKIMDPAKLLDILGNENRRKIIQILANRP   49
  AF1298 ------------------------------------------MKGLVPRGGEMDIEAETIMDVLGNESRRRILQLLSRKP   38
 PAB0208 ----------------------------------------------------MGEELDMLLDVLGNETRRRILLLLTKRP   28
  PH1744 MTMAILTFTSASLISFRSSLTFIILKFTENVFKSFSNSMLENAEIIKGGEREMGEELSKLLDVLGNETRRRILFLLTKRP   80
  PF1790 ----------------------------------------------------MGEELNRLLDVLGNETRRRILFLLTKRP   28
 MTH1288 ---------------------------------------------MMSGKSTENQNMEVILDVMGCKTRRDIINLLREEP   35
   ruler 1.......10.......20.......30.......40.......50.......60.......70.......80

                  HTH

          :*:*:*  :.:. **::.**  : .**::..   .  :    :*: * ..::::.: :.  :
    cmi2 CYVTEISEYLGVSPKAVIDHLRKLEDAGLIESRTDDQRR----KYFHISRNLRLEVNVSPYGFGAKSAYPPSPSLDMAGR  102
VNG1843C CYVTEISEYIGVSPKAVIDHLQQLDDAGLVESRVDDQRR----KYFHIAENLRLEVQLSPFGYGAKSAYPASTDLDLA-R  101
  MA4576 CYVSEISGRLGVGPKAIISHLSLLEQAGLIECSVDEQRR----KYFNIANNMRLEVSVSPYSY---TMSLQDINFDREKK   99
  MM1257 CYVSEISGRLGVGPKAIISHLSLLEQAGLIECSVDEQRR----KYFNIANNVRLEVSVSPYSY---TMALQDINFDREKK   99
METH0903 CYVSEISSRLGVGPKAIISHLGLLEQAGLIECSVDEQRR----KYFNIANNMRLEVSVSPYAY---TVTLQDVTFDRGNK  122
  AF1298 CYVSEISYYLGMAPKAVLEHLDKLVKAGIVSS-FEEGRK----RYYYIPRSLRLEVVISPHKFGAEIRDSENYDLPSLMK  113
 PAB0208 YFVSELSQELGVGQKAVLEHLRILEEAGLIESRIEKIPRGRPRKYYMIKRGLRLEILLTPTLFGSEMYEPKDIRKSPEYE  108
  PH1744 YFVSELSQELGVGQKAVLEHLRILEEAGLIESRIEKIPRGRPRKYYMIKRGLRLEILLTPTLFGSEMYEPREIRKSPEYE  160
  PF1790 YFVSELSRELGVGQKAVLEHLRILEEAGLIESRVEKIPRGRPRKYYMIKKGLRLEILLTPTLFGSEMYEAKGVRKSPEYE  108
 MTH1288 RFVSEISRELDIGQKAIIEHLRAMEEAGILSSFFRKIERGRPRKYYDISRDIHVSITINRNTFRVDVMDDLLNRK--QLP  113
   ruler ........90.......100.......110.......120.......130.......140.......150.......160

               .                    :       .     :                 :
    cmi2 CPHVTLEVEN-AGTDDVS----ELARELGKLEELDNELSLAQRWVQGRITDVLDRLNDRLGVDADSRFYAEILAALANGS  177
VNG1843C CQYVSVRIEERDSNAEVN----ELAAKLKELNELERELSMAQRWVQGQLADVQAALGDAIGDGPD-RLYADVLAALADDS  176
  MA4576 GEKSAVEKKETAARDESSCFFLKLSNRLQELKARQEELVQMQKQLQVEYTELMDRCLDSIEEVARNPVECELLFELLKNE  179
  MM1257 RESYAVENNGPATRDESSCFFLKLSERLRELRIKQEELAQMQKQLQAEYTELMDKCLDSIEDIARNPVECELLFELLKNE  179
METH0903 -EENPVEKEIPSG-EESSSFFLKLSNRLKELKKRHEELAQMQQQLQVEYTEAMDRCLDSIEEIARNPVECELLFELLKNE  200
  AF1298 EIRNEFNMIERLKAESIS----EIYRALKVAEDLQRRFSRVQSTLSMRFNQFVERMLNEIERVVSDDLERLVMLGLAKGL  189
 PAB0208 QARELIKSTEPIDVKMRE-----LAEFLHELDERIKEIIEEKRELEEVKILVETYIENVMRRMSEENRQTLEKIFKEIER  183
  PH1744 QARELIKSVEPIDIKMKE-----LAEFLHELDDRIKELIEEKRELEEVKILVETYIENVMRRISEENKEIVKKVFKDIEE  235
  PF1790 QAKELIKSQEPINVKMRE-----LAEFLHELNERIREIIEEKRELEEARILIETYIENTMRRLAEENRQIIEEIFRDIEK  183
 MTH1288 PGDEWSRLLNIENKIKKG-----QWEAVEELKNLIRLYDHLKR--RAEDLIMEAEIRRKME-------------------  167
   ruler .......170.......180.......190.......200.......210.......220.......230.......240

    cmi2 NTVRGIAKELNADPAVVDSALHHLAEGGLVAH---NGD-RWRID-------   217
VNG1843C RTARDVAEALEAPVPLIDGVLQTLADRGLVVE---RADGEYAIAE------   218
  MA4576 ATAAVLCYNLRLHPSIITSNLMDLAERGFVEYTIKNNQQYWRICETGVENK   230
  MM1257 ATAAVLCYNLRLHPSIINSNLMDLAERGFVEYTIKNSQQYWKICETGVENK   230
METH0903 TTLATLCYNLRLHPSIVNANLIDLTKRGFIEYGIKNNQYYWKIRETGVENE   251
  AF1298 RTAAEIAETFRLPYREVERALNSLHEKGLVEKEEKNGEVVWMIK-------   233
 PAB0208 ILPKDYARRIKEKFI-ENI--------------------------------   201
  PH1744 ILPPEYAKRIKEKFI-ENI--------------------------------   253
  PF1790 ILPPGYARSLKEKFLNINI--------------------------------   202
 MTH1288 ---------------------------------------------------   167
   ruler .......250.......260.......270.......280.......290.
```

FIG. 4. Multiple amino acid sequence alignments of putative heat shock regulatory proteins. The indicated sequences were aligned using ClustalW 1.83 with the default settings. The sequences were the following: AF1298, *Archaeoglobus fulgidus*; cmi2, *Haloferax volcanii*; VNG1843C, *Halobacterium* sp. (strain NRC-1); MA4576, *Methanosarcina acetivorans*; MM1257, *Methanosarcina mazei*; METH0903, *Methanosarcina barkeri*; PF1790, *Pyrococcus furiosus*; PAB0208, *Pyrococcus abyssi*; PH1744, *Pyrococcus horikoshii*; MTH1288, *Methanothermobacter thermautotrophicus* strain Delta H.

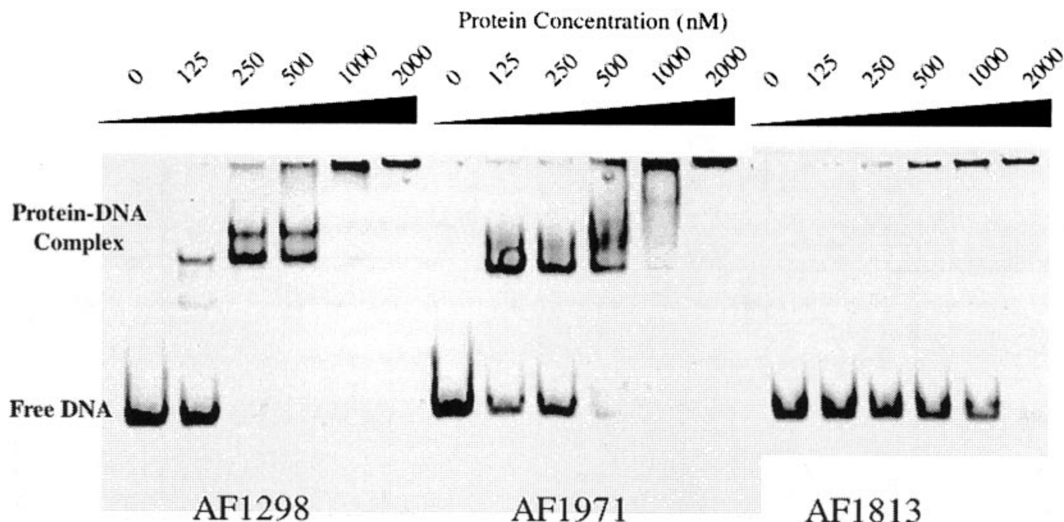

FIG. 5. Electrophoretic mobility shift assays for the AF1298, AF1971, and AF1813, DNA fragments. The concentration of HSR1 protein from left to right was 0, 125, 250, 500, 1,000, and 2000 nM. EMSA for the promoter regions from position bp −175 to +50 relative to the start of translation.

for approximately 10% of the 2,410 genes when cells were shifted from 78°C to 89°C by 5 min (Tables 1 and 2). In general, the *A. fulgidus* genes that were highly induced at 5 min remained induced during the 60-min duration of the heat shock experiment (Fig. 2). Of the 11 genes that were highly induced at 5 min (ca. 5- to 10-fold) (Fig. 2 and 3), 5 were previously annotated as heat shock genes. Two were not known to be heat shock induced (AF1813 and AF1323), and four encode hypothetical proteins (AF1298, AF0172, AF1526, and AF1835). Of the remaining annotated heat shock proteins in *A. fulgidus*, we observed that genes for the HSP60s, which form group II chaperonins referred to as thermosomes, and the genes for the small heat shock protein (sHSP20) were also heat induced (AF1296 and AF1971). While there are no HSP70 genes annotated in *A. fulgidus*, as in other Crenarchaeota, it has been suggested that a protein known as prefoldin may be its functional equivalent (27, 45). There are three prefoldin-related proteins in *A. fulgidus*, AF2063 (putative c-*myc* binding protein), AF1150 (putative ErpK), and AF0708 (a conserved hypothetical protein), predicted by COG to be a prefoldin. However, we observed that all of the prefoldin genes were down regulated following heat shock.

Other known heat shock genes in the *Bacteria* express ATP-dependent proteases, such as Clp, Lon, and HslUV. In *A. fulgidus* a Lon homolog without the ATP binding domain has been described (47), but we observed that the AF0364 gene was not differentially expressed upon heat shock. In *Thermoplasma acidophilum*, the protease complex known as the proteosome is essential for survival in heat stress (31), but we observed that the proteosome beta subunit, PsmB (AF0481), was slightly heat induced after 60 min. The alpha subunit, PsmA (AF0490), was decreased 2.5-fold by heat after 10 min. These transcriptome results are similar in several ways to *P. furiosus* (38), although in *P. furiosus* there are six heat-induced ATP-independent proteases and peptidases. Four of these have homologs in *A. fulgidus*, but only two were heat induced: AF1652 (subtilisin-like peptidases) and AF0235 (HtpX, heat

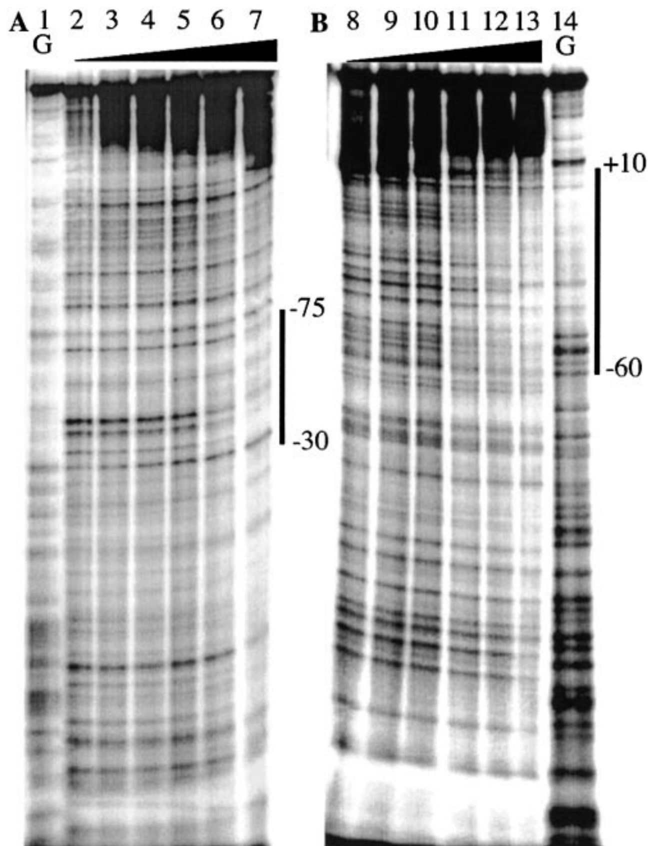

FIG. 6. DNase I footprint of AF1971 DNA on the coding and noncoding strands. Coding strand (lanes 1 to 7): lane 1, G reaction; lanes 2 to 7, increasing levels of HSR1 protein (from left to right) of 0, 125, 250, 500, 1,000, and 2,000 nM. Noncoding strand (lanes 8 to 14): lane 8 to 13, increasing levels of HSR1 protein (from left to right) of 0, 125, 250, 500, 1,000, and 2,000 nM; lane 14, G reaction. The numbering of the DNA is relative to the start of translation.

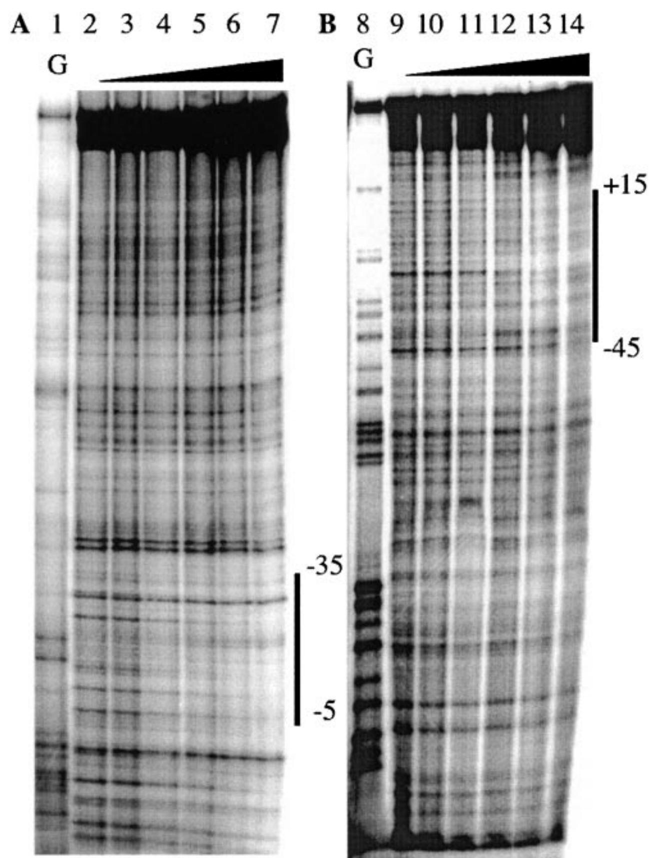

FIG. 7. DNase I footprinting of AF1298 DNA on the coding and noncoding strands. Coding strand (lanes 1 to 7): lane 1, G reaction: lanes 2 to 7, increasing concentrations of the HSR1 protein (from left to right) of 0, 125, 250, 500, 1,000, and 2,000 nM. Noncoding strand (lanes 8 to 14): lane 8, G reaction as ladder; lanes 9 to 14, increasing HSR1 protein (from left to right) of 0, 125, 250, 500, 1,000, and 2,000 nM. The numbering of the DNA is relative to the start of translation.

shock protein X). AF1652 was induced 1.8-fold at 30 min, in contrast to its counterpart in *P. furiosus*, which was induced almost 12-fold (38). *A. fulgidus* HtpX was induced twofold in 30 min. In *E. coli*, HtpX is regulated by CpxA-CpxR and is a membrane-bound metalloprotease with a cytoplasm-exposed active site (37). The role and location of HtpX in *A. fulgidus* has not yet been determined.

Role of AF1298 in *A. fulgidus* heat shock. The AF1298 gene product, designated here as HSR1, was demonstrated to bind to the promoter elements of two of the newly identified heat shock genes in *A. fulgidus*, AF1298 and AF1971 (Fig. 5 to 7). While it is not yet possible to assign the recognition sequence associated with HSR1 binding to DNA, an alignment of the two promoter regions reveals three regions with similarity. A 13-bp region contains a recognizable BRE box and a TATA box (Fig. 8). The third region is within the HSR1-proteced region and consists of a 17-bp region located 6 bp downstream of the TATA box. It has a palindromic motif, CTAAC-N5-GTTAG, where the central five nucleotides constitute a hypothetical spacer element. The HSR1-protected region is located immediately downstream of the putative TATA box, thus overlapping the transcriptional start sites of AF1298 and AF1971. The palindromic motif is absent in the AF1831 upstream region that failed to exhibit a HSR1 gel shift (Fig. 5).

To determine if the above palindrome was present elsewhere in the *A. fulgidus* genome, we searched all promoter regions consisting of 600 bp upstream to 100 bp downstream of each ORF start codon for the CTAAC-5N-GTTAG motif (where N is A, C, G, or T; see Material and Methods). We identified four occurrences near the promoter regions for AF1298, A1971, AF1626, and AF1792. At AF1626, the sequence has a similar location to those at AF1298 and AF1971, but there is no TATA box in close proximity to the potential HSR1 binding site. In the promoter of AF1792, the potential HSR1 binding site occurs 14 bp downstream of the annotated start codon. Correspondingly, neither the AF1626 nor the AF1792 mRNA level changed in response to heat shock as revealed by the microarray experiments described above. It is tempting to speculate that HSR1 serves a relatively limited role in the *A. fulgidus* heat shock response, given the lack of the above palindrome at any of the other 10 to 14% of genes exhibiting a heat shock response (Tables 1 and 2). The palindrome is also not present in the *P. furiosus* genome.

In our microarray analysis of the *A. fulgidus* heat shock response, we identified a self-regulating heat shock regulator (HSR1) with homology to one known and four potential new heat shock regulators in other Euryarchaeota species (Fig. 4). These include cmi2, VNG1843C, MA4576, and MM1257. The N-terminal region is the most highly conserved region and contains a potential HTH motif likely responsible for DNA recognition and binding by each protein. Interestingly, the AF1298, cmi2, VNG1843C, MA4576, and MM1257 homologs from two halophilic species and from two methanogen species also lack a 4-amino-acid spacer following the HTH region that is present in the more distantly related proteins, including Phr of *P. furiosus*. Similarly, the five AF1298 homologs also exhibit considerably reduced amino acid similarity in their C-terminal domains relative to Phr. Therefore, AF1298 appears to constitute a distantly related protein subfamily relative to Phr. It may also function differently. There is evidence that the HTH motif located in the N terminus may function as a negative regulator (28). By one plausible scenario, we propose that HSR1 binds DNA under normal cell growth temperatures (ca. 78°C), interferes with RNA polymerase interactions with TFB, and thereby inhibits transcription. At increased temperature, HSR1 is released from the DNA allowing transcription to proceed. A related control mechanism has been proposed for Phr in *P. furiosus* (46) and for HrcA in the gram-positive bacterium *Bacillus subtilis* (33). The prevention of recruitment of RNA polymerase is similar to the metal-dependent regulator MDR1 in *A. fulgidus*, where MDR1 with bound metal ions binds DNA to prevent RNA polymerase to start transcription (3). There are also similarities between HSR1 and HrcA in gram-positive bacteria, where the latter binds to palindromic *cis*-elements downstream of a sigma factor binding site to negatively regulate a small subset of heat-induced genes. A detailed mechanism of the heat shock regulation of AF1298 and AF1971 and the basis for HSR1 in this process remain to be elucidated.

Lipid metabolism. In other organisms, microarray analysis of heat shock has revealed a large number of differentially regulated ORFs, many of which have unknown or uncharac-

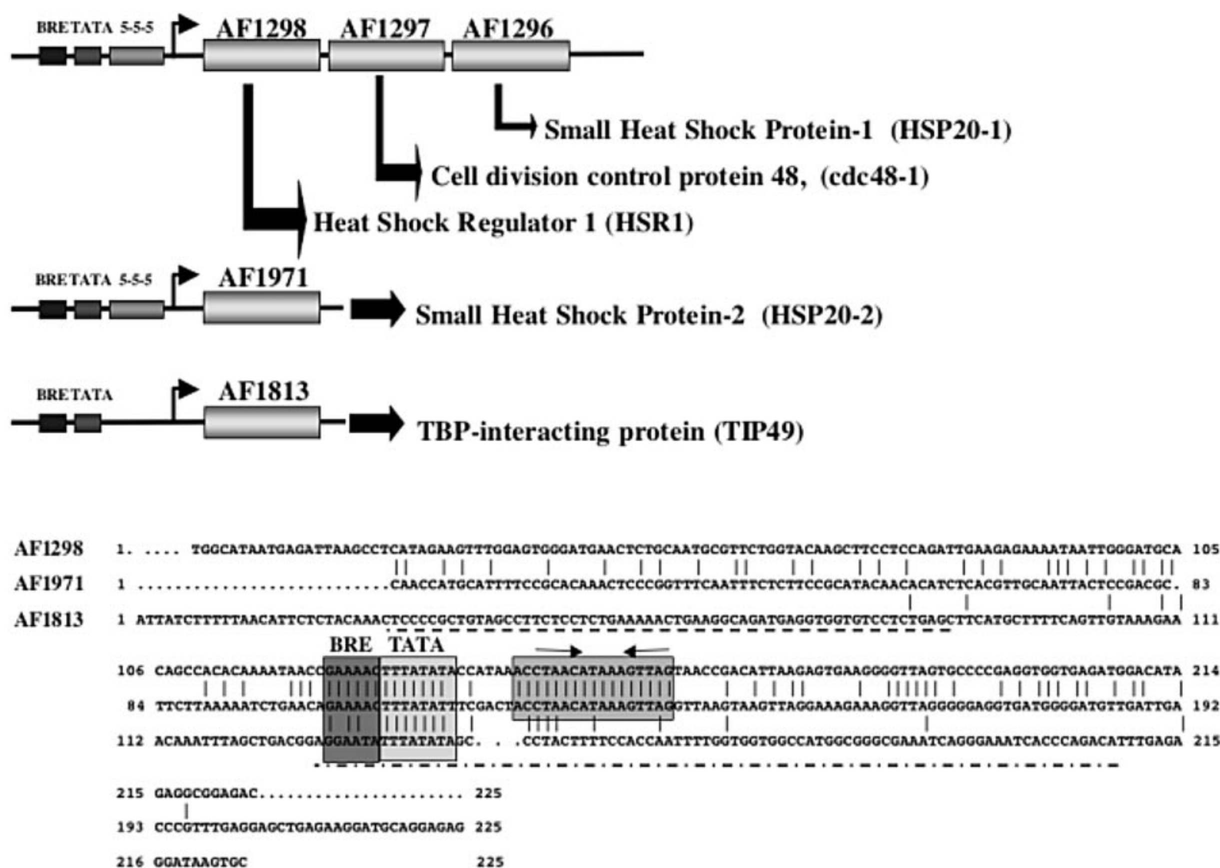

FIG. 8. Alignment of promoter DNA sequences for AF1298, AF1971, and AF1813. The sequences were aligned using GAP alignment from the GCG Wisconsin package 10.3 (Accelrys Inc., San Diego, CA). Three conserved regions are marked with gray boxes: the TATA box, the BRE interaction site, and a downstream potential binding site for HSR1. A conserved palindromic motif, CTAAC-N5-GTTAG, is indicated by the opposing arrows. The horizontal lines above (AF1298) and below (AF1971) the DNA sequences indicate the regions protected in the DNase I footprint experiments.

terized functions (14, 30, 40). In *A. fulgidus* we observed that heat induced or repressed the expression of genes related to energy production, amino acid metabolism, and lipid metabolism, thus indicating the importance of metabolic adaptation to heat stress (Tables 1 and 2). It is known that membrane composition, particularly the composition of isoprenoids in *Archaea*, may undergo significant changes during heat shock (6, 42). Among the known isoprenoid pathway genes, AF1736/MvaA (3-hydroxy-3-methylglutaryl-coenzyme A [HMG-CoA] reductase) is the only gene we observed to be heat induced in *A. fulgidus*. This gene encodes a rate-controlling enzyme in the mevalonate pathway leading to isoprenoid biosynthesis. Shimada et al. (36) overexpressed HMG-CoA in yeast and increased the carotenoid production two- to fourfold (36), indicating that in yeast HMG-CoA is a limiting step in the mevalonate pathway. It is therefore possible that increasing MvaA is sufficient to increase isoprenoid biosynthesis in Archaea.

Organic solutes. Organic solutes are also known to influence thermotolerance of hyperthermophilic Archaea (23). *A. fulgidus* responds to heat shock by increased synthesis of di-myo-inositol-1,1′(3,3′)-phosphate (DIP) and diglycerol phosphate by almost 30- and 2-fold, respectively (20). While the metabolic pathways for these organic solutes have not been fully elucidated, two different pathways for DIP synthesis have been proposed (5). In *Methanococcus igneus* (5), L-myo-inositol-1-phosphate reacts with CTP to form CDP-inositol, which reacts with myo-inositol to create DIP, and in *Pyrococcus woesei* (32) two L-myo-inositol-1-phosphates form a phosphodiester bond with the presumed hydrolysis of NTP (32). Both proposed pathways start with L-myo-inositol-1-phosphate synthesis from glucose-6-phosphate. In *A. fulgidus*, we observed that the myo-inositol-1-phosphate synthase (AF1794) was not differentially regulated by heat shock, although the gene in *P. furiosus* was clearly heat induced (38). We did observe that the gene for inositol monophosphatase (AF2373), which converts L-myo-inositol-1-phosphate to form myo-inositol, is heat induced 1.4-fold. If *A. fulgidus* uses the pathway proposed for *M. igneus* DIP synthesis, AF2315 encodes a candidate protein with a cytidylyltransferase domain since it is the only gene that is heat induced. This protein is currently categorized as a nicotinamide-nucleotide adenylyltransferase and has not been implicated in this pathway. It is possible that another heat-induced gene, such as AF1835, AF0172, AF0555, AF2160, or AF0267, could influence DIP synthesis, but this has yet to be determined.

Archaeal heat shock gene regulation. Compared to bacterial and eucaryal heat shock studies, relatively few microarray anal-

yses have been reported for the *Archaea* (1, 2, 34, 35, 38, 48). Among them, only one investigated the heat shock response of a hyperthermophilic archaeon, and this utilized a partial-genome array of *Pyrococcus furiosus* (38). When *P. furiosus* was shifted from its normal growth temperature of 90°C to a heat shock temperature of 105°C, 55 of the hand-picked 201 genes used in the microarray were differentially expressed. Thus, no conclusion could be made about the extent of the heat shock response in this microorganism. Thus, the current *A. fulgidus* experiments constitute the first whole-genome heat shock study in the *Archaea* and provide the foundation for comparing related processes in the *Bacteria* and *Eucarya*. Although a large set of genes exhibit differential mRNA abundance upon heat shock (ca. 14% of the genome), little is yet known about the means by which this is accomplished.

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